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**PROGNOSTIC INDICATORS IN CHOROIDAL AND  
CILIARY BODY MELANOMA**

**By**

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Academic Dissertation

To be publicly discussed, by permission of  
the Medical Faculty of the University of Helsinki,  
in the Auditorium of the Department of Ophthalmology,

Haartmaninkatu 4, Helsinki,

on June 13<sup>th</sup>, 2001, at 12 o'clock noon

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ISBN 952-91-3572-6 (nid.)

ISBN 952-10-0054-6 (PDF version, available at <http://ethesis.helsinki.fi>)

Yliopistopaino

Helsinki 2001

*To the memory of my grandfathers*

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## ORIGINAL PUBLICATIONS

This dissertation is based on the following publications as well as previously unpublished data on metastatic uveal melanoma. The original publications in the text will be referred to by their Roman numerals I-IV:

- I** Mäkitie T, Summanen P, Tarkkanen A, Kivelä T. Microvascular loops and networks as prognostic indicators in choroidal and ciliary body melanomas. *J. Natl. Cancer Inst.* 1999;91:359-367.
- II** Mäkitie T, Summanen P, Tarkkanen A, Kivelä T. Microvascular density in predicting survival of patients with choroidal and ciliary body melanomas. *Invest. Ophthalmol. Vis. Sci.* 1999;40:2471-80.
- III** Mäkitie T, Summanen P, Tarkkanen A, Kivelä T. Tumor-infiltrating macrophages (CD68<sup>+</sup> cells) and prognosis in malignant uveal melanoma. *Invest. Ophthalmol. Vis. Sci.* 2001;42:1414-1421.
- IV** Mäkitie T, Carpén O, Vaheri A, Kivelä T. Ezrin as a prognostic indicator and its relationship to tumor characteristics in malignant uveal melanoma. *Invest. Ophthalmol. Vis. Sci.* Under revision.

## **ABBREVIATIONS**

ABC	Avidin-biotinylated peroxidase complex
AFIP	The Armed Forces Institute of Pathology
BMDP	Statistical software package
BSA	Bovine serum albumin
CD	Cluster of differentiation
CI	Confidence interval
CK	Cytokeratin
c-met	The receptor of hepatocyte growth factor
COMS	The Collaborative Ocular Melanoma Study
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMA	Epithelial membrane antigen
ER	Estrogen receptor
FVIII-RAg	Factor VIII-related antigen
Gy	Gray, a unit of radiation
HGF	Hepatocyte growth factor
HLA	Human leukocyte-associated
HR	Hazard ratio
ICAM	Intercellular cell adhesion molecule
IHC	Immunohistochemistry
Ki-67	A proliferation-associated antigen
LBD	Largest basal tumor diameter
mAb	Monoclonal antibody
KDR	Receptor of vascular endothelial growth factor
MMP	Matrix metalloproteinase
MVD	Microvascular density
NCAM	Neural cell adhesion molecule
p	Short arm of a chromosome
PAS	Periodic acid-Schiff
PBS	Phosphate-buffered saline

## Abbreviations

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PC-10	A proliferation-associated antigen
q	Long arm of a chromosome
RD	Retinal detachment
SE	Standard error
SPSS	Statistical software package
TNM	System for classifying the extent of tumor spread
UEA-I	<i>Ulex europaeus</i> agglutinin I
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume

## **INTRODUCTION**

Cancer and blindness are among the most feared destinies.<sup>1,2</sup> These two miseries are combined in intraocular tumor. The most common intraocular tumors are benign melanocytic uveal nevi and secondary metastatic tumors.<sup>3</sup> Malignant melanoma of the uvea is, however, the most common primary malignant intraocular tumor in adults.<sup>4,5</sup>

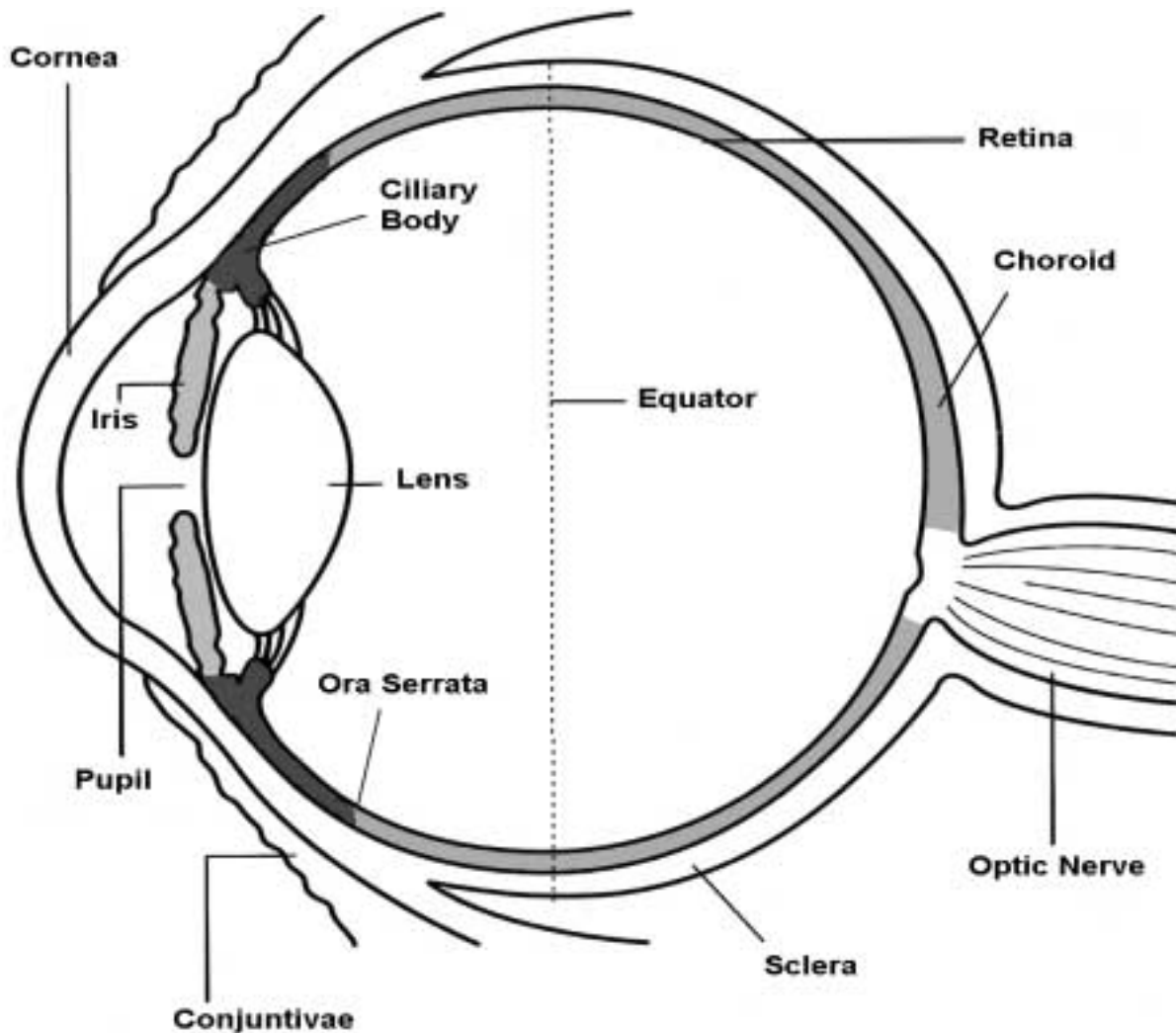
Uveal melanoma differs markedly from cutaneous melanoma, although both originate from melanocytes.<sup>3,6</sup> Unlike in cutaneous melanoma, UV light and hereditary predisposition have little or no effect on initiation of uveal melanoma.<sup>5-8</sup> Intraocular melanoma disseminates hematogeneously, because interior of the eye lacks lymphatics, and it has a great propensity to develop distant metastases, in particular in the liver, whereas cutaneous melanoma tends to disseminate in regional lymph nodes.<sup>2,9-12</sup> Recent studies have revealed substantial cytogenetic and molecular differences between these tumors, which probably explain their divergent biological behaviour.<sup>13-15</sup>

The uvea consists of three elements: the iris, ciliary body and choroid (Fig. 1).<sup>3</sup> Melanomas that arise from the iris differ markedly from those of the choroid and ciliary body. Due to their anatomic location that favours early diagnosis and, perhaps, their relatively benign cellular composition, deaths from iris melanoma are markedly less common than deaths from choroidal and ciliary body melanoma.<sup>3,16-18</sup> Consequently, iris melanomas are not included in the present study, and uveal melanoma in the present thesis refers exclusively to choroidal and ciliary body melanoma.

Malignant uveal melanomas were treated for decades by a prompt removal the eye; by 1970's conservative treatment methods such as plaque brachytherapy, charged particle irradiation and transscleral local resection came into daily clinical practice.<sup>19,20</sup> Since no convincing difference in survival has been detected between radical surgery of the eye and eye conserving therapies, functional, psychological, and cosmetic advantages of saving the eye have increased the popularity of conservative methods.<sup>20-27</sup> Even though new treatment options have shown their safety as compared to the traditional enucleation, no significant improvement has taken place in the outcome of patients with uveal melanoma.<sup>22,23</sup> Obviously in most cases, intraocular melanoma has already disseminated at the time when treatment of the primary tumor is given; the choice between radical and conservative therapy does not necessarily have a great impact on survival.<sup>23,28</sup> Manifest disseminated uveal melanoma is still an unbeatable challenge for oncologists; the median survival after metastatic disease is only from a few months to one year.<sup>29-31</sup>

In order to improve the survival of uveal melanoma patients, an effective and safe adjuvant therapy is needed, since high-risk and even many low-risk patients have micrometastases at the time of diagnosis of the primary tumor.<sup>28,29,32</sup> As our understanding of the metastatic process is far from complete, prognostic studies on uveal melanoma are warranted to recognise patients with high, moderate, or low risk for metastasis. Because little histopathological material is currently obtained as compared with the earlier era of routine enucleation, evaluation of prognosis at the time of treatment of the primary tumor has, in fact, become much more challenging after development of eye-conserving treatments.

**Figure 1.** Schematic drawing of the eye. The equator determines tumor location to anterior and posterior



## **REVIEW OF LITERATURE**

### **1. MALIGNANT UVEAL MELANOMA**

#### **1.1 Epidemiology**

Uveal melanoma is the most common malignant primary tumor of the eye in Caucasian adults.<sup>5</sup> The annual incidence of malignant uveal melanoma ranges from 6 to 8 new cases per million Caucasians, but its incidence is 15 to 50 times lower in Africans and Orientals.<sup>5</sup> Retinoblastoma exceeds uveal melanoma in frequency among non-Caucasians.<sup>33</sup> In a large epidemiological study conducted in Finland in 1977, Raivio reported an annual incidence of 5 per million persons, which means approximately 25 new melanoma patients per year in the 5 million population of Finland.<sup>34</sup> During recent years the incidence of uveal melanoma has been somewhat higher, ranging from 30 to 40 intraocular melanomas per year in Finland. This variation is most likely explained by incomplete early data collections of the Finnish Cancer Registry, founded in 1952. In two other Scandinavian populations, the Danish and the Swedish, the incidence is 7.1 and 7.2 per million inhabitants, respectively.<sup>35,36</sup> In contrast to the strikingly increasing incidence of cutaneous melanoma, the frequency of uveal melanoma has not changed during the last two decades.<sup>5</sup>

The incidence of uveal melanoma depends on age: uveal melanoma is very rare in childhood, but its incidence increases in older age groups achieving a peak in late middle age (50-60y).<sup>5</sup> Persons with lightly pigmented skin, hair, and irises, not uncommon features among the Scandinavians and the Finnish in particular, are at slightly increased risk of developing uveal melanoma.<sup>35,37,38</sup> A slight predominance of male gender is evident from large series of uveal melanoma, but the reason for the male predominance is unknown.<sup>5</sup>

#### **1.2 Aetiology and Predisposition**

Neuroectodermally derived melanocytes of the choroid, ciliary body and iris give rise to uveal melanoma.<sup>3</sup> The highly vascularised choroid is the largest part of the uveal tract and the most common site for intraocular melanoma.<sup>39</sup> Of uveal melanomas, 80% grow in the choroid, 15% involve both the choroid and the ciliary body, and 5% are confined to the ciliary body, iris, or both.<sup>39</sup> Diffuse uveal melanoma has an uncommon growth pattern, representing approximately 5% of posterior uveal

melanomas. It is named for its flat shape and small height and it may affect large areas of the choroid.<sup>40</sup> Variants of diffuse uveal melanoma are a ring melanoma that grows circumferentially in the ciliary body and a retinoinvasive melanoma that invades extensively retina and extends to optic nerve.<sup>41,42</sup> Rarely cutaneous melanoma can metastasize intraocularly.<sup>43</sup> These metastatic melanomas often grow in the retina and vitreous body that are rarely involved by uveal melanomas.<sup>42,43</sup>

In addition to age and race, one of the best-documented predisposing lesions is the relatively common choroidal nevus; 3-20% of the population has at least one choroidal nevus.<sup>3</sup> However, only a small minority of nevi (1 in 15000) transforms to malignant melanoma per year.<sup>3</sup> In contrast to skin melanoma, ultraviolet exposure does not correlate with development of uveal melanoma, but contradictory reports also exist.<sup>5,37</sup>

Congenitally increased number of melanocytes, known as congenital ocular melanocytosis and congenital oculodermal melanocytosis (the nevus of Ota) are more common among patients with uveal melanoma than in the general population.<sup>5</sup> The lifetime risk for developing uveal melanoma is 1:400 for a patient with ocular melanocytosis.<sup>44</sup> The dysplastic nevus syndrome is also associated with an increased risk of uveal melanoma.<sup>45,46</sup>

Hormonal factors, like childbearing history and hormonal therapy have been studied as etiological factors, but no convincing connection between hormonal factors and uveal melanoma has been established.<sup>47,48</sup>

Even though knowledge of cancer genetics has markedly increased, the genes involved in the development and progression of uveal melanoma are not yet known. Some frequent chromosomal changes, such as monosomy of chromosome 3 and structural alterations of chromosomes 6 and 8 are distinctly connected with uveal melanoma.<sup>49-51</sup> In spite of occasional uveal melanomas in first-degree relatives, no convincing evidence for familial inheritance exists.<sup>8,52</sup>

### **1.3 Diagnosis**

Most patients with uveal melanoma have symptoms of blurred vision, visual field loss, photopsia, floaters, and rarely, pain before diagnosis; 10-30% of uveal melanomas are diagnosed during routine ophthalmologic examination in the absence of any preceding symptoms.<sup>53-55</sup> Indirect ophthalmoscopy is widely used in making the diagnosis of choroidal melanoma.<sup>41</sup> Of medium-sized and large uveal

melanoma, up to 99% are diagnosed correctly by clinical methods.<sup>56</sup> Clinical diagnosis may be difficult, however, when differentiating small melanoma from nevi.<sup>20,41</sup> Documented growth is often needed to confirm the diagnosis of small melanomas, but also nevi may slowly grow in follow-up.<sup>20,41</sup> Exudative retinal detachment is very frequently associated with choroidal melanoma, which may help in diagnosis of a small melanoma, but it may also obscure an underlying larger tumor and delay diagnosis.<sup>57</sup>

Uveal melanoma may break two layers: the overlying Bruch's membrane and the underlying sclera.<sup>3</sup> Having broken the Bruch's membrane, choroidal melanoma has an access to the subretinal space, immediately achieving a shape of "collar button" or "mushroom", which are highly characteristic of, even pathognomic of choroidal melanoma (Fig. 2).<sup>3</sup> It may then also invade the overlying retina and extend to the vitreous cavity. The outermost layer of the eye, sclera, can resist local invasion to orbit for years, except close to the optic disc.

**Figure 2.** *Mushroom-shaped choroidal melanoma that has ruptured Bruch's membrane. Patient died of hepatic metastases three years after enucleation*



Transillumination of the eye helps to estimate the anterior margins of the tumor in lightly pigmented individuals. Development of B-scan ultrasonography has greatly helped ophthalmologists in making the diagnosis of choroidal tumors, and thus, the rate of misdiagnosis has markedly decreased during last decades.<sup>41,56</sup> High frequency ultrasonography helps in diagnosis of ciliary body tumors.<sup>58</sup> Computed tomography and magnetic resonance imaging are optional methods.<sup>41</sup> The cellular composition of the intraocular tumor can be assessed by fine-needle aspiration biopsy performed either via a transscleral or a transvitreal route, but this method has been so far limited to patients whose clinical diagnosis is in doubt and to experimental use.<sup>59,60</sup> “Double circulation” consisting of choroidal and tumor vessels that are leaky in late phases of a fluorescein fundus angiography is characteristic of uveal melanoma, but angiography does not differentiate a malignant lesion from a benign one reliably.<sup>41</sup>

The diagnosis of metastatic disease is conventionally based on liver function tests and chest x-ray examination.<sup>29,30</sup> However, screening for metastatic uveal melanoma by abdominal ultrasonography and computed tomography were found to be more sensitive methods for detecting metastatic disease.<sup>12</sup> Liver function tests, nevertheless, provide confirmatory evidence.<sup>12</sup>

#### **1.4 Treatment**

Enucleation was the standard and only treatment of uveal melanoma for decades until eye-conserving methods developed in the 1950's came to clinical use in late 1960's.<sup>61</sup> Enucleation is still an effective and acceptable treatment modality, especially for large melanomas and under circumstances in which the availability of conservative methods is limited, like in developing countries.<sup>41</sup> Enucleation has an excellent local tumor control, approaching to 100% if no extraocular extension is present at the time of surgery.<sup>62</sup> The American Collaborative Ocular Melanoma Study (COMS) was initiated in 1986 to resolve whether or not any difference in survival between enucleation and radiotherapy exists.<sup>63</sup> A large number of patients with medium-sized melanoma have been randomised either to enucleation or iodine plaque radiotherapy, and comparative survival statistics will be published by year 2003. Retrospective studies on effectiveness and safety of radiotherapy as compared to enucleation have shown that a significant difference between these two treatment methods is unlikely, which have already declined enucleation rates especially in European and American ophthalmic centers.<sup>22-26,41</sup>

Radiotherapy given either with episcleral radioactive plaques (cobalt<sup>60</sup>, iodine<sup>125</sup>, ruthenium<sup>106</sup>, iridium<sup>192</sup> or palladium<sup>103</sup>) or with accelerated protons or helium ions often saves some functional

vision in the eye with uveal melanoma particularly when the tumor is small.<sup>19,64-66</sup> Uveal melanoma is, however, resistant to markedly higher doses of irradiation than the retina and the optic nerve, which increases the risk for visual impairment due to radiation retinopathy and optic neuropathy.<sup>19,65-67</sup> Recurrences of uveal melanoma after radiotherapy range from 5 to 10% within 5 years and local recurrences may indicate even a 5-fold risk for metastatic disease.<sup>68,69</sup> It also remains a possibility that tumors that recur were initially more malignant. Low-dose (4-8 Gy) irradiation before enucleation has had no effect on survival.<sup>70,71</sup>

Thermotherapy given through dilated pupil (transpupillary thermotherapy), has been shown to be an effective treatment for selected small choroidal melanomas and in combination with brachytherapy also for larger ones.<sup>72-74</sup> Tumor resection under arterial hypotension in otherwise healthy patients is another type of eye conserving treatment of uveal melanoma.<sup>41,75</sup> Studies on gamma-knife techniques and photodynamic therapy have more recently been published.<sup>76,77</sup> Due to small sample sizes and short follow-up time their eventual place in clinical ophthalmic oncology are not yet established.

Treatment of metastatic uveal melanoma is often, if not always, unsatisfactory.<sup>29</sup> Chemotherapy and chemoembolization to the hepatic artery with or without interferon have potentially lengthened survival to a mean of 12 months and up to 20% of metastases have regressed at least partially during treatment, but the disease has always later progressed and killed the patient.<sup>29-31,78,79</sup> Only very few patients treated with surgery for a solitary, slow growing metastasis have survived for long term.<sup>80-82</sup> A preliminary non-randomised study on dietary biological adjuvant therapy for primary intraocular melanoma suggested an improved survival, but the series contained only nine patients and follow-up time was relatively short.<sup>83</sup>

### **1.5 Natural course of the disease**

Because uveal melanomas are treated promptly after diagnosis,<sup>20</sup> the natural course of uveal melanoma is a matter of dispute. In several series of treated patients the 10-year cumulative melanoma-specific survival has been close to 60%, and it has thereafter decreased by about 1% per year.<sup>84-86</sup> Deaths from metastatic uveal melanoma are regularly reported even decades after enucleation.<sup>87,88</sup> Uveal melanoma has a remarkably strong tendency to produce liver metastases.<sup>10,12,89,90</sup> Liver is involved as the only metastatic site in half of the patients, and only 5% of patients who have disseminated uveal melanoma do not have eventual metastasis in the liver.<sup>10,12,90</sup> Other preferred sites for metastases are skin, lung, and bone.<sup>10,12,89,91</sup> The median expected lifetime after metastatic uveal melanoma is diagnosed is in

most cases less than a year, but single patients with few, slowly growing metastases have survived even for three to four years.<sup>30,82</sup>

As documented in many series, the melanoma-specific mortality is highest within two to three years after enucleation, which lead Lorenz E. Zimmermann to suggest that removal of the eye might cause tumor dissemination and increase melanoma-specific mortality.<sup>92,93</sup> An inferred natural history of uveal melanoma is traditionally based on tumor size and how long it takes for a tumor to grow from a medium-sized to a large one, because large series of untreated patients have not been published.<sup>93,94</sup> The average survival time of patients who have refused all treatments for intraocular melanoma have been five years and some patients have survived even 15 years.<sup>34,94</sup> Ian McLean and collaborators have estimated that it takes 7 years for a small tumor (LBD <10 mm) to grow into a large one (LBD >15 mm).<sup>93</sup> Willem Manschot on the other hand, has strongly supported radical therapy (enucleation) even for small tumors (<7 mm), because they might be treated before dissemination has occurred.<sup>32,95,96</sup> Empirical data based on tumor doubling times of metastases, however, suggest that clinically undetectable micrometastases likely initiate on average two to three years before treatment for primary tumor is given.<sup>28</sup> To improve survival of patients with uveal melanoma, it seems prudent to minimise delays in treatment of primary tumor as much as possible and to screen high-risk patients for metastatic disease, hoping that eventual adjuvant therapy might be more efficient than current chemotherapy regimens are for distant metastasis.<sup>12,53,55</sup>

The natural course of the disease is, however, also modulated by tumor and host characteristics, because a patient with a large tumor may survive much longer than a patient with a small tumor and this also applies to other poor prognostic factors as well.

## **2. PROGNOSTIC INDICATORS OF UVEAL MELANOMA**

### **2.1 PATIENT-RELATED FACTORS**

#### **2.1.1 Age and sex**

Increasing age is associated with poorer melanoma-specific survival, which may result from a weakened immune system.<sup>97,98</sup> The short term prognosis (5-year) of young patients (< 20 years) is better than that of adults, but the long-term prognosis (15-year) is similar to that of adults.<sup>99,100</sup> Whether

women have better survival than men, as some studies suggest,<sup>101,102</sup> has remained controversial since many studies have not found such a relationship.<sup>103-106</sup>

### **2.1.2 Reproductive factors**

Some uveal melanomas have progressed during pregnancy, possibly by coincidence, but the childbearing history and the use of oral contraceptives do not increase the risk for metastatic disease.<sup>47,48,107</sup> In fact, a recent paper suggested that childbearing may even improve prognosis in uveal melanoma.<sup>101</sup> The protective influence of parity was highest in the early period following treatment and it even increased with the number of live births. In Cox regression, however, men and nulliparous women had only borderline higher risk for metastatic death than women who had given birth.<sup>101</sup>

### **2.1.3 Immunological factors**

The onset of clinical metastatic disease many years after enucleation and the rare spontaneous regression of uveal melanoma have been cited as indirect evidence for a role of the immune system in progression of this tumor.<sup>108</sup> This could be mediated by tumor-infiltrating and circulating immune cells. Of uveal melanoma infiltrating lymphocytes, the majority is CD4<sup>+</sup> and CD8<sup>+</sup> T-cells that may modulate the immune response and that may have direct cytotoxic effects on tumor cells.<sup>109,110</sup> A dense infiltration of lymphocytes has been associated with shorter survival time by univariate analysis.<sup>102,109</sup> Tumor-infiltrating macrophages have not been studied to the same extent than lymphocytes, but some reports have mentioned sparse to marked infiltration by CD68<sup>+</sup> macrophages in uveal melanoma.<sup>110-112</sup> The possible functions of macrophages in malignant tumors are thought to be variable and even contradictory; on one hand they might be tumoricidal, on the other hand they might promote tumor growth.<sup>113,114</sup> Other leukocytes, such as granulocytes, B-lymphocytes, and natural killer cells seem to be present to a smaller extent than T-lymphocytes and macrophages in these tumors and their role is even more uncertain.<sup>115</sup>

Human leukocyte-associated (HLA) antigens are essential for immune cells to recognise neoplastic cells.<sup>116</sup> Of HLA class I antigens, low expression of HLA-A and HLA-B antigens in uveal melanoma has been associated with a better survival.<sup>117,118</sup> This inverse correlation was explained by dual functions of HLA antigens. Whereas lymphocytes need HLA antigens to recognise tumor cells, presence of HLA class 1 antigens may block natural killer cell-mediated lysis of tumor cells.<sup>116,117</sup> A

high expression of HLA-B was associated with the epithelioid cell type and inversely correlated with expression of *c-myc* oncogene.<sup>119</sup>

#### 2.1.4 Miscellaneous factors

The presence of cutaneous dysplastic nevi not only increases the risk for developing primary uveal melanoma, but it may also be associated with the epithelioid cell type, a traditional poor prognostic indicator of uveal melanoma.<sup>45,120</sup> In a large series of proton beam irradiated uveal melanomas, patients with blue and gray irises were at 1.90 times higher risk of dying of metastatic uveal melanoma than patient with brown irises.<sup>38</sup> No relationship was found between smoking and risk of metastatic deaths.<sup>121</sup>

## 2.2 TUMOR-RELATED FACTORS

### 2.2.1 Tumor location

Anterior tumor location within the ciliary body or with ciliary body involvement has often been linked to increased melanoma-specific mortality.<sup>27,85,86,97,102,122</sup> Choroidal tumors extending to the ciliary body tend to be larger than posterior ones (posterior to the equator), but tumor size alone does not explain such an association, because ciliary body involvement has been an independent predictor of survival in many multivariate models in addition to LBD.<sup>86,97,122,123</sup> A high frequency of microvascular networks and chromosomal changes in some datasets, especially monosomy of chromosome 3 and gain of chromosome 8, have been put forward as factors underlying the more aggressive nature of ciliary body melanomas.<sup>49,106,123-125</sup> Iris melanomas on the other hand, metastasize very rarely and the reported 10-year mortality is only 5%.<sup>17</sup>

When a ciliochoroidal or iris melanoma that invades the ciliary body is diagnosed, the original uveal compartment where the tumor initiated may be difficult to resolve. The extent of ciliary body involvement graded by the proportion of the tumor base lying anterior to the ora serrata was recently put forward as a diagnostic criterion.<sup>27</sup> According to this study, a melanoma of presumed ciliary body origin (>50% tumor base anterior to the ora serrata) had 1.6 to 2.3 times higher chance to metastasize than a choroidal (<50% tumor base anterior to the ora serrata) tumor.<sup>27</sup> Of posterior tumors, those located adjacent to the optic disc may have a worse prognosis, related to more frequent extrascleral extension.<sup>57</sup>

### **2.2.2 Extraocular extension**

The fibrous outer layer of the eye, the sclera, resists the growth of intraocular melanoma effectively, but sometimes intraocular melanoma invades the orbit through emissaries such as posterior ciliary arteries and vortex veins.<sup>3</sup> Juxtapapillary choroidal melanoma may invade the orbit adjacent to the optic disc and, rarely, through the optic nerve.<sup>3</sup> Choroidal and ciliary body melanomas that infiltrate the chamber angle may grow to subconjunctival tissues to cause local lymph node metastases which otherwise do not develop from uveal melanoma.<sup>126</sup> Intraocular melanoma disseminates mostly hematogenously, and only when the anterior scleral wall is broken, tumor cells have access to conjunctival lymphatics.<sup>2,11</sup>

Extraocular extension is an unfavourable prognostic sign for which conservative treatment modalities are traditionally not recommended and radical surgery combined with irradiation therapy is often needed.<sup>127,128</sup> If extraocular extension is small (<3 mm), conservative methods such as plaque or charged particle irradiation may still be effective.<sup>129</sup>

### **2.2.3 Tumor size**

One of the most consistent prognostic indicators for metastases of uveal melanoma is tumor size.<sup>84,85,102,103,105,122,130,131</sup> Of various ways to classify according to tumor size (Table 1), largest basal tumor diameter (LBD) and tumor height are the most widely used.<sup>84,102,103,105,122</sup> According to LBD melanomas are divided into small ( $\leq 10$  mm), medium-sized (10-15 mm), and large ( $> 15$  mm) ones.<sup>102,103</sup> Evaluation of tumor volume and recently presented computer-assisted quantification of cross-sectional tumor area are other ways to measure tumor size.<sup>132,133</sup> The results have been most consistent for LBD and, from a clinical point of view, LBD can be conveniently measured by indirect ophthalmoscopy and B-scan ultrasonography.<sup>41</sup> LBD is also related to success of local resection and tumor height largely determines the dose in plaque brachytherapy.<sup>41</sup> Formal classifications, such as TNM system and COMS classification take also into account tumor height (Table 1). The 10-year melanoma-specific mortality is as high as 60% among large tumors, whereas it is only 10-15% among small melanomas.<sup>25,85,86,103,134</sup>

**Table 1.** Classifications of tumor size in choroidal and ciliary body melanomas

Classification	Category		
	Small	Medium-Sized	Large
TNM	LBD $\leq$ 10 mm and Height $\leq$ 3 mm (T1)	LBD >10-15 mm and Height >3-5 mm (T2)	LBD > 15 mm and Height > 5 mm (T3)
Collaborative Ocular Melanoma Study	LBD $\leq$ 16 mm and Height < 2.5 mm	LBD $\leq$ 16 mm and Height $\geq$ 2.5 but $\leq$ 10 mm	LBD > 16 mm and Height $\geq$ 2 mm or Height > 10 mm
Common Classification based on LBD <sup>102</sup>	LBD $\leq$ 10 mm	LBD >10-15 mm	LBD >15 mm
Tumor Cross- Sectional Area <sup>133</sup>	< 16 mm <sup>2</sup>	$\geq$ 16 mm <sup>2</sup> but < 61.4 mm <sup>2</sup>	$\geq$ 61.4 mm <sup>2</sup>

#### 2.2.4 Cell type

G. R. Callender described comprehensively the cytological features of uveal melanoma cells in 1931.<sup>135</sup> The classification that still carries his name is based on the shape and other cytological features of melanoma cells, but it has since then been modified by ophthalmic pathologists of the Armed Forces Institute of Pathology (AFIP). Callender defined two main cell types: the spindle and the epithelioid cells. Spindle cells typically grow in a compact cohesive fashion and they were originally subdivided into spindle-A and spindle-B type.<sup>3,135</sup> Patients with spindle-A type melanoma have a significantly more favourable clinical course than patients with spindle-B type melanoma.<sup>3,131</sup> In the modified AFIP classification, the spindle-A cell type melanoma was recorded a nevus rather than a malignant melanoma, and the diagnosis of spindle cell type melanomas required spindle-B cells.<sup>136</sup> The malignant potential of less cohesive epithelioid cells is well established in many independent studies.<sup>86,102,122,130,132</sup> In addition, the original Callender classification included mixed, fascicular, and

necrotic types of uveal melanoma. The presence of necrotic cell type indicated higher mortality than presence of non-necrotic cell types, but the association was non-significant after controlling for tumor size.<sup>137</sup>

A matter of controversy has been which amount of epithelioid cells is to be considered a sign of poor prognosis.<sup>138-140</sup> In recent prognostic studies, the mixed and epithelioid types have often been combined in the same category, and the presence of even a single well-defined epithelioid cell in a section may lead to classifying it as nonspindle instead of spindle.<sup>102,141,142</sup> This two-category classification is also conveniently handled in multivariate analysis.<sup>102</sup>

### 2.2.5 Tumor pigmentation

Heavy tumor pigmentation has been associated with decreased survival in several univariate studies, but different classifications of the amount of pigmentation make comparisons between cohorts difficult.<sup>85,105,131,132</sup> The relationship between heavy tumor pigmentation and two other prognostic indicators, epithelioid cell type and large LBD suggests that prognostic significance of tumor pigmentation may be secondary to other tumor characteristics.<sup>111</sup> Cutaneous melanomas with high melanin content are more resistant to irradiation than lightly pigmented ones, but the significance of this observation as regards to uveal melanoma is unknown.<sup>143</sup>

### 2.2.6 Microvascular patterns

Studies on tumor blood vessel morphology in uveal melanoma were introduced by Robert Folberg and colleagues in 1992 when they presented nine morphological types of microvessel architecture which they designated microvascular patterns.<sup>144</sup> The patterns were identified by fluorescein-conjugated *Ulex europaeus* I using laser scanning confocal microscopy and by periodic acid-Schiff stain (PAS) that stains basement membrane and collagen.<sup>144,145</sup> However, bleaching of melanin by permanganate and PAS staining without hematoxylin counterstain were found as effective and inexpensive methods of demonstrating microvessels.<sup>102,144</sup> PAS stains also melanoma cells, but a use of green filter enhances contrast of PAS-positive structures helping in recognising of microvessels.<sup>144,146</sup> According to the original classification<sup>144</sup>: the *normal pattern* consists of normal uncompressed choroidal vessels. The *silent pattern* contains no apparent tumor vessels. The *straight pattern* is comprised of randomly oriented straight vessels that are not linked with each other. The *parallel pattern* included straight vessels that are arranged parallel to one another. The *parallel with cross-link pattern* contains vessels

of parallel pattern that are also linked to each other. The *arcs* and *arcs with branching patterns* are curves of vessels that failed to form loops. The *loop pattern* consists of vessels that are completely closed. The diameter of loops ranged from 14  $\mu\text{m}$  to 157  $\mu\text{m}$  in dataset of 30 loops from different tumors.<sup>144</sup> The *network pattern* is composed of at least three back-to-back closed loops. By definition, if networks are present, loops are present.<sup>144</sup>

In the matched case-control study of 40 patients, patients who survived over 15 years had more frequently the normal and silent pattern than patients who died of metastatic uveal melanoma.<sup>144</sup> Furthermore, melanocytic nevi did not contain any parallel with cross-linking, arcs, arcs with branching, loops, or networks, consistent with the finding that these latter patterns would be associated with melanoma deaths.<sup>147</sup> In fact, patients who died of uveal melanoma had more frequently parallel, parallel with cross-link, loop, and network patterns than patients who had survived long.<sup>147</sup> These melanoma-associated microvascular patterns were found more frequently in ciliary body tumors than in choroidal ones.<sup>123</sup> A laboratory-based follow-up study of 234 patients who had an eye removed because of uveal melanoma showed melanoma-specific mortality to be higher among patients who had parallel vessels, parallel vessels with cross-links, arcs, arcs with branching, loops, and networks than among patients who lacked these microvascular patterns.<sup>102,123</sup> In multivariate analysis, presence of networks and parallel vessels with cross-links gained independent prognostic significance.<sup>102</sup> These two studies inspired a series of confirmatory studies from independent, sometimes sceptical groups that all substantiated the prognostic significance of microvascular patterns in uveal melanoma (Table 2).<sup>148</sup>  
104,141,149

In cutaneous and conjunctival melanoma parallel vessels with cross-linking or networks gained independent prognostic significance in multivariate analysis adjusted for the important conventional prognostic indicator, tumor thickness.<sup>150,151</sup> All nine microvascular patterns were found in these tumors proving that formation of these microvascular patterns is not restricted to intraocular melanoma.

Folberg's laboratory found that tumor cells of malignant uveal and metastatic cutaneous melanoma are able to form microvascular patterns, in particular microvascular loops and networks *in vitro* without presence of endothelial cells.<sup>152</sup> The active role of uveal melanoma cells in production and remodelling of ECM was supported by the observation that melanoma cells are capable of generating type VI collagen.<sup>153</sup> *In vitro* derived microvascular patterns were demonstrated to conduct dye and thus might be functional *in vivo*.<sup>152</sup> This concept of blood vessel formation without endothelial cells, now called *vasculogenic mimicry*, differs in many respects from the traditional view of tumor angiogenesis, in

which new vessels are formed from pre-existing ones by proliferation of endothelial cells.<sup>152</sup> Preliminary investigations have revealed differences in gene expression between melanomas that form microvascular networks and melanomas that are incapable of doing that.<sup>13</sup> Recently, a specific profile of protein tyrosine kinases was associated with the ability of uveal melanoma cells for vasculogenic mimicry.<sup>154</sup>

A panel of endothelial markers including *Ulex europaeus* I lectin, CD31, CD34, and KDR failed to reveal distinct positive immunostaining of the walls of uveal melanoma microvessels supporting a different nature from endothelial-lined tumor vessels.<sup>152</sup> The authors interpreted the positive immunoreaction they observed to reside in the vascular contents and in some cross-reacting tumor cells.<sup>152</sup> Tumor cell-lined blood channels are demonstrated by several independent groups,<sup>144,155,156</sup> but the functional evidence and the connection between tumor cell-lined channels and intratumoral microvessels are questioned.<sup>148,157,158</sup> In order to resolve the contradictory issues on microvascular mimicry and to define its generalizations, many experimental studies worldwide are in progress.

**Table 2.** Summary of prognostic studies on microvascular patterns in choroidal and ciliary body melanoma

Author Country	Sample Size	Inclusion Rate	Patterns Studied	Prognostic Significance	Difference in 10-year Mortality	Independent Indicator
<i>Folberg et al.</i> <sup>102</sup> USA 1993	234	0.70	All	L + N + Pa	0.36 (L) 0.38 (N)	N + Pa
<i>Sakamoto et al.</i> <sup>149</sup> Japan 1996	16	0.40	All	L + N	N/A	N/A
<i>McLean et al.</i> <sup>141</sup> USA 1997	496	N/A	L	L	0.34 (L)	L
<i>Foss et al.</i> <sup>148</sup> UK 1997	110	0.45	All	A + L + N + Pa	N/A	None
<i>Seregard et al.</i> <sup>104</sup> Sweden 1998	132	N/A	All	L + N + P	N/A	N

A = Arcs with branches

L = Loops

N = Networks

Pa = Parallel with cross-link

P = Parallel

N/A = not available

### 2.2.7 Microvascular density

The concept of microvascular density (MVD) and methods for measuring it from the highest area of vascularization were popularised in the early 1990's.<sup>159,160</sup> High numbers of immunolabeled microvessels counted from the area of densest vascularization called a "hot spot" indicated increased risk for metastatic death and this was taken as evidence of active angiogenesis in many types of cancer.<sup>159,161</sup> The majority of such studies have shown that high MVD is associated with shorter survival, but contradictory reports are not uncommon.<sup>161</sup> In particular, reports of cutaneous melanoma have been controversial.<sup>162,163</sup> Initial studies suggested that high vascularization has a significant influence on survival, but no consensus of its eventual significance has been achieved.<sup>162,163</sup> This may be due to the fact that cutaneous melanoma cells can also use lymphatic vessels in their dissemination, which likely blurs any association with the density of microvessels.<sup>164</sup> The theoretical background of MVD has been criticised for lack of well proved link with angiogenesis, poor reproducibility, and questionable specificity of some of the antibodies used in highlighting microvessels.<sup>2,163,165</sup>

Foss and colleagues reported that high MVD was associated with a shortened survival of patients with uveal melanoma.<sup>105</sup> The study population comprised of 116 patients with uveal melanoma and the series was enriched with an unspecified number of patients who had died of metastatic uveal melanoma.<sup>105</sup> They did not find any relationship between microvascular patterns and survival in their data set after adjusting for MVD.<sup>148</sup> It was found that MVD was higher in tumors with microvascular loops and networks, suggesting that the effect on prognosis of microvascular patterns might be secondary to high MVD.<sup>148</sup> Two other groups have presented opposite results on the prognostic significance of MVD in uveal melanoma.<sup>166,167</sup> A follow-up study of 63 tumors failed to document any survival difference between tumors with different microvessel counts,<sup>166</sup> which was also the case in a small follow-up study of 40 uveal melanomas.<sup>167</sup>

### 2.2.8 Cell-matrix interaction

Interaction between tumor cells and surrounding stroma are considered essential for tumor progression and dissemination. A decade ago it was documented that some uveal melanomas co-express vimentin (typical among melanocytes) and cytokeratin 8 and 18 (typical among carcinomas) intermediate filaments.<sup>142</sup> Recently it was shown *in vitro* that uveal melanoma cells coexpressing both types of intermediate filaments had a 6-fold capability of invasion as compared to melanoma cells that express only vimentin.<sup>168</sup> Cells that coexpress both intermediate filaments were named "interconverted"

cells.<sup>168,169</sup> Clinical evidence for an association between the interconverted phenotype and prognosis is still missing, perhaps due to the small number of tumors studied so far.<sup>142</sup>

Of cell adhesion molecules, neural cell adhesion molecule (NCAM) is preferentially expressed on rapidly metastasising uveal melanomas.<sup>170</sup> The HNK-1 epitope, which is part of NCAMs, was more frequently expressed in larger tumors, but seldomly in liver metastases.<sup>170</sup> Another report, however, failed to document any relationship between NCAM expression and survival, but instead reported that intercellular cell adhesion molecule-1 (ICAM-1) was linked to the development of metastases.<sup>171</sup> Expression of VLA-2 integrin receptor was associated with the presence of microvascular networks, but none of the integrin receptors studied (VLA-2, VLA-3, and alpha (v)) seemed to have prognostic significance.<sup>106</sup>

Both primary and metastatic uveal melanomas lack estrogen and progesterone receptors and potential effects of anti-estrogens on uveal melanoma can not be mediated by conventional mechanisms.<sup>112,172</sup> Positive cytoplasmic immunostaining with an antibody to estrogen receptors (ER) was shown to be from cross-reacting CD68-positive macrophages.<sup>112</sup> The expression of epidermal growth factor receptor (EGFR) in uveal melanoma cell lines correlated with metastatic potential and liver-targeted metastasis.<sup>173</sup> Moreover, EGFR expression correlated with metastatic death in a small clinical series of uveal melanomas.<sup>174</sup> However, EGFR expression in uveal melanoma may be confined to tumor-infiltrating macrophages.<sup>175</sup>

Expression of matrix metalloproteinase-2 (MMP-2) was recently linked to decreased uveal melanoma-specific survival, but the sample size was not large enough to test independent prognostic significance of MMP-2 in multivariate analysis.<sup>176</sup>

### **2.2.9 Markers of cell proliferation**

The development of antibodies to proteins, such as PC-10 and Ki-67, which characterise cells that are in the proliferative phase of the cell cycle, started a sequence of studies to resolve the prognostic significance of proliferating tumor cells in uveal melanoma and in other cancers.<sup>177-179</sup> A high fraction of PC-10 and Ki-67 immunopositive uveal melanoma cells has been associated with decreased melanoma-specific survival in many cohorts of uveal melanoma.<sup>177-180</sup> In one particular study, a high fraction of PC-10 immunopositive tumor cells was associated with a 40% increase in 10-year melanoma-specific mortality.<sup>179</sup> Also a high PC-10 count was an independent prognostic factor by

multivariate analysis adjusted for LBD, presence of microvascular networks, and the mean diameter of the largest nucleoli.<sup>104</sup> The high expression of cyclin D1, a protein involved in the cell cycle, indicates an unfavourable outcome in uveal melanoma.<sup>181</sup> Moreover, cyclin D1 positivity was associated with the epithelioid cell type and a high index of Ki-67 positive tumor cells.<sup>182,183</sup>

### **2.2.10 Nuclear morphometry**

A high number of mitoses has been associated with increased mortality in uveal melanoma.<sup>86,132,180</sup> Several studies have indicated that large indices of cytomorphometric parameters, such as measurements of nuclear and nucleolar area from randomly selected cells are associated with higher than average melanoma-specific mortality.<sup>84,184-187</sup> In particular, the mean of the ten largest nucleoli and the standard deviation of nucleolar area have retained independent prognostic value in multivariate analyses, and computer-assisted measurements have been reproducible between different laboratories.<sup>184-186</sup> However, the methodology of nuclear morphometry assessment is still controversial and recently silver-stained sections were found superior to HE-stained sections.<sup>188</sup> Regarding the relative importance of nuclear morphometry and other tumor characteristics, Pe'er and associates reported that the network and the parallel vessels with cross-link microvascular patterns were more powerful prognostic indicators than cytomorphometric indices, such as mean of the ten largest nucleoli, in their data set.<sup>189</sup>

## **2.3 CYTOGENETICS**

### **2.3.1 DNA aneuploidy**

Flow cytometry can be used to assess the DNA content and proliferative activity of tumor cells.<sup>190</sup> A DNA content deviating from the normal amount (diploid vs. aneuploid) reflects the genetic instability of tumor cells and impaired control of the cell cycle which are characteristics of malignant cells.<sup>190</sup> Aneuploid DNA and a high fraction of cells in the S-phase of the cell cycle have been associated with the epithelioid cell type and decreased survival in uveal melanoma.<sup>191,192</sup>

### 2.3.2 Chromosomal abnormalities

Specific chromosomal changes, in particular loss of one copy of chromosome 3 and gain of the long arm of chromosome 8 are emerging as particularly significant prognostic indicators in several independent studies of inconsecutive series of surgically treated uveal melanomas.<sup>51,124,193,194</sup> These chromosomal changes are more frequent in tumors with ciliary body involvement than in choroidal tumors, which may explain the worse prognosis of ciliary body melanomas as compared to choroidal melanomas.<sup>124,125</sup> So far, studies of chromosomal changes concern predominantly large melanomas, because small and medium-sized melanomas are mostly treated by irradiation.<sup>124,194</sup> Thus, the results can not yet be generalised to all uveal melanomas, but chromosomal changes certainly play an important role in tumor progression. A study based on a consecutive series of uveal melanomas that are studied regardless of tumor size is needed to establish the role and sequence of chromosomal changes among uveal melanomas of all sizes.

Recently, analysis of the partial deletion of the long and short arm of chromosome 3 suggested the presence of tumor suppressor gene locus at 3q24-q26 and 3p25.<sup>195</sup> The long-term follow-up data were not yet available for patients whose tumor had a partial deletion. The growth restricting effect of TGF $\beta$  may be lost in deletions involving chromosome 3p22 in which the gene for TGF $\beta$  receptor is located.<sup>196</sup> An abnormality of the TGF $\beta$  pathway were found in 61% of uveal melanomas, but authors did not determine monosomy 3 to TGF  $\beta$  function.<sup>196</sup>

## 2.4 MOLECULAR PATHOLOGY

### 2.4.1 Oncogene activation

A potential tumor-suppressor gene nm23 has been detected in uveal melanomas that were small in size and showed no extraocular extension.<sup>197,198</sup> In an animal model, the expression of nm23 gene was associated with reduced metastatic potential and fewer liver metastases.<sup>197</sup> No association was found between nm23 expression and conventional prognostic indicators, such as cell type and ciliary body involvement.<sup>198</sup>

A high expression of the *c-myc* oncogene is known to have an influence on survival in a number of cancers, including cutaneous melanoma. In case of uveal melanoma, the results have been opposite; a low expression of *c-myc* is associated with higher mortality.<sup>199,200</sup> Nuclear *c-myc* expression was found

in each of 71 studied uveal melanomas, but cytoplasmic *c-myc* expression was much more prominent than nuclear expression in another study.<sup>180,200</sup> The presence of *c-myc* positive cells was associated with decreased survival after adjusting for LBD and Ki-67 positive cells in multivariate analysis.<sup>180</sup>

#### **2.4.2 Programmed cell death**

Expression of Bcl-2 protein, a proto-oncogen that blocks programmed cell death (apoptosis), has been documented convincingly in uveal melanoma, but its prognostic significance is lacking.<sup>180,200-202</sup> On the other hand, very few melanomas have been shown to express p53, a nuclear phosphoprotein that induces apoptosis and cell-cycle arrest.<sup>200,202,203</sup> The treatment effect of irradiation and transpupillary thermotherapy may be mediated by apoptosis.<sup>200,203-205</sup> Dendritic cells stimulated effectively lymphocyte proliferation and phagocytosed melanoma cells after uveal melanoma cells had undergone apoptosis due to irradiation.<sup>204</sup> This finding suggested that dying intraocular melanoma can serve as an autologous stimulus for immune cells against tumor cells.<sup>204</sup> Recently, overexpression of  $\beta$ -Catenin, a molecule involved in cell adhesion and signal transduction, induced apoptosis in uveal melanoma cells.<sup>206</sup>

### **3. AIMS OF THE PRESENT STUDY**

The purpose of this study was to search for and evaluate new prognostic indicators for metastatic death in a well-documented and population-based series of Finnish patients with malignant choroidal and ciliary body melanoma.

In the first phase of the thesis, two hypothesis-confirmatory studies were designed and undertaken to confirm or contradict an association between melanoma-specific survival and presence of microvascular loops and networks and microvascular density.

In the second phase of the thesis, two hypothesis-generating studies were performed to find out whether tumor-infiltrating macrophages and ezrin immunoreactivity in melanoma cells are associated with prognosis.

## **4. MATERIALS AND METHODS**

### **4.1 PATIENTS**

#### **4.1.1 Recruitment and follow-up**

A consecutive cohort of uveal melanoma patients was collected from the diaries of the Ophthalmic Pathology Laboratory, Department of Ophthalmology, Helsinki University Central Hospital. A total of 170 consecutive patients, all Caucasian with a choroidal or ciliary body melanoma were enrolled from the years 1972 to 1981. During that period, enucleation was the standard treatment for all but the smallest uveal melanomas, and all eyes enucleated in the district of the Helsinki University Central Hospital were submitted to its Ophthalmic Pathology Laboratory. Even though a smaller number of uveal melanomas per year were treated in other hospitals than Helsinki University Hospital, the series, based on its files, is essentially unselected and representative of all malignant uveal melanomas diagnosed in its area.

Three patients were permanently excluded from the analysis: one patient due to metastatic cutaneous melanoma to the choroid, one patient due to the removal of choroidal melanoma under autopsy, and one patient because of iris melanoma. Thus, 167 patients with choroidal or ciliary body melanoma were left in the analysis.

Complete follow-up data for each patient was assembled in January 1997 from data retrieved from the Finnish Population and Cancer Registries, from patient charts of all hospitals where they had been treated for uveal melanoma, its metastases and other malignant tumors, from pathology laboratories, and from death certificates. A questionnaire concerning treatment for malignant tumors was sent to all living patients in 1997. The data on patients alive in 1997 was updated in December 1999 with the help of the Finnish Population Registry. Studies on ezrin and tumor-infiltrating macrophages are based on follow-up data updated in December 1999.

#### **4.1.2 Exclusion criteria**

The following exclusion criteria were adhered to throughout the study: Melanomas that were more than 50% necrotic, and, by analogy, specimens in which either less than 50% of the original melanoma remained or the remaining part was entirely on the vitreal side of Bruch's membrane. Altogether, 33

(20%) melanomas had to be excluded from the analysis, 15 (9%) melanomas because of more than 50% consisted of necrotic tumor, 16 (10%) melanomas either because of a small size of (<50%) remaining tumor or its location entirely on the vitreal side of Bruch's membrane, and two (1%) melanomas because of missing paraffin blocks. Thus, 134 (80%) patients were included in histopathological examination. The necrotic tumors were, however, included in the analysis of tumor-infiltrating macrophages. Patients who had a second primary tumor other than uveal melanoma were not excluded, because histopathologic confirmation of the cause of death was available for all of them.

#### 4.1.3 Reinvestigation of the cause of death

In order to analyse melanoma-specific mortality more reliably than in a cancer registry based-analysis, an attempt was made to differentiate all melanoma deaths from other deaths. To improve the accuracy, all tumor deaths irrespective of primary site and all other non-tumor deaths were personally reviewed. The clinical reliability of the cause of death was evaluated in the following descending order: autopsy, surgical biopsy, fine-needle biopsy, imaging, and clinical charts.

**Table 3.** Evidence for the cause of death among 130 patients who died during follow-up

	Metastatic Melanoma N=80 (%)	Other Tumor Deaths N=9 (%)	Other Deaths N=40 (%)
Autopsy	27 (34)	2 (22)	12 (30)
Biopsy	27 (34)	7 (78)	-
Fine-Needle Biopsy	13 (16)	-	-
Imaging	10 (12)	-	-
Clinical	3 (4)	-	30 (70)

The cause of death is unknown in one (1%) case

The most reliable evidence of tumor death was autopsy on 29 (33%) patients, a surgical biopsy on 34 patients (38%), a fine-needle aspiration biopsy on 13 (15%) patients, imaging on 10 (9%) patients (four by abdominal ultrasonography, three by liver scan, two by computed tomography of the liver, and one by chest x-ray), and clinical charts on three (3%) patients (Table 3). Histopathological material from

metastases could be retrieved from 32 of the 34 surgical biopsies and from 27 of the 29 autopsies performed on tumor patients. Moreover, no evidence of cancer was found in 12 autopsies that were performed on 40 patients who presumably died of other causes (Table 3). Altogether, autopsy was performed on 41 (32%) patients who died during follow-up period.

#### **4.1.4 Comparative analysis of primary and metastatic melanoma**

In order to correlate properties of primary tumors with metastatic tumors, a study on microvascular patterns and density was undertaken among patients who had died of metastatic uveal melanoma. Of 134 patients, who fulfilled the inclusion criteria for the study of MVD and microvascular loops and networks, 63 (47%) died of metastatic uveal melanoma during follow-up. Of these 63 patients, 42 (67%) subjects underwent either surgical biopsy or autopsy. Because of delayed fixation and autolysis, specimens taken at six autopsies had to be excluded from the analysis. The three smallest biopsies were not large enough to assess microvascular density from an area of  $0.313\text{mm}^2$  used in this thesis, leaving 33 of the 63 (52%) patients to the comparative study of microvascular properties of primary and metastatic uveal melanoma.

## **4.2 IMMUNOHISTOCHEMISTRY**

### **4.2.1 Monoclonal antibodies**

Primary mouse antibodies to human antigens were purchased, except the mAb 3C12 to ezrin that was received from the collaborators (Table 4). All used mAbs were previously documented to work in paraffin sections.

### **4.2.2 Immunoperoxidase staining**

The paraffin blocks were cut at  $5\ \mu\text{m}$  and the slides were randomly coded by an outside laboratory technician. The code was broken after both the final histopathologic and follow-up data were ready for analysis, all investigators being masked to the outcome of individual patients until that time.

The immunohistochemical staining was done using a commercial version (Vectastain ABC Elite Kit, Mouse IgG, Vector Laboratories, Burlingame, CA) of the avidin-biotinylated peroxidase complex (ABC) method.<sup>207</sup> The sections were deparaffinized in xylene and rehydrated in an ethanol series.

When heat-induced antigen retrieval was needed, the specimens were placed in a jar filled with 10 mM sodium citrate buffer (pH 6.0, adjusted with 2 N NaOH), and heated in a water-bath for 15 min at 95°C.<sup>208</sup> The jar was allowed to cool for 20 min at room temperature after heating. When proteolytic antigen retrieval was needed, the slides were treated with 0.4 % (w/v) pepsin (2500 FIP U/g, E. Merck, Darmstadt, Germany) in 0.01 M hydrochloric acid for 15 min at 37°C to reduce background and to enhance the intensity of specific staining.<sup>209</sup>

Endogenous peroxidase activity was consumed by treating the sections for 30-min in methanol containing 0.5 % (v/v) hydrogen peroxide. They were then incubated with normal horse or goat serum (Vectastain ABC Elite Kit, diluted 1:50) in a moist chamber for 30 min at room temperature. All immunoreagents were diluted with PBS (pH 7.0) containing 2.0 % (w/v) bovine serum albumin (BSA; E. Merck, Darmstadt, Germany). The sections were washed three times for 10 min in PBS between each step. Incubation with the primary mAbs was carried out in a moist chamber for overnight at 5°C. Subsequently, the sections were incubated with biotinylated horse anti-mouse or goat anti-rabbit IgG antiserum (Vectastain ABC Elite Kit; diluted 1:200) and then with the ABC (Vectastain ABC Elite Kit reagents A and B, both diluted 1:160) in a moist chamber for 30 min at 37°C. The peroxidase reaction was developed with 3',3'-diaminobenzidine tetrahydrochloride (Sigma; 150 mg in 16 ml dimethylsulfoxide and 200 ml PBS containing 0.03 % (v/v) hydrogen peroxide). Coverslips were mounted with Aquamount (BDH Chemicals, Poole, UK).

#### **4.2.3 Bleaching of melanin**

Chromogens, such as 3',3'-diaminobenzidine tetrahydrochloride and 3-amino-9-ethylcarbazole that yields a dark brown reaction and a brick red reaction product, respectively, can not be differentiated easily from melanin of pigmented tumors.<sup>112</sup> Melanin can be removed with 0.25% potassium permanganate followed by 5% oxalic acid,<sup>144,210</sup> but bleaching performed prior to immunoperoxidase staining often alters antigenicity and significant number of antibodies fail to work properly anymore.<sup>210</sup> Potassium permanganate bleaching is suitable, however, for removal of melanin prior to hematoxylin-eosin and periodic acid Schiff stainings.<sup>102,144</sup> Thus, in order to visualise positive immunoreaction in pigmented tumors reliably, melanin was bleached by incubating the sections in 3.0 % (v/v) hydrogen peroxide and 1.0 % (w/v) disodium hydrogen phosphate for 18 h at room temperature.<sup>211</sup> This procedure obviates any change in antigenicity due to the bleaching.<sup>211</sup>

**Table 4.** Primary mouse monoclonal antibodies to human antigens used in the thesis

Antigen	mAb	Lot	Dilution	Antigen Retrieval	Source
CD34	QBEND/10 <sup>212</sup>	121202	1:25	Pepsin	Novocastra Laboratories, Newcastle-upon-Tyne, UK
FVIII-RAg*		115	1:400	Pepsin	Dakopatts, Copenhagen, Denmark
$\alpha$ -smooth muscle actin	1A4 <sup>213</sup>	98F4808	1:8000	Pepsin	Sigma, St. Louis, MO
Immature melanosomes	HMB-45 <sup>214</sup>	0024b	1:200	Pepsin	DAKO, Copenhagen, Denmark
CK18	CY-90 <sup>142</sup>	49F-4815	1:3000	Pepsin	Sigma Chemical Co., USA
Vimentin	Vim 3B4 <sup>215</sup>	114544324	1:50	Pepsin	Boehringer-Mannheim GmbH, Mannheim, Germany
EMA	E29 <sup>142</sup>	078	1:25	Pepsin	DAKO, Copenhagen, Denmark
CD68	PG-M1 <sup>216</sup>	101	1:50	Pepsin	Dakopatts, Klostrup, Denmark
CD68	KP1 <sup>217</sup>	038	1:100	Heat	Dakopatts, Klostrup, Denmark
Macrophages	3A5 <sup>218</sup>	210501	1:50	Heat	Novocastra Laboratories, Newcastle-upon-Tyne, UK
Ezrin	3C12 <sup>219</sup>		1:1000	Heat	Dr. Olli Carpén, University of Helsinki, Finland

\* polyclonal rabbit antibody

### **4.3 ASSESSMENT OF TUMOR CHARACTERISTICS**

#### **4.3.1 Baseline characteristics**

The cell type was registered according to the modified Callender classification (spindle, mixed, epithelioid) from a hematoxylin-eosin stained section.<sup>136</sup> If the original histopathology report mentioned the presence of epithelioid cells in a tumor registered as spindle cell type, this tumor was reclassified to the nonspindle type. The location (choroidal, ciliary body with or without choroidal involvement), the largest basal tumor diameter (LBD) and the height of the tumor measured in mm, and the presence or absence of extraocular extension were taken from the original pathology reports and checked to be consistent with the sections studied. The degree of pigmentation of each tumor was graded semiquantitatively by sorting out unstained 5- $\mu$ m-thick paraffin-embedded sections of whole tumor into three groups that represented amelanotic to weak, intermediate, and strong pigmentation. The sorting was done simultaneously for the entire set of specimens on a sheet of white tissue paper under a light without magnification.

#### **4.3.2 Microvascular loops and networks**

Sections were stained with periodic acid-Schiff (PAS) without hematoxylin counterstain in order to identify loops and networks that were associated with increased melanoma-specific mortality in previous studies.<sup>102,104,141</sup> All sections irrespective of the amount of melanin were bleached by potassium permanganate before PAS staining. Two observers independently examined the slides under a light microscope using a dark green filter (Wratten No 58, Kodak, Rochester, NY), and recorded the presence or absence of loops and networks. Prior to analyzing the randomly coded study slides, the criteria to be used were agreed upon, and a set of slides, not included in the study series, was examined under a double-headed microscope. Disagreements in registering vascular patterns in the study series were resolved in the same manner.

#### **4.3.3 Microvascular density**

Microvessels were immunostained with mAb QBEND/10 to the CD34 epitope of endothelial cells and polyclonal rabbit antibodies to FVIII-RAg, and microvessels with a muscular layer were immunostained with mAb 1A4. Microvessels were counted from three separate, most highly

vascularized areas ("hot spots") according to Foss and colleagues.<sup>105</sup> Scanning the entire immunostained tumor at X100 magnification identified the three areas. Vessels were then counted at X200 magnification using an eyepiece with an etched square graticule (Olympus, WK 10x/20L-H, Tokyo, Japan). The area of the graticule was 0.313 mm<sup>2</sup> as measured with an object micrometer (Ernst Leitz GmbH, 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.<sup>105</sup> To assess intraobserver reproducibility, hot spots were reidentified in a masked fashion six months later from a subset of 31 slides (25%) immunostained with mAb QBEND/10, chosen on the basis of a random number table.

#### **4.3.4 Tumor-infiltrating macrophages**

In preliminary experiments, mAb 3A5 immunolabeled dendritic macrophages less effectively than mAb PG-M1, in line with a previous study.<sup>218</sup> Mab KP1 to the CD68 epitope cross-reacted with cytoplasm of some uveal melanomas, but such cross-reactivity was not found with mAb PG-M1. This mAb has shown a more restricted reactivity with the highly glycosylated and antigenically heterogeneous CD68 molecule than other mAbs to CD68.<sup>216</sup> Based on the facts above, PG-M1 to the CD68 epitope was chosen as default antibody of the study.

Macrophages were counted semiquantitatively in two ways based on standard photographs (Fig. 1 in III). The number of CD68-immunopositive cells was graded as few, moderate numbers, and many. The predominant morphological type of cells immunoreactive with mAb PG-M1 was graded as follows. Two groups consisted of tumors in which the majority (75% or more) of immunopositive cells were either dendritic or round in type. The third group consisted of tumors in which neither dendritic nor round CD68-immunopositive cells clearly predominated, or the morphology of immunopositive cells was intermediate between the dendritic and round type. Confluent immunopositive cells in necrotic areas did not influence the grading.

#### **4.3.5 Ezrin immunoreactivity**

Ezrin immunoreactivity was graded semiquantitatively into three groups under a double-headed microscope by two investigators according to predefined criteria: negative (no or only few convincingly immunopositive tumor cells), positive (faint to moderate, easily recognizable positive

granular immunoreaction in the majority of tumor cells), and strongly positive (stronger than average granular immunoreaction in the majority of tumor cells)(Fig. 1 in IV). The retina and choroid showed variable background, and neoplastic cells were graded immunopositive only if the reaction intensity was indisputably stronger than the background.

## **4.4 STATISTICAL ANALYSES**

### **4.4.1 General guidelines**

Descriptive statistics for normally distributed variables were given as mean and standard deviation and for other variables as median and range.<sup>220</sup> Confidence intervals (95%) were calculated for proportions. In the design phase of each study it was decided how variables will be divided into categories for hypothesis testing. P values less than 0.05 were taken as statistically significant and warranted the rejection of the null hypothesis. Multiple comparisons were adjusted for by the Bonferroni correction.<sup>220</sup>

### **4.4.2 Power and sample size calculation**

Calculation of the number of patients needed to prove a clinically significant difference in survival was done by simulation using a program that is available free of charge from Michael Borestein, New York, U.S.A.<sup>221</sup> The simulation was based on published survival statistics whenever such were available. In case of microvascular loops and networks, the simulation was based on Folberg's cohort study, in which 10-year cumulative melanoma-specific survival differed by 0.37 between patients with and without networks.<sup>102</sup> The corresponding difference for loops and no loops was 0.36.<sup>102</sup> The simulation indicated that a sample of 150 patients is needed to prove or disapprove of 0.20 difference in survival. The power of the study was 0.9 (for a two-tailed alpha of 0.05).

The calculation of the number of patients needed to prove a statistically significant difference between MVD and survival was based on the analysis of MVD by Foss and co-workers.<sup>105</sup> In their study the survival of patients with a lower and higher MVD than the median was 0.70 and 0.35, respectively, with a 0.35 difference in survival.<sup>105</sup> To detect such a difference with a power of 0.90 (given a two-sided alpha of 0.05), the simulation gave a minimum total sample size of 82 patients, divided equally between the two arms.

Because no previous studies either on macrophage infiltration or ezrin immunoreactivity and survival in uveal melanoma were available, formal sample size calculation for Kaplan-Meier analysis was not feasible. Power analysis by simulation indicated that the study of macrophages and ezrin had 80% power to detect a 0.25 difference and 95% power to detect a 0.32 difference in 20-year survival.

### **4.4.3 Group comparisons**

#### 4.4.3.1 Interrelationships between variables

For statistical comparisons continuous data, such as LBD and MVD (globally highest microvessel count obtained with mAb QBEND/10 to CD34) were categorized: the former in three categories (small  $\leq 10$  mm, medium-sized 10-15 mm, and large  $> 15$  mm) and the latter was divided in quartiles ( $\leq 24$  microvessels, 25-40 microvessels, 41-57 microvessels, and  $\geq 58$  microvessels). Cell type was divided into two categories according to the presence or absence of epithelioid cells (spindle, nonspindle) and tumor locations according to the presence or absence of ciliary body involvement. Degree of pigmentation was analyzed as an ordered three-category variable (amelanotic to weak, moderate, and strong pigmentation). Microvascular loops and networks were analyzed as an ordered variable that considered networks to be an advanced stage of loops (no loops, loops without networks, networks). The number of macrophages was analyzed as an ordered (few, moderate, many CD68-immunopositive cells) and the predominant morphologic type of macrophages as an unordered three-category variable (dendritic, intermediate, round CD68-immunopositive cells). Immunoreactivity with mAb 3C12 to ezrin was primarily analyzed as an ordered three-category variable (negative, positive, strongly positive) and secondarily as a two-category variable (negative and positive).

Fisher's exact and Pearson's chi-square tests were used to compare proportions in 2 x 2 and larger unordered contingency tables, respectively. Kruskal-Wallis and Jonckheere-Terpstra tests were used to compare proportions in singly ordered and in doubly ordered contingency tables, respectively. McNemar's test was used to compare paired 2 x 2 tables. Contingency tables were analyzed and exact probability distributions were computed with StatXact-3 (Cytel Software, Cambridge, MA).

Scattergrams of MVD and LBD against other variables were plotted. The nonparametric Mann-Whitney *U* test that does not require Normal distribution was used to analyze continuous variables between two independent groups, and the Wilcoxon rank-sum test was used to compare two paired samples of continuous data.

#### 4.4.3.2 Inter- and intraobserver agreement

Weighted kappa statistic was used to estimate chance-corrected interobserver agreement in identification of microvascular loops and networks, and in grading of the macrophages and ezrin immunoreactivity.<sup>220</sup> Moreover, the number of disagreements, and whether they were one or two-category disagreements, were recorded. Agreement between microvessel counts obtained from sections labeled with different antibodies and intraobserver reproducibility was assessed by plotting the difference between the two counts against their mean, and by calculating the mean difference with 95% limits of agreement.<sup>220</sup>

#### 4.4.4 Survival analyses

##### 4.4.4.1 Kaplan-Meier method

The survival analyses were performed with the BMDP PC-90 Statistical Software package (BMDP Statistical Software, Cork, Ireland) and with the SPSS for Windows 9.0.1 (SPSS Inc., Chicago, IL).

Survival analysis was based on Kaplan-Meier product-limit method, and melanoma-specific survival was compared with the Mantel-Cox test that gives equal weight to the entire survival curve.<sup>220,222</sup> Trend test was used if the categories analyzed were ordered.<sup>222</sup> Patients judged to die of other causes were censored at their time of death. To guard against the possibility that they were more or less likely to have progression than other patients, all-cause mortality was also analyzed. Because all-cause mortality in long- follow-up will always approach 100%, the Breslow test was the primary statistic in comparing all-cause mortality.<sup>222</sup>

##### 4.4.4.2 Cox multiple hazard regression

Cox proportional hazards regression was used to adjust the survival for the effect of previously identified independent predictors of prognosis. LBD and MVD were analyzed as continuous variables. MVD was square root-transformed to normalize its distribution.<sup>105</sup> In the first study, a best-fit model was obtained by forward stepwise regression.<sup>223</sup> In subsequent studies, the effect of new variables was adjusted for variables already present in the main model. The assumption of proportional hazards was assessed by adding each covariate by log-time interaction to the model and assessing the significance of the product term using the partial likelihood ratio test.<sup>223</sup> Variables that did not fulfil the assumption of proportional hazards were handled as stratification variables.<sup>222</sup>

## **5. RESULTS AND DISCUSSION**

### **5.1 DATA INTEGRITY**

#### **5.1.1 Confirmation of histopathological diagnosis**

A panel of antibodies with a known immunostaining profile was used to recognize melanomas from carcinomas: mAbs HMB-45 to immature melanosomes that labels more than 90% of primary and metastatic uveal melanomas,<sup>214,224</sup> mAb CY-90 to cytokeratin (CK) 18 that is expressed e.g. by adenocarcinomas, but can also be found in a minority of cells in uveal melanomas, mAb Vim 3B4 to vimentin that labels uveal melanomas but may also label cells in some carcinomas, and mAb E29 to EMA produced by adenocarcinomas but not by melanomas.<sup>142</sup> An amelanotic tumor was accepted as a melanoma if it showed matching histopathological features and a positive immunostaining reaction for HMB-45, vimentin, or both, but not for CK 18 and EMA, and as a carcinoma when the antigenic profile was the opposite.<sup>142</sup>

Immunolabeling with mAb HMB-45 against premature melanosomes was seen in 35 of 36 amelanotic primary melanomas (97%, 95% CI = 85-100). The only negative one reacted for vimentin with mAb Vim 3B4, and it did not react with mAb CY-90 to CK 18. When melanin pigment was visible in cytoplasm of tumor cells, melanoma diagnosis of primary tumor was accepted without the use of IHC.

The autopsy rate of 32% in present study is much higher than the rate of 6% in COMS, but the portion of patients who had histologically proven melanoma metastasis was comparable, 84% in present vs. 79% in COMS.<sup>91</sup> Of the 59 studied metastatic tumors, the original diagnosis was found to be incorrect in 5 (8%, 95% CI = 3-19). Three amelanotic metastases from uveal melanoma had been misdiagnosed as metastatic carcinoma. Conversely, a hepatic metastasis from a carcinoma had been misdiagnosed as metastatic melanoma. Finally, one biopsy had been read as metastatic mucocellular carcinoma, but specimens taken later at autopsy, as metastatic uveal melanoma. Antigenic profiles of misdiagnosed tumors are presented in Table 5. Of the 50 true metastases from uveal melanoma, 46 (92%, 95% CI = 80-98) were immunoreactive with mAb HMB-45.

**Table 5.** Antigenic profile of histopathologically misdiagnosed metastatic tumors

Site	Misdiagnosis	Antibodies				Correct Diagnosis
		HMB-45	Vim3B4	CY-90	E29	
Liver	Carcinoma	+	+	-	-	Melanoma
Liver	Carcinoma	+	+	-	-	Melanoma
Liver	Carcinoma	+	+	-	-	Melanoma
Liver	Melanoma	-	-	+	+	Carcinoma
Liver	Melanoma	-	-	+	+	Carcinoma

A misdiagnosis frequency of 8% among histopathologically diagnosed metastases before routine IHC is significant. Previously, one metastatic carcinoma incorrectly diagnosed as amelanotic malignant uveal melanoma was identified among the subset of primary tumors included in the present study.<sup>142</sup> Consequently, even without IHC, the rate of histopathologic misdiagnosis of primary uveal melanoma is very low, approximately 1/168 (0.6%, 95% CI = 0-3).

The results suggest a bias in retrospective studies based on cancer registry data. Histopathological diagnosis should be confirmed by immunohistochemistry whenever there is a possibility that amelanotic tumor might be melanoma, because an amelanotic epithelioid cell melanoma may be easily confused with adenocarcinoma and vice versa.<sup>142</sup> No single specific antibody for uveal melanoma exists. Thus a panel of antibodies that have a different staining pattern in melanoma and carcinoma provides the most reliable differentiation of these two entities.<sup>142</sup>

**5.1.2 Pattern of metastatic disease**

Of 80 patients who died of metastatic uveal melanoma, clinical charts were available from 77 (96%) patients to record metastatic disease in detail. Metastatic disease involved liver in all patients except one who died of brain metastasis. Other common metastatic sites were lung, skin, abdominal lymph nodes, and bone (Table 6). Surprisingly, among the 27 autopsied patients with disseminated uveal melanoma, 5 (19%) were autopsied without a suspicion of metastatic cancer. The number of known metastases increased under autopsy (Table 6) that was line with a recent report of COMS.<sup>91</sup> We previously stated that patients have not been selected to the autopsy based on extent of dissemination or presence of symptoms.<sup>90</sup> Case reports of “unconventional” sites of metastatic melanoma have

suggested that widespread melanoma is clinically underestimated unless autopsy has not been performed.<sup>35,90,91,225,226</sup>

Careful review of clinical charts and confirmation of histopathological diagnosis are warranted. Although the underlying cause of death of patients is available from registries in most Western countries, these registries are known to contain inaccuracies.<sup>227,228</sup> Inaccuracies seem to concern especially ocular melanoma, because even one a half of the deaths due to ocular melanoma can be missed if only death certificates are used.<sup>227</sup>

**Table 6.** Sites and frequencies of metastases on 77 of 80 patients who died of disseminated uveal melanoma

Sites	<i>Autopsied</i>				<i>Not Autopsied</i>		<i>All</i>	
	Prior to autopsy N=27		At Autopsy N=27		Clinical N=50		Total N=77	
	N (%)	95% CI	N (%)	95% CI	N (%)	95% CI	N (%)	95% CI
Liver	19 (73)	50-86	26 (96)	81-100	50 (100)	89-100	76 (99)	93-100
Lung	2 (7)	1-24	8 (30)	14-50	13 (26)	15-40	21 (27)	18-39
Skin	2 (7)	1-24	2 (7)	1-24	8 (16)	7-29	10 (13)	6-23
Lymph node	2 (7)	1-24	9 (33)	17-54	1 (2)	0-10	9 (12)	6-21
Bone	0 (-)	-	1 (4)	0-19	8 (16)	7-29	9 (12)	6-21
Heart	0 (-)	-	5 (19)	6-38	0 (-)	-	5 (7)	2-15
Adrenal gland	0 (-)	-	3 (11)	2-29	0 (-)	-	3 (4)	1-11
Pancreas	1 (4)	0-19	7 (26)	11-46	0 (-)	-	7 (9)	4-18
Thyroid gland	0 (-)	-	2 (7)	1-24	0 (-)	-	2 (3)	0-9
Ventricle	0 (-)	-	1 (4)	0-19	1 (2)	0-10	2 (3)	0-9
Brain	1 (4)	0-19	3 (11)	2-29	2 (4)	1-14	5 (7)	2-15
Spleen	0 (-)	-	2 (7)	1-24	2 (4)	1-14	4 (5)	1-13
Kidney	0 (-)	-	3 (11)	2-29	1 (2)	0-10	4 (5)	1-13
Bladder	0 (-)	-	2 (7)	1-24	0 (-)	-	2 (3)	0-9

## 5.2 CLINICAL AND HISTOPATHOLOGICAL TUMOR CHARACTERISTICS

### 5.2.1 General characteristics

Of the 167 patients 76 were (46%) men and 91 (54%) were women. The mean age at removal of the eye was 58 years (median, 61 years; range, 13-95 years). The tumor was entirely choroidal in 126 (75%) eyes and ciliochoroidal in 40 (24%) eyes. Of 40 ciliochoroidal tumors, 4 (10%) involved only the ciliary body by histopathology, which was too small a group for statistical analysis. The mean LBD was 13 mm (median, 13 mm; range, 6-25 mm) and the mean height was 8 mm (median, 7 mm; range 2-20 mm) and the series thus represented melanomas of all sizes. According to LBD, 44 (27%) tumors were classified as small ( $\leq 10$  mm), 69 (43%) as medium-sized ( $>10$ -15 mm), and 49 (30%) as large ( $>15$  mm). The tumor extended extraocularly in 18 cases (11%). Bruch's membrane was ruptured in 75 (48%) cases. The tumor consisted of spindle cells in 86 (64%) eyes, and 48 (36%) tumors contained epithelioid cells and were classified to be nonspindle type. No retinal detachment (RD) was present in 35 eyes (25%), subretinal fluid around the tumor was found in 23 (16%) eyes, RD involved 1-2 quadrants in 61 (43%) and 3-4 quadrants in 23 (16%) eyes. Tumor pigmentation was weak in 38 tumors (27%), moderate in 71 tumors (49%), and heavy in 35 (24%) tumors.

### 5.2.2 Microvascular loops and networks of primary tumors

At least one complete loop was detected in 80 tumors (60%, 95% CI = 51-68) and networks in 47 tumors (35%, 95% CI = 27-44). Networks were found somewhat more frequently in Folberg's series (45%; 95% CI = 49-52), and the frequency of networks was smaller in Foss' inconsecutive series (25%; 95% CI = 17-34).<sup>102,148</sup> The interpretation of a "loop" or "microvessel" in general may have varied between these groups, which may explain part of the difference in frequencies.<sup>102,148</sup> Foss and his group doubted the origin of microvascular patterns and claimed them to be fibrovascular tissue.<sup>148</sup> They were not able to demonstrate patterns well with PAS and a polyclonal antibody to FVIII-related antigen, and they concluded that microvascular patterns cannot be of microvascular origin.<sup>148</sup> McLean's group detected loops in only 157 of 496 tumors (32%, 95% CI = 28-36) but they used archival unbleached PAS slides that had been counterstained, which may obscure part of the microvascular patterns.<sup>141</sup> Likely explanations for varied frequencies are inclusion criteria and rates. Whereas 80% of patients were included in the present study, which can be considered a high rate in a retrospective study, the inclusion rate in the other studies mentioned ranged from a low rate of 11% to a medium rate of 55%.<sup>102,141</sup> Because the presence of loops and networks correlated with poor

prognosis, it is unlikely that the lower frequencies of loops and networks in Foss' study were due to random chance, because the series was enriched with tumors that have metastasized.<sup>148</sup> Random chance might apply to a small Japanese series that found loops and networks in 31% (95 % CI = 11-59) and 25% (95% CI = 7-52) of tumors, respectively.<sup>149</sup>

Interobserver agreement was good in the present study, the kappa statistic was 0.70 (95% CI = 0.57-0.82) for loops and 0.67 (95% CI = 0.53-0.80) for networks. Folberg and colleagues reached similar interobserver agreements in classifying loops and networks (kappa 0.67 and 0.59, respectively) indicating that assessment of microvascular loops and networks is reproducible across laboratories.<sup>102</sup>

The analysis of the distribution of prognostically significant microvascular patterns (parallel vessels with cross-linking and networks) across multiple levels of tumor showed that a single section taken from the central part of a tumor contained the pattern if it was found in the tumor.<sup>229</sup> In a few specimens some microvascular patterns disappeared from sections from the edge of tumor although they were present at other levels.<sup>229</sup> On the other hand, a study based on a single slide from the tumor suggested that parallel with cross-links and networks patterns have a preferential location in the tumor periphery.<sup>230</sup> The findings suggest that the inclusion criterion of requiring over 50% remaining tumor likely ensured that the sections were representative for detection of loops and networks.

### **5.2.3 Microvascular loops and networks of metastatic tumors**

Of 33 metastatic specimens studied, at least one microvascular loop was detected in 27 specimens (82%, 95% CI = 65-93). The overall frequency of loops was comparable in metastases and their corresponding primaries ( $P = 1.0$ , McNemar's test). The presence and absence of microvascular loops differed in 10 patients (30%, 95% CI = 16-49) in their primary tumor and metastatic sites: five times loops were absent from the primary tumor but existed in metastasis, and five times loops were present in the primary tumor but were absent from the metastatic tumor. In one case (3%, 95% CI = 0-16) the loop pattern, and thus also the network pattern, were absent from both the primary and the metastatic tumor.

Networks were detected in 18 metastatic specimens (55%, 95% CI = 36-72). The status of microvascular networks was different in 14 (42%, 95% CI 26-61) corresponding primary and metastatic tumors. In 6 patients (43%) networks were absent from primary tumor but they were found from metastatic specimen, and 8 patients (57%) had no networks in metastasis but had them in the

primary tumor. Presence of microvascular networks did not differ statistically between primary and metastatic tumors ( $P = .79$ , McNemar's test).

Previously microvascular patterns have been analyzed in hepatic metastases of five uveal melanoma patients and each of them contained microvascular networks (100%, 95% CI = 48-100).<sup>231</sup> The present study does not suggest that an enrichment of microvascular loops and networks would occur when metastasis develops, and the confidence intervals of the two studies overlap. Neither was the sample large enough to confirm small differences in frequencies statistically. Moreover, studying only one section of a single metastasis does not exclude the possibility that microvascular loops and networks might be present elsewhere in the metastasis or in other metastases. The differences in microvascular loops and networks between primary and metastatic tumors, when present, might reflect true variation in tumor characteristics between primary and metastatic sites, e.g. in the propensity of hepatic metastases to spread to tertiary sites.

Microvessels in metastases have clinical importance, because new therapeutic drugs targeted to blood vessels, such as angiogenesis inhibitors, have shown effect on controlling tumor growth *in vitro*.<sup>232</sup> Interestingly, drugs impacted, however, differently microvessels formed by tumor cells than vessels formed by endothelial cells.<sup>233</sup> The use of inhibitors of vasculogenic mimicry, such as inhibitors of epithelial cell kinase (e.g. Eck/EphA2), may offer novel therapeutic applications.<sup>154</sup>

#### 5.2.4 Microvascular density of primary tumors

The median MVD based on the globally highest count was 40 vessels/0.313mm<sup>2</sup> (range, 5-121), and the median MVD based on the three highest counts averaged was 33 vessels/0.313mm<sup>2</sup> (range, 5-113), as analyzed with antibodies to the CD34 epitope. The corresponding MVDs obtained with antibodies to FVIII-RAg were 33 (range, 6-102) and 27 (range, 4-84) vessels/0.313 mm<sup>2</sup>, respectively. Strong agreement existed between the mean of the three highest counts and the globally highest vessel count, and no obvious advantage was obtained from counting three separate areas.

Foss and associates used also FVIII-RAg and they obtained a median MVD of 40 microvessels, counted from a of 0.25 mm<sup>2</sup> single globally highest area of vascularization.<sup>105</sup> In two other data sets, however, MVD was reported to be much lower than in the present and Foss' studies.<sup>166,167</sup> In a case-control study of 63 tumors, two hot spots were counted from predetermined areas of tumor and the median MVD ranged from 5.7 to 11.4 vessels per counted area.<sup>166</sup> In a follow-up study of 40 uveal

melanomas, MVD was determined from five randomly chosen areas and the mean MVD ranged from 1 to 12.5 vessels per  $0.216\text{mm}^2$ .<sup>167</sup>

The main difference between these studies on MVD, and the most likely reason for different results, is the strategy of selecting areas to be counted. In the present study and in the series of Foss,<sup>105</sup> the analysis was based on the globally highest MVD (“hot spot”). Whereas, counting vessels from predetermined and random areas in the two other studies is likely to have had impact on MVDs, probably making them smaller and less variable than using the hot spot counting. The area to be counted should not be too small or too large to prevent exaggerating or dilution of the hot spot.<sup>234</sup> The method of visualizing microvessels also varied. Whereas our preferred marker was CD34 epitope, Lane used *Ulex europaeus* agglutinin I and the other two groups used FVIII-RAg.<sup>105,166,167</sup> The latter markers are thought to be less sensitive than the CD34 epitope, especially as regards to microvessels seen in malignant tumors, and weak or incomplete staining has been reported also in uveal melanoma.<sup>2,235</sup>

Intraobserver reproducibility in assessing MVD with antibodies to the CD34 epitope, as 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) on recounting based on the globally highest count, and 0.25 units less (95% CI = 0.02-0.48) on recounting based on the three highest counts averaged, corresponding to 3 and 1.7 vessels less per recounted area. The intraobserver reproducibility was regarded satisfactory.<sup>220</sup> Other investigators have reported good intraobserver and interobserver reproducibility for repeated counts, especially when based on hot spots, but the data were usually categorized and analyzed by kappa statistic.<sup>160</sup>

Because sections labeled with antibodies to the CD34 epitope were easier to read and thus more convenient to count than those labeled for FVIII-Rag (Fig. 1, A and B in II), and also yielded higher counts, it would seem reasonable to use the globally highest count obtained with antibodies to the CD34 epitope in further studies of uveal melanoma. The CD34 antigen is a cell-membrane associated glycoprotein that is frequently found on pluripotent hematopoietic progenitors, vascular endothelial cells, and embryonic fibroblasts.<sup>236</sup> The CD34 antigen has a large number of different glycosylation-dependent epitopes and mAb QBEND/10 to the CD34 recognizes its class II epitopes that are sensitive to enzymatic cleavage of the glycoprotease.<sup>236</sup>

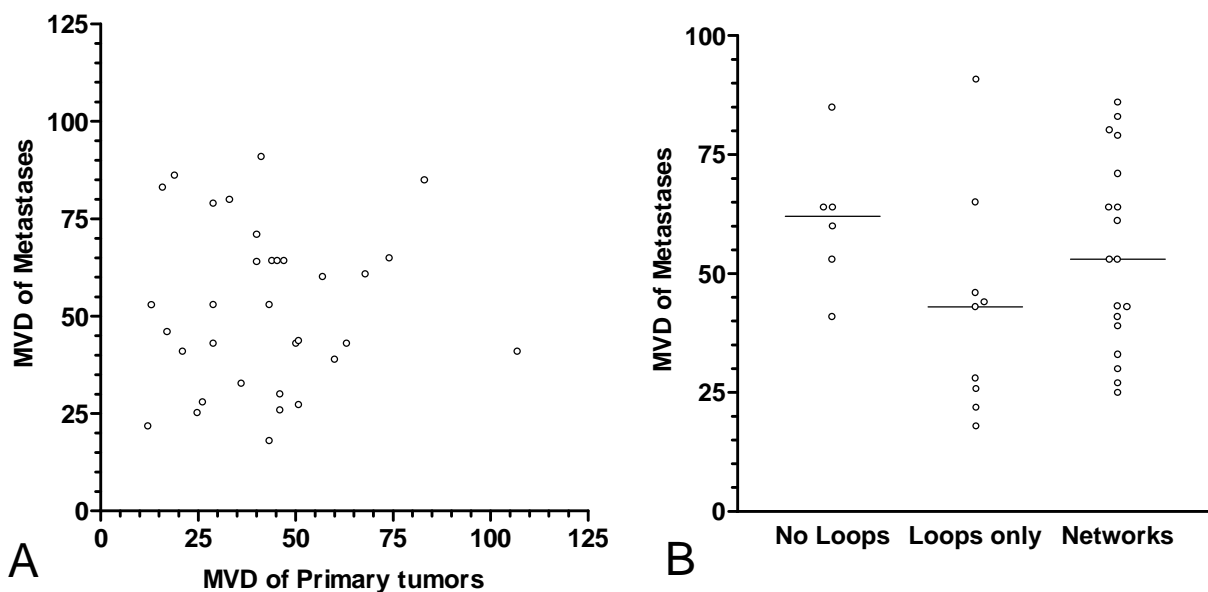
It must be realized that MVD as defined here is more of a rough index of relative densest vascularization in the hot spots rather than an exact measure of the number of microvessels present. It is also possible that not all microvessels are immunolabeled with above antibodies, and that not all positive immunoreaction is restricted to endothelial cells.<sup>152</sup>

### 5.2.5 Microvascular density of metastatic tumors

MVD in uveal melanoma metastases has not previously been reported. The mean MVD in metastatic specimens was 52 immunopositive vessels/0.313mm<sup>2</sup> (range 18-91 vessels) as obtained with mAb QBEND/10 to CD34 epitope. MVD tended to be higher and the range of variation smaller in metastatic tumors than in the corresponding primary tumors ( $P = .071$ , Wilcoxon signed ranks test), but overlapping was significant (Fig. 3A). On contrary to the primary tumors, MVD was not higher in samples that contain microvascular loops or networks (Fig 3B;  $P = .25$ , Kruskal-Wallis test).

The present series of 33 patients is one of the largest series of uveal melanoma in which corresponding primary and metastatic samples have been studied. Nevertheless, the small number of patients makes it impossible to detect anything but a large difference as statistically significant.

**Figure 3.** Scattergrams of MVD in metastases versus corresponding primary tumors and the presence of microvascular loops and networks (bars indicate median values)



### **5.2.6 Tumor-infiltrating macrophages**

The number of CD68-immunopositive cells was semiquantitatively graded as few in 24 tumors (17%; 95% CI = 11-25), moderate in 71 tumors (51%; 95% CI = 43-60), and many in 44 tumors (32%; 95% CI = 24-40). The predominant morphologic type of infiltrating cells was graded as dendritic type in 30 tumors (22%; 95% CI = 15-29), intermediate in 82 tumors (59%; 95% CI = 50-67), and round in 27 tumors (19%; 95% CI = 13-27). The interobserver agreement (weighted kappa) was 0.77 (95% CI = 0.69-0.85) for grading the number and 0.60 (95% CI = 0.49-0.71) for grading the type of CD68-immunopositive cells. These kappa values were considered moderate to good.

Even though it has long been recognized that uveal melanomas contain macrophages, especially melanophages, this type of tumor-infiltrating cell has not been addressed widely in literature.<sup>115</sup> In the ongoing Collaborative Ocular Melanoma Study (COMS), 1354 of 1526 (89%) enucleated melanomas had “none to minimal” or “scattered single small clumps”, and 172 tumors (11%) had “scattered single and larger aggregates” of macrophages, as judged by light microscopy.<sup>111</sup> In current analysis, especially round CD68-positive macrophages could amount to almost one quarter of the tumor cross-sectional area in nonnecrotic regions ( $>650$  cells/mm<sup>2</sup>) and the number of dendritic CD68 positive cells could be equivalent in other tumors. Tobal and associates have also noted a dense infiltration of CD68-positive macrophages (range 1200-1400 cells/mm<sup>2</sup>) in 16 uveal melanomas.<sup>110</sup> Taken together, it seems evident that light microscopic examination without labeling macrophages by immunohistochemistry and without bleaching of melanin underestimates the number of macrophages (CD68<sup>+</sup> cells).

The target antigen of antibodies to CD68, is an intracytoplasmic 110 kD glycoprotein that resides in lysosomal granules and is expressed by macrophages.<sup>216,217</sup> During differentiation of monocytes into macrophages the expression of CD68 increases markedly,<sup>237</sup> but the function of the CD68 epitope has remained unknown both in macrophages and in other cell types. The role of macrophages is currently completely open in uveal melanoma.

### **5.2.7 Ezrin immunoreactivity**

A granular, diffuse intracytoplasmic immunoreaction that frequently was concentrated focally along the cell surface was obtained with mAb 3C12 in uveal melanoma cells. The two investigators agreed on the grade of ezrin immunoreactivity in 82 of the 130 specimens (63%, 95 % CI = 54-71). After

consensus, 47 (36%, 95% CI = 28-45%) melanomas were classified as negative, 74 (57%, 95% CI = 48-66) as positive, and 9 (7%, 95% CI = 3-13) as strongly positive for ezrin. Some discrepancies were due to the presence of large numbers of immunopositive lymphocytes. A two-category discrepancy occurred once. The interobserver agreement (weighted kappa) was considered moderate and amounted to 0.50 (95% CI = 0.36-0.63).

The labeling intensity ranged from negative to strongly positive. When positive, the immunoreaction tended to be homogeneous throughout the tumor. However, in 4 of the 130 melanomas (3 %, 95% CI = 1-8) areas of weakly and strongly immunopositive tumor cells were found adjacent to each other. Adjacent sections immunostained with mAb PG-M1 and KP1 suggested that putative macrophages were typically not labeled by mAb 3C12.

### **5.2.8 Interrelationships between prognostic indicators**

#### **5.2.8.1 Microvascular loops and networks**

Tumors with nonspindle cells contained more often microvascular loops and networks, analyzed as a combined variable (no loops, loops without networks, and networks), than spindle cells melanomas ( $P = .003$ , Kruskal-Wallis test, Table 7). The association between microvascular loops and networks and epithelioid cell type is in line with the results of Folberg and coworkers.<sup>102</sup> Moreover, they found that epithelioid melanomas contained more often parallel, arcs, parallel with cross-linking, and arcs with branching patterns than spindle cell tumors.<sup>102</sup> Microvascular loops and networks tended to be more frequent in tumors with increasing LBD ( $P = .088$ , Jonckheere-Terpstra test). Microvascular loops were more common in tumors that involved the ciliary body ( $P = .040$ , Fisher's exact test), but such association was not observed in distribution of networks ( $P = 1.0$ , Fisher's exact test). Two other groups, however, have reported that ciliary body melanomas contained more often microvascular loops and networks than choroidal melanomas.<sup>106,123</sup> This discrepancy may result from small and biased sample in case of the recent study<sup>106</sup> or from differences in identification of microvascular loops and networks. One possible explanation might be misidentification of ciliary muscle bundles as networks as they can resemble networks in PAS staining (Fig. 1,G and H in I). The degree of tumor pigmentation, the number and type of tumor-infiltrating macrophages, and the ezrin immunoreactivity were not associated with the presence of microvascular loops and networks.

Interestingly, in the present study MVD was higher in tumors that contained microvascular loops and networks ( $P = .001$ , Jonckheere-Terpstra test), although globally highest microvessel count tended to be found from areas not involved by microvascular loops and networks. Moreover, no immunopositive microvessels were found inside the loops and networks. Foss and associates reported a higher MVD in tumors with parallel, parallel with cross-links, arcs, arcs with branches, and networks than in tumors that lacked these prognostically significant microvascular patterns.<sup>148</sup> Surprisingly, the MVD was lower in tumors with loops than in tumors that lacked loops.<sup>148</sup> They did not speculate on this finding, but addressed an incomplete immunostaining of microvascular patterns with FVIII-RAg and questioned the microvessel nature of microvascular patterns.<sup>148</sup>

#### 5.2.8.2 Microvascular density

The MVD was statistically significantly higher in melanomas that contained non-spindle cells ( $P = .011$ , Kruskal-Wallis test). Moreover, large tumors had higher MVD ( $P = .006$ , Jonckheere-Terpstra test), consistent with the study of Foss and coworkers (Table 7).<sup>105</sup> Substantial overlap was, however, observed especially as regards tumor size (Fig. 3, C and D in II). The connection between large tumor size and high microvascular density is true for other tumors as well, and may signify that tumor progression and growth are dependent on rich tumor circulation.<sup>159-161</sup> It seems also possible that a biologically significant association may exist between epithelioid cell type and specific microcirculation features in uveal melanoma. The biological significance of the observed statistical association between immunoreactivity for ezrin and high MVD remains unsolved ( $P = .004$ , Kruskal-Wallis test), as well as the relationship between high MVD and heavy tumor pigmentation ( $P = .013$ , Jonckheere-Terpstra test). MVD was not associated with ciliary body involvement, extraocular extension, sex, or age of the patient.

#### 5.2.8.3 Tumor-infiltrating macrophages

Melanomas with large LBD ( $P = .031$ , Jonckheere-Terpstra test), high MVD, heavy pigmentation ( $P = .001$ , Jonckheere-Terpstra test), and epithelioid cells ( $P = .025$ , Kruskal-Wallis test) had statistically significantly more macrophages than small and weakly pigmented melanomas and melanomas without epithelioid cells, respectively, but overlap between categories was notable (Fig. 2, A and B in III) (Table 7). In addition, females had statistically significantly more tumor-infiltrating macrophages than males ( $P = .010$ , Kruskal-Wallis test).

No association between the predominant type of CD68-immunopositive macrophages and LBD, the presence of epithelioid cells, MVD, and gender was observed. Weakly pigmented melanomas contained more often dendritic than round type of macrophages, whereas the reverse was true of heavily pigmented tumors ( $P = .001$ , Kruskal-Wallis test). The number and type of macrophages were interrelated ( $P = .005$ , Kruskal-Wallis test). When the number was small, the dendritic type predominated over the round type, and vice versa. The tumor pigmentation may have impact on morphology of tumor-infiltrating macrophages, but whether it has also impact on their function is unknown.

Neither the number nor the predominant type of immunopositive cells was statistically significantly associated with involvement of the ciliary body, presence of extraocular extension, and presence of over 50% necrosis. Ciliary body melanomas have, however, been reported to contain more melanophages than tumors confined to choroid, but the number of melanophages was assessed without IHC.<sup>111</sup>

#### 5.2.8.4 Ezrin immunoreactivity

The presence of immunoreactivity with mAb 3C12 was not statistically significantly associated with gender and LBD (Table 7). In contrast, melanomas with high number of tumor-infiltrating macrophages ( $P = .0006$ , Kruskal-Wallis test) were statistically significantly more often labeled with mAb 3C12 to ezrin than tumors with low number of tumor-infiltrating macrophages (Table 7).

**Table 7.** Summary of statistical associations between the clinical and histopathological variables analyzed in the present thesis

	LBD	Height	Cell Type	Patterns§	Ciliary Body	MVD	CD68 <sup>+</sup> Number	CD68 <sup>+</sup> Type	Pigmentation	Gender
Height	<b>.001*</b>	-	-	-	-	-	-	-	-	-
Cell Type	<b>.006†</b>	.10†	-	-	-	-	-	-	-	-
Patterns§	.088*	.12*	<b>.003†</b>	-	-	-	-	-	-	-
Ciliary Body	<b>.011†</b>	.27†	.096‡	.27†	-	-	-	-	-	-
MVD	<b>.006*</b>	<b>.009*</b>	<b>.011†</b>	<b>.001*</b>	.28†	-	-	-	-	-
CD68 <sup>+</sup> Number	<b>.031*</b>	.80*	<b>.025†</b>	.64*	.32†	<b>.001*</b>	-	-	-	-
CD68 <sup>+</sup> Type	.55†	.94†	.56¶	.34†	.41¶	.44†	<b>.005†</b>	-	-	-
Pigmentation	<b>.004*</b>	<b>.001*</b>	.11†	.79*	.14†	<b>.013*</b>	<b>.001*</b>	<b>.001†</b>	-	-
Gender	.90†	.23†	.59‡	.44†	.16‡	.27†	<b>.010†</b>	.47¶	.83†	-
Ezrin	.53†	.70†	.087‡	.14†	.30‡	<b>.003†</b>	<b>.0006†</b>	<b>.067¶</b>	.13†	.24‡

\* Jonckheere-Terpstra test, two-sided

† KruskalWallis test, two-sided

‡ Fisher's exact test, two-sided

¶ Chi-squared test for independence, two-sided

§ Patterns: no loops, loops without networks, and networks

Statistically significant ( $P < 0.05$ ) in **bold type** and trend ( $0.05 < P < 0.10$ ) in *italics*

### 5.3 MELANOMA-SPECIFIC AND ALL-CAUSE MORTALITY

#### 5.3.1 Overall mortality

At the end of the follow-up time (median 22 years, range 18-26 years), 37 of 167 patients (22%) were alive without evidence of metastatic melanoma and 130 (78%) patients had died. Of 130 deaths, 80 (62%) were due to metastatic uveal melanoma, 9 (7%) were caused by carcinomas, and 41 (31%) were other than tumor deaths. The follow-up time of 22 years was sufficiently long to capture total mortality as completely as possible, and the present series is representative of the entire population of patients with uveal melanoma.

The crude melanoma-specific mortality was 0.58 at the end of the median follow-up time of 22 years (Fig. 4A), which is in line with reports based on unselected series of uveal melanoma patients with long follow-up time.<sup>23,25,130</sup> The cumulative melanoma-specific mortality was highest during the first three years from enucleation, also in line with a number of survival studies,<sup>93,122</sup> being 0.30 at 5 years. Thereafter, melanoma-specific death rate remained relatively stable as long as follow-up continued, corresponding to 10-, 15- and 20-year mortalities of 0.41, 0.48, and 0.55 respectively (Fig. 4A). Metastatic melanoma remained the leading cause of death throughout the follow-up period. Based on this consecutive series, short follow-up times, such as 5 years, capture only about one half of all melanoma deaths. A statistically significant difference in melanoma-specific survival at 5-years of follow-up does not necessarily indicate that there will eventually be a significant difference in the long run.

Early metastasis might only reflect a more advanced tumor or a more rapid progression of tumor, and it does not necessary mean that other patients would be safe from metastasis. Apparently most, if not all, uveal melanomas disseminate, but depending on patient's age, competing diseases, and host factors, metastatic disease does not become clinically evident if the patient is censored because of unrelated death or short follow-up time. It is possible, but so far unexplored, that early melanoma deaths (e.g. <15 years from enucleation) and late melanoma deaths (e.g. >15 years from enucleation) may reflect differences in tumor biology and host response between patients. Consecutive and large representative series of uveal melanoma with extended follow-up time may shed light on the histopathological and host characteristics that determine progression to fatal disseminated disease within different time intervals.

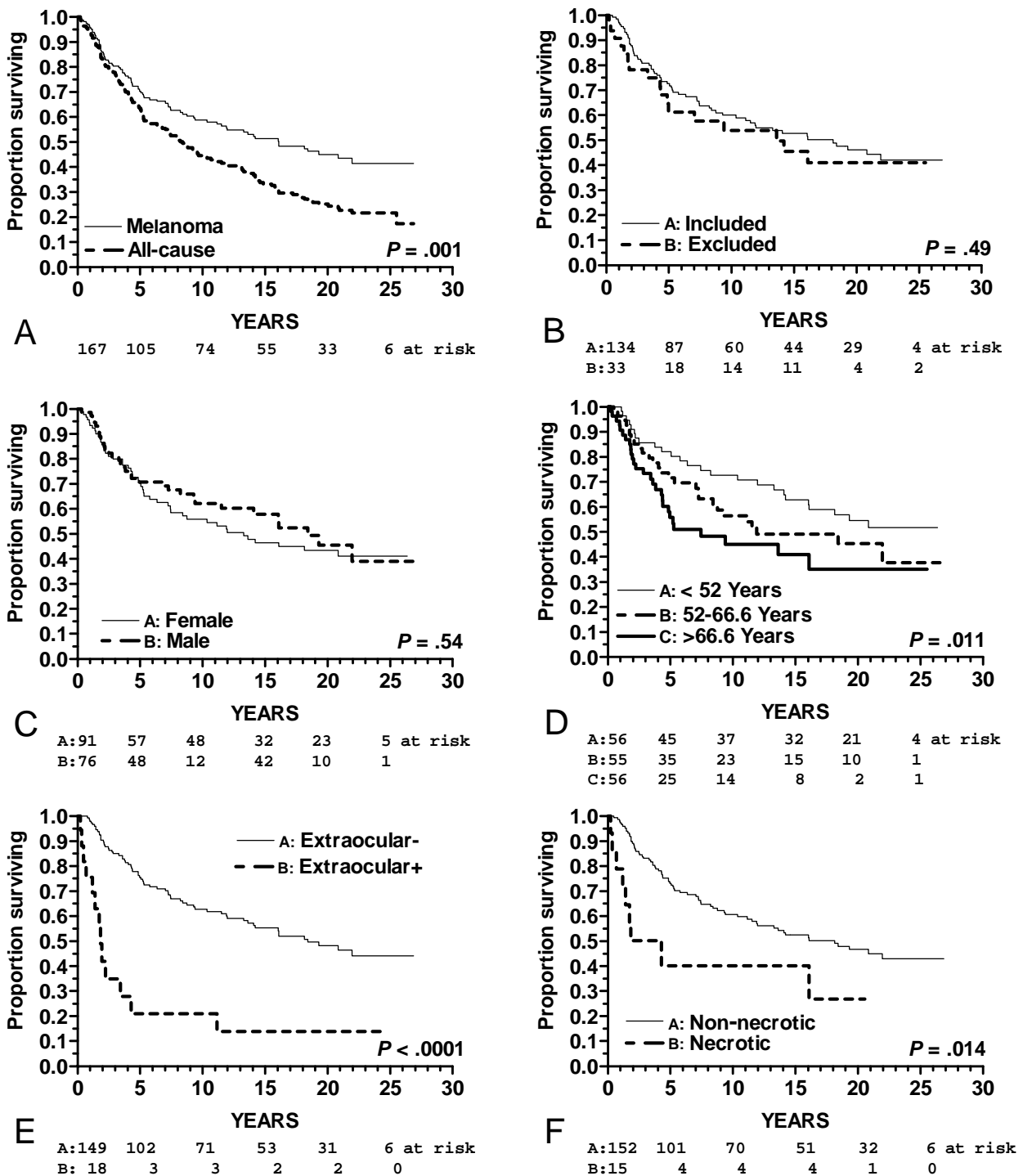
Because patients with uveal melanoma are usually middle-aged or older, they are also at risk of dying of other illnesses than metastatic uveal melanoma.<sup>98</sup> In reporting melanoma-specific mortality there is always a possibility that a patient with disseminated melanoma is censored from survival as being without an evidence of metastasis. Probably even performing a careful autopsy does not totally exclude micrometastatic disease, but as in present study, it may reveal clinically undetected metastases. To control for this source of bias, all-cause mortality is recommended as a secondary or even primary outcome measure in survival studies.<sup>98</sup> The cumulative all-cause mortalities of current series were 0.37 at 5 years, 0.56 at 10 years, 0.67 at 15 years, and 0.76 at 20 years (Fig. 4A). No difference in melanoma-specific mortality was observed between enrolled and excluded patients (Fig 4B;  $P = .49$ , Log-rank test).

### **5.3.2 Prognostic significance of baseline characteristics**

Melanoma-specific survival was similar among both sexes (Fig. 4C;  $P = .54$ , Log-rank test), but older patients died more often of metastatic melanoma than younger patients (Fig. 4D;  $P = .006$ , Log-rank test for trend). Patients with extraocularly extended melanoma had 0.42 higher 10-year melanoma-specific mortality than patients with entirely intraocular melanoma (Fig 4E;  $P < .0001$ , Log-rank test). Presence of large amount of necrosis (>50% of tumor section) increased melanoma mortality by 0.20 (Fig 4F;  $P = .014$ , Log-rank test).

The cumulative 10-year melanoma-specific mortality was 0.22 for small tumors ( $\leq 10$  mm), 0.44 for medium-sized tumors (10-15 mm), and 0.60 for large tumors ( $> 15$  mm) by Kaplan-Meier analysis (Fig 5A;  $P < .001$  Log-rank test for trend). The prognostic significance of tumor size in uveal melanoma is well documented in many survival studies (Table 8).<sup>84,85,102,103,105,122,130,131</sup> Large tumor size may indicate that the tumor has grown undetected for years inside the eye.<sup>93</sup> Alternatively, it may have had a rapid growth rate as a sign of aggressive nature.<sup>238</sup> In either case, a large tumor is more likely to be further advanced in tumor progression than a small one. Large tumors have sent micrometastases with a greater probability than small ones and micrometastases from large tumors may progress more often and more rapidly to clinically detectable metastases.

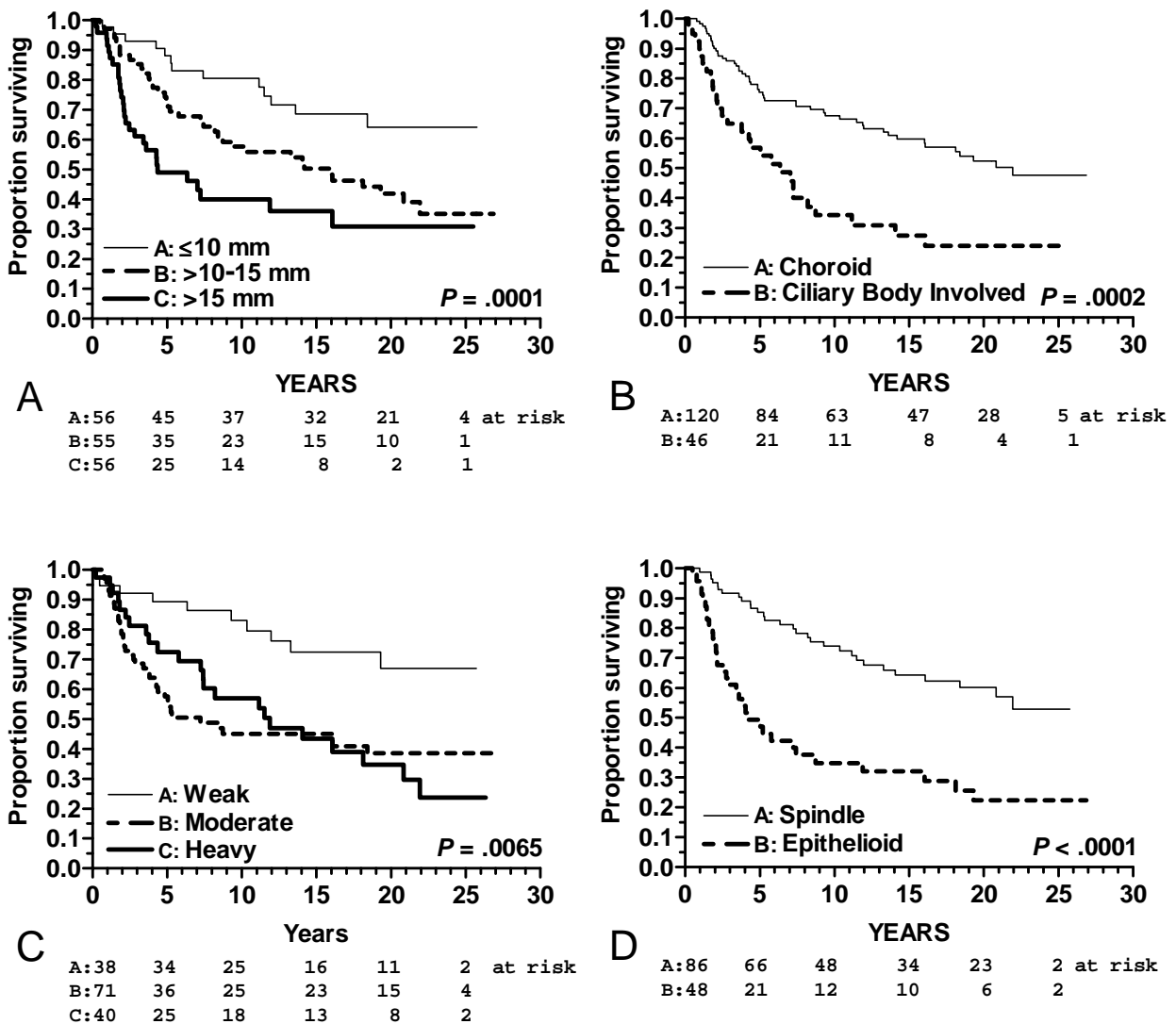
Figure 4. Kaplan-Meier curves of melanoma-specific survival.



Patients with melanoma extending to the ciliary body had 0.36 units higher 10-year melanoma-specific mortality than patients with choroidal melanoma (Fig. 5B;  $P = .0002$ , Log-rank test). Anterior tumor location is an important clinical prognostic indicator and recent studies have shown that ciliary body involved melanomas tend to contain more often chromosomal abnormalities and microvascular networks,<sup>49,106,123-125</sup> even though the present study failed to confirm the latter association.

The degree of tumor pigmentation was statistically significantly associated with melanoma-specific mortality (Fig 5C;  $P = .0065$ , Log-rank test for trend). The 10-year cumulative probability of survival was 0.83 for weak, 0.44 for moderate, and 0.57 for heavy tumor pigmentation.

**Figure 5.** Kaplan-Meier curves of melanoma-specific survival. LBD: small melanomas vs. medium-sized melanomas vs. large melanomas (A), Tumor location: choroidal melanomas vs. melanomas involving the ciliary body (B), Tumor pigmentation: weakly pigmented vs. moderately pigmented vs. heavily pigmented melanomas (C), Cell type: spindle cell melanomas vs. nonspindle melanomas (D).



Spindle cell tumors cause metastatic deaths, but at a lower rate than nonspindle tumors (Fig. 5D). The melanoma-specific mortality was higher after enucleation among patients with nonspindle cells than among patients with spindle tumors, and a survival difference of 0.35 to 0.38 remained after 5 years. The 10-year melanoma-specific mortalities were 0.26 for spindle cell and 0.64 for nonspindle tumors

(Fig. 5D;  $P < .001$  Log-rank test). Nonspindle cells are obviously of more malignant phenotype.<sup>178,192</sup> They have a less cohesive growth pattern, and more DNA abnormalities than spindle cells.<sup>3,178,192</sup> Cell type has been consistently associated with decreased survival in the vast majority of studies on uveal melanoma,<sup>86,102,122,130,132</sup> but a few recent ones have failed to document such an association in multivariate analysis.<sup>105,194</sup> Series based on histopathologic material collected during the era of eye-conserving treatment options may represent more advanced cases of intraocular melanomas, which may affect the possibility to detect associations.<sup>194</sup>

In a study based on present series of uveal melanoma patients, but not included in this thesis, suggested that increasing extent of exudative retinal detachment is associated with higher melanoma-specific mortality.<sup>239</sup> The association between RD and mortality disappeared when survival was controlled for LBD and presence of microvascular loops and networks.<sup>239</sup>

### **5.3.3 Prognostic significance of microvascular loops and networks**

This study was not undertaken to explore all nine microvascular patterns to avoid statistical problems from multiple comparisons of variables that may not be independent from each other. Primary aim of the present study was to confirm or contradict the prognostic significance of microvascular loops and networks, which had previously been the most powerful and consistent prognostic indicators of the nine microvascular patterns (Table 8).<sup>102,104,141</sup> This does not imply that the other seven patterns would not be clinically significant or interesting.<sup>240</sup> Need for multiple comparisons between microvascular patterns and conventional prognostic indicators, as well as the limited number of metastatic deaths ( $n = 80$ ) also precluded a study of all nine patterns.

The 10-year cumulative melanoma-specific mortality was 0.30 units higher for tumors with microvascular loops and 0.42 units higher for those with microvascular networks as compared to tumors without microvascular loops and networks (Fig. 6A;  $P < .001$ , Log-rank test). A 10-year melanoma-specific survival difference of 0.35 (range 0.31-0.38) between patients with presence of loops or networks and patients who lack these patterns is established by several independent studies (Table 2).<sup>102,104,141</sup> Results of multivariate analysis are more difficult to compare, because both the number of events and the number and type of other variables included in the model have a great impact on the statistics. Except for the study by Foss and co-workers,<sup>148</sup> either loops or networks, entered as an independent prognostic indicator into a multivariate model adjusted for conventional prognostic indicators: cell type, largest basal tumor diameter, and ciliary body involvement (Table 2).<sup>102,104,141</sup>

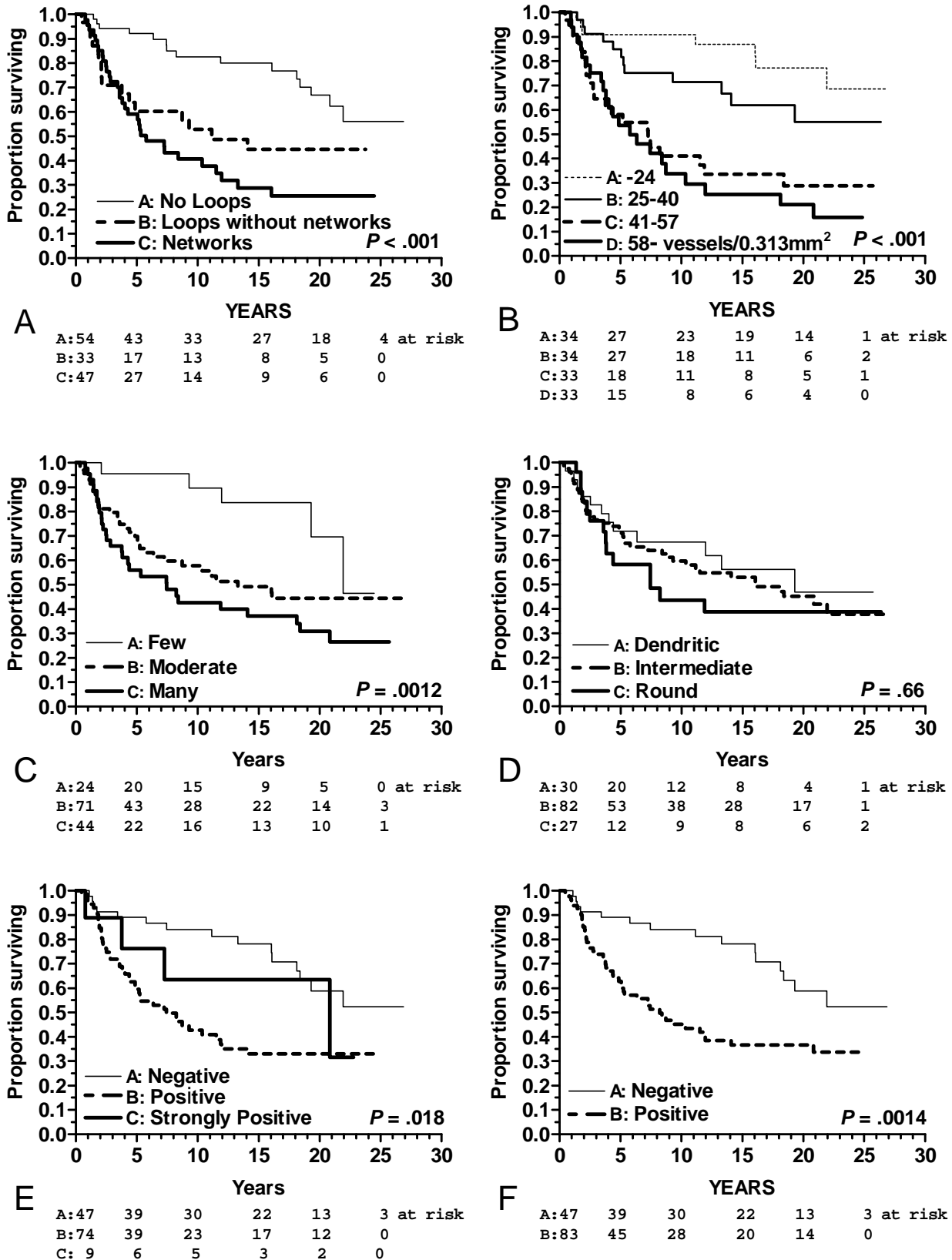
No melanoma deaths have occurred so far among 16 of 37 patients with microvascular loops or networks who survived 16 years or more from enucleation in the present series (Fig. 6A). At same time, 6 of 26 (23%) patients who lacked microvascular loops and networks and had survived at least for 16 years from enucleation, have died of metastatic uveal melanoma (Fig. 6A). Such delayed melanoma deaths took place also within each quartile of MVD. As regards to LBD, other investigators have observed that its prognostic significance varies with the follow-up.<sup>241</sup> These observations may indicate incorrect categorization or, perhaps more likely, as yet ill understood interplay with host and other tumor variables.

Since eye-preserving treatment modalities are now preferred to enucleation, less histopathological material is routinely available for evaluation of indicators of metastatic disease. It is unclear how representative specimens taken by fine-needle biopsy for assessing microvascular patterns are, but the suction of tumor cells into syringe and smearing them on the slide probably change the architecture of microvessels making evaluation inconsistent, and also the sample volume would be small. The same also applies to MVD. In order to evaluate microvascular architecture from eyes that are not removed, indocyanine green angiography with confocal scanning laser ophthalmoscopy and high-frequency ultrasound have been evaluated as tools for visualising tumor vasculature *in vivo*.<sup>242-244</sup> There is a clear demand for such methods, because identification of patients who are at high risk of dying of metastases is inaccurate if the evaluation is based on only traditional clinical prognostic factors, tumor size and tumor location.

#### 5.3.4 Prognostic significance of microvascular density

As analyzed by the globally highest MVD obtained with antibodies to the CD34 epitope, the 10-year cumulative melanoma-specific mortality increased when the MVD increased, being 0.09, 0.29, 0.59, and 0.66 for the four quartiles from lowest to highest density (Fig. 6B;  $P < .0001$  Log-rank test for trend). The above results are in line with an analysis recently conducted by Foss and co-workers,<sup>105</sup> who found a corresponding difference of 0.58 in 9-year probability of survival among 116 patients with choroidal and ciliary body melanoma using antibodies to FVIII-RAg. In both studies, the risk of death increased from quartile to quartile.<sup>105</sup>

**Figure 6.** Kaplan-Meier curves of melanoma-specific survival. Microvascular patterns: melanomas without loops vs. melanomas with loops but without networks vs. melanomas with networks (A), MVD: globally highest microvessel count (B), Number and Type of tumor-infiltrating macrophages (C, D), Ezrin immunoreactivity: three-category (E) and two-category (F) classifications.



Two other studies on uveal melanoma have failed to document any relationship between survival and MVD.<sup>166,167</sup> In addition to relatively small sample size, a modified methodology was used in assessing MVD, as already mentioned. Such negative studies have been published also from other cancers, but about three quarters of studies have shown an association between high MVD and tumor-specific survival, even in tumors such as breast cancer that also frequently spread via the lymphatic route.<sup>159,160,160,161</sup>

### 5.3.5 Prognostic significance of tumor-infiltrating macrophages

Melanoma-specific mortality was significantly associated with the number of CD68-immunopositive cells (Fig. 6C;  $P = .0012$ , Log-rank test for trend). The 10-year cumulative probability of survival was 0.90 for few, 0.58 for moderate numbers, and 0.43 for many immunopositive cells. In contrast, the predominant type of CD68-immunopositive cells identified with mAb PG-M1 was not significantly associated with melanoma-specific mortality (Fig. 6D;  $P = .66$ , Log-rank test)

The presence of a high number of macrophages in aggressive melanomas might either be an indication of a host response mounted against more malignant tumors or it might simply be indirect evidence of an aggressive tumor that has a high cell turnover rate and consequently a need for phagocytosing cells. Melanoma cells may modulate the infiltration of monocytes by secreting cytokines such as monocyte chemoattractant protein-1.<sup>245</sup>

### 5.3.6 Prognostic significance of ezrin immunoreactivity

The 10-year cumulative melanoma-specific probability of survival was 0.84 for melanomas not immunoreactive with mAb 3C12, 0.43 for tumors that were immunoreactive, and 0.63 for tumors that were strongly immunoreactive (Fig. 6E;  $P = .018$  Log-rank test for trend). Due to the small sample size of strongly ezrin immunoreactive tumors, strongly positive and positive tumors were combined as a secondary analysis and significant survival difference was observed as compared to ezrin immunonegative tumors (Fig. 6F;  $P = .0014$ , Log-rank test).

The expression of ezrin in endometrial carcinoma, pancreatic carcinoma, and hemangioblastoma cell lines has suggested its role as a modulator of tumor cell morphology, migration, and invasion.<sup>219,246-248</sup> Recently, ezrin immunoreactivity with mAb 3C12 was associated with increasing malignancy of

astrocytic tumors.<sup>249</sup> Evidence is lacking on functions of ezrin in uveal melanoma, but the study of invasive unconverted uveal melanoma cells indirectly suggests a role for ezrin in uveal melanoma: melanoma cells that expressed the transmembrane *c-met* receptor were highly responsive to the mitogenic effects of HGF.<sup>168</sup> *C-met* acts as a receptor of HGF and conducts the signal to intracellular ezrin which links the plasma membrane to the actin cytoskeleton.<sup>250,251</sup> Of other tumors, co-expression of HGF and *c-met* is documented in endometrial carcinoma, in which *c-met* expression also contributed to shorter survival.<sup>252</sup> Worth studying is whether uveal melanoma cells express *c-met*, whether *c-met* expression is increased in tumors with ezrin immunoreactivity, and whether *c-met* might be involved in liver-targeted metastasis. Ezrin might also modulate cell recognition by the immune system e.g. natural killer cells.<sup>253</sup>

## 5.4 COX PROPORTIONAL HAZARD REGRESSION ANALYSES

### 5.4.1 Univariate analysis

Risk for metastatic disease increased with increasing tumor size: the risk was 1.12 times greater for each mm that exceeded the mean LBD of 13 mm by univariate Cox regression (Table 8). Similarly, each mm over the mean height of 7 mm meant 1.13 times greater risk for metastatic death. The ciliary body involvement indicated 2.21 times higher risk for metastatic death (Table 8).

Because microvascular loops and networks are closely interrelated, the statisticians of the Journal of the National Cancer Institute encouraged us to analyze them as a combined variable: no loops, loops without networks, and networks. By Cox analysis, the risk for metastatic death was 1.81 times higher if a patient had microvascular loops, and 3.3 times higher if he or she had networks as compared to a patient who lacked loops and networks. Hazard rate for MVD was 1.33 for each unit change in square root transformed vessel count (Table 8). Ezrin immunoreactivity indicated a 2.5-fold risk for metastatic death. The number of macrophages did not fulfil the proportional hazards assumption ( $X^2 = 5.48$ ; 1 df;  $P = .019$ , indicating that the risk changes over time, and the hazard is not proportional) and this variable was modelled by stratification.

**Table 8.** Prognostic indicators of choroidal and ciliary body melanomas analyzed by univariate Cox regression in recently published papers.

	Rummelt et al. (n=234) <sup>123</sup>			Foss et al (n=116) <sup>148</sup>			Seregard et al. (n=132) <sup>104</sup>			Present study (n=134)		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
<i>Categorical variables</i>												
Male gender	1.54	0.95-2.50	.077	0.93	0.54-1.61	.82	0.80	0.33-1.26	.34	0.88	0.56-1.38	.58
Extraocular extension†	1.79	0.65-4.91	.26	2.27	1.30-4.00	.004	1.50	1.20-1.80	.008	2.40	1.03-5.60	.002
Nonspindle cell type †	2.63	1.57-4.42	<.001	2.02	1.35-3.03	.001	1.27	1.09-1.44	.008	2.94	1.78-4.85	.001
Ciliary body involvement‡	2.45	1.47-4.07	<.001	2.27	1.30-4.00	.004	1.36	1.03-1.68	.06	2.21	1.31-3.74	.0046
Heavy tumor pigmentation‡	N/A			1.41	1.07-1.84	.01	N/A			3.09	1.52-6.26	.0004
Ezrin¶	N/A			N/A			N/A			2.52	1.40-4.51	0.001
<i>Continuous Variables</i>												
Age	1.02	1.00-1.04	.025	1.01	0.99-1.03	.27	1.04	1.02-1.06	.001	1.02	1.01-1.04	.015
LBD	1.17	1.10-1.24	.001	1.15	1.08-1.23	<.001	1.14	1.08-1.20	.003	1.12	1.05-1.20	.001
Tumor height	N/A			N/A			1.08	0.90-1.16	.06	1.13	1.05-1.21	.0014
MVD§	N/A			1.56	1.33-1.84	<.001	N/A			1.33	1.16-1.51	.001

*Result and discussion*

	Rummelt et al. (n=234) <sup>123</sup>			Foss et al. (n=116) <sup>148</sup>			Seregard et al. (n=132) <sup>104</sup>			Present study (n=134)		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
<i>Microvascular patterns</i> †												
Normal	0.60	0.19-1.91	.39	0.40	0.22-0.72	.002	0.69	0.22-1.15	.12	N/A		
Silence	0.44	0.26-0.74	.002	0.93	0.49-1.72	.80	0.79	0.15-1.42	.48	N/A		
Straight	1.49	0.36-6.08	.58	1.23	0.70-2.17	.53	0.46	-0.11-1.04	.009	N/A		
Parallel	5.41	1.70-17.23	.004	1.41	0.79-2.50	.25	0.64	0.13-1.15	.09	N/A		
Parallel with cross-links	6.72	3.21-14.09	<.001	1.79	0.92-3.45	.09	0.78	0.12-1.44	.46	N/A		
Arcs	3.32	1.51-7.27	.003	2.13	1.19-3.85	.01	0.80	0.33-1.27	.34	N/A		
Arcs with branching	5.64	2.57-12.35	<.001	2.86	1.52-3.13	.001	0.82	0.32-1.32	.42	N/A		
Loops	5.71	2.98-10.92	<.001	2.86	1.59-5.26	<.001	1.72	1.25-2.19	.02	4.14	2.14-8.00	<.001
Networks	5.08	2.92-8.82	<.001	2.78	1.52-5.00	<.001	2.14	1.61-2.67	.005	2.81	1.67-4.70	<.001

HR = Hazard ratio

† Categories: No = 0, Yes = 1

‡ Categories: Negative = 0, Positive = 1

§ Categories: Weak = 0, Moderate = 1, Heavy = 2

§ Square root-transformed single globally highest vessel count/0.313mm<sup>2</sup> area, antibody QBEND10 to the CD34 epitope

### 5.4.2 Multivariate analysis

The multivariate model was built step by step, basing every new model on the previously obtained best model. Variables that independently indicated prognosis in the final main model were: the presence of epithelioid cells (HR 1.84), large tumor size (HR 1.14 for each mm increase in tumor diameter), high MVD (HR 1.20 for each unit increase in square root transformed vessel count), presence of microvascular loops and networks (HR 1.35 for each change in category), and ezrin immunoreactivity (HR 1.71) (Table 9). The number of macrophages was modelled as a stratification variable and it improved the models statistically significantly ( $P < .001$  Chi-squared test).

**Table 9.** Final multivariate Cox proportional hazards model stratified by the number of macrophages

Variable	Regression Coefficient (SE)	Likelihood Ratio	P	HR	(95% CI)
Epithelioid cells†	0.612 (0.282)	4.7	.030	1.84	(1.06-3.21)
Largest basal diameter	0.128 (0.040)	10.4	.0012	1.14	(1.05-1.23)
Microvascular patterns*	0.304 (0.161)	3.6	.057	1.35	(0.99-1.86)
Microvascular density§	0.179 (0.079)	5.1	.024	1.20	(1.02-1.40)
Ezrin¶	0.536 (0.325)	2.9	.087	1.71	(0.90-3.23)

\* Categories: No loops = 0, Loops without networks = 1, Networks = 2

Other symbols are given in Table 8

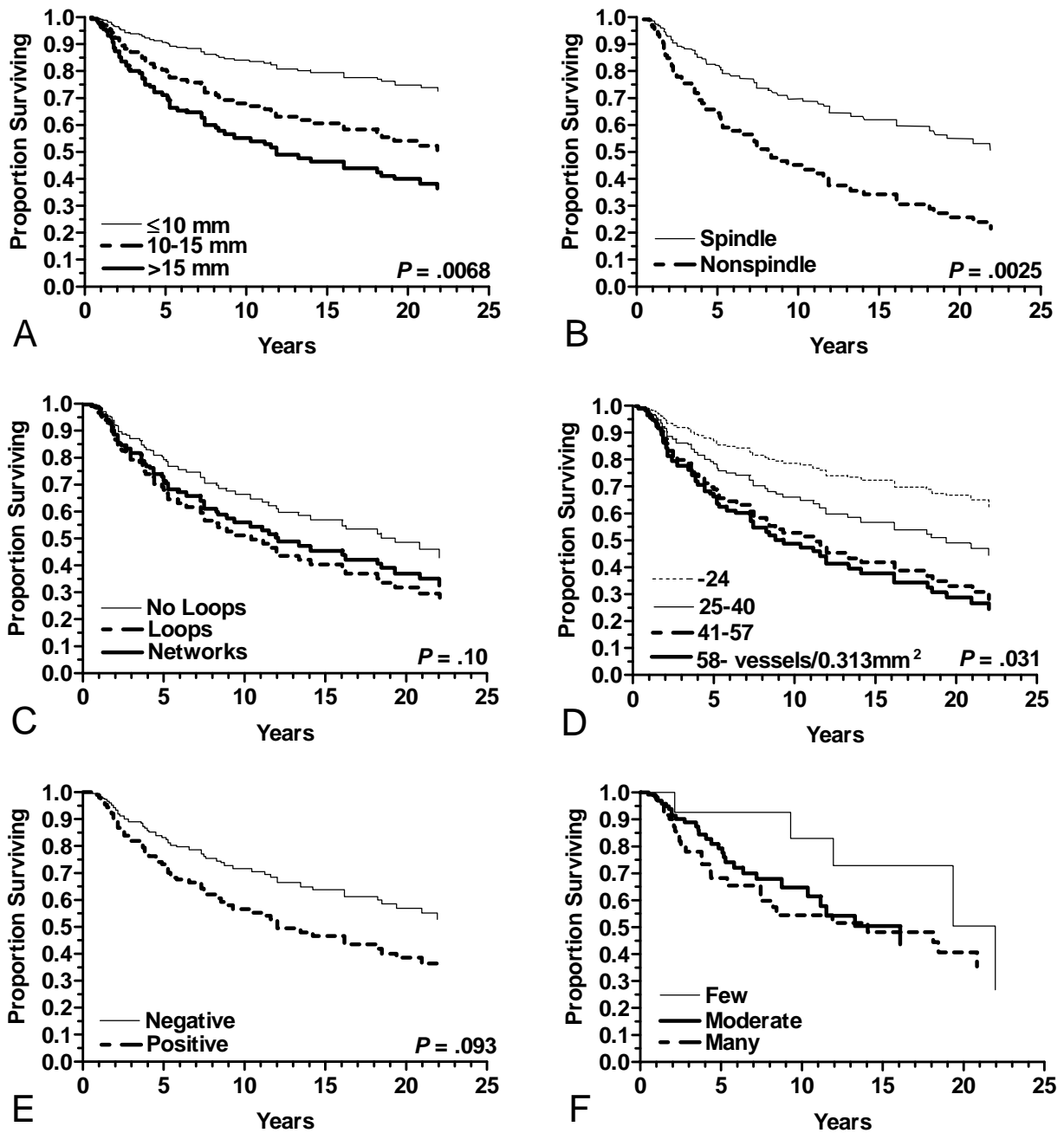
### 5.4.3 Adjusted survival

The melanoma-specific survival curves adjusted for other variables included in the final multivariate model were generated for LBD, cell type, microvascular loops and networks, MVD, and ezrin immunoreactivity. The 10-year melanoma-specific mortality increased (0.16 for small, 0.32 for medium-sized, and 0.45 for large) among large tumors even when survival was adjusted for the presence of epithelioid cells, microvascular loops and networks, MVD, and ezrin immunoreactivity (Fig. 7A; HR = 1.10,  $P = .0068$ ). The presence of epithelioid cells predicted 0.25 higher 10-year melanoma-specific mortality (0.30 for spindle cells, 0.55 for epithelioid cells) after its effect was adjusted for LBD, microvascular loops and networks, MVD, and ezrin immunoreactivity (Fig 7B; HR

= 2.25,  $P = .0025$ ). Tumors with loops or networks tended to have a higher 10-year melanoma-specific mortality than patients without loops (0.34 for absence of loops, 0.49 for presence of loops, 0.44 for presence of networks) after adjustment with LBD, cell type, MVD, ezrin immunoreactivity (Fig. 7C; HR = 1.30,  $P = .10$ ). Notably, melanomas with loops only and those with networks did not differ from each other in survival after adjustment. High microvascular density indicated increased 10-year melanoma-specific mortality (0.21 for MVD  $\leq 24$ , 0.34 for MVD 25-40, 0.47 for MVD 41-57, and 0.51 for MVD  $\geq 58$  vessels  $0.313\text{mm}^2$ ) after adjustment with LBD, cell type, and microvascular loops and networks, and ezrin immunoreactivity (Fig. 7D; HR = 1.18,  $P = .031$ ). Ezrin immunopositive tumors tended to have a higher 10-year melanoma-specific mortality than ezrin immunonegative tumors (0.29 for ezrin immunonegative, 0.45 for ezrin immunopositive) after adjustment with LBD, cell type, microvascular loops and networks, and MVD (Fig. 7E; HR = 1.70,  $P = .093$ ). The adjusted melanoma-specific mortality was 0.28 unit higher among patients with many macrophages than in patients with few macrophages (Fig. 7F; HR not available for macrophages).

The adjusted survival analysis indicated that LBD, cell type, and MVD have independent prognostic significance in the present, consecutive and unselected series of uveal melanoma patients. The adjusted survival curves allow estimation of the size of effect for each variable independently taking into account other variables. Cox regression analysis may also be applied to generate survival curves for an individual patient,<sup>254</sup> but 95% confidence intervals for such curves can not be calculated by conventional statistic programs.

**Figure 7.** The covariate-adjusted melanoma-specific survival curves generated by Cox regression analysis without stratification of macrophages (A-E). Adjusted survival curves separated for LBD (A), Cell type (B), Microvascular loops and networks (C), MVD (D), and Ezrin immunoreactivity (E). Cox regression analysis (Table 9) stratified by the Number of macrophages (F).



## **6. SUMMARY AND CONCLUSIONS**

Malignant melanoma of the uvea is a rare malignancy that has a well-documented capacity to metastasize hematogenously and to kill the patient.<sup>11</sup> Although the mortality of uveal melanoma has remained fairly constant for decades, the natural history of choroidal and ciliary body melanomas is not fully understood.<sup>93,95</sup> Important clinical prognostic factors for death from uveal melanoma are the size of the tumor, the location of tumor, and extrascleral tumor extension.<sup>97,255</sup> The onset of metastatic disease is an unfortunate occurrence and the patient's life expectancy is then only several months to a few years.<sup>29,30</sup> However, a significant portion of patients with uveal melanoma may survive even decades without evidence of metastasis. This clinicopathologic study was undertaken to improve understanding of factors that are associated with the development of uveal melanoma metastasis and which have an adverse effect on the clinical course of the disease. Factors related to tumor stroma (microvascular patterns and MVD), host response (macrophages), and tumor cells (ezrin) were investigated.

Most researchers wish that the results of their analyses could be generalized as widely as possible. A basic rule in the study design and generalizing statistical results is that the sample must represent the true underlying population. This is best ensured by enrolling consecutive and population based samples as in the current study, and if not possible, by random sampling. With minimal loss of follow-up data, the outcome of 167 Finnish patients with uveal melanoma showed that metastasis was their leading cause of death even 15 to 20 years after the removal of the eye. Because this series is exceptionally well documented, causes of death are well ascertained, and the inclusion rate is higher than in vast majority of studies, there is a reason to believe that, in spite of the relatively small number of tumors, the results should be well representative of the entire population of patients with uveal melanoma. In this regard, the present study compares favorably with many much larger series based on tertiary referral centre populations and cancer registry data.

Microcirculation is a prerequisite for tumor growth and metastasis.<sup>256</sup> Tumor vessels differ from vessels in nonneoplastic tissue; they are actively proliferating and remodelling.<sup>158</sup> The knowledge of the prognostic significance of microvascular factors has rapidly increased also in uveal melanoma.<sup>102,104,105,141,144,147,152</sup> The presence of specific microvascular patterns,<sup>104,141,144,147,152</sup> as well as high MVD have been associated with survival of uveal melanoma,<sup>105</sup> but controversies regarding their relation and even their existence and genesis have been prominent.<sup>148,157,165</sup> This controversy is about to stimulate research in many areas of tumor biology and several types of cancer. The current

study confirmed the relationship between presence of microvascular loops and networks and high melanoma-specific mortality. Using multivariate analysis, however, another microvessel parameter, MVD, showed independent prognostic significance in addition to loops and networks. In univariate analysis, high MVD was associated with presence of microvascular loops and networks, but their closer interrelationship remained unsolved and must be addressed on a qualitative level in future studies to find out what microvascular patterns are associated with “hot spots” of high MVD. Both MVD and microvascular patterns were associated with nonspindle cell type, which might suggest an interplay with an aggressive cell type and microvessels.

In addition to microvessels, tumor-infiltrating immune cells, such as macrophages and lymphocytes constitute a prominent stromal component within tumors.<sup>110,257</sup> The infiltration of macrophages has, however, been underreported in uveal melanoma: little attention has been paid on the function of macrophages and their potential clinical implications. The present study indicated that the infiltration of macrophages varies from low to high between uveal melanomas. Moreover, two morphological types, round and dendritic, of tumor-infiltrating macrophages were identified. The dense infiltration of macrophages was associated with decreased survival and high MVD, suggesting an active role in modulation of microvessels. The morphological type of macrophages did not show any prognostic significance. Tumor-infiltrating macrophages must also be remembered as a source of cross-reactivity.<sup>112,172,175</sup> They have been recently shown to express epidermal growth factor receptor and a cytoplasmic form of estrogen receptor in uveal melanoma, initially thought to reside in tumor cells.<sup>112,175</sup>

Uveal melanoma cells immunoreactive with mAb to ezrin, the ultimate function of which in tumors is unknown, were more often present in tumors that metastasized rapidly than in tumors that did not metastasize or metastasized later. The immunoreactivity of ezrin retained a borderline independent prognostic significance in multivariate analysis after adjusting for the presence of microvascular loops and networks, MVD, the presence of epithelioid cells, and LBD. The immunoreactivity of ezrin was associated with MVD and high numbers of tumor-infiltrating macrophages. This study is not able to answer, whether any biological relationship exists between MVD, ezrin, and macrophages, and an experimental approach will be needed. Macrophages are multifunctional cells that secrete e.g. HGF that modulates microvessels, and ezrin is a downstream target of HGF.<sup>250,251,258</sup> Further studies, in particular experimental ones, are needed to investigate the tumor biology of uveal melanoma. Host factors in particular have not been studied enough, and why an occasional patient with uveal melanoma of high-risk features may survive for a long time, remains a mystery.

## **ACKNOWLEDGEMENTS**

This study was carried out during the years 1996-2001 in the Ophthalmic Pathology Laboratory, Department of Ophthalmology, Helsinki University Central Hospital, Finland. I am sincerely grateful to Professor Leila Laatikainen, M.D., Head of the Department of Ophthalmology, for providing the good research facilities. I equally want to thank Professor Ahti Tarkkanen, M.D., the former Head of the Department of Ophthalmology, for continuous interest in my work. His profound and wide knowledge of ophthalmic research is admirable.

I am most grateful to my excellent supervisor, Docent Tero Kivelä, M.D., for introducing me to the world of ophthalmic pathology. It has been a privilege to work under his encouraging and tireless guidance and learn how to turn intriguing ideas into practice.

I am sincerely grateful for Docent Paula Summanen, M.D., for kind help during these years and her ability to see bright sides of all the things. I wish to thank Docent Lauri Merenmies, M.D., for educational and interesting discussions in Ophthalmic Pathology Laboratory. Docent Teppo Lyly, M.D., the Head of Finnish Cancer Registry, is gratefully acknowledged for help in collecting follow-up data. My co-workers, Professor Antti Vaheri, M.D., and Docent Olli Carpén, M.D., in Departments of Virology and Pathology, University of Helsinki, Finland, are gratefully acknowledged for providing their expertise in ezrin research.

I want to thank, in particular, my colleague and friend Sebastian Eskelin, M.D., for time that we shared in laboratory, especially for multiple topics of discussion from everyday life to science, which made those days unforgettable. It has also been a pleasure to collaborate with new talented members of the melanoma group: Seppo Tuomaala, M.D, to whom I am also grateful for the time that we have spent together in clinical practice, Päivi Toivonen, M.D., Ilkka Puusaari, M.D., and Emma Kujala, M.D. Mrs. Marjatta Koikkalainen, Mrs. Pirkko Yliharju, and Ms. Sirkka Elomaa are gratefully acknowledged for their expert technical assistance during this work.

I am also grateful to Mrs. Marja-Leena Ylivakkuri, M.A., of her help in gathering references, and to Pekka Rikonen, M.D., M.A., for the flexible revision of the English language of this thesis.

I would like to thank my colleagues Pasi Allinen, M.D., Tapio Stenborg, M.D., and Jouko Larinkari, M.D., for teaching me clinical ophthalmology in Kotka, and all my friends and colleagues at the

## *Acknowledgements*

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Department of Ophthalmology, Helsinki. I wish especially to thank Päivi Ranta, M.D., Mika Harju, M.D., Jouko Rinta-Rahko, M.D., Maria Rosenberg, M.D., and Jukka Moilanen, M.D for creating a friendly atmosphere. I warmly thank all my friends outside of ophthalmic clinic for long-lasting friendship.

My parents, Jukka and Mirja, deserve my deepest gratitude for their constant support and encouragement throughout my life. I also wish to thank my sister, Tuuli, and two brothers, Tuomo and Tuukka for interest in my project. Last, but definitely not least, I want thank dear Laura for everlasting love and patience, not only needed to tolerate this work.

This study was financially supported by grants from the Helsinki University Central Hospital (TYH8218 and TYH 0026). Personal support was provided by the Finnish Medical Foundation, the Eye Foundation, the Eye and Tissue Bank Foundation, the Friends of Blind, the Ida Montin Foundation, the Instrumentarium Science Foundation, the Cancer Foundation, the Biomedicum Helsinki Foundation, and the Research Foundation of Orion Corporation.

Helsinki, May 2001

## REFERENCES

1. Borland, R, Donaghue, N, Hill, D. Illnesses that Australians most feared in 1986 and 1993. *Aust J Public Health*. 1994;18:366-9.
2. Folberg, R, Hendrix, MJC, Maniotis, AJ. Vasculogenic mimicry and tumor angiogenesis. *Am J Pathol*. 2000;156:361-81.
3. Zimmerman, L. E. Malignant melanoma. In: Spencer, W. H., (ed). *Ophthalmic Pathology*. 3<sup>rd</sup> Ed. Philadelphia: W.B. Saunders Company; 1986:2072-2139.
4. Hungerford, J. Uveal melanoma. *Eur J Cancer*. 1993;29A:1365-8.
5. Egan, KM, Seddon, JM, Glynn, RJ, Gragoudas, ES, Albert, DM. Epidemiologic aspects of uveal melanoma. *Surv Ophthalmol*. 1988;32:239-51.
6. Mckee, PH. *Pathology of the Skin*. Philadelphia: J.B. Lippincott Company; 1989
7. Platz, A, Ringborg, U, Hansson, J. Hereditary cutaneous melanoma. *Semin Cancer Biol*. 2000;10:319-26.
8. Singh, AD, Shields, CL, De Potter P., Shields, JA, Trock, B, Cater, J, Pastore, D. Familial uveal melanoma. Clinical observations on 56 patients. *Arch Ophthalmol*. 1996;114:392-9.
9. Clarijs, R, Schalkwijk, L, Ruiter, DJ, de Waal, RMW. Lack of lymphangiogenesis despite coexpression of VEGF-C and its receptor flt-4 in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:1422-8.
10. Einhorn, LH, Burgess, MA, Gottlieb, JA. Metastatic patterns of choroidal melanoma. *Cancer*. 1974;34:1001-4.
11. McLean, IW. The biology of haematogenous metastasis in human uveal malignant melanoma. *Virchows Arch A Pathol Anat Histopathol*. 1993;422:433-7.
12. Eskelin, S, Pyrhönen, S, Summanen, P, Prause, JU, Kivelä, T. Screening for metastatic uveal melanoma revisited. *Cancer*. 1999;85:1151-9.
13. Bittner, M, Meltzer, P, Chen, Y, Jiang, Y, Seftor, E, Hendrix, MJ, Radmacher, M, Simon, R, Yakhini, Z, Ben-Dor, A, Sampas, N, Dougherty, E, Wang, E, Marincola, F, Gooden, C, Lueders, J, Glatfelter, A, Pollock, P, Carpten, J, Gillanders, E, Leja, D, Dietrich, K, Beaudry, C, Berens, M, Alberts, D, Sondak, V, Hayward, N, Trent, J. Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature*. 2000;406:536-40.
14. Cree, IA. Cell cycle and melanoma - two different tumours from the same cell type. *J Pathol*. 2000;191:112-4.
15. Naus, NC, Zuidervaart, W, Rayman, N, Slater, R, van Drunen, E, Ksander, B, Luyten, GP, Klein, A. Mutation analysis of the *PTEN* gene in uveal melanoma cell lines. *Int J Cancer*. 2000;87:151-3.
16. Jensen, OA. Malignant melanoma of the iris. A 25-year analysis of Danish cases. *Eur J Ophthalmol*. 1993;3:181-8.

17. Shields, CL, Shield, JA, Materin, M, Gershenbaum, E, Singh, AD, Smith, A. Iris melanoma. Risk factors for metastasis in 169 consecutive series. *Ophthalmology*. 2001;108:172-8.
18. Chowers, I, Folberg, R, Livni, N, Pe'er, J. Comparison of microcirculation patterns and MIB-1 immunoreactivity in iris and posterior uveal melanoma. *Ophthalmology*. 2001;108:367-71.
19. Finger, PT. Radiation therapy for choroidal melanoma. *Surv Ophthalmol*. 1997;42:215-32.
20. Shields, JA, Shields, CL, De Potter, P, Singh, AD. Diagnosis and treatment of uveal melanoma. *Sem Oncol*. 1996;23:763-7.
21. Seddon, JM, Gragoudas, ES, Albert, DM, Hsieh, CC, Polivogianis, L, Friedenber, GR. Comparison of survival rates for patients with uveal melanoma after treatment with proton beam irradiation or enucleation. *Am J Ophthalmol*. 1985;99:282-90.
22. Guthoff, R, Frischmuth, J, Jensen, OA, Bjerrum, K, Prause, JU. Das Aderhautmelanom. Eine retrospektive randomisierte Vergleichsstudie Ruthenium-Bestrahlung vs ENUKLEATION. *Klin Monatsbl Augenheilkd*. 1992;200:257-61.
23. Augsburger, JJ, Correa, ZM, Freire, J, Brady, LW. Long-term survival in choroidal and ciliary body melanoma after enucleation versus plaque radiation therapy. *Ophthalmology*. 1998;105:1670-8.
24. Char, DH, Kroll, SM, Castro, J. Ten-year follow-up of helium ion therapy for uveal melanoma. *Am J Ophthalmol*. 1998;125:81-9.
25. Seregard, S. Long-term survival after ruthenium plaque radiotherapy for uveal melanoma. A meta-analysis of studies including 1,066 patients. *Acta Ophthalmol Scand*. 1999;77:414-7.
26. Augsburger, JJ, Schneider, S, Freire, J, Brady, LW. Survival following enucleation versus plaque radiotherapy in statistically matched subgroups of patients with choroidal melanomas: results in patients treated between 1980 and 1987. *Graefes Arch Clin Exp Ophthalmol*. 1999;237:558-67.
27. Wenjun, L, Gragoudas, ES, Egan, KM. Metastatic melanoma death rates by anatomic site after proton beam irradiation for uveal melanoma. *Arch Ophthalmol*. 2000;118:1066-70.
28. Eskelin, S, Pyrhönen, S, Summanen, P, Hahka-Kemppinen, M, Kivelä, T. Tumor doubling times in metastatic malignant melanoma of the uvea: tumor progression before and after treatment. *Ophthalmology*. 2000;107:1443-9.
29. Gragoudas, ES, Egan, KM, Seddon, JM, Glynn, RJ, Walsh, SM, Finn, SM, Munzenrider, JE, Spar, MD. Survival of patients with metastases from uveal melanoma. *Ophthalmology*. 1991;98:383-9.
30. Albert, DM, Niffenegger, AS, Willson, JK. Treatment of metastatic uveal melanoma: review and recommendations. *Surv Ophthalmol*. 1992;36:429-38.
31. Pyrhönen, S. The treatment of metastatic uveal melanoma. *Eur J Cancer (Suppl)*. 1998;34:S27-S30.
32. Manschot, WA, Lee, WR, van Strik R. Uveal melanoma: updated considerations on current management modalities. *Int Ophthalmol*. 1996;19:203-9.

33. Lee, S-B, Au Eong, K-G, Saw, S-M, Chan, T-K, Lee, H-P. Eye cancer incidence in Singapore. *Br J Ophthalmol*. 2000;84:767-70.
34. Raivio, I. Uveal melanoma in Finland. An epidemiological, clinical, histological and prognostic study. *Acta Ophthalmol (Suppl)*. 1977;133:1-64.
35. Jensen, OA. Malignant melanomas of the uvea in Denmark 1943-1952. A clinical, histopathological, and prognostic study. *Acta Ophthalmol (Suppl)*. 1963;75:1-220.
36. Abrahamsson, M. Malignant melanoma of the choroid and the ciliary body 1956-1975 in Halland and Gothenburg. Incidence, histopathology and prognosis. *Acta Ophthalmol (Suppl)*. 1983;61:600-10.
37. Holly, EA, Aston, DA, Char, DH, Kristiansen, JJ, Ahn, DK. Uveal melanoma in relation to ultraviolet light exposure and host factors. *Cancer Res*. 1990;50:5773-7.
38. Regan, S, Judge, HE, Gragoudas, ES, Egan, KM. Iris color as a prognostic factor in ocular melanoma. *Arch Ophthalmol*. 1999;117:811-4.
39. Li, W, Jugde, H, Gragoudas, ES, Seddon, JM, Egan, KM. Patterns of tumor initiation in choroidal melanoma. *Cancer Res*. 2000;60:3757-60.
40. Shields, CL, Shields, JA, De Potter, P, Cater, J, Tardio, D, Barrett, J. Diffuse choroidal melanoma. Clinical features predictive of metastasis. *Arch Ophthalmol*. 1996;114:956-63.
41. Shields, JA, Shields, CL, Donoso, LA. Management of posterior uveal melanoma. *Surv Ophthalmol*. 1991;36:161-95.
42. Kivelä, T & Summanen, P. Retinoinvasive malignant melanoma of the uvea. *Br J Ophthalmol*. 1997;81:691-7.
43. Gunduz, K, Shields, JA, Shields, CL, Eagle, RC. Cutaneous melanoma metastatic to the vitreous cavity. *Ophthalmology*. 1998;105:600-5.
44. Singh, AD, De Potter P., Fijal, BA, Shields, CL, Shields, JA, Elston, RC. Lifetime prevalence of uveal melanoma in white patients with oculo(dermal) melanocytosis. *Ophthalmology*. 1998;105:195-8.
45. Hammer, H, Oláh, J, Tóth-Molnár, E. Dysplastic nevi are a risk factor for uveal melanoma. *Eur J Ophthalmol*. 1996;6:472-4.
46. van Hees, CL, de Boer, A, Jager, MJ, Bleeker, JC, Kakebeeke, HM, Crijns, MB, Vandenbroucke, JP, Bergman, W. Are atypical nevi a risk factor for uveal melanoma? A case-control study. *J Invest Dermatol*. 1994;103:202-5.
47. Egan, KM, Walsh, SM, Seddon, JM, Gragoudas, ES. An evaluation of the influence of reproductive factors on the risk of metastases from uveal melanoma. *Ophthalmology*. 1993;100:1160-5.
48. Holly, EA, Aston, DA, Ahn, DK, Kristiansen, JJ, Char, DH. Uveal melanoma, hormonal and reproductive factors in women. *Cancer Res*. 1991;51:1370-2.

49. Prescher, G, Bornfeld, N, Becher, R. Nonrandom chromosomal abnormalities in primary uveal melanoma. *J Natl Cancer Inst.* 1990;82:1765-9.
50. Sisley, K, Cottam, DW, Rennie, IG, Parsons, MA, Potter, AM, Potter, CW, Rees, RC. Non-random abnormalities of chromosomes 3, 6, and 8 associated with posterior uveal melanoma. *Genes Chromosomes Cancer.* 1992;5:197-200.
51. Aalto, Y, Eriksson, L, Seregard, S, Larsson, O, Knuutila, S. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosomes 1, 6, or 8 in metastasizing primary uveal melanoma. *Invest Ophthalmol Vis Sci.* 2001;42:313-7.
52. van Hees, CL, Jager, MJ, Bleeker, JC, Kemme, H, Bergman, W. Occurrence of cutaneous and uveal melanoma in patients with uveal melanoma and their first degree relatives. *Melanoma Res.* 1998;8:175-80.
53. Holden, R & Damato, BE. Preventable delays in the treatment of intraocular melanoma in the UK. *Eye.* 1996;10:127-9.
54. Ah-Fat, FG & Damato, BE. Delays in the diagnosis of uveal melanoma and effect on treatment. *Eye.* 1998;12:789-91.
55. Eskelin, S & Kivelä, T. Time from first health care contact to treatment of malignant uveal melanoma in Finland. *Ophthalmic Res (Suppl).* 2000;32:S134
56. Collaborative Ocular Melanoma Study Group. Accuracy of diagnosis of choroidal melanomas in the Collaborative Ocular Melanoma Study. *Arch Ophthalmol.* 1990;108:1268-73.
57. Shields, CL, Shields, JA, Kiratli, H, De Potter, P, Cater, JR. Risk factors for growth and metastasis of small choroidal melanocytic lesions. *Ophthalmology.* 1995;102:1351-61.
58. Maberly, DA, Pavlin, CJ, McGowan, HD, Foster, FS, Simpson, ER. Ultrasound biomicroscopic imaging of the anterior aspect of peripheral choroidal melanomas. *Am J Ophthalmol.* 1997;123:506-14.
59. Folberg, R, Augsburger, JJ, Gamel, JW, Shields, JA, Lang, WR. Fine-needle aspirates of uveal melanomas and prognosis. *Am J Ophthalmol.* 1985;100:654-7.
60. Char, DH, Kroll, SM, Stoloff, A, Crawford, JB, Miller, TR, Howes, EJ, Crawford, P. Cytomorphometry of uveal melanomas: fine needle aspiration biopsy versus standard histology. *Trans Am Ophthalmol Soc.* 1989;87:197-210.
61. Rennie, IG. The Ashton Lecture. Uveal melanoma: the past, the present and the future. *Eye.* 1997;11:255-64.
62. Collaborative Ocular Melanoma Study Group. The Collaborative Ocular Melanoma Study (COMS) randomized trial of pre-enucleation radiation of large choroidal melanoma III: local complications and observations following enucleation. *Am J Ophthalmol.* 1998;126:362-72.
63. Straatsma, BR, Fine, SL, Earle, JD, Hawkins, BS, Diener-West, M, McLaughlin, JA. Enucleation versus plaque irradiation for choroidal melanoma. *Ophthalmology.* 1988;95:1000-4.

64. Summanen, P, Immonen, I, Kivelä, T, Tommila, P, Heikkonen, J, Tarkkanen, A. Visual outcome of eyes with malignant melanoma of the uvea after ruthenium plaque radiotherapy. *Ophthalmic Surg Lasers*. 1995;26:449-60.
65. Shields, CL, Shields, JA, Cater, J, Gunduz, K, Miyamoto, C, Micaily, B, Brady, LW. Plaque radiotherapy for uveal melanoma. Long-term visual outcome in 1106 consecutive patients. *Arch Ophthalmol*. 2000;118:1219-28.
66. Gradoudas, ES, Lane, AM, Regan, S, Li, W, Judge, HE, Munzenreider, JE, Seddon, JM, Egan, KM. A randomised controlled trial of varying radiation doses in the treatment of choroidal melanoma. *Arch Ophthalmol*. 2000;118:773-8.
67. Summanen, P, Immonen, I, Kivelä, T, Tommila, P, Heikkonen, J, Tarkkanen, A. Radiation related complications after ruthenium plaque radiotherapy of uveal melanoma. *Br J Ophthalmol*. 1996;80:732-9.
68. Harbour, JW, Char, DH, Kroll, S, Quivey, JM, Castro, J. Metastatic risk for distinct patterns of postirradiation local recurrence of posterior uveal melanoma. *Ophthalmology*. 1997;104:1785-92.
69. Egan, KM, Ryan, LM, Gragoudas, ES. Survival implications of enucleation after definitive radiotherapy for choroidal melanoma: an example of regression on time-dependent covariates. *Arch Ophthalmol*. 1998;116:366-70.
70. Luyten, GP, Mooy, CM, Eijkenboom, WM, Stijnen, T, Hellemons, LP, Luider, TM, De Jong, PTVM. No demonstrated effect of pre-enucleation irradiation on survival of patients with uveal melanoma. *Am J Ophthalmol*. 1995;119:786-91.
71. Collaborative Ocular Melanoma Study Group. The Collaborative Ocular Melanoma Study (COMS) randomized trial of pre-enucleation radiation of large choroidal melanoma II: initial mortality findings. *Am J Ophthalmol*. 1998;125:779-96.
72. Oosterhuis, JA, Journée-de Korver, HG, Kakebeeke-Kemme, HM, Bleeker, JC. Transpupillary thermotherapy in choroidal melanomas. *Arch Ophthalmol*. 1995;113:315-21.
73. Shields, CL, Shields, JA, De Potter, P, Kheterpal, S. Transpupillary thermotherapy in the management of choroidal melanoma. *Ophthalmology*. 1996;103:1642-50.
74. Oosterhuis, JA, Journée-de Korver, HG, Keunen, JE. Transpupillary thermotherapy: results in 50 patients with choroidal melanoma. *Arch Ophthalmol*. 1998;116:157-62.
75. Damato, B, Groenewald, C, McGalliard, J, Wong, D. Endoresection of choroidal melanoma. *Br J Ophthalmol*. 1998;82:213-8.
76. Mueller, AJ, Talies, S, Schaller, UC, Horstmann, G, Wowra, B, Kampik, A. Stereotactic radiosurgery of large uveal melanomas with the gamma-knife. *Ophthalmology*. 2000;107:1381-8.
77. Favilla, I, Favilla, ML, Gosbell, AD, Barry, WR, Ellims, P, Hill, JS, Byrne, JR. Photodynamic therapy: a 5-year study of its effectiveness in the treatment of posterior uveal melanoma, and evaluation of haematoporphyrin uptake and photocytotoxicity of melanoma cells in tissue culture. *Melanoma Res*. 1995;5:355-64.
78. Pyrhönen, S, Hahka-Kemppainen, M, Muhonen, T. A promising interferon plus four-drug chemotherapy regimen for metastatic melanoma. *J Clin Oncol*. 1992;10:1919-26.

79. Leyvraz, S, Spataro, V, Bauer, J, Pampallona, S, Salmon, RJ, Dorval, T, Meuli, R, Gillet, M, Lejeune, F, Zografos, L. Treatment of ocular melanoma metastatic to the liver by hepatic arterial chemotherapy. *J Clin Oncol.* 1997;15:2589-95.
80. Fournier, GA, Albert, DM, Arrigg, CA, Cohen, AM, Lamping, KA, Seddon, JM. Resection of solitary metastasis. Approach to palliative treatment of hepatic involvement with choroidal melanoma. *Arch Ophthalmol.* 1984;102:80-2.
81. Salmon, RJ, Levy, C, Plancher, C, Dorval, T, Desjardins, L, Leyvraz, S, Pouillart, P, Schlienger, P, Servois, V, Asselain, B. Treatment of liver metastases from uveal melanoma by combined surgery-chemotherapy. *Eur J Surg Oncol.* 1998;24:127-30.
82. Aoyama, T, Mastrangelo, MJ, Berd, D, Nathan, FE, Shields, CL, Shields, JA, Rosato, EL, Rosato, FE, Sato, T. Protracted survival after resection of metastatic uveal melanoma. *Cancer.* 2000;89:1561-8.
83. Tallberg, T, Uusitalo, R, Sarna, S, Seregard, S, Werschnik, C. Improvement of the recurrence-free interval using biological adjuvant therapy in uveal melanoma. *Anticancer Res.* 2000;20:1969-76.
84. Gamel, JW, McLean, IW, McCurdy, JB. Biologic distinctions between cure and time to death in 2892 patients with intraocular melanoma. *Cancer.* 1993;71:2299-305.
85. Shammass, HF & Blodi, FC. Prognostic factors in choroidal and ciliary body melanomas. *Arch Ophthalmol.* 1977;95:63-9.
86. Seddon, JM, Albert, DM, Lavin, PT, Robinson, N. A prognostic factor study of disease-free interval and survival following enucleation for uveal melanoma. *Arch Ophthalmol.* 1983;101:1894-9.
87. Midena, E, de Belvis, V, Dei Tos, AP, Antonini, C. Isolated brain metastasis of malignant choroidal melanoma 27 years after enucleation. *Arch Ophthalmol.* 1999;117:1553-6.
88. Shields, JA, Augsburger, JJ, Donoso, LA, Bernardino, VJ, Portenar, M. Hepatic metastasis and orbital recurrence of uveal melanoma after 42 years. *Am J Ophthalmol.* 1985;100:666-8.
89. Char, DH. Metastatic choroidal melanoma. *Am J Ophthalmol.* 1978;86:76-80.
90. Mäkitie, T & Kivelä, T. Cardiac metastasis from uveal melanoma. *Arch Ophthalmol.* 2001;119:139-40.
91. Collaborative Ocular Melanoma Study Group. Assessment of metastatic disease status at death in 435 patients with large choroidal melanoma in the collaborative ocular melanoma study (COMS). *Arch Ophthalmol.* 2001;119:670-6.
92. Zimmerman, LE, McLean, IW, Foster, WD. Does enucleation of the eye containing a malignant melanoma prevent or accelerate the dissemination of tumour cells. *Br J Ophthalmol.* 1978;62:420-5.
93. McLean, IW, Foster, WD, Zimmerman, LE, Martin, DG. Inferred natural history of uveal melanoma. *Invest Ophthalmol Vis Sci.* 1980;19:760-70.

94. Zimmerman, LE & McLean, IW. Metastatic disease from untreated uveal melanomas. *Am J Ophthalmol.* 1979;88:524-34.
95. Manschot, WA. The natural history of uveal melanomas and its therapeutic consequences. *Doc Ophthalmol.* 1980;50:83-99.
96. Manschot, WA & van Strik R. Is irradiation a justifiable treatment of choroidal melanoma? Analysis of published results. *Br J Ophthalmol.* 1987;71:348-52.
97. Augsburger, JJ & Gamel, JW. Clinical prognostic factors in patients with posterior uveal malignant melanoma. *Cancer.* 1990;66:1596-600.
98. Kroll, S, Char, DH, Quivey, J, Castro, J. A comparison of cause-specific melanoma mortality and all-cause mortality in survival analyses after radiation treatment for uveal melanoma. *Ophthalmology.* 1998;105:2035-45.
99. Barr, CC, McLean, IW, Zimmerman, LE. Uveal melanoma in children and adolescents. *Arch Ophthalmol.* 1981;99:2133-6.
100. Singh, AD, Shields, CL, Shields, JA, Sato, T. Uveal melanoma in young patients. *Arch Ophthalmol.* 2000;118:918-23.
101. Egan, KM, Quinn, JL, Gragoudas, ES. Childbearing history associated with improved survival in choroidal melanoma. *Arch Ophthalmol.* 1999;117:939-42.
102. Folberg, R, Rummelt, V, Parys-van Genderdeuren, R, Hwang, T, Woolson, RF, Pe'er, J, Gruman, LM. The prognostic value of tumor blood vessel morphology in primary uveal melanoma. *Ophthalmology.* 1993;100:1389-98.
103. Coleman, K, Baak, JP, Van Diest, P, Mullaney, J, Farrell, M, Fenton, M. Prognostic factors following enucleation of 111 uveal melanomas. *Br J Ophthalmol.* 1993;77:688-92.
104. Seregard, S, Spångberg, B, Juul, C, Oskarsson, M. Prognostic accuracy of the mean of the largest nucleoli, vascular patterns, and PC-10 in posterior uveal melanoma. *Ophthalmology.* 1998;105:485-91.
105. Foss, AJE, Alexander, RA, Jefferies, LW, Hungerford, JL, Harris, AL, Lightman, S. Microvessel count predicts survival in uveal melanoma. *Cancer Res.* 1996;56:2900-3.
106. Anastassiou, G, Schilling, H, Djakovic, S, Bornfeld, N. Expression of VLA-2, VLA-3, and  $\alpha_v$  integrin receptors in uveal melanoma: associations with microvascular architecture of the tumour and prognostic value. *Br J Ophthalmol.* 2000;84:899-902.
107. Seddon, JM, MacLaughlin, DT, Albert, DM, Gragoudas, ES, Ference, M. Uveal melanomas presenting during pregnancy and the investigation of oestrogen receptors in melanomas. *Br J Ophthalmol.* 1982;66:695-704.
108. Lambert, SR, Char, DH, Howes, EJ, Crawford, JB, Wells, J. Spontaneous regression of a choroidal melanoma. *Arch Ophthalmol.* 1986;104:732-4.
109. Durie, FH, Campbell, AM, Lee, WR, Damato, BE. Analysis of lymphocytic infiltration in uveal melanoma. *Invest Ophthalmol Vis Sci.* 1990;31:2106-10.

110. Tobal, K, Deuble, K, McCartney, A, Lightman, S. Characterization of cellular infiltration in choroidal melanoma. *Melanoma Res.* 1993;3:63-5.
111. Collaborative Ocular Melanoma Study Group. Histopathologic characteristics of uveal melanomas in eyes enucleated from the Collaborative Ocular Melanoma Study. *Am J Ophthalmol.* 1998;125:745-66.
112. Mäkitie, T, Tarkkanen, A, Kivelä, T. Comparative immunohistochemical oestrogen receptor analysis in primary and metastatic uveal melanoma. *Graefes Arch Clin Exp Ophthalmol.* 1998;236:415-9.
113. Sunderkotter, C, Steinbrink, K, Goebeler, M, Bhardwaj, R, Sorg, C. Macrophages and angiogenesis. *J Leukoc Biol.* 1994;55:410-22.
114. Mantovani, A. Tumor-associated macrophages in neoplastic progression: a paradigm for the in vivo function of chemokines. *Lab Invest.* 1994;71:5-16.
115. de Waard-Siebinga, I, Hilders, CG, Hansen, BE, van Delft, JL, Jager, MJ. HLA expression and tumor-infiltrating immune cells in uveal melanoma. *Graefes Arch Clin Exp Ophthalmol.* 1996;234:34-42.
116. Ma, D & Niederkorn, JY. Transforming growth factor-beta down-regulates major histocompatibility complex class I antigen expression and increases the susceptibility of uveal melanoma cells to natural killer cell-mediated cytotoxicity. *Immunology.* 1995;86:263-9.
117. Blom, D-JR, Luyten, GP, Mooy, C, Kerkvliet, S, Zwinderman, AH, Jager, MJ. Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. *Invest Ophthalmol Vis Sci.* 1997;38:1865-72.
118. Jager, MJ, Völker-Dieben, HJ, de Wolff-Rouendaal, D, Kakebeeke-Kemme, H, D'Amato, J. Possible relation between HLA and ABO type and prognosis of uveal melanoma. *Doc Ophthalmol.* 1992;82:43-7.
119. Blom, DJ, Mooy, CM, Luyten, GP, Kerkvliet, S, Ouwkerk, I, Zwinderman, AH, Schrier, PI, Jager, MJ. Inverse correlation between expression of HLA-B and c-myc in uveal melanoma. *J Pathol.* 1997;181:75-9.
120. Tóth-Molnár, E, Hammer, H, Oláh, J. Cutaneous dysplastic naevi in uveal melanoma patients: markers for prognosis? *Melanoma Res.* 2000;10:36-9.
121. Egan, KM, Gragoudas, ES, Seddon, JM, Walsh, SM. Smoking and the risk of early metastases from uveal melanoma. *Ophthalmology.* 1992;99:537-41.
122. Seregard, S & Kock, E. Prognostic indicators following enucleation for posterior uveal melanoma. A multivariate analysis of long-term survival with minimized loss to follow-up. *Acta Ophthalmol Scand.* 1995;73:340-4.
123. Rummelt, V, Folberg, R, Woolson, RF, Hwang, T, Pe'er, J. Relation between the microcirculation architecture and the aggressive behavior of ciliary body melanomas. *Ophthalmology.* 1995;102:844-51.

124. Sisley, K, Rennie, IG, Parsons, MA, Jacques, R, Hammond, DW, Bell, SM, Potter, AM, Rees, RC. Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997;19:22-8.
125. Sisley, K, Parsons, MA, Garnham, J, Potter, AM, Curtis, D, Rees, RC, Rennie, IG. Association of specific chromosome alterations with tumour phenotype in posterior uveal melanoma. *Br J Cancer*. 2000;82:330-8.
126. Dithmar, S, Diaz, CE, Grossniklaus, HE. Intraocular melanoma spread to regional lymph nodes: report of two cases. *Retina*. 2000;20:76-9.
127. Affeldt, JC, Minckler, DS, Azen, SP, Yeh, L. Prognosis in uveal melanoma with extrascleral extension. *Arch Ophthalmol*. 1980;98:1975-9.
128. Pach, JM, Robertson, DM, Taney, BS, Martin, JA, Campbell, RJ, O'Brien, PC. Prognostic factors in choroidal and ciliary body melanomas with extrascleral extension. *Am J Ophthalmol*. 1986;101:325-31.
129. Gunduz, K, Shields, CL, Shields, JA, Cater, J, Brady, LW. Plaque radiotherapy for management of ciliary body and choroidal melanoma with extraocular extension. *Am J Ophthalmol*. 2000;130:97-102.
130. McLean, IW, Foster, WD, Zimmerman, LE. Uveal melanoma: location, size, cell type, and enucleation as risk factors in metastasis. *Hum Pathol*. 1982;13:123-32.
131. Miller, MV, Herdson, PB, Hitchcock, GC. Malignant melanoma of the uveal tract - a review of the Auckland experience. *Pathology*. 1985;17:281-4.
132. McLean, MJ, Foster, WD, Zimmerman, LE. Prognostic factors in small malignant melanomas of choroid and ciliary body. *Arch Ophthalmol*. 1977;95:48-58.
133. Mehaffey, MG, Folberg, R, Meyer, M, Bentler, SE, Hwang, T, Woolson, R, Moore, KC. Relative importance of quantifying area and vascular patterns in uveal melanomas. *Am J Ophthalmol*. 1997;123:798-809.
134. Collaborative Ocular Melanoma Study Group. Mortality in patients with small choroidal melanoma. *Arch Ophthalmol*. 1997;115:886-93.
135. Callender, GR. Malignant melanotic tumors of the eye. A study of histologic types in 111 cases. *Trans Am Acad Ophthalmol Otolaryngol*. 1931;36:131-42.
136. McLean, IW, Foster, WD, Zimmerman, LE, Gamel, JW. Modifications of Callender's classification of uveal melanoma at the Armed Forces Institute of Pathology. *Am J Ophthalmol*. 1983;96:502-9.
137. Moshari, A, Cheeseman, EW, McLean, IW. Totally necrotic choroidal and ciliary body melanomas: associations with prognosis, episcleritis, and scleritis. *Am J Ophthalmol*. 2001;131:232-6.
138. Seddon, JM, Polivogianis, L, Hsieh, CC, Albert, DM, Gamel, JW, Gragoudas, ES. Death from uveal melanoma. Number of epithelioid cells and inverse SD of nucleolar area as prognostic factors. *Arch Ophthalmol*. 1987;105:801-6.

139. Char, DH, Kroll, SM, Miller, T, Castro, J, Quivey, J. Irradiated uveal melanomas: cytopathologic correlation with prognosis. *Am J Ophthalmol.* 1996;122:509-13.
140. Bechrakis, NE, Weng Sehu, K, Lee, WR, Damato, B, Foerster, MH. Transformation of cell type in uveal melanomas. A quantitative histologic analysis. *Arch Ophthalmol.* 2000;188:1406-12.
141. McLean, IW, Keefe, KS, Burnier, MN. Uveal melanoma: comparison of the prognostic value of fibrovascular loops, mean of the ten largest nucleoli, cell type, and tumor size. *Ophthalmology.* 1997;104:777-80.
142. Fuchs, U, Kivelä, T, Summanen, P, Immonen, I, Tarkkanen, A. An immunohistochemical and prognostic analysis of cytokeratin expression in malignant uveal melanoma. *Am J Pathol.* 1992;141:169-81.
143. Kinnaert, E, Morandini, R, Simon, S, Hill, HZ, Ghanem, G, van Houtte, P. The degree of pigmentation modulates the radiosensitivity of human melanoma cells. *Radiation Res.* 2000;154:497-502.
144. Folberg, R, Pe'er, J, Gruman, LM, Woolson, RF, Jeng, G, Montague, PR, Moninger, TO, Yi, H, Moore, KC. The morphologic characteristics of tumor blood vessels as a marker of tumor progression in primary human uveal melanoma: a matched case-control study. *Hum Pathol.* 1992;23:1298-305.
145. Rummelt, V, Gardner, LM, Folberg, R, Beck, S, Knosp, B, Moninger, TO, Moore, KC. Three-dimensional relationships between tumor cells and microcirculation with double cyanine immunolabeling, laser scanning confocal microscopy, and computer-assisted reconstruction: an alternative to cast corrosion preparations. *J Histochem Cytochem.* 1994;42:681-6.
146. Nowak, MA, Fatteh, SM, Campbell, TE. Glycogen-rich malignant melanomas and glycogen-rich balloon cell malignant melanomas: frequency and pattern of PAS positivity in primary and metastatic melanoma. *Arch Pathol Lab Med.* 1998;122:353-60.
147. Rummelt, V, Folberg, R, Rummelt, C, Gruman, LM, Hwang, T, Woolson, RF, Yi, H, Naumann, GO. Microcirculation architecture of melanocytic nevi and malignant melanomas of the ciliary body and choroid: a comparative histopathologic and ultrastructural study. *Ophthalmology.* 1994;101:718-27.
148. Foss, AJE, Alexander, RA, Hungerford, JL, Harris, AL, Cree, IA, Lightman, S. Reassessment of the PAS patterns in uveal melanoma. *Br J Ophthalmol.* 1997;81:240-6.
149. Sakamoto, T, Sakamoto, M, Yoshikawa, H, Hata, Y, Ishibashi, T, Ohnishi, Y, et, a. Histologic findings and prognosis of uveal malignant melanoma in Japanese patients. *Am J Ophthalmol.* 1996;121:276-83.
150. Warso, MA, Maniotis, AJ, Chen, X, Majumdar, D, Patel, MK, Shilkaitis, A, Das Gupta, TK, Folberg, R. Prognostic significance of periodic acid-Schiff positive patterns in primary cutaneous melanoma. *Clin Cancer Res.* 2001;7:473-7.
151. Rummelt, V, Axmacher, K, Folberg, R, Naumann, GOH. Microcirculation patterns as prognostic factor in conjunctival melanoma. *Invest Ophthalmol Vis Sci [ARVO Abstract].* 1997;38:S807

152. Maniotis, AJ, Folberg, R, Hess, A, Seftor, EA, Gardner, LMG, Pe'er, J, Trent, JM, Meltzer, PS, Hendrix, MJC. Vascular channel formation by human melanoma cells in vivo and in vitro: Vasculogenic mimicry. *Am J Pathol.* 1999;155:739-52.
153. Daniels, KJ, Boldt, HC, Martin, JA, Gardner, LM, Meyer, M, Folberg, R. Expression of type VI collagen in uveal melanoma: its role in pattern formation and tumor progression. *Lab Invest.* 1996;75:55-66.
154. Hess, AG, Seftor, EA, Gardner, LMG, Carles-Kinch, K, Schneider, GB, Seftor, REB, Kinch, MS, Hendrix, MJC. Molecular regulation of tumor cell vasculogenic mimicry by tyrosine phosphorylation: role of epithelial cell kinase (Eck/EphA2). *Cancer Res.* 2001;61:3250-5.
155. Yanko, L. An angiographic and histologic study of the vasculature of choroidal malignant melanoma. *Acta Ophthalmol.* 1973;51:12-24.
156. Tímár, J & Tóth, J. Tumor sinuses - vascular channels. *Pathol Oncol Res.* 2000;6:83-6.
157. McDonald, DM, Munn, L, Jain, RK. Vasculogenic mimicry: How convincing, how novel, and how significant? *Am J Pathol.* 2000;156:383-8.
158. McDonald, DM & Foss, AJE. Endothelial cells of tumor vessels: abnormal but not absent. *Cancer Metastasis Rev.* 2000;19:109-20.
159. Weidner, N. Intratumor microvessel density as a prognostic factor in cancer. *Am J Pathol.* 1995;147:9-19.
160. Weidner, N, Semple, JP, Welch, WR, Folkman, J. Tumor angiogenesis and metastasis - correlation in invasive breast carcinoma. *N Engl J Med.* 1991;324:1-8.
161. Weidner, N. Angiogenesis as predictor of a clinical outcome in cancer patients. *Hum Pathol.* 2000;31:403-5.
162. Graham, CH, Rivers, J, Kerbel, RS, Stankiewich, KS, White, WL. Extent of vascularization as a prognostic indicator in thin (<0.76 mm) malignant melanomas. *Am J Pathol.* 1994;145:510-4.
163. Busam, KJ, Berwick, M, Blessing, K, Fandrey, K, Kang, S, Karaoli, T, Fine, J, Cochran, AJ, White, WL, Rivers, J, Elder, DE, Po Wen, D-R, Heyman, BH, Barnhill, R. Tumor vascularity is not a prognostic factor for malignant melanoma of the skin. *Am J Pathol.* 1995;147:1049-56.
164. de Waal, RMW, van Altena, MC, Erhard, H, Weidle, UH, Nooijen, PTGA, Ruiter, DJ. Lack of lymphangiogenesis in human primary cutaneous melanoma. Consequences for the mechanism of lymphatic dissemination. *Am J Pathol.* 1997;150:1951-7.
165. Folberg, R. Discussion of paper by Foss *et al.* *Br J Ophthalmol.* 1997;81:247-8.
166. Lane, AM, Egan, KM, Yang, J, Saornil, MA, Alroy, J, Albert, D, Gragoudas, ES. An evaluation of tumour vascularity as a prognostic indicator in uveal melanoma. *Melanoma Res.* 1997;7:237-42.
167. Schaling, D. F., Van der Pol, J. P., Schlingemann, R. O., Parys-van Ginderdeuren, R., Ruiter, D. J., and Jager, M. J. Vascular density and vascular patterns in the prognosis of choroidal melanoma. In: *Thesis D.F. Schaling: Radionuclides and radiolabelled antibodies in choroidal melanoma (diagnosis and therapy)*. Leiden: Rijksuniversiteit te Leiden; 1996:43-54.

168. Hendrix, MJ, Seftor, EA, Seftor, REB, Kirschmann, DA, Gardner, LM, Boldt, HC, Meyer, M, Pe'er, J, Folberg, R. Regulation of uveal melanoma interconverted phenotype by hepatocyte growth factor/scatter factor (HGF/SF). *Am J Pathol.* 1998;152:855-63.
169. Hendrix, MJ, Seftor, EA, Seftor, RE, Gardner, LM, Boldt, HC, Meyer, M, Pe'er, J, Folberg, R. Biologic determinants of uveal melanoma metastatic phenotype: role of intermediate filaments as predictive markers. *Lab Invest.* 1998;78:153-63.
170. Mooy, CM, Luyten, GP, de Jong, PT, Jensen, OA, Luider, TM, van der Ham, F, Bosman, FT. Neural cell adhesion molecule distribution in primary and metastatic uveal melanoma. *Hum Pathol.* 1995;26:1185-90.
171. Anastassiou, G, Schilling, H, Stang, A, Djakovic, S, Heiligenhaus, A, Bornfeld, N. Expression of the cell adhesion molecules ICAM-1, VCAM-1 and NCAM in uveal melanoma: A clinicopathological study. *Oncology.* 2000;58:83-8.
172. Foss, AJ, Alexander, RA, Guille, MJ, Hungerford, JL, McCartney, AC, Lightman, S. Estrogen and progesterone receptor analysis in ocular melanomas. *Ophthalmology.* 1995;102:431-5.
173. Ma, D & Nieder Korn, JY. Role of epidermal growth factor receptor in the metastasis of intraocular melanomas. *Invest Ophthalmol Vis Sci.* 1998;39:1067-75.
174. Hurks, HMH, Metzelaar-Blok, JAW, Barthen, ER, Zwinderman, AH, de Wolff-Rouendaal, D, Keunen, JE, Jager, MJ. Expression of epidermal growth factor receptor: risk factor in uveal melanoma. *Invest Ophthalmol Vis Sci.* 2000;41:2023-7.
175. Scholes, AGM, Hagan, S, Hiscott, P, Damato, BE, Grierson, I. Overexpression of epidermal growth factor receptor restricted to macrophages in uveal melanoma. *Arch Ophthalmol.* 2001;119:373-7.
176. Väisänen, A, Kallioinen, M, von Dickhoff, K, Laatikainen, L, Höyhty, M, Turpeenniemi-Hujanen, T. Matrix metalloproteinase-2 (MMP-2) immunoreactive protein - A new prognostic marker in uveal melanoma? *J Pathol.* 1999;188:56-62.
177. Pe'er, J, Gnessin, H, Shargal, Y, Livni, N. PC-10 immunostaining of proliferating cell nuclear antigen in posterior uveal melanoma. Enucleation versus enucleation postirradiation groups. *Ophthalmology.* 1994;101:56-62.
178. Karlsson, M, Boeryd, B, Carstensen, J, Franlund, B, Gustafsson, B, Kågedal, B, Sun, XF, Wingren, S. Correlations of Ki-67 and PCNA to DNA ploidy, S-phase fraction and survival in uveal melanoma. *Eur J Cancer.* 1996;32A:357-62.
179. Seregard, S, Oskarsson, M, Spångberg, B. PC-10 as a predictor of prognosis after antigen retrieval in posterior uveal melanoma. *Invest Ophthalmol Vis Sci.* 1996;37:1451-8.
180. Mooy, CM, Luyten, GP, de Jong, PT, Luider, TM, Stijnen, T, van der Ham, F, van Vroonhoven, CC, Bosman, FT. Immunohistochemical and prognostic analysis of apoptosis and proliferation in uveal melanoma. *Am J Pathol.* 1995;147:1097-104.
181. Coupland, SE, Anastassiou, G, Stang, A, Schilling, H, Anagnostopoulos, I, Bornfeld, N, Stein, H. The prognostic value of cyclin D1, p53, and MDM2 protein expression in uveal melanoma. *J Pathol.* 2000;191:120-6.

182. Coupland, SE, Bechrakis, N, Schuler, A, Anagnostopoulos, I, Hummel, M, Bornfeld, N, Stein, H. Expression patterns of cyclin D1 and related proteins regulating G1-S phase transition in uveal melanoma and retinoblastoma. *Br J Ophthalmol.* 1998;82:961-70.
183. Ghazvini, S, Kroll, S, Char, DH, Frigillana, H. Comparative analysis of proliferating cell nuclear antigen, bromodeoxyuridine, and mitotic index in uveal melanoma. *Invest Ophthalmol Vis Sci.* 1995;36:2762-7.
184. Sørensen, FB, Gamel, JW, McCurdy, J. Stereologic estimation of nucleolar volume in ocular melanoma: a comparative study of size estimators with prognostic impact. *Hum Pathol.* 1993;24:513-8.
185. Gamel, JW, McCurdy, JB, McLean, IW. A comparison of prognostic covariates for uveal melanoma. *Invest Ophthalmol Vis Sci.* 1992;33:1919-22.
186. McLean, IW, Sibug, ME, Becker, RL, McCurdy, JB. Uveal melanoma: the importance of large nucleoli in predicting patient outcome: an automated image analysis study. *Cancer.* 1997;79:982-8.
187. Coleman, K, Baak, JP, van Diest, PJ, Mullaney, J. Prognostic value of morphometric features and the Callender classification in uveal melanomas. *Ophthalmology.* 1996;103:1634-41.
188. Moshari, A & McLean, IW. Uveal melanoma: mean of the longest nucleoli measured on silver-stained sections. *Invest Ophthalmol Vis Sci.* 2001;42:1160-3.
189. Pe'er, J, Rummelt, V, Mawn, L, Hwang, T, Woolson, RF, Folberg, R. Mean of the ten largest nucleoli, microcirculation architecture, and prognosis of ciliochoroidal melanomas. *Ophthalmology.* 1994;101:1227-35.
190. Hodge, WG, Duclos, AJ, Rocha, G, Anteck, E, Baines, MG, Corriveau, C, Brownstein, S, Deschenes, J. DNA index and S phase fraction in uveal malignant melanomas. *Br J Ophthalmol.* 1995;79:521-6.
191. Meecham, WJ & Char, DH. DNA content abnormalities and prognosis in uveal melanoma. *Arch Ophthalmol.* 1986;104:1626-9.
192. Toti, P, Greco, G, Mangiavacchi, P, Bruni, A, Palmeri, MLD, Luzzi, P. DNA ploidy pattern in choroidal melanoma: correlation with survival. A flow cytometry study on archival material. *Br J Ophthalmol.* 1998;82:1433-7.
193. Horsthemke, B, Prescher, G, Bornfeld, N, Becher, R. Loss of chromosome 3 alleles and multiplication of chromosome 8 alleles in uveal melanoma. *Genes Chromosomes Cancer.* 1992;4:217-21.
194. Prescher, G, Bornfeld, N, Hirche, H, Horsthemke, B, Jockel, KH, Becher, R. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet.* 1996;347:1222-5.
195. Tschentscher, F, Prescher, G, Horsman, DE, White, VA, Rieder, H, Anastassiou, G, Schilling, H, Bornfeld, N, Bartz-Schmidt, KU, Horsthemke, B, Lohmann, DR, Zeschnigk, M. Partial deletions of the long and short arm of chromosome 3 point to two tumor suppressor genes in uveal melanoma. *Cancer Res.* 2001;61:3439-42.

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196. Myatt, N, Aristodemou, P, Neale, MH, Foss, AJE, Hungerford, JL, Bhattacharya, S, Cree, IA. Abnormalities of the transforming growth factor-beta pathway in ocular melanoma. *J Pathol.* 2000;192:511-8.
197. Ma, D, Luyten, GP, Luider, TM, Jager, MJ, Niederkorn, JY. Association between NM23-H1 gene expression and metastasis of human uveal melanoma in an animal model. *Invest Ophthalmol Vis Sci.* 1996;37:2293-301.
198. Greco, IM, Calvisi, G, Ventura, L, Cerrito, F. An immunohistochemical analysis of nm23 gene product expression in uveal melanoma. *Melanoma Res.* 1997;7:231-6.
199. Chana, JS, Cree, IA, Foss, AJ, Hungerford, JL, Wilson, GD. The prognostic significance of c-myc oncogene expression in uveal melanoma. *Melanoma Res.* 1998;8:139-44.
200. Chana, JS, Wilson, GD, Cree, IA, Alexander, RA, Myatt, N, Neale, M, Foss, AJE, Hungerford, JL. *c-myc*, *p53*, and *Bcl-2* expression and clinical outcome in uveal melanoma. *Br J Ophthalmol.* 1999;83:110-4.
201. Jay, V, Yi, Q, Hunter, WS, Zielenska, M. Expression of bcl-2 in uveal malignant melanoma. *Arch Pathol Lab Med.* 1996;120:497-8.
202. Brantley, MA & Harbour, JW. Deregulation of the Rb and p53 pathways in uveal melanoma. *Am J Pathol.* 2000;157:1795-801.
203. Kishore, K, Ghazvini, S, Char, DH, Kroll, S, Selle, J. p53 gene and cell cycling in uveal melanoma. *Am J Ophthalmol.* 1996;121:561-7.
204. Shaif-Muthana, M, McIntyre, C, Sisley, K, Rennie, I, Murray, A. Dead or alive: immunogenicity of human melanoma cells when presented by dendritic cells. *Cancer Res.* 2000;60:6441-7.
205. Schurmans, LRHM, Blom, DJR, de Waard-Siebinga, I, Keunen, JEE, Prause, JU, Jager, MJ. Effects of transpupillary thermotherapy on immunological parameters and apoptosis in a case of primary uveal melanoma. *Melanoma Res.* 1999;9:297-302.
206. Kim, K, Pang, KM, Evans, M, Hay, ED. Overexpression of  $\beta$ -Catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. *Mol Biol Cell.* 2000;11:3509-23.
207. Hsu, S-M. & Raine, L. The use of avidin-biotin-peroxidase complex (ABC) in diagnostic and research pathology. In: DeLellis, R. A., (ed). *Advances in immunohistochemistry*. New York: Masson Publishing, USA; 1984:31-42.
208. Kivelä, T, Jääskeläinen, J, Vaheri, A, Carpén, O. Ezrin, a membrane-organizing protein, as a polarization marker of the retinal pigment epithelium in vertebrates. *Cell Tissue Res.* 2000;301:217-23.
209. Brozman, M. Immunohistochemical analysis of formaldehyde- and trypsin- or pepsin-treated material. *Acta Histochemica.* 1978;63:251-60.
210. Foss, AJE, Alexander, RA, Jefferies, LW, Lightman, SL. The effect of melanin bleaching on immunohistochemical techniques. *Br J Biomed Sci.* 1995;52:22-5.

211. Kivelä, T. Immunohistochemical staining followed by bleaching of melanin: a practical method for ophthalmic pathology. *Br J Biomed Sci.* 1995;52:325-6.
212. Ramani, P, Bradley, NJ, Fletcher, CDM. QBEND/10, a new monoclonal antibody to endothelium: assessment of its diagnostic utility in paraffin sections. *Histopathology.* 1990;17:237-42.
213. Skalli, O, Ropraz, P, Trzeciak, A, Benzouana, G, Gillensen, D, Gabbiani, G. A monoclonal antibody against  $\alpha$ -smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol.* 1986;103:2787-96.
214. Luyten, GP, Mooy, CM, Post, J, Jensen, OA, Luider, TM, de Jong, PT. Metastatic uveal melanoma. A morphologic and immunohistochemical analysis. *Cancer.* 1996;78:1967-71.
215. Bodey, B, Kaiser, HE, Goldfarb, RH. Immunophenotypically varied cell subpopulations in primary and metastatic human melanomas. Monoclonal antibodies for diagnosis, detection of neoplastic progression and receptor directed immunotherapy. *Anticancer Res.* 1996;16:517-31.
216. Falini, B, Flenghi, L, Pileri, S, Gambacorta, M, Bigerna, B, Durkop, H, Eitelbach, F, Thiele, J, Pacini, R, Cavaliere, A. PG-M1: a new monoclonal antibody directed against a fixative-resistant epitope on the macrophage-restricted form of the CD68 molecule. *Am J Pathol.* 1993;142:1359-72.
217. Pulford, KA, Rigney, EM, Micklem, KJ, Jones, M, Stross, WP, Gatter, KC, Mason, DY. KP1: a new monoclonal antibody that detects a monocyte/macrophage associated antigen in routinely processed tissue sections. *J Clin Pathol.* 1989;42:414-21.
218. Jaspars, EH, Bloemena, E, Bonnet, P, Scheper, RJ, Kaiserling, E, Meijer, CJ. A new monoclonal antibody (3A5) that recognises a fixative resistant epitope on tissue macrophages and monocytes. *J Clin Pathol.* 1994;47:248-52.
219. Böhling, T, Turunen, O, Jääskeläinen, J, Carpén, O, Sainio, M, Wahlström, T, Vaehri, A, Haltia, M. Ezrin expression in stromal cells of capillary hemangioblastoma. An immunohistochemical survey of brain tumors. *Am J Pathol.* 1996;148:367-73.
220. Altman, DG. *Practical Statistics for Medical Research.* 1<sup>st</sup> Ed. London: Chapman & Hall; 1991
221. Borenstein, M. Planning for precision in survival studies. *J Clin Epidemiol.* 1994;47:1277-85.
222. Parmar, MKB & Machin, D. *Survival Analysis. A Practical Approach.* Chichester: John Wiley & Sons; 1996
223. Kleinbaum, DG. *Survival analysis: a self-learning text.* New York: Springer-Verlag; 1996
224. Steuhl, KP, Rohrbach, JM, Knorr, M, Thiel, HJ. Significance, specificity, and ultrastructural localization of HMB-45 antigen in pigmented ocular tumors. *Ophthalmology.* 1993;100:208-15.
225. Ruiz, RS, El Harazi, S, Albert, DM, Bryar, PJ. Cardiac metastasis of choroidal melanoma. *Arch Ophthalmol.* 1999;117:1558-9.
226. Rosario, RT, DiMaio, DJ, Lapham, RL, Sweeney, M, Smalling, R, Barasch, E. Metastatic ocular melanoma to the left ventricle inducing near-syncope attacks in an 84-year-old woman. *Chest.* 2000;118:551-3.

227. Percy, C, Stanek, E, Gloeckler, L. Accuracy of cancer death certificates and its effect on cancer mortality statistics. *Am J Public Health*. 1981;71:242-50.
228. Engel, LW, Strauchen, JA, Chiazze, L, Heid, M. Accuracy of death certification in an autopsied population with specific attention to malignant neoplasms and vascular disease. *Am J Epidemiol*. 1980;111:99-112.
229. Mehaffey, MG, Gardner, LM, Folberg, R. Distribution of prognostically important vascular patterns across multiple levels in ciliary body and choroidal melanomas. *Am J Ophthalmol*. 1998;126:373-8.
230. Folberg, R, Fleck, M, Mehaffey, MG, Meyer, M, Bentler, SE, Woolson, RF, Pe'er, J. Mapping the location of prognostically significant microcirculatory patterns in ciliary body and choroidal melanoma. *Pathol Oncol Res*. 1996;2:229-36.
231. Rummelt, V, Mehaffey, MG, Campbell, RJ, Pe'er, J, Bentler, SE, Woolson, RF, Naumann, GO, Folberg, R. Microcirculation architecture of metastases from primary ciliary body and choroidal melanomas. *Am J Ophthalmol*. 1998;126:303-5.
232. O'Reilly, MS, Boehm, T, Shing, Y, Fukai, N, Vasios, G, Lane, WS, Flynn, E, Birkhead, JR, Olsen, BR, Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*. 1997;88:277-85.
233. Rybak, SM, Sanovich, E, Hollingshead, M, Newton, DL, Kaur, G, Sausville, EA. Differential effects of drugs on vascular channels formed by tumor cells vs vascular channels formed by endothelial cells. *Cancer Detection Prevention (Suppl)*. 2000;24:194
234. Fox, SB, Leek, RD, Weekes, MP, Whitehouse, RM, Gatter, KC, Harris, AL. Quantification and prognostic value of breast cancer angiogenesis: comparisons of microvessel density, Chalkey count, and computer image analysis. *J Pathol*. 1995;177:275-83.
235. Anthony, PP & Ramani, P. Endothelial markers in malignant vascular tumours of the liver: superiority of QB-END/10 over von Willebrand factor and Ulex europaeus agglutinin 1. *J Clin Pathol*. 1991;44:29-32.
236. Sovalat, H, Racadot, E, Hénon, P, Fuchs, P, Lewandowski, H, Billot, M. Comparative analysis of class I, II and III epitope detecting CD34 monoclonal antibodies by quantitative flow cytometry. *Hematology Cell Therapy*. 1998;40:259-68.
237. van der Kooij, MA, von der Mark, EM, Kruijt, JK, van Velzen, A, van Berkel, TJ, Morand, OH. Human monocyte-derived macrophages express an approximately 120-kD Ox-LDL binding protein with strong identity to CD68. *Arterioscler Thromb Vasc Biol*. 1997;17:3107-16.
238. Char, DH, Kroll, S, Phillips, TL. Uveal melanoma. Growth rate and prognosis. *Arch Ophthalmol*. 1997;115:1014-8.
239. Kivelä, T, Eskelin, S, Mäkitie, T, Summanen, P. Exudative retinal detachment from malignant uveal melanoma: predictors and prognostic significance. *Invest Ophthalmol Vis Sci*. 2001;
240. Folberg, R, Chen, X, Boldt, HC, Pe'er, J, Brown, K, Woolson, RF, Maniotis, AJ. Microcirculation patterns other than loops and networks in choroidal and ciliary body melanomas. *Ophthalmology*. 2001;108:996-1001.

241. Gamel, JW, McLean, IW, Greenberg, RA. Interval-by-interval Cox model analysis of 3680 cases of intraocular melanoma shows a decline in the prognostic value of size and cell type over time after tumor excision. *Cancer*. 1988;61:574-9.
242. Mueller, AJ, Bartsch, DU, Folberg, R, Mehaffey, MG, Boldt, HC, Meyer, M, Gardner, LM, Goldbaum, MH, Pe'er, J, Freeman, WR. Imaging the microvasculature of choroidal melanomas with confocal indocyanine green scanning laser ophthalmoscopy. *Arch Ophthalmol*. 1998;116:31-9.
243. Coleman, DJ, Rondeau, MJ, Silverman, RH, Folberg, R, Rummelt, V, Woods, S, Lizzi, FL. Correlation of microcirculation architecture with ultrasound backscatter parameters of uveal melanoma. *Eur J Ophthalmol*. 1995;5:96-106.
244. Cicaloni, B & Pattera, N. The properties of intratumoral blood flow as a possible prognostic index in uveal melanoma: a study using color Doppler imaging. *Ann Ophthalmol*. 1997;29:225-30.
245. Graves, DT, Barnhill, R, Galanopoulos, T, Antoniadis, HN. Expression of monocyte chemotactic protein-1 in human melanoma in vivo. *Am J Pathol*. 1992;140:9-14.
246. Ohtani, K, Sakamoto, H, Rutherford, T, Chen, Z, Satoh, K, Naftolin, F. Ezrin, a membrane-cytoskeletal linking protein, is involved in the process of invasion of endometrial cancer cells. *Cancer Letters*. 1999;147:31-8.
247. Akisawa, N, Nishimori, I, Iwamura, T, Onishi, S, Hollingsworth, MA. High levels of ezrin expressed by human pancreatic adenocarcinoma cell lines with high metastatic potential. *Biochem Biophys Res Commun*. 1999;258:395-400.
248. Vaheri, A, Carpén, O, Heiska, L, Helander, TS, Jääskeläinen, J, Majander-Nordenswan, P, Sainio, M, Timonen, T, Turunen, O. The ezrin protein family: membrane-cytoskeleton interactions and disease associations. *Curr Opin Cell Biol*. 1997;9:659-66.
249. Geiger, KD, Stoldt, P, Schlote, W, Derouiche, A. Ezrin immunoreactivity is associated with increasing malignancy of astrocytic tumors but it is absent oligodendrogliomas. *Am J Pathol*. 2000;157:1785-93.
250. Jiang, WG, Hiscox, S, Singhrao, SK, Puntis, MCA, Nakamura, T, Mansel, RE, Hallett, MB. Induction of tyrosine phosphorylation and translocation of ezrin by hepatocyte growth factor/scatter factor. *Biochem Biophys Res Commun*. 1995;217:1062-9.
251. Crepaldi, T, Gautreau, A, Comoglio, PM, Louvard, D, Arpin, M. Ezrin is an effector of hepatocyte growth factor-mediated migration and morphogenesis in epithelial cells. *J Cell Biol*. 1997;138:423-34.
252. Wagatsuma, S, Konno, R, Sato, S, Yajima, A. Tumor angiogenesis, hepatocyte growth factor, and *c-met* expression in endometrial carcinoma. *Cancer*. 1998;82:520-30.
253. Helander, TS, Carpén, O, Turunen, O, Kovanen, PE, Vaheri, A, Timonen, T. ICAM-2 redistributed by ezrin as a target for killer cells. *Nature*. 1996;382:265-8.
254. Mäkitie, T & Kivelä, T. Predicting the long-term prognosis of a young woman with a malignant choroidal melanoma. *Ophthalmic Res (Suppl)*. 2000;32:S142

255. Mooy, CM & de Jong, PT. Prognostic parameters in uveal melanoma: a review. *Surv Ophthalmol.* 1996;41:215-28.
256. Folkman, J. Clinical applications of research on angiogenesis. *N Engl J Med.* 1995;333:1757-63.
257. van Ravenswaay Claasen, HH, Kluin, PM, Fleuren, GJ. Tumor infiltrating cells in human cancer. On the possible role of CD16+ macrophages in antitumor cytotoxicity. *Lab Invest.* 1992;67:166-74.
258. Kodelja, V, Muller, C, Tenorio, S, Schebesch, C, Orfanos, CE, Goerd, S. Differences in angiogenic potential of classically vs alternatively activated macrophages. *Immunobiology.* 1997;197:478-93.