RELATIVE CONTRIBUTION OF CELL PROLIFERATION AND MICROVASCULARITY TO METASTASIS IN MALIGNANT UVEAL MELANOMA

By

Rana’a Tayseer Al-Jamil

ACADEMIC DISSERTATION
To be publicly discussed, with the permission of the Medical Faculty of the University of Helsinki, In the Lecture Hall of the Skin and Allergy Hospital, Meilahtentie 2, Helsinki, on September 16th, 2011, at 12 noon.

Helsinki 2011
To my parents Tayseer and Amal
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<tr>
<td>ABC</td>
<td>Avidin-biotinylated peroxidase complex</td>
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<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CM</td>
<td>Conjunctival melanoma</td>
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<tr>
<td>CMM</td>
<td>Cutaneous malignant melanoma</td>
<td></td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>COMS</td>
<td>The Collaborative Ocular Melanoma Study</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>FAG</td>
<td>Fluorescein angiography</td>
<td></td>
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<td>FNAB</td>
<td>Fine-needle aspiration biopsy</td>
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<tr>
<td>HE</td>
<td>Hematoxylin-eosin</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>High power field</td>
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<td>HR</td>
<td>Hazard ratio</td>
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<tr>
<td>ICG</td>
<td>Indocyanine green angiography</td>
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<tr>
<td>IGF-1R</td>
<td>The Insulin-like Growth Factor 1 Receptor</td>
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<tr>
<td>UICC</td>
<td>The Union for International Cancer Control</td>
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<td>Ki-67</td>
<td>A proliferation-associated antigen</td>
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<tr>
<td>LBD</td>
<td>Largest basal tumor diameter</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<td>MLN</td>
<td>Mean diameter of the ten largest nucleoli</td>
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<tr>
<td>MVD</td>
<td>Microvascular density</td>
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<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PBT</td>
<td>Proton beam therapy</td>
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<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
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<td>PC-10</td>
<td>A proliferation-associated antigen</td>
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<tr>
<td>PHH3</td>
<td>Phospho-Histone H3 Ser10</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>TFSOM</td>
<td>To Find Small Ocular Melanoma (a mnemonic)</td>
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<tr>
<td>TNM</td>
<td>System for staging cancer Tumor, Node, Metastasis</td>
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# Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>TTT</td>
<td>Transpupillary thermotherapy</td>
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<tr>
<td>UM</td>
<td>Uveal melanoma</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
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<tr>
<td>wt/vol</td>
<td>Weight/volume</td>
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1. ABSTRACT

The purpose of my dissertation was to study the relationship between prognostic factors such as tumor cell nucleolar size, proliferation, extravascular matrix patterns, and dissemination of uveal melanoma, and to assess to what extent there is a relationship to metastasis.

The secondary goal was to develop a multivariate model which includes MLN and cell proliferation in addition to MVD, and which would fit better with population-based, melanoma-related survival data than previous models.

I. A population-based, retrospective cohort study of melanoma-related and all-cause mortality in 167 consecutive patients with enucleated eye due to choroidal and ciliary body melanoma from 1972 through 1981. MLN was measured from silver stained slides. MLN and extravascular matrix loops and networks were unrelated, independent predictors of survival. MLN and MVD were found to be partially interrelated. Multivariate models that included MVD in addition to MLN fitted better with observed melanoma-related survival than models that excluded MVD.

II. A population-based, retrospective cohort study of 167 consecutive choroidal and ciliary body melanomas enucleated from 1972 to 1981. Mouse monoclonal antibody Ki-67 was used to identify proliferating cells. High cell proliferation index was associated with presence of epithelioid cells and with a higher risk of metastatic death independent of MLN, MVD, and presence of extravascular matrix loops and networks.

III. A cross-sectional histopathological analysis of 37 metastases from corresponding primary choroidal and ciliary body melanomas enucleated from 1961 to 1981 was conducted. Hepatic metastases had more frequent epithelioid cells and a higher MVD than their corresponding primary tumors. Hepatic metastases tended to have a smaller MLN than the corresponding primaries. MLN in hepatic metastases was not associated with presence of epithelioid cells and MVD. The results suggest that MLN is not a useful marker for assessing prognosis after diagnosis of hepatic metastasis from uveal melanoma.

IV. A population-based retrospective, cohort study of IGF-1R, 167 consecutive patients with choroidal and ciliary body melanoma enucleated from 1972 to 1981. More heavy
pigmentation, larger number of macrophages, and higher MVD were associated with a higher percentage of tumor area that was immunopositive for IGF-1R. Immunoreactivity for IGF-1R did not independently predict metastasis from primary uveal melanoma. Partial loss of antigenicity could not be ruled out as a confounding factor because no frozen sections were available. The results of earlier studies have likewise been inconsistent, suggesting that immunohistochemical determination of IGF-1R from formalin-fixed, paraffin-embedded specimens may not be practical as a routine test.
2. INTRODUCTION

Uveal melanoma (UM) is the second most common primary intraocular cancer worldwide.\textsuperscript{1} It is a relatively rare cancer, but still the second most common type of primary malignant melanoma in humans.\textsuperscript{2} UM is a slowly growing tumor, and gives rise to distant metastasis mainly to the liver via the bloodstream.\textsuperscript{3}

UM arises from melanocytes of the choroid, the ciliary body, and the iris.\textsuperscript{4} These cells are of neuroectodermal origin and related to melanocytes found in the skin and the conjunctiva.

The choroid is the most common location for UM, then the ciliary body and finally the iris.\textsuperscript{5} Usually patients are asymptomatic in the early stages but as the tumor increases in size symptoms appear (e.g. blurred vision, visual field loss, floaters, eventual pain).\textsuperscript{5} Enucleation was the treatment traditionally offered to all patients with ciliochoroidal melanomas.\textsuperscript{6} With increasing evidence showing that eye preserving forms of treatment are not associated with increased mortality,\textsuperscript{7,8} enucleation has been largely replaced by radiotherapy, especially brachytherapy using Ru\textsuperscript{106} and I\textsuperscript{125} plaques. Since the late 1970s in institutions with access to a nuclear accelerator, charged particle radiation using protons\textsuperscript{9} and helium ions\textsuperscript{10} has been used. Other current forms of eye-preserving treatment of UM include stereotactic radiotherapy,\textsuperscript{11,12} endoresection,\textsuperscript{13,14} local transscleral resection,\textsuperscript{15-17} and transpupillary thermotherapy.\textsuperscript{18-20}

UM differs from both cutaneous malignant melanoma (CMM) and conjunctival melanoma (CM) in that it almost always metastasizes hematogenously because there are no lymph vessels inside the eye and the orbit. Only in the rare case that the anterior sclera is penetrated by UM, may it disseminate through conjunctival lymphatics to the regional lymph nodes.\textsuperscript{21,22} According to one study optic nerve invasion in UM is found in 1 in 20 patients.\textsuperscript{23} In more than 90% of cases, the liver is involved when metastatic disease is diagnosed.\textsuperscript{3,24}

About 40% of patients with UM die of metastatic disease within 10 years of diagnosis, irrespective of the type of treatment.\textsuperscript{25,26} UM can be classified according to its cell type into spindle cell, mixed cell, and epithelioid cell melanomas.\textsuperscript{27,28} The prognostic implications of this classification have been known since 1931.\textsuperscript{27} Tumors containing epithelioid cells grow faster and have a poorer prognosis.\textsuperscript{27,29-31}

During the last decade, two main lines of research have aimed to achieve enhanced understanding of the metastasis process and accurate prognosis of patients with UM. One emphasizes the characteristics of tumor cells, particularly their nucleoli,\textsuperscript{32-48} and markers of proliferation,\textsuperscript{44,46,49-52} and the other the characteristics of tumor blood vessels.\textsuperscript{29,30,40,41,46,53,54}
Of several morphometric measurements, the mean diameter of the ten largest nucleoli (MLN) has become the most widely applied. A large MLN has consistently been associated with high likelihood of dying from UM. Many methods have been used to stain and sample the largest nucleoli and no agreement has yet been reached as to which combination is the best.

Blood vessels are of paramount importance in metastasis of UM. Different extravascular matrix patterns can be seen in UM, like loops (of any size enclosing melanoma cells) and networks (which are composed of 3 back-to-back loops). This presence is associated with death from metastatic melanoma. However, the density of microvessels is also of prognostic importance.

MLN has been studied in general by many authors, but essentially none of them have studied both extravascular matrix patterns and MLN and their interrelationship, which is a main topic of my experiments. To the best of my knowledge MVD has never been studied in relation to MLN.
3. REVIEW OF THE LITERATURE

3.1 Uveal Melanoma as a Clinical Problem

3.1.1 Epidemiology of Uveal Melanoma

UM is the commonest intraocular malignancy in adult Caucasians, as they are at more than 8 times greater risk of developing the disease than are Africans.\textsuperscript{60} According to recently published data from the European Cancer Registry, its age-standardized incidence in different European countries varied from 2 new cases in Spain and Southern Italy to 8 new cases in Norway and Denmark, and up to 6-8 new cases in Finland per million per year between 1983 and 1994.\textsuperscript{61,62} The mean age at the time of diagnosis is between 50 and 60 years.\textsuperscript{60,63-65}

The frequency of UM depends on age, usually it is very rare in childhood, but its incidence increases in elderly after the age of 40-45, it reaches the apex in late middle age, usually between 55-60 years.\textsuperscript{26,60} The risk of UM increases with age, but in one study it seems to level off after the age of 70, more in females.\textsuperscript{65} Which differs from other adult cancers, as the risk increases with age.\textsuperscript{60}

According to various studies, 64% to 90% of UM originates in the choroid,\textsuperscript{63} and about 10% and 3% in the ciliary body and the iris respectively.\textsuperscript{65,66} UM limited to the iris (iris melanoma) is normally discussed as a separate entity because of its favorable prognosis compared to melanomas.\textsuperscript{39,67,68}

UM is almost always unilateral. Less than 0.002% of UM patients have it bilaterally.\textsuperscript{81} Clinical metastases are present in 0.2 – 2.5% of patients at diagnosis of primary UM.\textsuperscript{69-74} But up to 50% develop them later.\textsuperscript{26}

3.1.2 Etiology and Predisposition

The etiology of UM remains unknown. Light blue eyes are also predisposing factors for increased risk for UM.\textsuperscript{60} People with lightly pigmented skin, hair, and irises are at a slightly increased risk of developing UM.\textsuperscript{66,75,76}

Even though lightly pigmented skin and light iris color are established risk factors, the role of sunlight and other ultraviolet light exposure which have been implicated in the etiology of cutaneous melanoma, is vague in UM.\textsuperscript{75,77-79}
A slight predominance of male gender is evident from large series of UM, but the reason for this male predominance is unknown.60,64,65 No risk factors were associated with occupation, tobacco, and hormonal causes.60,75,80

Congenital ocular melanocytosis (nevus of Ota), which is a pigmentary disorder where the number of melanocytes are increased in ocular tissues, is associated with an increased risk for UM.82 It has been noted also in patients with bilateral UM.81

One tenth of UM develops from a previously diagnosed choroidal nevus.82-85 Singh et al.84 estimated the prevalence of choroidal nevi to range from 4.6% to 7.9%. Whereas Kivellä et al.87 obtained confidence limits for the cumulative lifetime risk estimate based on the calculations of Singh et al.,84 by estimating that the lifetime risk of UM from a choroidal nevus for a patient living to be 90 years old would be 0.04% (confidence interval [CI], 0.03%–0.06%) by the age of 40 years, 0.28% by 60 (CI, 0.20%–0.34%), and 0.78% by 80 (CI, 0.58%–1.0%). The lifetime risk estimate would approach 1%, which is not an insignificant figure.87

The detection and treatment of choroidal melanoma early in its natural course is critical for optimal prognosis.86 Studies of tumor doubling time have indicated that metastasis from choroidal melanoma may occur quite early in the course of the disease, when the tumor is about 3.0 mm in basal dimension and 1.5 mm in thickness.86,87 Clinical studies have shown that, at 5 years, metastasis occurs in 16% of patients with small choroidal melanomas (less than 4 mm thick), compared with 32% of those with medium-sized (4-8 mm thick) choroidal melanomas and in 53% of those with large (more than 8 mm thick) choroidal melanomas.86

The difficulty with early detection of choroidal melanoma relates to its clinical similarity to benign choroidal nevus. Factors that assist in differentiating small choroidal melanoma from choroidal nevus can be remembered using the mnemonic "TFSOM" (To Find Small Ocular Melanoma),88,89 where T = thickness greater than 2 mm, F = subretinal fluid, S = symptoms, O = orange pigment and M = margin touching optic disc. Any one of these factors increases the risk for growth, which often is a sign of malignancy, the more so when occurring together.

Choroidal melanocytic tumors that display none of these factors have a 3% risk of growth into melanoma at 5 years and most likely represent choroidal nevi.88 Tumors that display one factor have a 38% risk of growth, and those with two or more factors show growth in over 50% of cases. Shields et al. calculated the relative risk for growth to be 1.9 for one factor, 3.8 for 2 factors, 7.4 for 3 factors, 14.1 for 4 factors, and 27.1 for all 5 of their risk factors combined.88
The COMS group has identified as additional risk factors larger basal diameter, and absence of drusen and retinal pigment epithelial changes adjacent to the tumor. Most tumors with two or more risk factors probably represent small choroidal melanomas, and early treatment is generally indicated. Therefore, ophthalmologists should be aware of the clinical factors that identify small choroidal melanoma thereby ensuring their patients early treatment and better prognosis.

UM in general is not hereditary, although some families with two or more patients have been reported. The most identified prognostic factor yet is the partial or total loss of chromosome 3, (monosomy 3). Other known chromosomal anomalies involve chromosomes 8 and 6.

3.1.3 Clinical Diagnosis

As UM grows slowly, the symptoms are usually not specific. The commonest symptoms are blurred vision (50-69%), visual field defect (9-30%) and photopsia (18-26%). In 25-30% of patients the diagnosis is made during a normal exam, while a minimum 50% of them are asymptomatic.

Accuracy in the diagnosis of UM has improved in the past three decades. The diagnosis of UM is most often based on the typical appearance of the tumor on fundus examination and B-scan ultrasound. The key clinical features are size, shape, pigmentation, exudative retinal detachment and orange lipofuscin pigment. (Figure A)

In contrast to most other cancers, histopathologic specimens are rarely obtained for diagnosing UM. As this might increase the risk of local spread and vision loss, beside the sample might not represent the entire tumor. Fine-needle aspiration biopsy (FNAB) is nevertheless used to confirm difficult diagnoses. Moreover, today biopsies are also performed for prognostic purposes, thereby increasing the diagnostic accuracy.

Clinical diagnosis might be difficult, differentiating small melanoma from nevi. Documented growth is usually required to confirm the diagnosis of small melanomas, despite the fact that nevi might grow slowly during follow-up period.

The inner parts of the tumor gives a low-reflective shadow in B-scan ultrasound. (Figure B) This examination may detect extrascleral extensions as well. Transillumination may show tumor edges extending anterior to the ora serrata, which can be useful to detect melanomas in the ciliary body. Although, it is best seen with high-frequency ultrasound.
Figure A:

Typical fundus photograph of the left eye in a patient with UM, showing a pigmented lesion and orange lipofuscin pigment.

Figure B:

B-scan ultrasonography showing a low-reflective tumor in a patient with UM.
Fluorescein angiography (FAG) is now rarely used, but it has been used earlier to identify the double circulation pattern typical of UM, and is useful in the differential diagnosis of a vascular tumor. Indocyanine green angiography (ICG), a more recent diagnostic method, has been shown to be superior to FAG in imaging tumor vascularization.

Optical coherence tomography (OCT) may provide information about the retinal structure overlying prominent tumors and the extent of adjacent retinal detachment. In its present state of development, OCT is especially valuable in the differential diagnosis of small choroidal tumors in detecting incipient retinal detachment. Its potential value for the follow-up of shallow tumors needs further investigation.

3.1.4 Histopathological Diagnosis

Histologic factors play an important role in the diagnosis and estimation of prognosis in UM, and staining techniques have developed greatly in recent years. The tumors are classified according to cell type by the modified Callender classification to; spindle cell, mixed cell, and epithelioid cell melanomas (Figures C, D). Spindle cell melanomas grow more slowly and have better prognosis than other types. Epithelioid tumors show a faster growth and usually metastasize.

Figure C: Hematoxylin-eosin slide showing a predominantly spindle cell UM with single epithelioid cells.

Figure D: Hematoxylin-eosin slide showing epithelioid cell UM.
3.1.4.1 Extravascular Matrix Patterns

The presence of extravascular matrix patterns (Figure E) in UM and their association with metastatic death are well established.\textsuperscript{54}

Tumor extravascular morphology in UM was first described by Folberg et al. in 1992.\textsuperscript{54} Folberg classified “vascular patterns” into nine morphological types\textsuperscript{54}: The normal pattern consists of normal uncompressed choroidal vessels. The silent pattern contains no apparent tumor vessels. The straight pattern is composed of randomly oriented straight vessels that are not linked with each other. The parallel pattern includes 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 ranges from 14 µm to 157 µm in different tumors.\textsuperscript{54} The network pattern is composed of at least three back-to-back closed loops. By definition, if networks are present, loops are present. Of the nine different patterns described, closed extravascular matrix loops and networks have been most extensively studied.\textsuperscript{29,40,53-56,115-121}

Figure E: 
*Periodic acid-Schiff slide showing extravascular matrix patterns of the network type in UM.*

Analysis of some of these patterns by multivariate models has shown them to be better prognostic indicators than conventional clinical or histopathological characteristics,\textsuperscript{122} including tumor size,\textsuperscript{54,123} involvement of the ciliary body,\textsuperscript{40,54} the cell type,\textsuperscript{54,123} and the mean diameter of the 10 largest nucleoli.\textsuperscript{39}

Melanocytic nevi do not have any parallel with cross-linking, arcs, arcs with branching, loops, or networks, consistent with the finding that these patterns may be associated with melanoma.\textsuperscript{118,119,124,125}

In a study of 40 patients, those who survived more than 15 years had more often the normal and silent patterns than those who died of the metastatic disease.\textsuperscript{54}
A follow-up study of 234 patients showed melanoma-related mortality to be higher among patients with parallel vessels, parallel vessels with cross-links, arcs, arcs with branching, loops, and networks than among patients without these extravascular matrix patterns. These patterns were found more often in ciliary body than in choroidal melanomas. Loops and networks are particularly useful in differentiating spindle cell and choroidal melanomas with better and worse prognoses.

Extravascular matrix patterns are also seen in cutaneous melanoma. Parallel vessels with cross-linking or networks carried independent prognostic significance in multivariate models when adjusting for the known strong prognostic indicator, tumor thickness. Tumor cells of UM and metastatic cutaneous melanoma are able to form extravascular matrix patterns, in particular extravascular matrix loops and networks, in vitro without the presence of endothelial cells.

3.1.4.2 Microvascular Density (MVD)

Microvascular density (MVD) is a morphologic measure of the densest area of vascularization. (Figure F) The number of immunolabeled microvessels counted from the densest vascularized area called a “hot spot” is associated with an increased risk for metastatic death. High MVD is taken as evidence of active angiogenesis in many types of cancer, including tumors that frequently spread through the lymphatic route, such as breast cancer. Some studies on MVD have failed to document a relationship with metastatic death with other cancers.

Some studies have shown that high MVD is associated with shorter survival in UM. Foss et al. first reported this relationship. They did not find any relationship between extravascular matrix patterns and survival in their data set after adjusting for MVD. MVD was higher in tumors with extravascular matrix loops and networks, suggesting that the effect of extravascular matrix patterns on prognosis may be secondary to high MVD. On the contrary, Mäkitie et al. found in a population based study of 167 patients a statistically significant association between high MVD and the presence of extravascular matrix loops and networks, presence of epithelioid cells, and largest basal tumor diameter. This was confirmed by Chen et al.

Figure F: High MVD in UM evaluated from a “hot spot” by antibodies to CD34 epitope.
3.1.4.3 Macrophages

Presence of tumor infiltrating macrophages is statistically associated with high MVD rather than with loops and networks.\textsuperscript{55,59,135} High numbers of macrophages also predict a high likelihood of metastasis from UM. It is unknown whether macrophages are causally related to tumor progression and formation of extravascular matrix patterns e.g. by secreting growth-promoting cytokines, or whether they are merely scavengers that reflect high cell turnover in rapidly growing, aggressive melanomas.\textsuperscript{136}

3.1.4.4 Mitotic Count

Some authors have reported an association between mitosis and high mortality rates in UM.\textsuperscript{31,137,138} The mitotic count of UM correlates with the risk of metastatic death.\textsuperscript{139}

Conventionally, mitotic count is established by counting the number of mitoses per 40 high-power fields (HPF) using the 40x objective in HE stained sections.\textsuperscript{137} However, Agni et al.\textsuperscript{139} recently reported that because of difficulties in identifying mitotic count reliably with HE stained sections, they now identify mitotic count by using immunohistochemistry with the mitosis-specific marker Phospho-Histone H3 Ser10 (PHH3).\textsuperscript{139}

3.1.4.5 Cell Proliferation Markers

PC-10 and Ki-67 are known antibodies for detecting proliferation of tumor cells, PC-10 monoclonal antibody detects an epitope on the proliferating cell nuclear antigen, a 36-kDa nuclear protein associated with the cell cycle.\textsuperscript{140} The concentration of the proliferating cell nuclear antigen increases during the S-phase of the cell cycle,\textsuperscript{141} and flow cytometry studies confirm that the PC-10 clone may be used as an S-phase marker.\textsuperscript{142} The Ki-67 detects cells in late G1, S, G2, and M phases of the cell cycle; it does not detect cells in the G0 phase.\textsuperscript{143,144}

Some studies have reported a prognostic association between proliferating tumor cells and mortality in UM and in other cancers.\textsuperscript{50,51,145} A high proportion of PC-10 and Ki-67 immunopositive UM cells were associated with decreased survival.\textsuperscript{50,51,138,145} Seregard et al. found that a high fractions of PC-10 immunopositive tumor cells are associated with a 40% increase in 10-year melanoma-related mortality.\textsuperscript{51} They also found that a high PC-10 count was an independent prognostic factor by multivariate analysis adjusted for LBD, presence of extravascular matrix patterns, and MLN.\textsuperscript{46}
3.1.4.6 Nuclear Morphology

Many papers have indicated that cytomorphometric parameters, such as the nucleolar area (Figure G) measured from selected cells of UM are associated with higher mortality.\textsuperscript{25,36,38,42,146} In general, MLN was an independent prognostic parameter in multivariate models, among different research laboratories.\textsuperscript{36,38,42} Different methods of measuring MLN are still a matter of controversy. Currently silver-stained sections are considered superior to HE-stained sections.\textsuperscript{48}

Moshari et al. summarized and compared the various histologic methodologies for using nucleoli to assess the malignant potential of UM. He found that the silver-stained nucleoli define the nucleolar boundaries more clearly.\textsuperscript{48}

Pe’er et al. reported that the network and the parallel vessels with cross-link extravascular matrix patterns were more powerful prognostic indicators than cytomorphometric indices, such as the mean of the ten largest nucleoli in their data set.\textsuperscript{39} But they were unable to confirm the use of the mean of the ten largest nucleoli as a significant prognostic factor in the outcome of patients whose eyes have been removed due to ciliary body or choroidal melanomas.\textsuperscript{39} Gamel et al. reported that MLN from a single routine hematoxylin and eosin-stained section can be a useful cytologic index of the malignant potential of uveal melanomas.\textsuperscript{36} Sørensen et al. in their multivariate models found that MLN and nucleolar volume pleomorphism had independent prognostic value.\textsuperscript{37} Mclean et al. have published on the importance of large nucleoli in predicting patient outcome.\textsuperscript{42}

Seregard and coauthors found that the PC-10 count retains a prognostic value in UM when adjusting for the effect of the MLN and diverse vascular patterns.\textsuperscript{46}

Bechrakis et al. described cytologic transformation and tumor progression in a series of 15 UM which was treated by primary transscleral local resection without primary adjuvant treatment, and needed enucleation because of local tumor recurrence. They found that there was an increase in the epithelioid cells in the tumor recurrence group, and that the nucleolar area was significantly increased in all cases, which demonstrates that in UM cytologic phenotype changes considerably even after a relatively short time, resulting in increased mortality.\textsuperscript{147}

Figure G:

\textit{Nucleoli of UM evaluated using the silver staining method.}
3.1.4.7 IGF-1R

The insulin-like growth factor (IGF) family consists of ligands (IGF-I, IGF-II, insulin), and several receptors (including IGF-1R). Members of this family regulate key cellular activities and they also play an important role in the development and progression of cancer.\textsuperscript{148}

IGF-1 and its receptor (IGF-1R), a plasma membrane glycoprotein composed of four subunits linked by disulfide bonds,\textsuperscript{149,150} are involved in tumorigenesis and may also protect against apoptotic cell death.\textsuperscript{150,151} In a series of papers, a relationship between IGF-1R expression and metastatic death from UM has been proposed.\textsuperscript{152-156} The experiments also suggested that the viability of UM cells would be decreased after inhibition of IGF-1R activity,\textsuperscript{155,156} making this a particularly interesting tumor marker.

The fact that the liver is the principal target organ for metastasis in UM\textsuperscript{157} raises the suspicion that hepatic environmental factors such as the growth factors largely produced in the liver – hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin like growth factor-1 (IGF-1) – and their receptors might have a role in the homing and survival of these metastatic melanoma cells.

3.1.4.8 TNM Classification

The Tumor, Node, Metastasis (TNM) System is an international classification developed in collaboration by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) to aid in the management, research and assessment of prognosis for different forms of cancer. The TNM classification for UM has evolved in recent and the latest edition will be used worldwide.\textsuperscript{158} The current TNM 7th Edition provides clinical useful definitions of tumor size, location, and metastatic disease for almost all eye cancers including UM. The use of this classification will allow ocular oncologists to compare treatments on equivalently sized and “staged” tumors. The introduction of this staging system will improve participation in clinical trials and compliance with cancer center status.\textsuperscript{159}

For tumors of the iris there are four T categories based on tumor extension to the iris, ciliary body, choroid or both, scleral extension, association with glaucoma, and extrascleral extension. For ciliary body and choroidal tumors there are four T categories and four subcategories (Tables A and B), with or without ciliary body involvement and extraocular involvement. The regional lymph node involvement is marked with N1, and distant metastasis
with M1. The latter is divided into 3 groups, largest diameter of the metastasis 3 cm or less, between 3.1 cm to 8 cm, and more than 8 cm.\textsuperscript{159,160}

Table A: TNM classification of ciliary body and choroidal melanomas according to anatomical extent

<table>
<thead>
<tr>
<th>T1</th>
<th>Tumor size category 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1a</td>
<td>T1 category without ciliary body involvement or extraocular extension</td>
</tr>
<tr>
<td>T1b</td>
<td>T1 category with ciliary body involvement</td>
</tr>
<tr>
<td>T1c</td>
<td>T1 category without ciliary body but with extraocular extension ( \leq 5 \text{ mm} )</td>
</tr>
<tr>
<td>T1d</td>
<td>T1 category with ciliary body and extraocular extension ( \leq 5 \text{ mm} )</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>T2</th>
<th>Tumor size category 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2a-d</td>
<td>T2 category and similar sub-categories as for T1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>Tumor size category 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3a-d</td>
<td>T3 category and similar sub-categories as for T1</td>
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</table>

<table>
<thead>
<tr>
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<th>Tumor size category 4</th>
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</thead>
<tbody>
<tr>
<td>T4a-d</td>
<td>T4 category and similar sub-categories as for T1</td>
</tr>
<tr>
<td>T4e</td>
<td>Any tumor size category with extraocular extension ( &gt; 5 \text{ mm} )</td>
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</table>

Table B: TNM staging of uveal melanoma according to prognostic groups

<table>
<thead>
<tr>
<th>Stage</th>
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<th>M0</th>
</tr>
</thead>
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<td>M0</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T1b-d</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>T2a</td>
<td>N0</td>
<td>M0</td>
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<tr>
<td>Stage IIIA</td>
<td>T2b</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>T3a</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIC</td>
<td>T3b-c</td>
<td>N0</td>
<td>M0</td>
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<td>T4a</td>
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<td>M0</td>
</tr>
<tr>
<td>Stage V</td>
<td>Any T</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>Stage VI</td>
<td>Any T</td>
<td>Any N</td>
<td>M1a-c</td>
</tr>
</tbody>
</table>
3.1.5 TREATMENT

3.1.5.1 Enucleation

Enucleation used to be the only treatment for UM until the late 1960’s, when eye-conserving methods were introduced. Enucleation is still an option in many developing countries and for some patients when the tumor has progressed to a large size and the chances of saving the eye and vision are limited.

In 1978, Zimmerman et al. published an article suggesting that enucleation accelerated metastatic death by physically disseminating tumor cells from the eye into the general circulation. This hypothesis was based largely on the observation that the mortality rate peaks in the second post-operative year.

Enucleation has some advantages. It is an easy procedure to perform at most ophthalmic units. It prevents local tumor recurrences if there has been no extrascleral extension prior to surgery. Besides, it provides the ophthalmic pathologist with large samples for future analysis. The disadvantage is an immediate loss of the eye and remaining vision. No significant difference in survival has been observed between equivalent groups of patients with a primary choroidal or ciliary body melanoma treated by enucleation versus plaque radiation therapy.

3.1.5.2 Radiotherapy

Radiotherapy is a type of treatment given either through episcleral radioactive plaques (Co$^{60}$, I$^{125}$, Ru$^{106}$, Ir$^{192}$ or Pd$^{103}$) or through accelerated particles such as protons or helium ions. The advantage of these methods is to preserve some vision in the affected eye, especially in eyes with small tumors.

In general UM is quite resistant to high doses of radiation, which leads to visual complications because of accompanying cataract, radiation retinopathy, and optic neuropathy. Recurrence rate of UM post radiotherapy ranges from 5 to 10% over 5 years, local recurrences may also increase the risks of distant metastasis up to five times.
3.1.5.3 Proton Beam Therapy (PBT)

PBT delivers a homogenous dose of radiation within the tumor volume, whereas in brachytherapy the tumor base receives several times the dose of the tumor apex.\textsuperscript{175} PBT avoids any risk of radiation exposure to the surgeon’s hands.\textsuperscript{175} In some centers, it is selected for all UM undergoing radiotherapy treatment.\textsuperscript{175} It is expensive compared to brachytherapy, and may cause significant side effects in ocular and extra-ocular structures.\textsuperscript{176} It is usually used for a special group of patients.\textsuperscript{176} It permits radiotherapy in larger tumors not suitable for brachytherapy and may be preferred for tumors located close to the optic disc or macula.\textsuperscript{175}

3.1.5.4 Stereotactic Radiotherapy

Stereotactic radiotherapy, which is based on collimated beams of gamma rays or gamma-knife electronic linear accelerator which converge to an exact volume.\textsuperscript{12,177,178} It can be used to treat small, medium sized and some large UM.\textsuperscript{12,179}

3.1.5.5 Transpupillary Thermotherapy (TTT)

In selected cases primary TTT may help to preserve vision by arresting the progression of tumor growth. The trade-off between potential loss of vision and the risk of local tumor recurrence and metastasis must be carefully discussed with each patient. Current data indicate that TTT as primary therapy even for small choroidal melanocytic lesions is not as effective as radiation.\textsuperscript{180} The overall effect of primary TTT on survival is not currently known.\textsuperscript{180,181}

3.1.5.6 Resection

Transscleral resection or endoresection is another method of treating UM.\textsuperscript{14,104} The tumor is removed through a scleral incision or vitrector, respectively, the advantage of this method being that only limited radiation from a plaque used as a safety measure is needed, and therefore risk of neovascular glaucoma and optic neuropathy is lower, the chances of preservation of vision are higher.\textsuperscript{182} Bechrakis et al. recently has reported that transscleral resection could be an alternative to enucleation for the treatment of large UM,\textsuperscript{15} and if
combined with adjuvant radiotherapy could be the treatment of choice in cases of large tumors.\textsuperscript{183}

This method also has disadvantages as it carries a higher risk of local recurrence.\textsuperscript{14} Besides, it requires a special type of hypotensive anesthesia which is not safe for all patients.

### 3.2 Prognosis

The 10-year cumulative melanoma-related survival is about 60\%, and then decreases about 1\% each year.\textsuperscript{25,31,184} Death is mainly due to metastasis, which in some series has been documented even decades after enucleation.\textsuperscript{185,186} UM has a predilection to metastasize to the liver.\textsuperscript{3,187-189} In 50\% of cases the liver is the only organ involved in metastasis.\textsuperscript{3,189,190} Annual screening with liver function tests and abdominal ultrasound will identify 59\% of patients while they are still asymptomatic.\textsuperscript{3} Other more frequent sites involved are skin, lung, bone and the central nervous system (CNS).\textsuperscript{3,188,190-192}

The median expected survival after metastasis is about 12 months, although there have been some reports regarding slower growing metastases in patients who have survived between 36 and 48 months.\textsuperscript{193,194}

Treating metastatic UM is frequently disappointing.\textsuperscript{195} Chemotherapy, chemoimmunotherapy, and chemoembolization may have increased survival to a mean of over one year.\textsuperscript{193,195-199} This may, however, be due to lead time bias.\textsuperscript{200} Patients treated with surgery for slow growing metastases have rarely survived for long periods.\textsuperscript{194,201,202}

The course of UM is modulated by both tumor and host, some patients with large tumor may live longer than those with smaller tumor; this applies to other prognostic factors as well.
4. AIMS OF THE PRESENT STUDY

This study was undertaken to determine the relationship between microvessels, tumor nucleoli and cell dissemination in malignant UM, to add to what is known about them individually from earlier studies. What is not known is how they independently relate to the growth rate of the tumor compared to the ability of tumor cells to generate micrometastasis.

While the work for this dissertating was ongoing several studies on Insulin-like Growth Factor 1 Receptor (IGF-1R) in UM were published, but from only two laboratories, thus it was important to check whether it holds true for other material as well.
5. MATERIALS AND METHODS

5.1 Patients

There were a total of 167 patients, 76 (46%) were males and 91 (54%) were females. Mean age at enucleation was 58 years (median, 61 years; range, 13-95 years). UM was choroidal in 126 (76%) globes and ciliochoroidal in 40 (24%) globes, of which 4 (10%) were located in the ciliary body and were confirmed by histopathology.

5.1.1 Eligibility Criteria and Follow-up (I, II, and IV)

A cross-sectional, population-based cohort of patients with UM previously used for analysis of extravascular matrix loops and networks was studied.

A total of 170 consecutive patients with UM were identified from the medical records of the Ophthalmic Pathology Laboratory, Department of Ophthalmology, Helsinki University Central Hospital. All patients with a choroidal or ciliary body melanoma from the period 1972 to 1981 were included in the study. During that period, enucleation was the only treatment modality for patients with UM except those with the smallest melanomas during this period. These were first followed-up for growth, making the series essentially population-based and unselected. All enucleated globes were submitted to the Ophthalmic Pathology Laboratory. Three patients were excluded from the analysis: one patient with cutaneous melanoma metastatic to the choroid, one patient due to removal of the eye with choroidal melanoma in autopsy, and one patient because of a miscoded iris melanoma. Therefore, 167 patients with choroidal or ciliary body melanoma were left for study. Inclusion criteria were that at least 50% of the primary tumor remained in the tissue block and the remainder was not entirely on the vitreal side of Bruch’s membrane.30

Complete follow-up data for each patient was gathered in December 2001 from the Finnish Population and Cancer Registries, patient records, from pathology laboratories, and death certificates.

In order to analyze melanoma-related mortality more reliably than in a cancer registry based-analysis, an attempt was made to differentiate as reliably as possible all melanoma deaths from other deaths.26

The cause of death was validated by reviewing all patient records relating to malignant tumors and death, crosschecking with the Finnish Population and Cancer Registries, and by
acquiring all histopathological material available from primary tumors, metastases, and second cancers.\textsuperscript{30,53,136} The study followed the principles of the Declaration of Helsinki and was approved by the Institutional Review Board.

Histopathological diagnosis of all amelanotic primary tumors, all nine secondary cancers, and 49 of 53 specimens of metastases from UM were previously reconfirmed by immunohistochemistry.\textsuperscript{26,30,53}

To improve the accuracy, all tumor deaths irrespective of the primary site and all other non-tumor deaths were 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.

\textbf{5.1.2 Eligibility Criteria and Follow-up (III)}

All patients with a choroidal and ciliary body melanoma enucleated between 1962 and 1981 and which metastasized were eligible to increase the number of biopsied metastases. Inclusion criteria were that at least 50\% of the primary tumor remained in the tissue block and the remaining part was not entirely on the vitreal side of Bruch’s membrane,\textsuperscript{30} and that one or more core needle biopsy, biopsy or autopsy specimens, with a surface area of at least 0.35 mm\textsuperscript{2} was available from hepatic metastases. This is roughly the minimum area needed to measure MVD.\textsuperscript{30}

During the study period, 292 consecutive patients had an eye with a choroidal and ciliary body melanoma removed, and 145 of these patients developed metastases that were cytologically or histologically confirmed in 92 of them; 48 pairs of primary tumors and hepatic metastases fulfilled the inclusion criteria in a prior study.\textsuperscript{203}

Of the 48 specimens, only 37 were available for restaining, because one of the primary tumors was necrotic, four had indistinct nucleoli, and six had very small specimen samples to be analyzed. (Table 1; Paper III)

Of the 37 sections, 1 (3\%) was a core needle biopsy, 12 (32\%) were surgical biopsies, and 24 (65\%) were autopsy specimens. The metastases had been detected by liver imaging or laparoscopy after they caused symptoms. The original size of the biopsied and autopsied metastases was not recorded and, except in one case, the entire metastasis was not present in the specimen. The largest diameter in the biopsy was measured from the sections with a caliper. The ranges of specimen sizes for core needle biopsy, surgical biopsy, and autopsy were 1.5 mm, 9 mm (range, 4-19), and 20 mm (range, 6.5-25) respectively.
5.1.3 Bleaching of Melanin

Potassium permanganate bleaching is suitable for the removal of melanin prior to hematoxylin-eosin (HE) and periodic acid-Schiff staining.\textsuperscript{29,54} This method was used in the analysis of extracellular matrix patterns.

Chromogens, such as 3',3'-diaminobenzidine tetrahydrochloride and 3-amino-9 ethylcarbazole that yield a dark brown reaction and a brick red reaction product respectively cannot be differentiated easily from melanin in pigmented tumors.\textsuperscript{204} If bleaching is performed prior to immunoperoxidase staining it often alters antigenicity and a significant number of antibodies fail to work properly.\textsuperscript{205} Thus, in order to visualize positive immunoreaction in pigmented tumors reliably, we bleached melanin by incubating the sections in 3.0\% (v/v) hydrogen peroxide and 1.0\% (wt/v) disodium hydrogen phosphate for 18 h at room temperature after immunostaining.\textsuperscript{206} This procedure obviates any change in antigenicity due to the bleaching.\textsuperscript{206}

5.1.4 Assessment of Nucleolar Size (silver staining)

Both HE staining\textsuperscript{33,35-39,41,42,46} and silver staining,\textsuperscript{42,48} originally designed for labeling nucleolar organizing regions, were used to identify nucleoli for measurement. The silver stain provides high contrast between nucleoli and other structures, allowing accurate discrimination of nucleoli.\textsuperscript{42,48} A comparative study found that measurements from silver-stained slides were easier to make and provided prognostically more significant results than those from HE slides.\textsuperscript{48} The most frequent field selection for sampling has been a 5-mm-long linear strip from the center of the melanoma. Linear sampling has been reported to be comparable to scanning nucleoli from the entire tumor section in predicting outcome.\textsuperscript{48} For these reasons, I chose silver staining and linear sampling.

Sections were cut at 5 \( \mu \)m on chromium-gelatin–treated glass slides\textsuperscript{207} and randomly coded. The code was broken only after all MLN measurements had been obtained. After deparaffinization, the sections were bleached with 0.25\% (wt/vol) potassium permanganate for 1 hour and 5.0\% (wt/vol) oxalic acid in distilled water for 5 minutes.

One-step silver staining was performed using two solutions\textsuperscript{42,48} first, 2.0\% (wt/vol) gelatin (Bacto Gelatin; Difco Laboratories, Detroit, MI) and 0.88\% (vol/vol) formic acid in distilled water; second, 50\% (wt/vol) silver nitrate in distilled water. The solutions were mixed 1:2 in
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The dark and poured into a dish to cover the specimens for 30 minutes. The sections were washed in distilled water and dehydrated, and coverslips were mounted (Mountex; Histolab Products AB, Göteborg, Sweden).

Each slide was examined under a light microscope (BH-2; Olympus, Tokyo, Japan) at 20X magnification to orient the central longest axis of the tumor parallel to the tumor base for digital photography (DP-10; Olympus). A photograph under low magnification was first taken for documentation of orientation. A series of color photographs under 400X optical magnification were obtained to image the nucleoli along this axis. A 5-mm strip was photographed, divided into 25 slightly overlapping images (resolution, 1280 X 1024 pixels, image area 218 X 75 µm). If the tumor was less than 5 mm by largest basal diameter (LBD), the entire central axis of the tumor was photographed.

From each of the 25 photographs, the largest nucleoli were measured by image-analysis software (Olympus DP-10 Soft, ver. 3.0; Soft Imaging System GmbH, Münster, Germany). A strip one screen high at 300% digital magnification (final magnification on screen, X4700; corresponding to 41 µm) was scanned from the top of each photograph. Measurements were taken along the longest axis of the nucleoli. The number of nucleoli measured was one to five per image (13 to 80 per section; total area, 0.205 mm²). The mean of the 10 largest nucleoli was calculated.

The images from a randomly drawn set of 63 (50%) slides were remeasured after 6 weeks by the same observer, and a second observer also graded these same images to assess intraobserver and interobserver variability respectively.

5.1.5 Immunohistochemistry

The paraffin blocks were cut at 5 µm thickness. 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. 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 ml sodium citrate buffer (pH 6.0, adjusted with 2 N NaOH), and heated in a water-bath for 15 min at 95°C. The jar was allowed to cool for 20 min at room temperature after heating.

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% (wt/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 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).

5.1.6 Assessment of Microvascular Factors

Closed extravascular matrix loops and networks consisting of at least three back-to-back loops, were identified according to Folberg et al.\textsuperscript{29,54} from sections bleached with potassium permanganate and oxalic acid and stained with periodic acid–Schiff without counterstain.\textsuperscript{30,53} They were viewed under a green filter (Wratten No. 58; Eastman Kodak, Rochester, NY). Loops of all sizes were taken into account.

Microvessels were identified with the monoclonal antibody QBEND/10 to the CD34 epitope of endothelial cells (lot 121202; Novocastra Laboratories, Newcastle-upon-Tyne, UK; diluted 1:25).\textsuperscript{210} They were counted at 400X magnification from the most highly vascularized area (hot spot), using an eyepiece with an etched graticule corresponding to 0.313 mm\(^2\) (WK 10x/20L-H; Olympus).\textsuperscript{30} Any immunolabeled element, clearly separate from adjacent ones and totally inside the graticule or touching its top or left border, was counted as a microvessel.\textsuperscript{134}

5.1.7 Assessment of Ki-67 Immunoreactivity

Mouse monoclonal antibody (mAb) Ki-67\textsuperscript{211} (clone 7B11, lot 30276010C; Zymed Laboratories, San Francisco, CA, USA), diluted 1:10, was used for identifying cells in late G1, S, G2, and M phases of the cell cycle; it does not detect cells in the G0 phase.\textsuperscript{143} Another Ki-67 antibody (clone MIB-1, lot 012(201), DAKO A/S, Glostrup, Denmark), tested in pilot series, did not produce an equally good immunoreaction. An mAb to proliferating cell nuclear
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antigen (clone PC10, lot 71K4870, Sigma Chemical Co., St. Louis, MO, USA) produced unduly heavy background. The slides were stained in duplicate and examined under a light microscope (magnification, 20X). Cells with immunopositive nuclei were counted.

To determine the cross-sectional area of the tumor, the slides were photographed with a digital camera (CoolPix 950, Nikon Corporation, Tokyo, Japan) and tumor area was measured with image analysis software (Olympus DP-10 Soft). A set of the same slides was stained using HE. Each slide was photographed under a light microscope at 20X magnification. Nuclei per image area (width 432 µm, height 346 µm) were counted using the image analysis software.

Two orbital lymphomas processed identically to the melanoma slides were used as external positive controls in the immunohistochemical staining. A large number of proliferating cells was found in both. In addition, limbal corneal epithelium was used as an internal positive control, whenever present in the slide.

5.1.8 Assessment of IGF-1R Immunoreactivity

Rabbit polyclonal antibodies against a peptide mapping at the N-terminus of IGF-1Rα (N-20; sc-712, Santa Cruz Biotechnology, CA, diluted 1:500) were used. The staining observed in the lens fibers was considered to represent the background level of staining, and tumor areas which had a stronger immunoreaction than that were considered positive. Any cystic or necrotic parts of the tumor were excluded from the tumor area. The lens epithelium and non-pigmented ciliary epithelium, which are known to express IGF-1R were used as internal positive controls in the immunohistochemical staining.

5.1.9 Assessment of Tumor-Infiltrating Macrophages

MAb PG-M1 (IgG3; lot 101; Dakopatts; diluted 1:50) to the CD68 epitope, a 110-kDa glycoprotein of lysosomal granules expressed by macrophages in most human tissues, was used to identify tumor-infiltrating macrophages. The number of immunopositive cells was graded semiquantitatively by comparing CD68 immunostained sections to standard photographs (few vs. moderate vs. high number of cells).
### Materials and Methods

**Summary of the antibodies used including dilutions and manufacturer information**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone/Lot</th>
<th>Purpose of use</th>
<th>Manufacturer</th>
<th>Country</th>
<th>Dilution</th>
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<td>Clone 7B11 Lot 30276010C</td>
<td>Cell proliferation assessment</td>
<td>Zymed Laboratories</td>
<td>San Francisco, CA, USA</td>
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<td>Cell proliferation assessment</td>
<td>DAKO A/S</td>
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<td>Different concentrations</td>
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<td>Mouse monoclonal Anti-PCNA</td>
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<td>St. Louis, MO, USA</td>
<td>Different concentrations</td>
</tr>
<tr>
<td>Rabbit polyclonal antibody of IGF-1Ra</td>
<td>N-20 SC-712</td>
<td>Tumorigenesis assessment</td>
<td>Santa Cruz Biotechnology</td>
<td>CA, USA</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse monoclonal antibody (mAb) PG-M1 to CD68 epitope</td>
<td>IgG3 Lot 101</td>
<td>Macrophages assessment</td>
<td>DAKO A/S</td>
<td>Glostrup, Denmark</td>
<td>1:50</td>
</tr>
<tr>
<td>Mouse monoclonal antibody (mAb) to CD34 epitope</td>
<td>cloneQBEND/10 Lot 121202</td>
<td>Microvessels assessment</td>
<td>Novocastra Laboratories</td>
<td>Newcastle-upon-Tyne, UK</td>
<td>1:25</td>
</tr>
</tbody>
</table>
6. STATISTICAL METHODS AND DATA ANALYSIS

All analyses were performed with Stata® (ver. 7.0 -10.0; Stata Co, College Station, TX), and StatXact-3 (Cytel Co, Cambridge, MA) statistical software.

Descriptive statistics for normally distributed variables are given as means and standard deviations and for other variables as medians and ranges. P < 0.05 was considered statistically significant. All tests were two-tailed. Median and range are given as descriptive statistics.

Kruskal-Wallis test was used to compare continuous variables between categories. Spearman’s rank correlation was used to analyze interrelationships between two continuous variables. The Wilcoxon signed-rank test or non-parametric test for trend was used to compare distributions of paired continuous data, and the Stuart-Maxwell test to compare unordered paired contingency tables, respectively.

Intraobserver agreement in measuring MLN was assessed by plotting the difference between the measurements against their mean and by calculating the mean difference with 95% confidence limits. Interobserver reproducibility was similarly assessed.

Overall survival was calculated as the time from the date of diagnosis of metastases to death. Survival was analyzed with the Kaplan-Meier product-limit method and log-rank test. Survival was modeled using Cox proportional hazard regression.

Univariate analyses of survival time data were based on the Kaplan-Meier product-limit method without taking competing risks into account. Patients judged to have died from causes other than UM were censored. Cell type and tumor location were dichotomized according to the presence of epithelioid cells (spindle, nonspindle) and ciliary body involvement (not involved, involved). Extravascular matrix loops and networks were coded as a three-category variable, considering networks to be an advanced stage of loops (no loops, loops without networks, networks). Largest basal tumor diameter (LBD) was coded in three categories (<10, >10–15, and >15 mm). MVD and MLN were divided in two or three categories according to their median and tertiles, respectively. Extravascular matrix loops and networks were coded as a three-category variable, considering networks to be an advanced stage of loops (no loops, loops without networks, networks). Ki-67 immunoreactivity was analyzed in two (absent, present) or three categories (absent, lower than median, higher than median).

Cox proportional hazards regression was used to adjust survival time data for the effect of other prognostic factors. LBD and MVD were modeled as continuous variables and MLN alternatively as divided in tertiles to assess robustness of results. MVD was square-root transformed to obtain normal distribution. Independent variables were allowed in the model if P < 0.10, and different models were compared with the likelihood ratio test.
number of variables was restricted to four; based on a rule to have at least 15 to 20 events for each additional variable.\textsuperscript{216} The regression coefficients and hazard ratios (HR) with 95% confidence intervals (CI) were calculated. The assumption of proportional hazards was tested by the method of Therneau and Grambsch.\textsuperscript{218}

LBD and MVD were modeled as continuous variables. MVD was square-root transformed to obtain normal distribution.\textsuperscript{30,134} Independent variables were allowed in the model if $p < 0.10$, and different models were compared with the likelihood ratio test.\textsuperscript{217} The number of variables was restricted to four, based on a rule to have at least 15 to 20 events per each additional variable.\textsuperscript{216} The assumption of proportional hazards was tested by the method of Therneau and Grambsch.\textsuperscript{218}

For the analysis of MLN the minimum sample size was calculated on the basis of a previous consecutive series, which reported the cumulative 10-year probability of survival to be 0.69 and 0.22 for patients who had a melanoma in which MLN was lower and higher than the median respectively, corresponding to a survival difference of 0.47.\textsuperscript{6} Power analysis indicated that to detect a similar difference as significant with a power of 80%, the study should have a minimum of 58 patients (Power and Precision, ver. 2.0; Biostat, Englewood, NJ). The other studies were more exploratory in nature and were not based on calculations after previously published data.
7. RESULTS

7.1 Clinical and Histopathological Tumor Characteristics

7.1.1 MLN in primary tumors

Nucleoli were identified in 126 (75%) of the 167 slides (Figure 1A Paper I). The median MLN was 4.05 µm (range, 2.60–6.18), and the mean was 4.06 µm (SD 0.54), which was comparable with other studies.37-39,46,48 (Table 1; Paper I)

The mean difference for reevaluating MLN using the Bland-Altman method for intraobserver comparison was +0.097 µm (95% CI, –0.025 to +0.22) (Figure 1B; Paper I), and the mean for interobserver comparison was –0.38 µm (95% CI, –0.51 to –0.25; Figure 1C; Paper I). The greater difference may be due to personal preference in caliper positioning.

7.1.2 MLN in metastatic tumors and their corresponding primaries

Nucleoli were identified in all 37 metastatic melanoma specimens, and in the 26 corresponding primary tumor specimens using the silver staining method. The median MLN in the matched primary UM was 4.1 µm (range, 3.3–5.2), and the mean was 4.2 µm (SD 0.52) (Table 1; Paper III).

The specimens of metastases fell into three subcategories, specimens from surgical biopsies (12), specimens from autopsies (24), and a specimen from a core needle biopsy (1). (Table 1) The median MLN in the surgical biopsies was 3.9 µm (range 3.2– 6.4), and the mean was 4.3 µm (SD 1.14), in the autopsy specimens the median was 3.5 µm (range 2.7– 5.7) and the mean 3.7 µm (SD 0.72) (Figure 1B; Paper III). The MLN in the single core needle biopsy was 3.8 µm. In the autopsy specimens MLN values were smaller in comparison to the surgical specimens (P = 0.065, Kruskal–Wallis test). (Table 1)

In the metastatic group of autopsied patients, three patients had received either chemotherapy or irradiation for their metastases while 12 did not receive any treatment. In the treated group the mean MLN was 3.6 µm and for those who did not receive any
Results

treatment the mean was 3.9 µm, although the median MLN for both groups was 3.6 µm. (Table 1).

The MLN in the autopsy group had a trend towards a smaller median value, which could be due to a post mortem artifact. To clarify this, the MLN was measured from normal hepatocytes in liver tissue available in two surgical and four autopsy specimens and compared. The median MLN was 2.2 µm in the surgical specimen, and 2.7 µm in the autopsy specimen, indicating that smaller MLN in metastases was not likely to be a systematic post mortem artifact.

Generally, hepatic metastases in all types of specimens combined had a smaller MLN than the corresponding primary UM, the median MLN was 3.6 µm (range 2.7–6.3) and mean 3.9 µm (SD 0.91), the mean difference between pairs being 0.55 µm (P = 0.066, Wilcoxon signed rank test) (Figure 1 C; Paper III).

**TABLE 1. Comparison of MLN measured from different specimens of hepatic metastases**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Surgical biopsies (n=12)</th>
<th>Autopsy (n=24)</th>
<th>Needle biopsy (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median MLN, (range)</td>
<td>3.9 (3.2– 6.4)</td>
<td>3.5 (2.7– 5.7)</td>
<td>3.8†</td>
</tr>
<tr>
<td>Mean MLN (SD)</td>
<td>4.3 (1.14)</td>
<td>3.7 (0.72)</td>
<td>3.8†</td>
</tr>
<tr>
<td>Median MLN of hepatocytes</td>
<td>2.2</td>
<td>2.7</td>
<td>-</td>
</tr>
<tr>
<td>Treatment given</td>
<td>-</td>
<td>3.6</td>
<td>-</td>
</tr>
<tr>
<td>No treatment</td>
<td>-</td>
<td>3.9</td>
<td>-</td>
</tr>
</tbody>
</table>

† Single specimen
Results

7.1.3 Microvascular factors in primary tumors

MVD was identified in 113 (68%) of the 167 slides. The median MVD was 32 counts/0.313 mm² (range, 5–121), and the mean was 37 counts/0.313 mm² (SD 23). Likewise extravascular matrix patterns were identified from 115 (69%) of the 167 slides. They were divided into three groups: no loops (40%), loops only (23%), and networks (37%).

7.1.4 Microvascular factors in metastatic and matched primary tumors

MVD was identified from 37 metastatic UM, and from 36 of the 37 corresponding primary tumors (Table 1; Paper III). The median of MVD in the matched primary UM was 44.5 counts/0.313 mm². In the metastatic specimens, MVD was 56 counts/0.313 mm², which was significantly higher than in the primaries (median difference, 7.5 counts/0.313 mm² more, range 38 counts less to 70 counts more; \( P = 0.044 \), Wilcoxon signed rank test) (Figure 1E; Paper III). MVD in metastases was not associated with specimen diameter (\( P = 0.62 \), Spearman’s rank correlation) (Figure 1F; Paper III), which was thus unlikely to be a confounding factor.

7.1.5 Ki-67 Immunopositivity in primary tumors

The tumor cross-sectional area was successfully measured from 137 (82%) of the 167 slides. Its median was 29 mm² (range, 1.2–135). The nuclei were successfully identified and counted in 141 (84%) of the slides. The median number of cells was 1,655 cells/mm² (range, 315–5,554), based on the number of nuclei identified.

Immunostaining of tumor slides with mAb Ki-67 was successful in 141 (84%) of the 167 slides (Figures 1C and D; Paper II). The median number of immunopositive Ki-67 cells was 8 per tumor cross-section (range, 0–272) and the mean was 32 cells (SD, 50), corresponding to a median of 0.30 cells/mm² (range, 0–53) and a mean of 1.7 cells/mm² (SD 5.2). The median percentage of Ki-67 immunopositive cells of all cells identified (proliferation index) was 0.02% (range 0–3.2) and the mean was 0.1% (SD 0.3).
7.1.6 IGF-1R Immunopositivity in primary tumors

The tumor was successfully measured from 129 (78%) of the 166 slides of primary UM. Immunoreactivity for IGF-1R varied from cell to cell and between tumor areas. The median tumor area was 34 mm² (range, 2-145). The median percentage of tumor area that was graded as immunopositive was 68% (range, 0-100) and its mean was 60% (SD, 39). Of the 129 tumors, 21 did not stain for IGF-1R.

7.2 Interrelationships between prognostic indicators

7.2.1 MLN in primary tumors

When epithelioid cells existed in the tumor, MLN was greater than if it consisted of spindle cells only (difference, 0.31 µm; \( P = 0.017 \), Kruskal-Wallis test; Figure 2A Paper I), MLN also rose with rising MVD (\( P = 0.0053 \), Spearman’s correlation; Figure 2B Paper I). MLN was not associated with gender (\( P = 0.21 \), Kruskal-Wallis test), ciliary body involvement (\( P = 0.44 \), Kruskal-Wallis test), LBD (\( P = 0.24 \), nonparametric test for trend; Figure 2C Paper I), pigmentation of the tumor (\( P = 0.84 \)), presence of tumor-infiltrating macrophages (\( P = 0.78 \)), and extravascular matrix loops and networks (\( P = 0.62 \); Figure 2D; Table 2; Paper I).

7.2.2 Ki-67 Immunopositivity in primary tumors

When the tumor type was non-spindle, the percentage of Ki-67 immunopositivity was higher than if the tumor consisted exclusively of spindle cells (median, 0.028% vs. 0%; \( P = 0.089 \) Kruskal-Wallis test). Proliferation index plot against tumor cross-sectional area suggested that this index is smaller in larger tumors (Figure 2B; Paper II). No similarities were seen between Ki-67 immunopositivity and gender, ciliary body involvement, LBD, pigmentation of the tumor, presence of tumor-infiltrating macrophages, MLN (Figure 2C; Paper; II), MVD, and the presence of extravascular matrix loops and networks (Table 1; Paper II).
7.2.3 IGF-1R Immunopositivity in primary tumors

High MVD appeared to be associated with the percentage of the tumor area which was immunopositive for IGF-R1 ($P = 0.060$) (Table 1; Paper IV). Heavy pigmentation and greater number of macrophages were also associated with the percentage of tumor area which was immunopositive ($P = 0.001$ and $P = 0.003$ respectively). Extrasclerally extending tumors had small proportion of immunopositivity for IGF-1R ($P = 0.049$). (Table 1; Paper IV).

No significant association was found between the proportion with IGF-1R positivity and involvement of the ciliary body, LBD, MLN, cell type, and extravascular matrix patterns (Table 1; Paper IV).

7.3 Melanoma-specific Mortality

7.3.1 Survival Outcome

In the study of MLN in primary tumors (Paper I), 21% of 126 patients were alive at the end of follow-up, 48% had died of metastatic UM, 6% had died of second cancer confirmed by histopathology, 25% had died of other diseases not related to malignancy, and one had died of unknown causes (Table 2).

Regarding Ki-67 in primary tumors (Paper II), 20% of 128 patients were still alive at the end of follow-up, 52% had died of metastatic UM, 5% of a second cancer confirmed by histopathology, 23% of unrelated disease, and one patient had died of unknown causes (Table 2).

For the IGF-1R study in primary melanomas (Paper IV), 17% of 129 patients were still alive by the end of the follow-up, 50% had died of metastatic UM, 6% of a second cancer confirmed by histopathology, and 27% had died of other causes (Table 2).
Results

**Table 2. Survival outcome of the patients included in the three studies of primary UM**

<table>
<thead>
<tr>
<th>Survival outcome</th>
<th>MLN (n=126)</th>
<th>Ki-67 (n=128)</th>
<th>IGF-1R (n=129)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients still alive</td>
<td>26 (21%)</td>
<td>26 (20%)</td>
<td>22 (17%)</td>
</tr>
<tr>
<td>UM metastatic death</td>
<td>60 (48%)</td>
<td>66 (52%)</td>
<td>64 (50%)</td>
</tr>
<tr>
<td>Second cancer death</td>
<td>8 (6%)</td>
<td>6 (5%)</td>
<td>8 (6%)</td>
</tr>
<tr>
<td>Other cause of death</td>
<td>31 (25%)</td>
<td>29 (23%)</td>
<td>35 (27%)</td>
</tr>
<tr>
<td>Unknown cause of death</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

**7.3.2 Prognostic significance of MLN, Ki-67, and IGF-1R by Kaplan-Meier analysis**

Melanoma-related mortality was associated with LBD ($P = 0.0007$ log-rank test for trend; Figure 3C Paper I, and $P = 0.0065$ Table II Paper II respectively), presence of epithelioid cells ($P = 0.0001$ log-rank test; Figure 3D Paper I, and $P < 0.0001$ Table II; Paper II respectively). As analyzed in the Ki-67 study, melanoma-related mortality was also associated with ciliary body involvement ($P = 0.0065$), degree of tumor pigmentation ($P = 0.0067$), presence of tumor-infiltrating macrophages ($P = 0.013$) and MLN ($P = 0.06$) by Kaplan-Meier analysis (Table 2; Paper II).

Likewise, melanoma-related mortality was associated with microvascular loops and networks ($P = 0.0001$ log-rank test for trend; Figure 3E Paper I, and $P = 0.0001$ Table II; Paper II respectively), and MVD ($P = 0.0001$ log-rank test for trend; Figure 3F Paper I, and $P < 0.0001$ Table II; Paper II respectively).

The Kaplan-Meier estimate for 10-year survival was 0.80 (95% CI, 0.64–0.90) if no loops, 0.48 (95% CI, 0.27–0.67) if loops were present without networks, and 0.40 (95% CI, 0.25–0.55) if loops forming networks were present (Paper I). The corresponding estimates according to tertiles of MVD were 0.86 (95% CI, 0.70–0.94), 0.50 (95% CI, 0.33–0.65), and 0.41 (95% CI, 0.24–0.57) respectively.
Melanoma-related mortality rates were significantly associated with MLN ($P = 0.0060$, log-rank test for trend; Figure 3A, Paper I). The 10-year Kaplan-Meier estimate for survival was 0.74 (95% CI, 0.56–0.85) for small, 0.60 (95% CI, 0.44–0.74) for medium, and 0.42 (95% CI, 0.25–0.58) for large MLN.

When Ki-67 immunopositive cells were present, the melanoma-related mortality was statistically higher than if they were absent ($P = 0.050$, log-rank test; Figure 3A; Paper II). The 10-year Kaplan-Meier estimate of mortality was 0.38 (95% CI, 0.26–0.52) for tumors which did not include Ki-67 immunopositive cells, and 0.50 (95% CI, 0.38–0.73) for tumors that contained Ki-67 immunopositive cells.

The Kaplan-Meier estimate for melanoma-related mortality was comparable, however, for both immunopositive groups when divided into three categories according to the median number of Ki-67 immunopositive cells (first category was negative for Ki-67, second was below the median, and the third was above the median). ($P = 0.80$, log-rank test; Figure 3B; Paper II).

Melanoma-related mortality was not associated with the area of IGF-1R immunopositivity by Kaplan-Meier analysis when divided in four categories ($P = 0.832$), (Table 2; Paper II).

7.3.3 Prognostic significance of MLN in metastatic tumors

Overall survival rates between patients having metastasis with a smaller versus larger MLN than the median value were comparable (1.1 months versus 1.8 months; $P = 0.95$, log-rank test) (Figure 2A; Paper III). Overall survival was longer if the metastases had a smaller MVD value than the median (6.5 months versus 1.5 months; $P = 0.096$, log-rank test) (Figure 2B; Paper III).

7.4 Cox Proportional Hazard Regression Analyses of MLN, Ki-67, and IGF-1R in Primary Tumors

7.4.1 Univariate analysis

MLN was significantly associated with melanoma-related mortality as a continuous variable ($P = 0.016$; HR = 1.82 for each micrometer increase), and was also significant as a categorical variable ($P = 0.007$; HR = 1.57 for each category increase). (Paper I)
Results

Ki-67 immunoreactivity was associated with melanoma-related mortality ($P = 0.052; HR = 1.65$ for tumors with immunopositive cells).

By Cox regression analysis, tumor area immunopositive for IGF-1R was not associated with survival in univariate analysis (HR 1.01 for each 10 percentage point increase in immunopositive tumor area; 95% CI 0.95-1.08, $P = 0.70$).

Other variables associated with melanoma-related mortality were ciliary body involvement ($P = 0.003; HR = 2.34$ in Paper I, $P = 0.008; HR = 2.04$ in Paper II), large LBD ($P = 0.001; HR = 1.13$ in Paper I, $P < 0.001; HR = 2.55$ in Paper II), presence of epitheliod cells ($P < 0.001; HR = 2.28$ in Paper I, $P < 0.001; HR = 3.22$ in Paper II), grade of pigmentation ($P = 0.017; HR = 1.54$ in Paper I), presence of microvascular loops and networks ($P < 0.001; HR = 1.80$ in Paper I, $P < 0.001; HR = 1.72$ in Paper II) and high MVD ($P < 0.001; HR = 1.80$ in Paper I, $P < 0.001; HR = 1.30$ in Paper II) (Table 3, Paper I; and Table 2, Paper II respectively).

7.4.2 Multivariate analysis

The bivariate and multivariate models showed that MLN was a significant predictor of prognosis, both as continuous and categorical variable, when adjusted for the effect of ciliary body involvement, LBD, presence of epitheliod cells, and microvascular loops and networks (Table 3; Paper I). MLN was of borderline significance when adjusted as a continuous variable for MVD ($P = 0.11$) (Table 3; Paper I).

Of all the bivariate models tested, two models which linked MLN with cell type and MLN with MVD predicted best melanoma-specific survival, and of these two, the one which included MVD was the best predictor (Table 3; Paper I). The other model linking MLN with microvascular loops and networks was better than the models linking MLN with ciliary body involvement, and MLN with LBD. When the four best predictors were combined in multivariate models, MLN lost its statistical significance when modeled as a continuous variable ($P = 0.11–0.18$) but retained its significance as a categorical one ($P = 0.023–0.067$) (Table 3; Paper I).

Ki-67 immunopositive cells remained an independent predictor of prognosis when adjusted in turn for the effect of MLN and MVD in bivariate models (Table 2; Paper II). The models which combined Ki-67 immunoreactivity with cell type and MLN best predicted melanoma-related mortality among all six models tested (models 3 & 4; Table 2; Paper II), and of these
two models number three was the preferred one (Table 2; Paper II; -2 log likelihood = 482.7 vs. 526.5; difference, 43.8; \( P < 0.0001 \) \( \chi^2 \) test).

When Ki-67 immunopositivity and MLN were combined with presence of epithelioid cells (model 7 Table 2; Paper II), extravascular patterns (model 8 Table 2; Paper II), and MVD (model 9 Table 2; Paper II), the fit improved, and the model with cell type provided the best fit (-2 log likelihood = 447.9 vs. 460.5; difference, 12.6; \( P = 0.0004 \)). Ki-67 immunopositivity and MLN decreased in significance in a model with four variables (Table 2; Paper II), but the general model fit improved (–2 log likelihood = 434.9 vs. 447.9; difference, 12.4; \( P = 0.0004 \)).

IGF-1R immunoreactivity was not an independent predictor of prognosis when including IGF-1R tumor area and MLN in a bivariate model (-2 log likelihood = 446.5, \( P = 0.937 \); Model 1; Table 3). The same applies to a bivariate model which included IGF-1R tumor area and MVD (-2 log likelihood = 447.8, \( P = 0.86 \); Model 2; Table 3). Nor did IGF-1R predict the prognosis in a trivariate model which combined IGF-1R immunopositive tumor area, MLN, and MVD (-2 log likelihood = 414.0, \( P = 0.44 \); Model 3; Table 3).
### TABLE 3. Multivariate model of IGF-1R immunoreactivity and melanoma-related mortality of patients with UM

IGF-1R immunoreactivity modeled as a categorical variable

<table>
<thead>
<tr>
<th>Regression</th>
<th>Wald Coefficient (SE)</th>
<th>Wald chi-square</th>
<th>Wald $P$</th>
<th>Wald Hazard ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLN†</td>
<td>0.545 (0.298)</td>
<td>3.35</td>
<td>0.067</td>
<td>1.72 (0.97-3.09)</td>
</tr>
</tbody>
</table>

**BIVARIATE ANALYSIS**

**Model 1, -2 log likelihood = 446.5**

<table>
<thead>
<tr>
<th>Regression</th>
<th>Wald Coefficient (SE)</th>
<th>Wald chi-square</th>
<th>Wald $P$</th>
<th>Wald Hazard ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1R area*</td>
<td>0.003 (0.038)</td>
<td>0.006</td>
<td>0.937</td>
<td>1.00 (0.93-1.08)</td>
</tr>
<tr>
<td>MLN†</td>
<td>0.545 (0.298)</td>
<td>3.35</td>
<td>0.067</td>
<td>1.72 (0.97-3.09)</td>
</tr>
</tbody>
</table>

**Model 2, -2 log likelihood = 447.8**

<table>
<thead>
<tr>
<th>Regression</th>
<th>Wald Coefficient (SE)</th>
<th>Wald chi-square</th>
<th>Wald $P$</th>
<th>Wald Hazard ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1R area*</td>
<td>-0.006 (0.037)</td>
<td>0.029</td>
<td>0.865</td>
<td>0.99 (0.92-1.07)</td>
</tr>
<tr>
<td>MVD‡</td>
<td>0.319 (0.077)</td>
<td>17.057</td>
<td>0.000</td>
<td>1.37 (1.18-1.60)</td>
</tr>
</tbody>
</table>
**MULTIVARIATE ANALYSIS**

Model 3, -2 log likelihood = 414.0

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (SE)</th>
<th>Exp(B)</th>
<th>P-value</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1R area*</td>
<td>-0.031 (0.409)</td>
<td>0.592</td>
<td>0.441</td>
<td>0.97 (0.89-1.05)</td>
</tr>
<tr>
<td>MLN†</td>
<td>0.414 (0.311)</td>
<td>1.769</td>
<td>0.184</td>
<td>1.51 (0.82-2.79)</td>
</tr>
<tr>
<td>MVD‡</td>
<td>0.299 (0.083)</td>
<td>13.032</td>
<td>0.000</td>
<td>1.35 (1.15-1.59)</td>
</tr>
</tbody>
</table>

---

SE = standard error  
CI = confidence interval  
* Continuous variable, 10 percentage points  
† Coding: First tertile = 0, Second tertile = 1, Third tertile = 2  
‡ Continuous variable, square–root transformed vessel count/0.313mm\(^2\)
8. DISCUSSION

8.1. Strengths and Limitations of the Study

There are some limitations in this study, mainly as some of the specimens were not satisfactory due to technical reasons. For example, it was technically difficult to evaluate the tumor area in some specimens due to loss of tissue, folds, or artifacts. On the other hand, some tumors were very small (to evaluate), or simply necrotic. In some heavily pigmented tumors it was difficult to identify the nucleoli or the immunoreaction despite bleaching of melanin.

As mentioned, specimens may lose some immunoreactivity during the laboratory process; e.g. because of formalin-induced cross-linking; and sometimes the intensity of retrieved immunoreactivity might vary from batch to batch. In addition, it was sometimes difficult to distinguish the borderline of true immunopositivity from the background, despite the use of positive controls.

Therefore, future projects using fresh UM material should be considered, to rule out the possibility of technical issues related to formalin-fixed, paraffin-embedded material. Because of the rarity of UM, this likely will necessitate prospectively collected, collaborative tissue banks.

The strength of this study resides in the fact that it is a consecutive, population-based cohort study, with good quality survival data and a long follow-up time. All the 167 malignant UM patients who had an eye enucleated because of choroidal and ciliary body melanoma were verified from the records of the ophthalmic pathology laboratory, Helsinki University Central Hospital, the main referral center which manages almost all uveal melanomas in Finland.

Cause of death was validated by reviewing all patient records relating to malignant tumors and death, crosschecking with the Finnish Population and Cancer Registries, and by acquiring all histopathological material available from primary tumors, metastases, and second cancers during this and earlier studies undertaken in this research laboratory.30,53,136

8.2 Main findings and their implications

A main goal of this dissertation was to study MLN, and microvascular factors in primary UM and their association with metastasis using a population-based retrospective cohort data
Discussion

set, and to evaluate whether these factors are independent predictors of survival in primary UM. Additionally, the association of cell proliferation in the same data set was evaluated by using the Ki-67 antibody in conjugation with MLN and other prognostic factors. Finally, the association between immunoreactivity for IGF-1R in primary UM and metastatic death was investigated, because proliferation of tumor cells in vitro can be reduced with substances which inhibit this receptor.\(^{153}\)

The mean MLN in primary UM was greater than previously published figures (Table 1; Paper I).\(^{37-39,46,48}\) This difference between studies could be multifactorial. One possibility is the effect of the staining substance. Silver was used in this study, and this is known to display nucleoli as larger than HE, thereby causing a difference in size.\(^{48}\) Because silver staining defines the boundaries of the nucleolus sharper than in HE staining, it facilitates the detection of irregularly shaped nucleoli allowing accurate discrimination of nucleoli.\(^{42,48}\) One study found that measurements from silver stained slides were easier to make and provided better prognosis than those from hematoxylin-eosin slides.\(^{48}\)

Another factor is the method used for measurement, because MLN will be smaller if the horizontal diameter of the nucleoli is measured instead of their longest diameter.\(^{46}\) Sampling techniques may well explain part of the difference in MLN between various studies. In the present study the sample was taken from the central 5-mm of the tumor, rather than from the whole tumor area, which has also been reported in another study to result in a larger MLN mean value.\(^{48}\)

Given the association between death and MLN in UM,\(^{33,36-38,41,46,48}\) selecting the data may have affected the MLN mean values. Earlier studies selected the patients in two groups, the first group containing half of the patients who died because of metastatic UM, while the other half had survived for a minimum of 10 years without any evidence of metastasis.\(^{33,35,38,48}\)

As these studies excluded patients surviving for a short time and possibly still at risk for developing metastasis, the mean MLN could have been biased towards a smaller value, as these patients were excluded from the analysis while they were still at risk of developing metastatic disease. Lastly, interobserver measurement variations may contribute to difference in mean MLN, as reported\(^{39}\) and as also was the case in my study.

In the present study MLN was strongly associated with death from UM, and the 10-year cumulative proportion for those who died was 0.32 units larger if the patients had UM with a large rather than a small MLN. Likewise, the risk of death was 3.1 times higher by Cox regression analysis if UM had a large MLN. One study reported the 10-year survival difference as 0.47,\(^{37}\) which is broadly comparable with my result.
MLN was significantly larger in UM which contained epithelioid cells,\textsuperscript{46,48} but no significant difference in MLN was found according to ciliary body involvement,\textsuperscript{46} and the presence of extravascular matrix loops and networks.\textsuperscript{39,46} MLN in this study was not associated with LBD as reported in other studies.\textsuperscript{39,46,48} Adding a new discovery, not reported earlier this study suggests that MLN is significantly associated with MVD.

In spite of the connection between MLN and the presence of epithelioid cells, both independently predicted survival by bivariate Cox regression, confirming some\textsuperscript{36} but not all previous analyses.\textsuperscript{33} Additionally, the fit of this model to the data was significantly better than that of competing models including ciliary body involvement, LBD, and microvascular loops and networks. The findings that MLN maintain significance when adjusting for LBD,\textsuperscript{33,35,36} loops,\textsuperscript{41} and networks\textsuperscript{46} concurs with earlier multivariate studies. It is appropriately well established that MLN and microvascular loops and networks in UM are unrelated, but other microvascular patterns have not been evaluated in this regard.

MVD is likewise known to be an important prognostic factor in UM, independent of extravascular matrix loops and networks.\textsuperscript{30,59,134} The association of MVD with prognosis may reflect the aggressiveness of UM tumor cells which either promote angiogenesis or share features with endothelial cells.\textsuperscript{59} It was found that multivariate models which included MVD and MLN fitted better with survival than models which had excluded MVD.

Given that MLN and extravascular matrix patterns predict the prognosis in UM may be in line with the theory that they could represent different stages possibly related to the metastatic capability, such as their ability to metastasize and proliferate. Along with their ability to express the degree of malignancy in melanoma cells,\textsuperscript{117} extravascular matrix loops and networks and hot spots which correlate to high MVD harbor the vascular channels, which may be involved when the UM cells pass through the blood circulation. One could speculate that nucleolar size is connected to tumor growth, as high metabolism is connected to active transcription, translation and gene activation. The possibility to send metastases and to proliferate is probably interrelated;\textsuperscript{219} it was observed in this study that MVD illustrates some prognostic association with MLN, and because UM cells express some antigens occasionally, which can be measured based on MVD,\textsuperscript{59} future investigations of nucleoli in UM which share these antigens might reveal a relationship at the cellular level.

To sum up, in this data set it was not only proven that MLN and extravascular matrix loops and networks are independent predictors of survival in UM,\textsuperscript{41,46} but it was also found that adding MVD to multivariate models with MLN fitted better with survival.
In addition to studying nucleolar size and microvascular factors in primary UM, this study investigated these factors in corresponding hepatic metastases. Generally the median MLN turned out to be smaller than in the corresponding primary tumors; whereas the opposite was true for MVD, which was larger in the metastases than in corresponding primary tumors.

Another interesting finding was that the MLN values from the autopsy specimens tended to be smaller than those of surgical biopsies. This was initially thought to be due to post mortem changes or artifacts. To verify this, MLN was measured from hepatocytes in normal liver tissue and the same groups of specimens were compared, but no difference was found. Thus, the difference was not likely to be due to post mortem artifacts. In spite of the mean MLN being smaller in metastases, MLN in certain metastases was nevertheless larger than in the corresponding primary UM, another possibility might be that most of the sampled metastases showed a minority which were dormant, hence the smaller nucleoli.

The median MVD in the primary tumors was lower than in the corresponding hepatic metastases. The MVD in the metastasis was not associated with specimen diameter; although the smaller the specimen is the more likely it is that such a sample does not represent the “hot spot” of densest microvascularization in the biopsied metastasis.

MVD is a well known prognostic indicator associated with shorter survival after diagnosis of primary UM. In the primary UM, MVD may indicate tumor angiogenesis and aggressiveness. High MVD in the primary UM is associated with the presence of epithelioid cells, which turned out to be true for the matched metastasis as well.

In line with the fact that the presence of high MVD in primary UM is associated with a shorter time for developing metastases than the presence of low MVD, high MVD in metastasis was associated with short survival after diagnosis of metastasis.

Once hepatic metastases were diagnosed, the survival was similar regardless of high or low MLN in the metastases, and therefore MLN in metastasis was not a prognostic indicator of survival.

Folberg et al. suggested that because no current effective treatments are available for metastatic UM, modeling the behavior of UM in the liver is important in order to study the biology of metastatic disease and to treat future metastasis.

Many cell proliferation markers are available commercially, such as PC-10, and Ki-67. Ki-67 recognizes a nuclear antigen present in proliferating cells, but does not detect cells in resting phase. Ki-67 has been studied by several authors as a tool to detect proliferating cells in UM. Some of the factors known to be associated with Ki-67 immunopositivity include p53 expression, larger tumor size, and shorter survival.
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PC-10 on the other hand is a cell cycling marker\textsuperscript{226} and has been studied for its role in evaluating cell proliferation in UM\textsuperscript{50,51,226} but it failed to work reliably with my paraffin sections, therefore Ki-67 was used as my primary antibody to study the association of cell proliferation in UM with MLN, microvascular factors, and survival.

In the present study the median Ki-67 proliferation index was 0.02\% (range, 0.7\% - 1.7\%) and less than in some published reports\textsuperscript{49,211,222,223,227} (Table 3; Paper II). The percentage of immunopositive tumors was also lower, 56\% versus 87\% -100\% in other studies.\textsuperscript{49,52,211,222-225,227}

A significant overlap was observed among the ranges in the published papers (Table 3; Paper II). A considerable number of factors could affect the median proliferation index. In this study the whole tumor area was evaluated for nuclear immunopositivity, while some authors have calculated the proliferation from random areas of the tumor\textsuperscript{211,223,227} or tumor “hot spots”\textsuperscript{52,224}. The latter method obviously might explain the higher mitotic index obtained (Table 3; Paper II).

Another factor worth considering is the counting methodology (Table 3; Paper II). The percentage of immunopositive cells was calculated in this study relative to all cells in the tumor, while others have calculated the fraction of immunopositive cells relative to cells counted from 10 to 20 high power fields (HPF).\textsuperscript{49,211,222,223,227} Some authors have simply counted immunopositive cells within an HPF irrespective of differences in the total number of cells in these fields.\textsuperscript{52,224,225} The present study showed that proliferation estimated by two methods from the same tumors may result in different conclusions.

Proliferation index values may also be affected by factors related to specimen processing; for example the specimen may lose its immunoreactivity during the staining process due to formalin-induced cross-linking, but this can be overcome by using antigen retrieval methods, such as heat treatment. These, however, often differ across laboratories (Table 3; Paper II). The effectiveness of reagents may vary depending on the batch used. Use of controls may contribute to the interpretation of true immunopositivity as well; in some studies, only negative controls have been used,\textsuperscript{52,211,223-225,227} which may cause uncertainty in interpreting the immunoreactivity. Lymphoma tissue was used as a positive control in this study, because it had large numbers of immunopositive cells and helped differentiate true Ki-67 immunopositivity from the background. Moreover, in some specimens when the tumor cell nuclei were not immunopositive for Ki-67, the limbal epithelial served as an internal positive control.
The presence of epithelioid cells was associated with high proliferation index in the present study, as indeed in earlier reports,\textsuperscript{222,223} an observation which is in line with the fact that the presence of epithelioid cells is clinically associated with shorter tumor doubling times.\textsuperscript{114,228} However, another study which had used the “hot-spot” sampling method reported an absence of any relationship with proliferation index and cell type.\textsuperscript{52}

In the present study, extravascular matrix loops and networks and Ki-67 immunopositivity were interrelated, similar to previous studies.\textsuperscript{224,225} This would support the hypothesis that the presence of extravascular matrix patterns is evidence of the aggressiveness of UM cells.\textsuperscript{117} Likewise Ki-67 proliferation index and ciliary body involvement were interrelated, which was also noted in another study.\textsuperscript{222} Ciliary body melanomas indeed metastasize more often than choroidal tumors which do not involve the ciliary body.

Differences between the studies mentioned in noticing the associations above may be due to the generally small number of specimens studied, and the variations in counting the proliferation index with Ki-67 antibodies which, as mentioned, are subject to differences in tissue processing techniques between different laboratories. Results from larger studies,\textsuperscript{52,222-224} such as the present one, must still be analyzed and further studies are needed to resolve the remaining discrepancies by staining more specimens from different laboratories.

The association of Ki-67 immunopositivity with the survival of patients with UM has not been studied earlier. Previous work has generally addressed the proliferation index after irradiation.\textsuperscript{49,52,211,223,225,227} In this study, a proliferation index higher than zero corresponded to 1.6 times higher mortality than absence of Ki-67 immunopositivity.

Ki-67 immunopositivity fitted best with the survival data when combined with MLN and the presence of epithelioid cells, and both remained independent indicators of high metastatic risk. In contrast, Ki-67 immunopositivity was not clearly connected with MVD or the presence of extravascular matrix loops and networks.

Cell proliferation index has been evaluated earlier by using other proliferation markers (such as PCNA), and a high index has been associated with shorter survival in patients with UM.\textsuperscript{46} Proliferation index, LBD, MLN, and extravascular matrix loops and networks were independent predictors of melanoma-related mortality in that study, whereas the cell type was not.\textsuperscript{46} Unfortunately, PCNA failed to work properly in my hands. These findings taken together nevertheless support the hypothesis that a high cell proliferation index is associated with a high risk of metastasis independent of MLN and extravascular matrix loops and networks.
Primary UM can now be divided into two classes based on the gene expression profile, in class 1 the tumor rarely metastasizes, whereas in class 2 there is a high risk for metastasis.\textsuperscript{94,229-231} Regarding the association between Ki-67 and gene expression profile, a recently published study\textsuperscript{232} reported a significant association between higher Ki-67 immunopositivity and class 2 tumors compared to class 1 tumors. This study may explain why class 2 tumors have strong tendency to rapid metastasis, and it also independently confirmed that Ki-67 positivity is strongly associated with metastatic risk.\textsuperscript{232}

Liver is the main target for metastasis in UM, therefore some growth factors are thought to be produced there; of which the insulin-like growth factor-1 (IGF-1) and its receptor (IGF-1R) are important.\textsuperscript{149,150} It is thought as well that IGF-1R is involved in tumorigenesis,\textsuperscript{150,151} and may have a role in metastasis in malignant tumors.\textsuperscript{152}

In my study of prognostic associations of IGF-R in primary UM, the percentage of tumors immunopositive for IGF-1R was 84%, which was comparable to findings of other studies\textsuperscript{153,212} (76%-95%). These studies were based on 36 and 132 surgically removed melanomas respectively.\textsuperscript{153,212} In another study of 40 enucleated UM specimens varying in size from medium to large immunopositivity was found in only 20%.\textsuperscript{152}

Obvious variations were noticed among all these studies regarding their methods, the only common factor between them being use of the same primary antibody.

In this study, the immunopositive tumor area was considered to be that which stained stronger than the background seen in lens fibers. Other studies have used the percentage of immunopositive cells instead, regardless of staining intensity.\textsuperscript{153,212} In another study, tumor grading was not based on the presence or absence of cytoplasmic immunopositivity, but on the presence of membranous immunostaining only.\textsuperscript{152}

Another factor is the loss of immunoreactivity due to formalin induced cross-linking, which was discussed earlier in this chapter (Chapter 8.2). One laboratory treated the slides with microwaves or digested them with pronase,\textsuperscript{153} whereas in another study there was no mention of antigen retrieval.\textsuperscript{212} A third study used microwaves and a citrate buffer.\textsuperscript{152} In our research laboratory, water bath treatment at 95ºC in a citrate buffer has been the preferred method as it often preserves ocular tissues better than cooking with microwaves because it does not involve boiling.

After immunostaining, melanin was bleached because sometimes the granules could interfere with recognition of immunoreaction.\textsuperscript{206} This step has also varied between studies, and no bleaching of melanin was mentioned in one study,\textsuperscript{153} whereas in another bleaching was done with hydrogen peroxide and disodium hydrogen phosphate\textsuperscript{212} before immunostaining,
which could have altered the immunoreactivity.\textsuperscript{206} Bleaching before immunostaining could well have caused the low percentage of immunopositivity in another study.\textsuperscript{152}

None of the published studies reported any relationship between ciliary body involvement or tumor size and IGF-1R immunoreactivity (Table 2; Paper IV). In one study, higher IGF-1R immunopositivity was seen in tumors which contained epithelioid rather than spindle cells ($P = 0.046$).\textsuperscript{153}

Darkly pigmented tumors were more frequently immunopositive than lightly pigmented ones in some studies (Table 2; Paper IV).\textsuperscript{152} Additionally it was noted that larger numbers of macrophages and a higher MVD were associated with larger IGF-1R immunopositive tumor areas. These factors have not been studied earlier. An association with heavy lymphocyte infiltration was, however, observed previously ($P = 0.039$, Fisher’s exact test), which was able to be calculated from data in the study of Topcu-Yilmaz et al.\textsuperscript{152} (Table 1; Paper IV)

Mäkitie et al. found that heavy pigmentation, larger numbers macrophages and high MVD were associated with one another.\textsuperscript{136} On the other hand macrophages and MVD, extravascular matrix loops and networks were not associated with IGF-1R. Nor did these patterns show any association with the number of macrophages,\textsuperscript{136} but they were associated with high MVD.\textsuperscript{30}

Taken together, the findings connect IGF-1R immunoreactivity more with presence macrophages than microvascular factors.

All-Ericsson et al.\textsuperscript{153} proposed that higher IGF-1R expression was associated with shorter survival rates, the 10-year rates of metastasis were reported as 33\% vs. 71\% for low vs. high expression\textsuperscript{153} respectively. This conclusion could be biased, as the study was exploratory. In a later study they reported a smaller survival difference, 30\% vs. 44\% vs. 59\% for no vs. low vs. moderate to high expression respectively,\textsuperscript{154} but it was not possible to prove such a large difference in my study.

Future collaboration using fresh tumor material to avoid using formalin-induced cross-linking from paraffin-embedded blocks should be considered to reassess the role of IGF-1R in predicting survival in UM before discarding this factor now because of contradictory results, because cyclolignan picropodophyllin inhibits the growth of UM both in cell culture and in xenografts.\textsuperscript{155,233} and could be applicable clinically. Taking all these factors together, however, assessing IGF-1R immunopositivity from formalin-fixed, paraffin-embedded material is not a practical method.
UM in general is a rare cancer, but it has the ability to metastasize hematogenously and shorten survival.\textsuperscript{24} The course of UM is poorly understood.\textsuperscript{234,235} It is well known that tumor size, location, and extrascleral extension are valuable clinical prognostic factors for diminished survival from UM.\textsuperscript{236} This study was undertaken to help to understand some histopathological factors which might contribute to developing metastasis in UM patients. Factors which could be related to tumor progression to metastasis disease, namely nucleolar size, MLN, MVD, cell proliferation, and IGF-1R were investigated.

Of the 167 patients with UM, who developed metastasis even after a very long time following removal of the eye, metastatic disease was the main cause of death, as documented in the Finnish Cancer Registry and on death certificates.

Using an independent population-based data set, it was confirmed that MLN and extravascular matrix loops and networks were unrelated, independent predictors of survival in UM.\textsuperscript{41,46} Also, it has been found that multivariate models including MVD in addition to MLN fitted significantly better with survival data than models which excluded MVD. This supports the idea that both the characteristics of the blood vessels and the cells are important, and the future direction would be to look for the gene expression profile, whether it is associated more with MVD or MLN.\textsuperscript{237,238} The former relates to the host response to the tumor and may not be as tightly associated with the gene expression profile, yet most likely involved in the process of hematogenous metastasis. Because fresh tumor material is needed for reliable genetic analysis, such analysis could not be performed.

Although noninvasive detection of certain extravascular matrix patterns is now technically possible\textsuperscript{124,239,240} in managing patients with UM,\textsuperscript{241} this study and tumor genetics suggest that such noninvasive methods will not fully capture the process of clinical metastasis. Progress in resection and biopsy techniques is likely in the near future to result in fresh material for the ophthalmic pathologist to correlate angiographic data, histopathological characteristics such as MLN, and genetic data.

This study supported the theory that tumors containing epithelioid cells grow faster and have poorer prognosis\textsuperscript{27,29-31} when studied by cell proliferation in UM based on Ki-67 immunoreactivity. Cell proliferation index fitted best with the survival data when combined with MVD, MLN, and presence of epithelioid cells.

The relationship between prognosis and MLN as measured from biopsy and autopsy specimens of hepatic metastases was significantly weaker than that of survival and MLN.
measured from primary tumors. Survival rates after the diagnosis of metastatic disease were comparable whether metastases had a high or a low MLN, and MLN in metastatic specimens was thus not a useful prognostic indicator.

Analogous with the finding that high MVD in primary UM is associated with shorter time to metastasis than low MVD, high MVD in hepatic metastasis tends to be associated with shorter survival after diagnosis of metastasis. A future direction would be to measure in the clinical settings the MVD of metastasis when planning treatment or estimating the prognosis after metastasis, as this is not currently done.

Because the liver is the main organ for metastasis from UM, growth factors largely produced in the liver – hepatocyte growth factor, epidermal growth factor and insulin-like growth factor-1 (IGF-1) – together with their receptors may have a role in the homing and survival of metastatic cells. Therefore the association between immunoreactivity for IGF-1R in primary UM and metastatic death was studied. It was found that immunoreactivity for IGF-IR did not independently predict metastasis from primary UM in my series. Partial loss of antigenicity could not be ruled out as a confounding factor because no frozen sections were available. Results of earlier studies have likewise been inconsistent, suggesting that immunohistochemical determination of IGF-1R from formalin-fixed, paraffin-embedded specimens is not practical as a routine test. It may be still useful to test inhibitors of IGF-1R clinically in spite of the noncontributory histopathological findings.
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11. REFERENCES


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