Macrophages in Regressed and Progressed Uveal Melanoma

By
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Academic Dissertation

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The Medical Faculty of the University of Helsinki
In Auditorium Areena, Folkhälsan
Topeliuksenkatu 20, Helsinki
On September 23th, 2011, at 12 noon.

Helsinki 2011
To the memory of my Father
To Jukka, Iikka, Tilda, Ahti, and Arvi
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This dissertation is based on the following original publications on non-irradiated, irradiated, and metastatic uveal melanoma. The original publications in the text will be referred to by their Roman numerals I-IV:

I

II

III

IV
## Abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ABC</td>
<td>Avidin-biotinylated peroxidase complex</td>
</tr>
<tr>
<td>ACAID</td>
<td>Anterior chamber-associated immune deviation</td>
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<td>APC</td>
<td>Antigen-presenting cell</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>COMS</td>
<td>The Collaborative Ocular Melanoma Study</td>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EVM</td>
<td>Extravascular matrix</td>
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<tr>
<td>EMAP</td>
<td>Endothelial monocyte-activating polypeptide</td>
</tr>
<tr>
<td>EORTC</td>
<td>European Organization for Research and Treatment of Cancer</td>
</tr>
<tr>
<td>FAG</td>
<td>Fluorescein angiography</td>
</tr>
<tr>
<td>FNAB</td>
<td>Fine needle aspiration biopsy</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
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<tr>
<td>HUCH</td>
<td>Helsinki University Central Hospital</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IOP</td>
<td>Intraocular pressure</td>
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<tr>
<td>LBD</td>
<td>Largest basal diameter</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage-migration-inhibitory factor</td>
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<tr>
<td>MLN</td>
<td>Mean diameter of the ten largest nucleoli</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MVD</td>
<td>Microvascular density</td>
</tr>
<tr>
<td>N/A</td>
<td>Not applicable</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>PAD</td>
<td>Pathologic-anatomical diagnosis</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-Schiff</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>TNM</td>
<td>Tumor, node, metastasis classification, a cancer staging system</td>
</tr>
<tr>
<td>TTT</td>
<td>Transpupillary thermotherapy</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasonography</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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1. ABSTRACT

This study was undertaken to better understand the behavior of macrophages during regression and progression of uveal melanoma. The study was divided into three histopathological parts (I, II, and IV) and one clinical part (III). The first study (I) aimed to find out how irradiation and subsequent regression of the tumor tissue affects the number and type of tumor-infiltrating macrophages and microcirculation attributes in uveal melanoma. The second study (II) was carried out to understand the relationship between tumor-infiltrating macrophages and microcirculation attributes in primary uveal melanoma and corresponding hepatic metastases. The purpose was to find out how progression of the tumor affects these variables and to investigate whether microvascular attributes influence the survival. The third study (III) described the evolution and addressed the origin of pigmented episcleral deposits found after brachytherapy and investigated their relationship to survival. The last study (IV) concentrated on the number of macrophages in normal extratumoral tissues in eyes with the uveal melanoma to chart the migration of macrophages.

I. Irradiation is known to influence tumor cells and blood vessels. I studied 56 eyes enucleated after brachytherapy: for 34 of which, it was possible to find a matched pair from 292 primarily-enucleated uveal melanomas. These 34 matched pairs of irradiated, secondarily-enucleated and primarily-enucleated uveal melanomas were stained with mAb PG-M1, which binds to the CD68 epitope in macrophages, with PAS to detect extravascular matrix loops and networks, and with mAb QBEND/10 to the CD34 epitope to determine MVD. Case-control analyses of irradiated uveal melanomas and primarily-enucleated eyes revealed lower MVD in irradiated uveal melanomas. The average number of macrophages remained unchanged after regression caused by brachytherapy.

II. From 292 primarily enucleated uveal melanomas, tumors with corresponding liver metastases were identified. A cross-sectional histopathologic analysis of 48 pairs of primary tumor and their metastases was carried out by staining both specimens in a way similar to the first study (I). The relationship between microcirculation attributes and melanoma-related mortality was also studied. MVD was higher in hepatic metastases than in corresponding primary tumors, and the survival of the patient after diagnosis of disseminated disease tended to be shorter if hepatic metastases had a higher MVD. Hepatic metastases had also a lower grade of pigmentation, more epithelioid cells, and more dendritic macrophages than the primary uveal melanomas which spawned the metastases.

III. This clinical study was a noncomparative clinical case series of 211 choroidal and ciliary body melanoma eyes, which were treated by a single ruthenium or iodine plaque brachytherapy. Eighty-eight eyes were treated prospectively during the study. The number and location of pigmented episcleral deposits were recorded under the slit lamp during each visit after brachytherapy. The association of the deposits with tumor characteristics and survival was analyzed with logistic regression and Kaplan-Meier analysis. During the study period, one eye with multiple pigmented episcleral deposits was enucleated because of irradiation complications and several hundred sections were stained immunohistochemically to detect the pigmented deposits. The study described for the first time pigmented episcleral deposits, which are found in most uveal melanoma eyes after brachytherapy and proved that
Abstract

the deposits are macrophage-related. This knowledge may save patients from unnecessary enucleation, because episcleral pigmented deposits might be mistaken for extrascleral tumor growth. The presence of pigmented macrophage-related episcleral deposits was associated with plaque size and isotope rather than with tumor size, suggesting that radiation atrophy of retinal pigment epithelium and choroid in addition to tumor regression contributes to the formation of the deposits.

IV. This was a case-control study of the same 34 matched pairs used in the first study (I). The purpose was to find out how irradiation affects the number and migration of macrophages in extratumoral tissues in uveal melanoma eyes. The number of macrophages was counted in the normal sclera beneath the tumor base, in the choroid adjacent to the tumor, and in the ciliary body from mAb PG-M1 stained uveal melanoma eyes. The number of macrophage-related deposits was counted in limbal episclera, ipsi- and contralateral to the tumor. The study confirmed that resident macrophages are present in extratumoral tissues in uveal melanoma eyes. Brachytherapy appeared to increase the number of infiltrating macrophages in the sclera and the number of histopathologically detectable episcleral aggregates of macrophages close to the limbus. The latter may be clinically visible as episcleral deposits in irradiated eyes. The distribution of macrophages suggests that, after irradiation, these cells migrate to the anterior segment of the eye along the sclera rather than along the uveal tract as in non-irradiated eyes. The presence of macrophages reflects local inflammatory responses and detailed knowledge of their behavior and distribution might help to develop biological tools against uveal melanoma in the future.
2. INTRODUCTION

Uveal melanoma is one of the two most common primary malignancies within the eye\(^1\) and the second most common type of primary malignant melanoma in humans. It is thought to develop from melanocytes in the uvea, which can be anatomically divided into three parts: the highly vascularized choroid, the ciliary body, and the iris (Fig. 1).\(^2\) The choroid lies in the posterior segment of the eye between the hard white sclera and the sensory retina, and the ciliary body supports the lens of the eye anteriorly and produces intraocular aqueous humor. This thesis covers choroidal and ciliary body, but not iris melanomas because of their divergent biological behavior compared with choroidal and ciliary body melanomas.\(^3\)

Figure 1. Cross-section of an enucleated eye with uveal melanoma (the star). Small arrows point out the iris, big arrow-heads ciliary body, and small arrow-heads choroid which in this section has partly detached from the sclera.
Uveal melanoma threatens both vision and survival. Vision is at risk because of both the tumor itself and as a consequence of different treatments. Survival has hardly improved over the decades despite intensive research in the oncology field. One explanation for this is that because the eye is an immunologically sheltered organ, the primary tumor may grow without interference within the eye. The larger the tumor, the poorer the survival and conservation of vision. Uveal melanoma disseminates purely hematogenously if the conjunctiva is not invaded, and it has a tendency to metastasize to the liver. In addition, dissemination in the form of micrometastases is believed to take place several years before diagnosis. These micrometastases may stay dormant for several years and are clinically undetectable. Once they progress to macrometastases within the liver or elsewhere, which can be seen on imaging studies, the remaining lifetime of the patient is usually short. Approximately half of the patients die within 15 years after diagnosis of the primary tumor, when analyzed by Kaplan-Meier method. By cumulative incidence estimates, which take competing risks into account, the mortality of 50% is reached by 30 years.

Until 1970s, uveal melanomas were treated by enucleation i.e. removal of the eye. Thereafter, eye-conserving treatment methods based on both irradiation and surgery came into clinical practice. These conservative treatments have proved to be as safe for the patient as enucleation, and in most cases with small to medium-sized melanomas, eyes with useful visual acuity can be achieved with these techniques. Currently, among the most common treatments for choroidal and ciliary body melanoma is brachytherapy, especially in Europe and North America. Other conservative treatment modalities include charged particle irradiation, fractionated stereotactic radiotherapy, gamma-knife radiosurgery, and local transscleral resection. Patients in this thesis were treated by primary enucleation or brachytherapy using cobalt, ruthenium, and iodine plaques.

This study was mainly designed to evaluate the histopathological events of the regression and progression of uveal melanoma. The former is induced by primary brachytherapy of the tumor, and the latter is evidenced by development of metastases from the primary uveal melanoma. Understanding the biological behavior of uveal melanomas both in the state of regression and the state of progression might guide us in finding new treatment modalities against this disease, which is fatal far too often.
3. REVIEW OF THE LITERATURE

3.1. EPIDEMIOLOGY OF UVEAL MELANOMA

Uveal melanoma arises annually in 4 to 11 people per million inhabitants in Caucasian populations.\textsuperscript{23-25} In the world population, the annual number of uveal melanomas is estimated to range from about 6700 to 7100.\textsuperscript{1} The incidence of uveal melanoma in Finland between 1955 and 1994 varied from 6.9 to 11 per million people and was somewhat higher among males than females for reasons unknown.\textsuperscript{26} In Sweden, the incidence is similar to that in Finland.\textsuperscript{23} In the United States, the recently reported overall mean age-adjusted incidence was lower, being 4.3 per million,\textsuperscript{25} similar to that in Central Europe but higher than in Southern Europe.\textsuperscript{27}

Even though the incidence of cutaneous and conjunctival melanoma\textsuperscript{28} has been increasing over the last decades (possibly due to increasing exposure to ultraviolet radiation), the incidence of uveal melanoma has been essentially stable.\textsuperscript{23,25}

Uveal melanoma is usually unilateral. A bilateral disease (i.e. a primary tumor in both eyes) is a rarity, occurring in less than 2 in 1000 patients with uveal melanoma.\textsuperscript{29,30}

3.2. PATHOGENESIS

3.2.1. Etiology

The etiology of uveal melanoma is still a largely unsolved puzzle. During the last two decades genetic investigations have identified several chromosomal defects associated with uveal melanoma, the most important of which seems to be the combination of monosomy of chromosome 3 and partial gain of chromosome 8.\textsuperscript{31-35} Several predisposing factors have been investigated, some of which are still controversial.

Sunlight has been suspected of increasing the risk for uveal melanoma,\textsuperscript{36} as it does for skin and conjunctival melanoma.\textsuperscript{28} However, there is no firm scientific evidence to support this hypothesis. Instead, geographic latitude is strongly associated with the incidence of uveal melanoma.\textsuperscript{27,37,38}

3.2.2. Predisposing factors

3.2.2.1. Age

Uveal melanoma is rare in young patients but can occur as early as in teenagers.\textsuperscript{39} The risk for it increases with age, especially after the age of 45 years until the age of 70, after which the risk-curve reaches a plateau.\textsuperscript{14,25} The median age at diagnosis is 55-65 years.\textsuperscript{24,40}

3.2.2.2. Race

Uveal melanoma has been estimated to be 9-72 times more common in Caucasians than in Africans and Orientals.\textsuperscript{25,40,41} Light-colored skin and iris color are also risk factors for uveal melanoma.\textsuperscript{42,43}

3.2.2.3. Nevi

A choroidal nevus can be found in 3 - 20% of normal Caucasian populations.\textsuperscript{2,44} Progression of these common nevi into malignant uveal melanoma is rare and it has been estimated that about 1 of 8800 choroidal nevi becomes malignant annually.\textsuperscript{2,44} Lifetime risk maybe about
Review of the literature

1%. It may be difficult to recognize a nevus from a small choroidal melanoma because they often share characteristics. Characteristics of nevi likely to grow, or of small choroidal melanomas, which suggest a high probability of malignancy, have been identified. These include: presence of symptoms and subretinal fluid; tumor thickness greater than 2 mm; orange lipofuscin pigment over the tumor; and tumor margin touching the optic disc. 

The COMS group has found additional factors, such as larger basal diameter, absence of drusen, and absence of retinal pigment epithelial changes that are predictive for growth. Recently, Shields et al added three more “helpful hints”, which could help the clinician to find a small melanoma at an earlier stage: ultrasonographic hollowness, and absence of both drusen and halo around the tumor. Any one of these factors raises the risk for growth, and the risk increases with increasing number of characteristics. These features (thickness greater than 2 mm, fluid, symptoms, orange pigment, margin touching optic disc, ultrasonographic hollowness, halo absence, and drusen absence) predicting growth and malignancy can be remembered with the mnemonic “To find small ocular melanomas using helpful hints daily”. 

3.2.2.4. Ocular and oculodermal melanocytosis

Ocular melanocytosis (OM) is a congenital pigmentary anomaly in which unusually large numbers of melanocytes have migrated to the uveal tract, episclera, sclera, orbital tissues, and sometimes to the meninges. If the periocular skin also is involved, the condition is termed oculodermal melanocytosis (nevus of Ota). Rarely melanocytosis can be associated with Sturge-Weber syndrome. OM and nevus of Ota are usually unilateral and nonhereditary. Both conditions are fairly common in Asians but the risk for uveal melanoma is small among them. In whites, the prevalence is about 0.04% and an association with uveal melanoma is clear. It has been estimated that OM increases the risk for uveal melanoma over 20-fold as compared with the normal population, and that the lifetime risk of developing uveal melanoma in patients with OM is 1 in 400. On the other hand, about 1.4% of Caucasian patients with uveal melanoma have OM.

3.2.2.5. Other factors

Smoking and hormonal factors have been suspected of increasing the risk for uveal melanoma or the growth of its metastases in the past, but no conclusive evidence exists.

3.2.3. Heredity

Even though uveal melanoma most often occurs sporadically, some families with more than one member affected with uveal melanoma exist. Familial uveal melanoma may in some cases be associated with other cancers. Families with both uveal and cutaneous melanomas suffer often from the familial atypical multiple mole-melanoma syndrome. The possible underlying genetic alterations and environmental factors in families with uveal melanoma and other primary cancers are not fully understood.

3.2.4. Growth pattern

Most uveal melanomas grow slowly and without causing inflammation because of the special immunological environment within the eye. At earlier stages, a small choroidal melanoma is flat and it may be difficult to distinguish it from a benign choroidal nevus. Ciliary body and choroidal melanomas generally grow both in diameter and height. The diffuse variant grows mainly in diameter. It has been estimated that it takes approximately 7 years for a medium-
sized melanoma (LBD < 10 mm) to become a large melanoma (LBD > 15 mm). Most choroidal melanomas display a dome or mushroom-shaped growth pattern. In choroidal melanomas, the pathognomic mushroom-shape emerges when the tumor thickness increases and the tumor finally breaks through Bruch’s membrane into the subretinal space. In some eyes, the tumor will also grow through the retina into the vitreous. Rupture of Bruch’s membrane is seen in 40-87% of enucleated uveal melanoma eyes. Extraretinal tumor growth to the orbit is also possible and is reported in 2-17% of uveal melanomas. Additionally, uveal melanomas sometimes grow into the optic nerve. This is seen approximately in 2-5% of all enucleated eyes with uveal melanoma, being even more frequent in uveal melanomas located adjacent to the optic nerve.

Ciliary body melanomas grow either in a circumscribed or in a diffuse pattern. A characteristic form of the latter is a circumferential growth, resulting in a so-called “ring melanoma”. Prognosis of ring melanomas is poor due to the difficulties in diagnosis because of their hidden growth pattern. In very rare cases, a ciliary body melanoma may grow in a retinoinvasive manner, which means that the tumor invades through the vitreous and non-adjacent retina into the retrobulbar optic nerve.

3.2.5. Metastasis
Like most malignant tumors, uveal melanoma has a tendency to metastasize. It has been calculated that primary uveal melanomas may micrometastasize several years before treatment. Progression of uveal melanoma into metastatic disease depends on several patient- and tumor-related factors. Because there are no lymphatic vessels within the eye, uveal melanoma disseminates hematogenously. However, dissemination via lymphatics is possible, if the tumor has invaded the conjunctiva and its lymphatics. The most common site for metastasis is the liver, being involved in more than 90% of cases of metastatic uveal melanoma. Liver is often also the only metastatic site (in up to 56% of patients) but metastases may typically develop later also in the lung, skin, bone, and rarely the brain.

Despite effective current treatments for the primary tumor, metastatic disease still develops in about 40-50% of uveal melanoma patients within 10-15 years, as analyzed by the Kaplan-Meier method; but metastasis even as late as 40 years after diagnosis of the primary tumor has been reported. After detection of metastases, the prognosis is poor and death usually occurs within 12 months. In 2003, Eskelin et al presented a working formulation, which took into account the Karnofsky index (a measure of general health of the patient); the largest dimension of the largest metastasis; and serum level of alkaline phosphatase (AP) of metastatic uveal melanoma patients. Depending on these variables, the patients’ survival could be categorized into three groups: group A corresponded to a predicted survival of at least 12 months; group B predicted a survival of 6-11 months; and group C a survival less than 6 months. Current therapies for metastatic uveal melanoma have only slightly prolonged the survival of patients, and even this improvement may partly be due to lead time bias.
3.3. DIAGNOSIS

To minimize ocular morbidity and to improve survival, early diagnosis of uveal melanoma is desirable. Depending on tumor location, the symptoms may vary or be even absent for several years. Most of them are unspecific. In Finland, 13% of the patients diagnosed with uveal melanoma are entirely asymptomatic and contact the ophthalmologist mostly in order to change spectacles. Approximately 10% of uveal melanomas seem to arise from known presumed nevi. Follow-up is often needed when the tumor is small in order to verify growth. The presence of high risk characteristics is increasingly being considered to be an indication to initiate treatment, especially if the tumor is located distant from the macula and optic nerve.

3.3.1. Symptoms

Most typical symptoms before diagnosis are blurred vision, visual field defect, photopsia, and floaters, which are often caused by a secondary exudative retinal detachment adjacent to a choroidal tumor. Intravitreal hemorrhages, caused by tumor growth through the retina, may also lead to sudden visual loss.

Irritation and ocular or periocular pain are possible, if uveal melanoma affects the ciliary body. Additionally, ciliary body melanomas may sometimes present with glaucoma, sector cataract or uveitis. Mechanisms behind these symptoms are tumor invasion of the chamber angle, contact with the lens, and inflammation caused by a large tumor. If a choroidal melanoma causes glaucoma, the mechanism is either angle closure by the tumor or iris neovascularisation.

3.3.2. Clinical diagnosis

The diagnosis is usually made by a retinal specialist or ocular oncologist using slit lamp biomicroscopy and indirect ophthalmoscopy. Typically, the diagnosis is based on fundus examination and B-scan ultrasound, but other supportive diagnostic methods are A-scan ultrasonography, fluorescein angiography (FAG), indocyanine green angiography (ICG), optical coherence tomography (OCT), orbital CT and MRI, and positron emission tomography/computed tomography (PET/CT) scanning. The latter utilizes 18-fluoro-2-deoxyglucose (FDG), which is a radioactive form of glucose that accumulates in metabolically active tumor cells.

Uveal melanomas have some characteristic clinical features. Their surface, particularly in the posterior pole, often shows a patchy orange pigmentation caused by lipofuscin in macrophages and retinal pigment epithelium. The pathognomic form of uveal melanoma is the mushroom-shape, which results from a rupture in Bruch’s membrane. Pigmentation of the tumor may vary from amelanotic to darkly pigmented, even within the tumor. Exudative retinal detachment (RD) surrounding or covering the tumor tissue is typical. OCT may be helpful in diagnosing an incipient RD.

Uveal melanoma has distinct echogenic structure with decreasing reflectivity within the tumor in contrast to other uveal tumors. Ultrasound is also an excellent tool in follow-up to detect growth of observed small tumors and regression or progression of treated tumors, including extrascleral growth. High-frequency ultrasound helps to detect ciliary body melanomas, which can sometimes be visualized also by transillumination.

FAG and ICG cannot distinguish a malignant from a benign choroidal tumor and their diagnostic value is limited. A typical “double circulation” pattern can often nevertheless be
seen in uveal melanoma. If a vascular tumor is suspected in the differential diagnosis of an amelanotic melanoma, FAG can still be useful. ICG uses infrared light, which penetrates the choroid more efficiently, helping to identify vascularization within the tumor better than with FAG. It may even delineate fluid-conducting extravascular matrix patterns, some of which are known prognostic parameters, within the tumor tissue.

The diagnosis of uveal melanoma may remain equivocal: for example, if the tumor is amelanotic and thus resembles a metastasis. In that case, systemic investigations to rule out a primary tumor or widespread metastases elsewhere are then useful and CT and MRI may give further information.

Fine-needle aspiration biopsy (FNAB), which is widely used in the diagnosis of tumors, is regularly used only in atypical cases with difficulties in a definite diagnosis. A feared consequence of this procedure is local spread of tumor cells through the site of scleral perforation. However, with current small needles and technique, such spread is exceptional, especially if the biopsy is immediately followed by the radioactive plaque brachytherapy. Currently, ocular oncology centers have started to biopsy also to obtain prognostic information.

The main differential diagnoses for uveal melanoma are choroidal nevus, melanocytoma, hemangioma, osteoma, and metastasis to the eye. The latter most often originate from breast and lung cancer in females and males, respectively. In the Collaborative Ocular Melanoma Study (COMS), it was noted that, a possibility for second primary cancer is good to keep in mind, particularly amongst smokers. The most common sites for the second primary tumor in the COMS series were the prostate (23%) and the breast (17%). Generally, up to 10% of patients with uveal melanoma have or develop later a second cancer.

3.4. TREATMENT OF PRIMARY TUMOR

Treatment of primary uveal melanoma can roughly be divided in radical and conservative treatments. The former consists of enucleation (i.e. removal of the eye) or exenteration if the tumor extends into the orbit. Conservative treatments consist of several different treatment options, which all aim to save the eye and any remaining useful vision.

These conservative treatment options for small melanomas include observation, laser photocoagulation, transpupillary thermotherapy (TTT), and plaque brachytherapy; for medium-sized tumors plaque brachytherapy, local transscleral resection, charged particle irradiation (mainly proton beam therapy) and stereotactic radiotherapy; and for large melanomas endoresection, local transscleral resection, charged particle irradiation, and plaque brachytherapy.

3.4.1. Enucleation

Enucleation was the only treatment until the 1960’s when eye-conserving treatments came to daily clinical use. Development of conservative treatments was hastened by Zimmerman, McLean, and Foster, who published articles in which they questioned the benefits of enucleation and suggested the possibility for accelerated dissemination of tumor cells by this procedure. Later, The Collaborative Ocular Melanoma Study (COMS) Group ventured to find out whether or not any difference in all-cause mortality rates after treatment of uveal melanoma with enucleation versus brachytherapy existed. In 2001, the COMS Group reported similar 10-year survival rates for patients with medium-sized melanomas undergoing either enucleation or iodine brachytherapy. This randomized multi-center study also showed
that irradiation (20 Gy) of large melanomas preoperatively does not improve survival, although it does decrease the risk of orbital recurrence.\textsuperscript{103,104}

Enucleation remains the most frequent primary treatment in the case of very large tumors with little hope of saving the eye and useful vision. However, plaque brachytherapy may offer a chance of preserving useful vision at least short-term: for every 6 patients with large, irradiated uveal melanoma, one preserves some useful vision in the tumor eye for at least 2 years.\textsuperscript{18}

As a secondary treatment, enucleation is performed after conservative treatments should tumor re-growth or major treatment complications occur. COMS reported a 12.5\% cumulative proportion estimate of secondary enucleation at 5 years after primary treatment of medium-sized uveal melanomas with brachytherapy using Kaplan-Meier analysis.\textsuperscript{105} For large tumors primarily treated with iodine brachytherapy, the corresponding figure was 16\%.\textsuperscript{18,106}

3.4.2. Plaque brachytherapy

Brachytherapy, which is radiotherapy delivered with concave plaques containing radioactive material, is currently the most common treatment of uveal melanoma in developed countries. The plaque is sutured against the outer wall of the globe (i.e. sclera) over the tumor base and is left there for a pre-calculated time depending on the height of the tumor and the age of the plaque.\textsuperscript{107} When the required dose, generally at least 80-100 Gy at tumor apex, has been delivered, the plaque is removed. This usually takes 1-14 days.

Radioisotopes used in plaque brachytherapy are shown in Table 1. In Finland, the first isotope used was cobalt-60, which scatters more radiation to the healthy, surrounding tissues and thus generates numerous radiation-related complications.\textsuperscript{108} Consequently, this $\gamma$-ray source has been replaced by safer ones, such as iodine-125 and palladium-103 $\gamma$-ray sources as well as the ruthenium-106 $\beta$-ray source. Iodine-125 is the most widely used isotope in the world and one of two isotopes used in Finland. It is suitable for the treatment of medium- and even large-sized melanomas, and its use has been widely documented.\textsuperscript{17,18,105,109-113} Iodine also was the isotope used in the COMS study.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Symbol</th>
<th>Type</th>
<th>Energy</th>
<th>Half-Life</th>
<th>Introduced*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobalt</td>
<td>Co-60</td>
<td>Gamma / Beta</td>
<td>1.3 MeV / 320 keV</td>
<td>5.2 years</td>
<td>1948</td>
</tr>
<tr>
<td>Ruthenium</td>
<td>Ru-106</td>
<td>Beta</td>
<td>293 keV</td>
<td>373 days</td>
<td>1964</td>
</tr>
<tr>
<td>Iodine</td>
<td>I-125</td>
<td>Gamma</td>
<td>27-35 keV</td>
<td>60 days</td>
<td>1975</td>
</tr>
<tr>
<td>Strontium</td>
<td>Sr-90</td>
<td>Beta</td>
<td>546 keV</td>
<td>29 years</td>
<td>1983</td>
</tr>
<tr>
<td>Iridium</td>
<td>Ir-192</td>
<td>Gamma / Beta</td>
<td>600 keV / 370 keV</td>
<td>74 days</td>
<td>1983</td>
</tr>
<tr>
<td>Palladium</td>
<td>Pd-103</td>
<td>Gamma</td>
<td>21 keV</td>
<td>50 days†</td>
<td>1986</td>
</tr>
</tbody>
</table>

* First used in ophthalmology
† In practice, the half-life of Pd-103 is 17 days because of the dramatic drop in energy emission after that
The other isotope used in Finland is ruthenium-106, which is suited only for the treatment of small and medium-sized melanomas because it has lower tissue penetration (up to 6 mm). Ruthenium-106 was first applied by Lommatzsch and Vollmar in 1964, and is nowadays in common use especially in Europe.\textsuperscript{114-121}

The most common treatment complications after brachytherapy are cataract, radiation retinopathy, maculopathy, optic neuropathy, retinal or vitreous hemorrhages and exudative retinal detachment.\textsuperscript{112;123;124} Dry eye, scleral melting, keratopathy, episcleritis, and strabismus are also possible. If radiation retinopathy or optic neuropathy becomes severe or persists, neovascular glaucoma may develop and the patient may end up with a blind and painful eye requiring enucleation.

Radiation complications depend on a number of factors, which are patient-related (e.g. diabetes), tumor-related (e.g. tumor size, location), and irradiation-related (e.g. isotope, total dose). With specific positioning of radioactive seeds and collimating plaque design, the risk for radiation complications may decrease.\textsuperscript{113;125} Fortunately, the more serious complications typically appear with a delay 2-4 years after brachytherapy. It has been estimated that 89\% of patients treated by conservative therapy (not only brachytherapy) succeed in saving their eye for 5 years.\textsuperscript{126}

At 5 and 10 years after ruthenium brachytherapy, the local recurrence rate for choroidal and ciliary body melanomas, including also large tumors, has been estimated to be as high as 22-24\%.\textsuperscript{127} Large LBD and rupture of Bruch’s membrane predict local recurrence. For small or medium-size melanomas (LBD ≤ 16 mm and height ≤ 8 mm), a local tumor control rate as good as 96\% has reported at 5 years after ruthenium brachytherapy.\textsuperscript{119} In a recently-published English study of 189 patients with posterior uveal melanoma, 14 patients developed a recurrence and 13 did not respond to ruthenium brachytherapy.\textsuperscript{128} Thus, the overall control rate was approximately 86\%. The recurrences appeared at a median of 25 months after treatment (range, 12 to 71 months).

After iodine brachytherapy for large uveal melanomas, the 5-year incidence of local tumor recurrence has reported to be 6-7\% depending on tumor dimensions.\textsuperscript{18;106} In the case of juxtapapillary choroidal melanomas, the corresponding estimates for tumor recurrence have been 14\% and 21\% at 5 and 10 years, respectively.\textsuperscript{129} Most (95\%) of these juxtapapillary cases were treated with iodine brachytherapy.

3.5. TREATMENT OF METASTASES

Metastatic uveal melanoma is almost invariably fatal mainly due to its preferential metastatic site, the liver, and its resistance to chemotherapy. Especially liver metastases have proven to be resistant to available systemic chemo- and immunotherapies.\textsuperscript{6-8;130} Difficulties in controlling liver metastases by intravenous treatments have led to an urge to develop regional treatment modalities, including surgical resection,\textsuperscript{131} hepatic intra-arterial chemotherapy, chemoembolization, isolated hepatic perfusion, regional immunotherapy, and percutaneous hepatic perfusion.\textsuperscript{132} If extrahepatic metastases exist, systemic chemo- and or immunotherapy are additionally given.\textsuperscript{81} In rare cases, treatment of metastases may result in long-term event-free survival.\textsuperscript{133;134}
3.6. PROGNOSIS

Prognosis of uveal melanoma is still approximately the same as that reported decades ago despite many efforts to detect primary tumors early, to screen for metastasis, to develop efficient and safe treatment options both for the primary tumor and its metastases, and to understand the biological behavior of the tumor. Several patient- and tumor-related factors influence the risk for metastasis and death.

3.6.1. TNM classification

One useful tool for categorizing cancer patients into different prognostic groups is the Tumor, Node, Metastasis (TNM) classification. It is widely used for classifying solid tumors (i.e. carcinomas). Such classification helps in planning appropriate treatment, prognosticating, and estimating treatment results. Furthermore, a valid classification supports research and facilitates participation in multicenter clinical trials.

The very first effort to classify uveal melanomas was that of Knapp in the late 19th century. He divided tumors according to symptoms, extraocular growth, and metastasis. Subsequently, Callender classified uveal melanomas based on the morphology of tumor cells in 1931 (see 3.7.2.4.). Warren classified uveal melanomas according to their size, and his staging system, with later modifications, became widely used in the United States and formed the basis for the first TNM system (first included in its 4th edition).

In the COMS trial, tumors were divided into “small”, “medium-sized”, and “large”, depending mainly on tumor height and LBD, but this system was not a true classification. It represented inclusion criteria in different arms of this particular study.

In 2003, the 6th edition of TNM classification (TNM6) adopted the size-categories created by the COMS Group. This new TNM system neglected ciliary body involvement, which was an important independent predictor for prognosis, and classified most tumors as medium-sized. Inspired by criticism to TNM6, the Ophthalmic Oncology Task Force, consisting of 43 physicians from 11 countries, was created to revise the TNM system in order to make it evidence-based. In the 7th edition (TNM7), the definitions of T1-T4 have been modified by considering ciliary body involvement and revising handling of extraocular extension (without, equal to or less than 5 mm, and greater than 5 mm).

Taking tumor size, ciliary body involvement and extraocular extension into account, the TNM7 categories were regrouped according to survival into stages. Ten-year survival rates for the seven TNM7 stages I, IIA-B, IIIA-C, and IV were 88%, 80%, 68%, 45%, 26%, 21%, and 0%, respectively.

3.7. PROGNOSTIC FACTORS

3.7.1. Patient-related factors

The older the patient, the worse the prognosis has been a common conclusion based on Cox regression analyses. However, if competing risks, which are frequent in older age-groups, are considered, increasing age is no longer a significant independent predictor of melanoma-related shortened survival.

Other patient-related factors, which may be associated with increased risk of metastatic disease in uveal melanoma, include light irises and the cutaneous dysplastic nevi syndrome.
3.7.2. Tumor-related factors

3.7.2.1. Tumor size

It has been known for several decades that the bigger the uveal melanoma, the shorter the survival. However, exactly how to group tumor size has been a controversial issue. The TNM7 will hopefully change this diversity into a uniform practice. The present T1-T4 categories are shown in Table 2. According to the TNM7, based on more than 7000 patients with uveal melanoma, ten-year survival rates for the size categories T1-T4 were 90%, 78%, 58%, and 40%, respectively.

In 2004, it was proposed that tumor volume would be a better prognostic indicator than LBD and height. The authors calculated tumor volume with the formula \((3/4 \pi a^2 b)/2\) in which \(a\) is the tumor diameter divided by 2 and \(b\) is the tumor height, based on the assumption that tumors are rotated ellipsoids. In their data set with seven events (i.e. deaths) in survival analysis, they claimed to have confirmed their hypothesis. We tested this hypothesis in our population-based data set of 289 patients with ciliary body and choroidal melanoma, of whom 145 died during the follow up. In our dataset, LBD and tumor height in a Cox regression multivariate model fitted to survival data significantly better than tumor volume. Further, LBD was the best parameter to predict survival alone. Hence, calculation of tumor volume with present formulations, which are only assumptions, does not give us more valuable information about tumor size than measuring LBD and tumor height.

<table>
<thead>
<tr>
<th>Height (mm)</th>
<th>&gt; 15.0</th>
<th>12.1-15.0</th>
<th>9.1-12.0</th>
<th>6.1-9.0</th>
<th>3.1-6.0</th>
<th>≤ 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
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<td></td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Largest basal diameter (mm)</td>
<td>&gt; 15.0</td>
<td>12.1-15.0</td>
<td>9.1-12.0</td>
<td>6.1-9.0</td>
<td>3.1-6.0</td>
<td>≤ 3.0</td>
</tr>
</tbody>
</table>

Table 2. The present TNM classification categories based on tumor height and largest basal diameter of choroidal and ciliary body melanoma
3.7.2.2. Tumor location

Uveal melanomas confined to the iris carry the best prognosis, followed by those located in the choroid. Ciliary body involvement, on the contrary, is associated with a shorter survival. One reason may be that because of their relatively hidden location, the diagnosis may be delayed. Ciliary body melanomas may also contain more extravascular matrix networks, which, in turn, are associated with shorter survival. Other indicators of poor prognosis found to be overrepresented in ciliary body melanomas are monosomy 3 and partial gain of chromosome 8.

3.7.2.3. Presence of extraocular extension

Extraocular extension of uveal melanoma indicates a poorer prognosis for survival. It also seems that the larger the extraocular extension, the greater the chance of fatal metastasis.

Extraocular spread is more likely in advanced tumors and can occur directly through the sclera, via optic nerve, vortex veins, ciliary nerves and arteries, and Schlemm’s canal. Extraocular spread correlates with the presence of epithelioid cells, large LBD, anterior tumor extension, closed loops, high mitotic rate, and monosomy 3, all of which are indicators for a more malignant type of tumor. The same study also showed that each route of spread decreased survival.

3.7.2.4. Cell type

In 1931, Callender classified uveal melanomas for the first time based on the morphology of tumor cells and described the two main cell types in uveal melanoma: spindle and epithelioid. Spindle cells tend to grow close to each other and they have ovoid nuclei, while epithelioid cells grow more loosely, they have larger nuclei and nucleoli, are more irregular and larger in size with abundant typically acidophilic cytoplasm. Many uveal melanomas contain both spindle and epithelioid cells.

Callender’s classification was modified by ophthalmic pathologists of the Armed Forces Institute of Pathology (AFIP), and this modified version is still used widely for the histomorphological subtyping of uveal melanomas. However, the identification of the cell type is inherently subjective among ophthalmic pathologists. In the modified version, tumors are divided into spindle, mixed, and epithelioid tumors; however, no consensus exists regarding what proportion of epithelioid cells determines whether the tumor is categorized as epithelioid or mixed. Several ophthalmic pathologists and researchers have now come to the conclusion that if a single epithelioid cell is found within a section, the tumor should in fact be classified as epithelioid.

I have used this dichotomous classification in my thesis. Epithelioid tumor cells are more aggressive than spindle ones. Thus, epithelioid tumors seem to grow faster and be associated with shorter survival. It seems that irradiated tumors secondarily-enucleated because of complications or tumor re-growth (I, IV) are more often mixed or epithelioid in type than primarily-enucleated tumors are (65% vs. 36%, respectively). In the COMS study, eyes enucleated after brachytherapy contained significantly more often epithelioid tumors than primarily-enucleated eyes (9% vs. 3%, P=.001).

The presence of epithelioid cells has shown to be associated with other adverse prognostic factors, such as high numbers of macrophages, monosomy 3, and extraocular extension of the tumor.
3.7.2.5. Grade of tumor pigmentation
Pigmentation of uveal melanoma is classified in different ways, and one single tumor may contain both totally amelanotic and heavily pigmented areas. Hence, it may be challenging to draw conclusions from it as an independent prognostic factor. Several univariate studies have proposed that heavy pigmentation might be associated with shorter survival. Heavier pigmentation in the primary tumors has shown to be associated with high numbers of tumor-infiltrating macrophages and the round type of macrophages.

3.7.2.6. Microcirculatory factors
In 1992, microcirculatory factors of uveal melanoma were brought to attention by Robert Folberg and coworkers in their studies on tumor blood vessel architecture. They found that depending on the grade of malignancy of the tumor, the arrangements of microvessels and extracellular matrix, initially known as "microvascular patterns", varied within the tumor. The nevi and “good” uveal melanomas contained certain patterns, while “bad” tumors had arrangements of microvessels, which predicted increased risk for metastatic disease. These patterns were divided into nine categories, of which the most adverse are “closed loops” and “networks”. The patterns can be visualized histologically using the periodic acid-Schiff (PAS) stain, and clinically to some extent by confocal indocyanine green angiography. What is particularly interesting about these microvascular patterns is evidence that suggests that they may represent fluid-conducting spaces, and that they could represent one form of microcirculation of the tumor known as "vasculogenic mimicry".

Extravascular matrix (EVM) loops and networks have been found to be associated with other prognostic factors such as the presence of epithelioid cells and high microvascular density (MVD). The association with macrophages is also interesting: sometimes tumor-infiltrating macrophages seem to cluster around or even within these patterns; however, a high macrophage density is not associated with presence of EVM loops and networks.

What happens with these matrix patterns upon tumor dissemination? One study investigated EVM patterns in metastases and found that the patterns were associated with a high risk of metastatic disease in primary tumors. In this study, EVM loops and networks were present in 81% of 10 hepatic; 83% of 5 pulmonary; and variably in 50-100% of metastases at other sites. However, the metastases were not from the same patients as the primary tumors, and so this study was incapable of showing what actually may have changed during progression of a particular tumor.

What happens to the EVM loops and networks during regression caused by brachytherapy? Histopathologic studies on uveal melanomas secondarily-enucleated after brachytherapy have shown changes such as sclerosis and hyalinization of vessel walls, plumped endothelial cells, partial obliteration, and thrombosis in tumor vessels. However, the alterations in EVM loops and networks in regressed uveal melanomas after brachytherapy has not been described previously.

Another type of microcirculatory factor with prognostic significance is MVD, which can be determined with immunohistochemical staining using antibodies or lectins that bind to vascular endothelial cells. The antibodies used recognize the CD31 or CD34 epitopes or Factor-VIII related antigen. MVD is generally counted from the densest areas of immunopositive elements, so called “hot spots”, as suggested by Foss et al. Hot spots may be associated with extravascular matrix patterns, but more often are located away from them. MVD is believed to represent density of true microvessels of the tumor, although it
has been claimed that even melanoma cells may be stained with the antibody used, and thus potentially influence the number of immunopositive elements.

High MVD was first found to be associated with shorter survival in many non-ocular cancers. Foss et al reported first the association between high MVD and mortality in patients with uveal melanoma. In this study, Factor-VIII related antigen was identified immunohistochemically in 116 enucleated eyes with uveal melanoma and MVD was evaluated. In the Kaplan-Meier analysis for survival, patients were divided into quartiles according to the maximum MVD, and a strong association between higher MVD and shorter survival was found ($P < 0.00005$). Two later studies reported a negative association but in these studies MVD was counted from predetermined or random areas of the tumor, instead of from the hot spots. Confirmatory evidence of MVD being a strong, independent prognostic factor was published in 1999 by Mäkitie et al. In this study, the threshold count of CD34-immunopositive elements, which divided patients into low and high risk of melanoma-related death, was 39 vessels/0.313 mm². High MVD was also significantly associated with the presence of EVM loops and networks. However, high MVD was found sometimes even in tumors which did not contain EVM loops or networks. Additionally, the MVD was higher in uveal melanomas which had epithelioid cells, large LBD and tumor height. A subsequent study further showed an association between a high number of tumor-infiltrating macrophages and high MVD. In 2002, Chen and coworkers independently confirmed MVD to be a prognostically significant factor. They stained 200 sections of uveal melanoma with an antibody to the CD34 epitope. In Kaplan-Meier analysis, a statistically significant association with poorer survival was found ($P = 0.0007$). In Cox proportional hazards models with different tumor characteristics, the result for square-root transformed MVD (HR 1.23, 95% CI 1.06-1.44) was almost exactly the same as reported by Mäkitie et al (HR 1.23, 95% CI 1.06-1.43). EVM patterns were also an independent prognostic factor in this study, further confirming the findings of Mäkitie et al. Sections were double-labeled for melanoma markers (S100 protein and Melan-A) and the CD34 epitope to determine whether melanoma cells might stain for CD34. Indeed, diffuse expression of CD34 in tumor cells was observed in some uveal melanomas indicating that MVD may not be a specific marker of tumor vascularity.

What happens to MVD in uveal melanomas during regression caused by brachytherapy and progression to metastatic disease has to the best of knowledge not been studied before my thesis.

3.7.2.7. Tumor-infiltrating macrophages

In the 1990’s, several studies showed that tumor-infiltrating macrophages were present in uveal melanomas. In the COMS study, 89% of enucleated uveal melanomas had “none to minimal” or “scattered single small clumps”, and 11% had “scattered single and larger aggregates” of macrophages by light microscopy without immunohistochemical stainings. Subsequently, several mAbs specific for macrophages have been used in other studies, e.g. mAb PG-M1 to the CD68 epitope has been shown to work well.

In 2001, Mäkitie et al showed that a high number of macrophages is associated with a shorter survival of patients with primarily-enucleated uveal melanomas. Semiquantitatively-graded macrophage density was few in 17%, moderate in 51%, and many in 32% of the tumors. They also subtyped the type of macrophages by the predominant morphologic type among the immunopositive cells, and found it to be dendritic in 22%, intermediate in 59%, and round in 19% of the tumors. They also showed that high numbers of
tumor-infiltrating macrophages were significantly associated with the presence of epithelioid cells \((P=0.025)\), heavy pigmentation \((P=0.001)\), large LBD \((P=0.031)\), and high MVD \((P=0.001)\).\(^{168}\)

Other studies have confirmed the presence of CD68-positive macrophages in uveal melanomas. Polak et al studied in more detailed dendritic cells (DCs) in uveal melanomas and found that Factor XIIIa, a marker expressed by DCs irrespective of their maturity, stained a population of cells in 70% of tumors. Coexpression with CD68 and human leucocyte antigen (HLA)-DR existed, suggesting that characteristics of DCs and macrophages overlap.\(^{187}\) HLA-DR is essential for antigen-presenting cells and is expressed by activated macrophages.\(^{188}\)

In 2008, Maat et al showed that a high number of tumor-infiltrating macrophages was associated with several other prognostic indicators, such as monosomy 3 \((P=0.001)\), LBD \((P=0.045)\), and a positive HLA Class I \((P=0.017)\) and II \((P=0.001)\).\(^{35;188}\)

What happens to tumor-infiltrating macrophages in uveal melanomas during regression after brachytherapy and progression from primary tumor to metastasis is largely uncharted. One study found some CD68-positive elements in liver and skin metastases from one patient.\(^{189}\)

3.7.2.8. Extracellular environment
The interaction between tumor cells and the surrounding tissue is relevant for tumor cell behavior in all states from regression to progression. During progression processes such as tumor cell migration, adhesion, reorganization of ECM, and invasion to ECM are involved. Many enzymes are involved in these steps. Matrix metalloproteinases (MMPs) are proteolytic enzymes important in degradation of ECM, modulation of cell-cell adhesion, and angiogenesis. Little is known about their association with tumor progression and invasion in uveal melanoma, but some studies have suggested that MMP-2 and -9 are associated with poorer prognosis in uveal melanoma patients.\(^{190;191}\) In a recently published study of 18 primarily-enucleated uveal melanomas, MMP-1 expression was also found to be present in all tumors, in addition to MMP-2 and -9. MMP-2 seemed to be consistently expressed by tumor vasculature; in contrast, MMP-1 and -9 immunoreactivity was inconsistent or heterogeneous in tumor blood vessels.

It has been suggested that macrophages can produce a wide range of MMPs,\(^{193}\) and in uveal melanoma co-expression of CD68 and MMP-2 has been found.\(^{194}\)

Ezrin is a protein that is involved in cell migration and it has been suggested to influence cell-cell adhesion.\(^{195}\) Positive immunoreactivity with a mAb to ezrin was found to be associated with higher numbers of tumor-infiltrating macrophages, MVD, and higher mortality in patients with uveal melanoma.\(^{196}\)

Other possible regulators of adhesion of uveal melanoma cells to ECM proteins with prognostic significance are insulin-like growth factor 1 (IGF-1) and its receptor IGF-1R.\(^{197}\)

3.7.2.9. Tumor cell proliferation
Activity of uveal melanoma cells has been measured by determining the mean diameter of the 10 largest nucleoli (MLN) from silver-stained specimens.\(^{198;199}\) Large MLN has been reported to be an independent predictor of shortened survival with associations to presence of epithelioid cells and high MVD in primary uveal melanomas.\(^{199}\) In contrast, no difference in survival rates was found between low or high MLN in hepatic metastases.\(^{200}\)

Cell proliferation can also be evaluated by counting mitoses in 40 high-power fields\(^{201}\) and by staining for the Ki-67 antigen. The latter is expressed during the active phases of the cell
cycle and has been found to be associated with the presence of epithelioid cells in uveal melanoma.  

3.7.3.0. Cytogenetics
In uveal melanoma, the most important chromosomal changes that are associated with the development of metastatic disease are partial or total loss of one chromosome 3 (i.e. monosomy 3) and partial gain of chromosome 8. Chromosome 6p abnormalities are in contrast seen in tumors at low metastatic risk.  

In 2008, Maat et al reported an association between monosomy 3 and the presence of epithelioid cells, a high number of macrophages, and a higher expression of HLA class I and II in enucleated uveal melanomas. In their study, extravascular matrix loops and networks were unrelated to presence of monosomy 3, contrary to the studies conducted by Scholes et al and Kilic et al.

It has been suggested that based on their gene expression profile, uveal melanomas could be divided even more reliably into two groups based on their metastatic risk. Class II tumors have shown to carry high metastatic risk and there seems to be correlation between class II gene profile and monosomy 3. In one study, class I tumors showed 95% survival, while class II tumors were associated with 31% survival at 8 years of follow up. Recently, Onken et al reported that class II uveal melanomas were significantly associated with Ki-67 positivity and, thus, had a higher proliferative rate than class I tumors.

3.8. INFLAMMATORY PHENOTYPE OF UVEAL MELANOMA

In 1996, De Waard-Siebinga et al reported that uveal melanomas contain different types of infiltrating leucocytes. These included lymphocytes positive for CD3, CD4, and CD8-epitopes; monocytes/macrophages positive for CD11b; and granulocytes positive for CD15. The latter were scarcely present, but macrophages were found in about 90% of the tumors and were together with CD3-positive cells associated with a high expression of HLA class I. Only one tumor had B cells.

A proposed inflammatory phenotype of uveal melanoma is characterized by immune cells, such as macrophages, T and B lymphocytes, natural killer (NK) cells, and by HLA expression on tumor cells. Unlike in several other cancers, T-cell infiltration in uveal melanoma has shown to be associated with a shorter survival. A poorer prognosis has also reported for tumors with a higher expression of HLA class I antigens. A high level of HLA I and II expression, together with high numbers of macrophages and lymphocytes, has been reported to represent an “inflammatory phenotype” characteristic of aggressive tumors with a poor prognosis. This “inflammatory phenotype” was associated with presence of epithelioid cells and a high MVD.

The inflammatory infiltrate in uveal melanoma is a complex issue with many unsolved questions. The main focus of my thesis is macrophages in uveal melanoma.

3.8.1. Macrophages
Macrophages are white blood cells derived from bone marrow monocytes and they are present in all tissues and in the lymph fluid. In different tissues, they differentiate into multifarious cells. Indeed, depending on their location they express variable characteristics. Their appearance depends also on the state of their activation. Within the normal eye, macrophages are present mainly in the uveal tract and in the cornea. In the retina, they are called
“microglia”, and they express different characteristics than macrophages elsewhere in the eye.\textsuperscript{216}

Macrophages have several diverse roles. They are important in wound healing, muscle fiber repair, phagocytosis, angiogenesis, tumor growth, regulation of cell migration, and regulation of immune responses.\textsuperscript{188,214} They have been found to migrate from the eye to the spleen and other lymphoid organs and act there as antigen-presenting cells (APCs), thus playing a role in so-called anterior chamber-associated immune deviation (AICAID).\textsuperscript{217,218} In ACAID, both peripheral and local immune tolerance is induced by APCs.\textsuperscript{219} This helps to protect delicate structures of the eye from devastating effects of inflammation caused by immunologic reactions.\textsuperscript{220} Tumor-infiltrating macrophages function differently depending on their response to variable signals from the surrounding microenvironment.\textsuperscript{221}

3.8.1.1. Different types of macrophages in uveal melanoma
MAb PG-M1 to CD68-epitope immunostains macrophages uniformly and reliably.\textsuperscript{168} Bleaching of melanin at the end of the immunohistochemical staining enables a better evaluation of heavily pigmented tumors and their infiltrating macrophages. As mentioned above, (see 3.7.2.7) Mäkitie et al. graded CD68-positive macrophages according to their morphology, which ranged from round to dendritic.\textsuperscript{168} Possibly, variability in the morphology of macrophages reflects disparate states of activation. Mantovani et al have divided macrophages into two types, M1 and M2, depending on their action and phenotype.\textsuperscript{221} M1 macrophages are classical, highly antigen-presenting cells with effective cytotoxic activities against foreign antibodies and tumor cells. Thus, they activate type I T-cell responses. M2 macrophages have a poor capacity in antigen-presenting, but are believed to enhance tumor progression and invasion by promoting angiogenesis and tissue remodelling. Instead of activating the immune responses, M2 cells seem to suppress them.\textsuperscript{221-223}

Whether round tumor-infiltrating macrophages are mainly of the M1 type and dendritic ones of the M2 type is not known. Current markers for macrophages and DCs are overlapping.\textsuperscript{187}

CD11b-positive macrophages in uveal melanoma were reported in 1996 by De Waard-Siebinga et al,\textsuperscript{185} as mentioned earlier. CD11b-positive macrophages have been found to stimulate lymphangiogenesis and angiogenesis.\textsuperscript{224} Recently, McKenna et al reported of the presence of CD11b-positive cells circulating in the blood of patients with uveal melanoma.\textsuperscript{225} Generally, tumor-infiltrating macrophages in uveal melanoma are believed to be mainly M2 macrophages,\textsuperscript{188} but a thorough knowledge of the different type of macrophages with different states of function, is still lacking.

3.8.1.2. Migration of macrophages
Several chemotactic cytokines, such as monocyte chemotactic protein-1 (MCP-1), macrophage colony stimulating factor (M-CSF), and vascular endothelial growth factor (VEGF) are involved in the recruitment of macrophages in human tumors.\textsuperscript{193} Some of the factors contributing the migration have been discussed above (see 3.7.2.8).

In uveal melanoma, Claris et al.\textsuperscript{226} reported that accumulation of macrophages occurred especially near EVM loops and networks and was associated with endothelial monocyte-activating polypeptide (EMAP)-II expression of tumor cells. A strong EMAP-II positivity correlated also with a high immunopositivity of intracellular adhesion molecule (ICAM)-1, expressed on endothelial cells. Consequently, they suggested that tumor cells may regulate the presence of macrophages via EMAP-II, which in turn induces ICAM-1 expression on
endothelial cells. Generally, ICAM-1 is believed to be involved in infiltration of monocytes in tissues.\textsuperscript{188}

The secretion of VEGFs is generally stimulated by hypoxia.\textsuperscript{227-230} Their role in the migration of macrophages in uveal melanoma is not fully understood. In the study mentioned above, VEGF-C, which was expressed in half of the tumors, was unassociated with macrophages.\textsuperscript{226} Tumors were also immunonegative for VEGF-A, which generally is produced by different type of tumor cells and has been shown to play a role in angiogenesis and in monocyte activation and recruitment.\textsuperscript{231}

Contrary to this immunohistochemical study of uveal melanomas, uveal melanoma cell lines expressed VEGF-A in a study conducted by Ijland et al.\textsuperscript{232} Missotten et al.\textsuperscript{233} showed that eyes with uveal melanoma showed higher VEGF-A concentrations in the aqueous than normal eyes. To localize the source of VEGF-A, they performed \textit{in situ} hybridization, western blot analysis, and enzyme-linked immunosorbent assay which all showed that both retinal and tumor tissues contained VEGF-A.\textsuperscript{233}

The main role of VEGFs, the family of which is large, is in angiogenesis.\textsuperscript{234} Conventionally, VEGF isoforms, which are formed by differential splicing of pre-mRNA,\textsuperscript{235-237} have been shown to act as \textit{pro}-angiogenic cytokines.\textsuperscript{238} In 2002, Bates et al found a new isoform of VEGF-A and they termed it VEGF\textsubscript{165b}.\textsuperscript{235} Interestingly, this novel isoform was down-regulated in renal cell carcinoma even though the tumor tissue generally is an angiogenic environment. Subsequently, expression of VEGF\textsubscript{165b} was also down-regulated in two other angiogenic conditions, such as prostate cancer\textsuperscript{236} and the vitreous of diabetic retinopathy patients.\textsuperscript{239} The former of these studies identified new alternative isoforms corresponding to VEGF\textsubscript{165b}, and together this family was termed the VEGF\textsubscript{xxxb} family. In two different angiogenesis models, VEGF\textsubscript{165b} was found to inhibit VEGF\textsubscript{165}-mediated angiogenesis in animals. In human retina, it has shown to inhibit angiogenesis caused by hypoxia.\textsuperscript{240} Consequently, the VEGF\textsubscript{xxxb} family has been termed the \textit{anti}-angiogenic family of VEGF isoforms. Recently, these inhibitory VEGFs\textsubscript{xxxb} were studied in malignant skin melanomas.\textsuperscript{237} The expression of them was down-regulated in melanomas with metastatic disease as compared to those without metastases, suggesting that switch in splicing VEGF isoforms from anti-angiogenic (i.e. VEGF\textsubscript{xxxb}) to \textit{pro}-angiogenic (i.e. VEGF\textsubscript{xxx}) within tumor microenvironment, could play role in progression to metastatic disease. In uveal melanoma, preliminary studies on anti-angiogenic family of VEGF isoforms are ongoing, but to date their importance in the progression of uveal melanoma is unknown.\textsuperscript{241}

Macrophage-Migration-Inhibitory Factor (MIF) is a cytokine produced by both tumor cells and macrophages.\textsuperscript{193} It inhibits the migration of macrophages and also regulates different functions of macrophages such as phagocytosis and release of e.g. tumor necrosis factor alpha (TNF\textalpha). TNF\textalpha is a toxic factor important in anti-tumor functions of macrophages. In 2000, Repp et al.\textsuperscript{242} showed that uveal melanoma cell lines produce MIF. Levels of MIF expression were highest in cell lines that were isolated from metastases of uveal melanoma.

In conclusion, many issues remain to be discovered concerning the regulation of migration of macrophages in uveal melanoma.
4. AIMS OF THE PRESENT STUDY

The purpose of this study was to:

1. Investigate how brachytherapy affects microcirculation and tumor-infiltrating macrophages in primary uveal melanoma and to assess interrelationships between microcirculation attributes and macrophages.

2. Test the hypothesis that microcirculation and tumor-infiltrating macrophages increase in grade with progression from primary uveal melanoma to metastasis.

3. Characterize pigmented episcleral deposits found in eyes with primary uveal melanoma after brachytherapy and determine whether their number can be predicted by characteristics of the irradiated tumor.

4. Assess whether episcleral deposits are associated with melanoma-related mortality.

5. Compare in eyes with irradiated and non-irradiated primary uveal melanoma the number of macrophages infiltrating extratumoral tissues, to gain insights into their routes of migration after brachytherapy.
5. PATIENTS AND METHODS

5.1. ELIGIBILITY CRITERIA AND STUDY POPULATION

This thesis followed the tenets of the Declaration of Helsinki and was approved by the departmental Institutional Review Board of the Helsinki University Central Hospital (HUCH). Patients were ascertained from the files of the Department of Ophthalmology, HUCH, which is a tertiary referral unit that manages over 90% of uveal melanoma patients in Finland.

5.1.1. Paired cross-sectional, retrospective studies (I, II, and IV)

5.1.1.1. Studies I and IV

All eyes with a choroidal and ciliary body melanoma removed after brachytherapy given with cobalt, ruthenium, and iodine plaques between 1981 and 2002 at HUCH were eligible irrespective of the extent of necrosis, provided that tumor tissue remained in the block and that a matched pair from non-irradiated, primarily-enucleated melanomas was found.

Files of the Ophthalmic Pathology Laboratory were searched from March 1981, when brachytherapy of uveal melanoma was for the first time used in this center, to August 2002. A total of 56 consecutively enucleated, irradiated eyes were identified. In two cases no residual tumor remained in the blocks, leaving 54 of the 56 tumors for matching (Fig. 2).

Matched pairs for the irradiated, secondarily-enucleated tumors were drawn from a consecutive series of primary uveal melanomas in the files of the Ophthalmic Pathology Laboratory, enucleated between 1962 and 1981, before brachytherapy was available. Enucleation was the standard treatment for all but the smallest uveal melanomas during this period. All eyes enucleated in the district were submitted to this laboratory, making the series essentially population-based and unselected. Altogether 292 consecutive patients who had an eye with choroidal and ciliary body melanoma removed during these years were ascertained from the archives (Fig. 2).
Patients and methods

Fig 2. Diagram of the material included and excluded in the four studies of this thesis.

All choroidal and ciliary body melanomas treated with brachytherapy
1981-2002

Primarily-enucleated choroidal and ciliary body melanomas
n = 292
1962-1981

Choroidal and ciliary body melanomas enucleated after brachytherapy
n = 56

Registration of pigmented episcleral deposits
n = 212
1999 - 2002

Excluded: No residual tumor left
n = 2

Excluded: Two choroidal melanomas in one eye
n = 1

Matching

No match
n = 20

With metastases
n = 145

No histopathologically confirmed metastases
n = 53

Histopathologically confirmed metastases
n = 92

34 pairs (I, IV)
• 4 cobalt
• 21 ruthenium
• 9 iodine

48 pairs (II)
• 3 core needle biopsy
• 18 surgical biopsy
• 27 autopsy

Excluded:
• < 50% of remaining primary tumor in the tissue block OR
• primary tumor entirely on the vitreal side of Bruch’s membrane OR
• an area of hepatic metastasis available < 0.35 mm²
n = 44

The assumption was made that the effect of irradiation would be qualitatively similar irrespective of the isotope used. In a pilot data set of 48 irradiated uveal melanomas, I found no significant association between the type of isotope and the presence of extravascular matrix loops and networks (P=0.47, Kruskal-Wallis test) and MVD (P=0.74).
Patients and methods

Matching was based on four variables associated with the presence of extravascular matrix loops and networks; MVD; and tumor-infiltrating macrophages:166,168

1. Tumor location: (1) ciliary body involved versus (2) uninvolved. Hematoxylin-eosin (H&E)-stained sections confirmed the ciliary body involvement.

2. Height of tumor at primary treatment: (1) <8 mm versus (2) ≥8 mm. In both groups, the height at the time of primary treatment (i.e. before brachytherapy or from the slides after primary enucleation) was used in matching. A- and B-scan ultrasonography, clinical examination, or both, determined the height depending on availability of the ultrasonography and the location of the tumor.

Efficient matching variables should be strongly associated with outcome variables but unassociated with each other.243 Because largest basal diameter (LBD) and tumor height are related, LBD was not matched, expecting that matching for height would sufficiently reduce the bias caused by LBD.

3. Cell type of the tumor at enucleation: (1) spindle versus (2) nonspindle [mixed or epithelioid] versus (3) necrotic, evaluated from bleached hematoxylin-eosin stained sections.

4. Pigmentation at enucleation: (1) amelanotic to weak versus (2) moderate versus (3) strong, determined by sorting unstained sections on white tissue paper under incandescent light.168

A four-digit index for each patient was computer-generated according to the categories of the four matching variables, and the patients were matched on the basis of this index masked to other pre-treatment data and outcome. In case of several available matches, the one with the most similar tumor height was selected.

From the 292 primarily enucleated eyes, a matched pair was found for 34 of the 54 irradiated eyes (inclusion ratio, 63%). Of the 34 tumors, 4 (12%) were treated with a cobalt-60 plaque, 21 (62%) with a ruthenium-106 plaque, and 9 (26%) with an iodine-125 plaque (Fig 2); seven of these irradiated tumors underwent retreatment later.

5.1.1.2. Study II
All cases of choroidal and ciliary body melanoma treated by enucleation in the district of the Helsinki University Central Hospital between 1962 and 1981 and later metastasized were eligible. Inclusion criteria were: 1) at least 50% of the primary tumor remained in the tissue block; 2) the remaining part was not entirely on the vitreal side of Bruch’s membrane;95 and 3) one or more core-needle biopsy, surgical biopsy or autopsy specimens with a surface area of at least 0.35 mm² were available from the corresponding hepatic metastases. The surface area above corresponds roughly to the minimum area needed to measure MVD.166

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. Forty-eight pairs of primary tumors and hepatic metastases that fulfilled the inclusion criteria were identified (Fig 2; inclusion ratio, 33% of all patients with metastases; 29 women and 19 men).

The metastases had been recognized by liver imaging or laparoscopy after they had caused symptoms. The original size of the biopsied and autopsied metastases was not recorded. In one case, the metastasis was not present in the specimen. The largest diameter in the biopsy was measured from the sections with a pair of calipers. Of the 48 sections, 3 (6%) were obtained by core needle biopsy, 18 (38%) by surgical biopsy, and 27 (56%) at autopsy. The
Means and ranges of specimen sizes for these three groups were 2 mm (range, 1.5-3), 7 mm (range, 2-18.5), and 20 mm (range, 6.5-35), respectively.

5.1.2. Noncomparative cross-sectional and longitudinal case series (III)
All the patients with a choroidal and ciliary body melanoma that were treated once with brachytherapy using ruthenium and iodine plaques between March 1981 and April 2002 in HUCH, and who were under follow up in this unit, were eligible for the cross-sectional and follow-up study. During the study period, more than 90% of all uveal melanomas managed with brachytherapy in Finland were treated and followed up at 1, 3, and 6 months, and thereafter once a year for a minimum of three years in the HUCH. Subsequent follow-up one to two times a year was shared with 15 regional central hospitals.

Systematic registration of pigmented episcleral deposits after brachytherapy began in May 1999. A total of 212 irradiated eyes had been screened for episcleral deposits by April 2002 (Fig 2). One patient with two choroidal melanomas in the same eye was excluded from the study. Of the 211 enrolled patients, 88 (42%) were managed and prospectively followed up during the study. No effort was made to enroll patients who had been treated more than three years before the start of the study, and who lived outside the catchment area of HUCH (and thus were followed up elsewhere). Data were not available from patients who had died before this study started.

5.2. CLINICAL DATA
The date of the diagnosis (the date of enucleation and for irradiated patients the date of treatment decision; I, III, IV), and the dates of all visits to the ocular oncology service following brachytherapy were registered (III). Disease-free interval was calculated as the time from diagnosis of primary tumor to diagnosis of metastases, and overall survival as the time from the date of diagnosis of metastases to death. These intervals could be calculated for the 22 patients who had undergone a biopsy of their metastasis before death (II).

5.2.1. Tumor characteristics
The height and largest basal diameter (LBD) of the tumor at diagnosis and the height during each subsequent visit were based on A- and B-scan ultrasonography, clinical examination, or both (I, III, IV). The last measurement preceding enucleation and, for tumors managed by primary enucleation, macroscopic measurement made by the pathologist were taken to be tumor height at enucleation (I, II, IV). The theoretical volume of the tumor was calculated with the formula \( \frac{3}{4} \pi d^2 h / 2 \) in which \( d \) is tumor diameter divided by 2 and \( h \) is tumor height, based on the assumption that tumors are rotated ellipsoids (III).\textsuperscript{154}

Presence or absence of tumor growth through Bruch’s membrane was taken from the patient charts and was based on A- and B-scan ultrasonography, clinical examination with binocular indirect ophthalmoscopy, or both. The grade of tumor pigmentation before irradiation was registered clinically (amelanotic to weak, moderate, strong, and variable).

The location of the tumor was registered by dividing the eye into eight sectors (III, Fig. 1, p. 866) and by noting the location of the anterior and posterior tumor border relative to the optic disc, foveola, equator, ora serrata, ciliary body, and iris.
Patients and methods

Fig. 3. The pigmented episcleral deposits were counted in eight sectors and the numbers were registered to the database.

5.2.2. Clinical characteristics
5.2.2.1. Intraocular pressure (III)
Intraocular pressure (IOP) was measured with applanation tonometry at each examination.

5.2.2.2. Pigmented episcleral deposits (III)
The anterior segment of the eye was divided into eight sectors and the number of pigmented deposits (0, 1, 2, 3, 4, 5, 6-10, 11-15, 16-20, and >20) was counted in each sector by a single observer under 16× magnification at each visit to the ocular oncology service (Fig. 3).

5.2.2.3. Radiation (I, III, and IV)
Ruthenium plaques were bought from BEBIG Isotopen- und Medizintechnik GmbH (Berlin, Germany). Iodine applicators were crafted to conform with the ruthenium plaques. Cobalt treatment was performed by Stallard’s 60/Co-60 applicator. Five 0.5 mm-thick unrimmed, non-collimating ruthenium and iodine plaques were used: CCA (diameter 15 mm, circular), CCB (20 mm, circular), CCC (25 mm, circular), COB (20 mm, notch for the optic nerve) and CIB (20 mm, notch for the limbus). Iodine seeds were attached with silicone rubber that increased plaque thickness to 1.0 to 1.5 mm. The diameters of the cobalt plaques used for four patients (I, IV), were 15 mm (CKA-3) and 20 mm (CKA-4).

The dose to tumor apex was calculated with commercial brachytherapy software (Cadplan, Varian Dosetek, Helsinki, Finland). Prescription point was tumor height plus 1 mm for the sclera. Prescription dose for ruthenium plaques was 100 Gy, and 120 Gy if the tumor was very thin. Prescription dose with iodine was initially 100 Gy, and 80 Gy if the tumor was very thick, to limit complications. Since 1997, the prescription dose for iodine has been 80 Gy to the apex, and very thick tumors have received a prescription dose of 70 to 60 Gy.
The tumor was localized with transillumination using a fiberoptic probe, indirect ophthalmoscopy with scleral indentation, or both. A minimum safety margin of 2 mm around the tumor was desirable, but not an absolute requirement. Tumors close to the optic disc and macula were often irradiated with a smaller or no safety margin toward these structures. The position of the plaque relative to tumor margins, the macula and the optic disc was checked at the end of the procedure by indenting the plaque, by transscleral illumination with a bent vitrectomy light probe positioned in contact with the plaque margin, or by both methods.

The type and diameter of the plaque used were registered, and the radiation dose to tumor apex and base was calculated from the dose rate and treatment time. If the patient had received multiple treatments, the cumulative dose was used (I, IV).

In study III, seven (3%) patients underwent local argon laser photocoagulation around tumor margins prior to brachytherapy. Eight (4%) enrolled patients subsequently underwent secondary brachytherapy and were censored from the analysis at that time. To cover the entire tumor, three (1%) patients had two simultaneous or sequential plaques as part of the primary treatment.

5.2.2.4. Survival data (III)
Charts relating to terminal illness were retrieved and death certificates were obtained from Statistics Finland. Of the 211 patients, 43 (20%) had died by the end of 2004; the survival status of 5 (2%) patients was restricted in Statistics Finland and remained therefore unknown. Deaths were coded as melanoma-related, if the diagnosis of metastatic melanoma was confirmed (in our laboratory) by immunohistochemistry, or, if the original histopathologic report of metastasis documented unequivocal melanin. Patients without histopathologic analysis who had evidence of liver metastasis and a progressive course with no evidence of a second cancer, were also considered to have died because of metastatic melanoma.

At the end of the follow-up, 163 (79%) of 206 patients were alive, 34 (16%) had died of metastatic melanoma, one had died of second cancer, and eight (4%) had died of other causes.

5.3. IMMUNOHISTOCHEMISTRY

5.3.1. Monoclonal antibodies
All primary mouse antibodies (mAb) used were purchased and they were previously documented to work in paraffin sections in our laboratory (Table 3).

5.3.2. Immunoperoxidase staining (I-IV)
The paraffin blocks were cut at 5 µm. Immunostaining of tumor cells, macrophages, and microvessels was performed using the avidin-biotinylated peroxidase complex method (Vectastain ABC Elite Kit; Mouse IgG, Vector Laboratories, Burlingame, CA).

At first, the sections were deparaffinized in xylene and rehydrated in ethanol series. Between each of the following steps, the sections were washed three times for 10 min in PBS (pH 7.0) and all immunoreagents were diluted with PBS containing 2.0% (v/v) bovine serum albumin (BSA; E. Merck, Darmstadt, Germany). As a proteolytic pretreatment for antigen retrieval, the slides were treated with 0.4% (w/v) pepsin (250FIP-U/g E. Merck, Darmstadt, Germany) in 0.01 M hydrochloric acid for 15 min at 37°C to reduce background and to enhance the intensity of specific staining. Next, the sections were incubated for 30 min in methanol-containing 0.5% (v/v) hydrogen peroxide to consume endogenous peroxidase
activity, after which they were incubated with normal horse serum (Vectastain ABC Elite Kit, diluted 1:50) in a moist chamber for 30 min at room temperature. Incubation with the primary mAbs was performed overnight at 5°C in a moist chamber. The following morning, the sections were first incubated with biotinylated horse anti-mouse IgG antiserum (Vectastain ABC Elite Kit, diluted 1:200) and subsequently, with the ABC (Vectastain ABC Elite Kit, reagents A and B, both diluted 1:160 and mixed 30 min before use) in a moist chamber for 30 min at 37°C, respectively. Chromogen 3,3′-diaminobenzidine tetrahydrochloride (Sigma; 150 mg in 16 ml dimethylsulfoxide and 200 ml PBS containing 0.03% (vol/vol) hydrogen peroxide) was subsequently used for developing the peroxidase reaction.

5.3.3. Bleaching of melanin (I-IV)
All sections were bleached regardless of the grade of pigmentation. After immunoperoxidase staining, the sections were incubated with 3.0% (vol/vol) hydrogen peroxide and 1.0% (wt/vol) disodium hydrogen phosphate for 18 h at room temperature. Next morning, the sections were rinsed carefully one by one and finally, the coverslips were mounted to the sections with Aquamount (BDH Chemicals, Poole, UK).

<table>
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<th>Antigen</th>
<th>mAb</th>
<th>Lot</th>
<th>Dilution</th>
<th>Antigen Retrieval</th>
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<td>Boehringer-Mannheim GmbH, Mannheim, Germany</td>
</tr>
</tbody>
</table>
5.4. HISTOPATHOLOGIC DATA

5.4.1. Light microscopy
Outcome variables were assessed from non-necrotic areas. The grade of pigmentation (amelanotic to weak versus moderate versus strong) was estimated by sorting unstained sections on white tissue paper under incandescent light (I, II, IV).\textsuperscript{168} Cell type was registered from hematoxylin-eosin stained sections as spindle if no epithelioid cells were found, nonspindle if one or more typical epithelioid cells were present (I, II, IV), and necrotic in the case of essentially complete necrosis (I, IV). Ciliary body involvement (present versus absent) and the area of necrosis as percentage from the whole tumor were also recorded from hematoxylin-eosin stained sections (I, II, IV). The number of mitotic figures per 10 high power fields (HPF; total area, 2.6 mm\textsuperscript{2}) was counted from hematoxylin-eosin stained sections when the specimen was at least of this size (II).

5.4.2. Extravascular matrix (EVM) loops and networks (I, II)
Sections were bleached with 0.25% potassium permanganate and 5% oxalic acid and stained with periodic acid-Schiff without counterstain.\textsuperscript{147,171} EVM loops and networks were identified, according to the criteria of Folberg et al.\textsuperscript{147,171} by two independent observers. Loops and networks were identified under a green filter (Wratten No 58; Kodak, Rochester, NY). By definition, networks consisted of at least three back-to-back loops.\textsuperscript{171} Disagreements were resolved by consensus of the two observers and the senior ocular pathologist using a double-headed microscope.

5.4.3. Microvascular density (MVD; I, II)
Microvascular elements were immunostained with QBEND/10 to the CD34 epitope of endothelial cells. MVD was evaluated from the densest immunopositive area (“hot spot”) identified by scanning the entire CD34-immunostained tumor at ×100 magnification according to Foss et al.\textsuperscript{179} Immunolabeled elements were then counted at ×200 magnification using an eyepiece with an etched square graticule (WK10x/20L-H; Olympus, Tokyo, Japan) corresponding to an area of 0.313 mm\textsuperscript{2},\textsuperscript{166} as measured with an object micrometer (Ernst Leitz GmbH, Wetzlar, Germany). Any immunopositive component, clearly separate from an adjacent one and either totally inside the graticule or touching its top or left border, was counted as a microvascular element.\textsuperscript{166,179}

Hot spots were re-identified by the same observer at a later date. The intraobserver agreement, evaluated as the difference between square root-transformed counts,\textsuperscript{166} was 0.046 (SD 0.71) units more on recounting, corresponding to a mean systematic difference of no more than one count per recounted area.

5.4.4. Tumor-infiltrating macrophages (I, II, IV)
MAb PG-M1 to the CD68 epitope was used to label macrophages. CD68 is an intracytoplasmic 110-kDa glycoprotein of lysosomal granules, which is expressed by macrophages in most human tissues. PG-M1 was chosen to this study because it immunostains tumor-infiltrating macrophages in uveal melanoma more consistently than other tested anti-CD68 antibodies.\textsuperscript{168}
The number of tumor-infiltrating macrophages was evaluated semiquantitatively according to Mäkitie et al. The density of CD68-positive cells in non-necrotic areas of the tumor was compared to published standard photographs and graded as few, moderate, and high numbers of cells (Fig 4).

**Fig. 4.** The number of macrophages graded as few (A, D), moderate (B, E), and many (C, F). The predominant type of macrophages is round in figures A-C and dendritic in figures D-F.
The predominant morphologic type of CD68-positive cells was likewise divided into three groups according to standard photographs \(^{168}\) and graded as follows: two groups of tumors in which the majority (75% or more) of immunopositive cells were either round or dendritic (Fig 4), and the third group consisted of tumors in which neither the dendritic nor the round type predominated or the morphology of immunopositive cells was intermediate.\(^{168}\)

Confluent immunopositive cells in necrotic areas did not influence the grading. Analyses of tumor-infiltrating macrophages included all sizes of specimens, because these cells infiltrate uveal melanomas more or less diffusely.\(^{168}\)

5.4.5. Macrophages in normal intraocular tissues (III and IV)
In Study III, one eye, which had numerous episcleral deposits and was enucleated because of radiation-related complications 2 years and 3 months after brachytherapy, was used to verify the nature of the episcleral deposits. Four hundred serial sections were cut at 5-µm intervals and alternately were stained with hematoxylin-eosin and 2 monoclonal antibodies: mAb PG-M1 to the CD68 epitope to label macrophages and mAb HMB-45 to immature melanosomes to identify tumor cells. The sections were scanned under a light microscope (Olympus BH-2, Olympus, Tokyo, Japan) to identify immunopositive deposits in the episclera.

The matched set of 34 irradiated and primarily-enucleated eyes was stained with mAb PG-M1 to the CD68 epitope and analyzed using a light microscope to count CD68-positive macrophages within the sclera, choroid, ciliary body, and episclera (IV).

5.4.5.1. Intrascleral macrophages under the tumor
The area with visually densest immunopositive elements within the sclera underneath the tumor base was identified under 10× magnification and photographed for counting under 40× magnification (area, 218 x 174 µm).

5.4.5.2. Macrophages in the choroid adjacent to the tumor
The outermost etched rectangle of the photography eyepiece (Olympus WK 10x/20) was aligned with the edge of the tumor on both sides under 10× magnification (Fig. 5). The center crosshair identified the area of the choroid to be photographed for counting under 40× magnification (distance from tumor edge, 0.65 mm). The thickness of the choroid was also measured if the choroid did not fill the entire image height. This count was excluded if this area coincided with the optic disc or ciliary body.
Fig. 5. PG-M1 staining of an irradiated, secondarily enucleated eye with a uveal melanoma with an image of Olympus WK 10x/20-eyepiece to illustrate how the outermost etched rectangle was aligned with the edge of the tumor. To attain the best possible elucidation, the magnification here is smaller than the one used in the studies.

5.4.5.3. Macrophages in the ciliary body
The outermost etched rectangle was aligned with the chamber angle under 10× magnification, and the crosshair identified the area of the ciliary body to be photographed for counting under 40× magnification (distance from chamber angle, 0.65 mm). Immunopositive elements were counted primarily from the ciliary body ipsilateral to the tumor. In case of equal distance to tumor margins, both sides were photographed and the mean count was used for analysis. When the tumor infiltrated the ciliary body, the contralateral ciliary body was evaluated.

For each of the three areas photographed, all CD68-immunopositive elements at least 3 μm in size and clearly separate from one another were counted from the digital photographs using image analysis software (Olympus DP-10 Soft, vers. 3.0, Soft Imaging System GmbH, Münster, Germany).

5.4.5.4. Episcleral macrophages adjacent to the limbus
The outermost etched rectangle was aligned with the chamber angle ipsilateral and contralateral to the tumor under 2× magnification. The episclera and the conjunctiva, when present, and the outermost sclera coinciding with the crosshair were photographed under 40× magnification (distance from chamber angle, 3.2 mm).
Patients and methods

All CD68-immunopositive elements and clusters at least 8 μm in size were counted as aggregates of cells potentially visible clinically as deposits. The size limit was based on knowledge that erythrocytes, the diameter of which is 8 μm, can be routinely visualized with biomicroscopy. The ipsilateral and contralateral counts were analyzed separately.

5.5. STATISTICAL ANALYSES

5.5.1. Descriptive statistics (I - IV)
All data were collected and analyzed using the statistical software packages Stata (release 7.0, Stata Co., College Station, TX), StatXact-3 (Cytel Software, Cambridge, MA), GraphPad Prism (release 3.01 and 4.0; GraphPad Software, San Diego, CA).

Mean and standard deviation are given for normally distributed variables, and median and range for other variables as descriptive statistics. The 95% confidence intervals (CI) were calculated for proportions. \( P \) values less than 0.05 were considered statistically significant, and all tests were two-tailed.

In Study III, the total number of pigmented episcleral deposits was summated over all 8 sectors (using the mean value in calculation if recorded as a range). This variable was skewed, and it was square-root transformed to obtain a graph displaying the evolution of deposits over time. Tumor reduction was calculated as a percentage relative to its height at diagnosis and was similarly plotted (III). To describe the association between the location of the pigmented episcleral deposits and the location of the tumor, the sectors clockwise and counterclockwise from the tumor center were coded “1”, “2”, “3”, and “opposite” (III, Fig. 1, p. 866). The mean number of deposits in corresponding sectors was calculated and plotted (III).

5.5.2. Matched pairs analysis (I, II, IV)
The Wilcoxon signed-rank test was used to compare distributions of paired continuous data, and the Stuart-Maxwell test and its trend version to compare unordered and ordered paired contingency tables, respectively. Spearman’s rank correlation was used to analyze interrelationships between two variables (I, II, IV).

5.5.2.1. Interrelationships in case-control studies (I, IV)
When analyzing the interrelationships of two variables, Spearman rank correlation coefficient, nonparametric test for trend, and Kruskal-Wallis test were used to compare continuous variables. Kruskal-Wallis test was used to compare singly ordered contingency tables (I, IV). Pearson’s chi-square test and Jonckheere-Terpstra test were used to compare unordered and doubly-ordered contingency tables, respectively (I). Because these were explorative studies, no adjustment was made for multiple comparisons.

The number of macrophages was given per 1 μm². For tabulation, tumor necrosis was divided in tertiles (<5%, 5-29%, 30-99%) and \( P \)-values for both categorized and continuous data analysis were given, when appropriate (IV).

Scattergrams of the area of necrosis, the morphologic type and the number of tumor-infiltrating CD68-positive macrophages, EVM loops and networks, and MVD were plotted for the matched pairs (I). Scattergrams of CD68-positive macrophages in normal extratumoral tissues were likewise plotted (IV).
5.5.3. Survival analysis (II, III)

5.5.3.1. Disease-free interval and survival after metastasis (II)
The Mann-Whitney $U$-test was used to compare the disease-free interval between categories. Overall survival was analyzed with the Kaplan-Meier product-limit method and log-rank test. MVD was divided in two groups according to its median. Cox proportional hazards regression was used to adjust for size differences of the metastatic specimen.

5.5.3.2. Pigmented deposits and melanoma-related mortality (III)
To assess the association between the number of pigmented deposits and melanoma-related mortality, the Kaplan-Meier method and log-rank test were used, conditional to surviving to the follow-up visit when the deposits were counted. Eyes were divided in 2 groups according to the median deposit count at 1 and 2 years after therapy. Patients who died of causes unrelated to uveal melanoma were censored at the time of death.

5.5.3.3. Power calculation (III)
Power of the analysis was estimated a posteriori from sample size tables. This study had 80% power to rule out a 0.35 difference in survival at 1 year ($P<0.05$) and 2 years ($P<0.10$) given the number of events observed.

5.5.4. Univariate and multivariate logistic regression (III)
To analyze the association between the total number of deposits and tumor and treatment characteristics, univariate and multivariate logistic regression at 1 and 3 years after brachytherapy was undertaken. These time points were chosen because the data set was largest, and most deposits had appeared within 3 years. Because logistic regression requires a 2-category dependent variable, the eyes were divided in 2 groups based on the median number of deposits. This categorization provided groups of approximately equal size, which makes logistic regression efficient. In addition to variables with a $P<0.10$, tumor pigmentation as a confounding variable was allowed in models. For regression analysis, the groups of variable and moderate tumor pigmentation were combined. Plaque diameter was coded at 5-mm steps to reflect the availability of plaques. Plaque area was coded at 25-mm² steps. Tumor reduction was determined in millimeters. Univariate models were used to identify independent variables for multivariate modelling. Different multivariate logistic regression models were compared with the likelihood ratio test.
6. RESULTS AND DISCUSSION

6.1. MACROPHAGES IN REGRESSED AND PROGRESSED CHOROIDAL AND CILIARY BODY MELANOMAS

The matched case-control analysis of choroidal and ciliary body melanomas that were enucleated primarily and after brachytherapy investigated how regression caused by brachytherapy affects macrophages, while the effect of progression on macrophages was studied in a paired analysis of primarily-enucleated melanomas and their corresponding hepatic metastases. Regression after irradiation resulted in more necrosis, while progression of the tumor to metastasis was associated with a more frequent presence of epithelioid cells, dendritic shaped (rather than round) tumor-infiltrating CD68-immunopositive cells, and less intense pigmentation.

6.1.1. General characteristics of the matched pairs in the regression arm

The gender distribution was equal in the matched pairs. The median dose to tumor apex was 86 Gy and it took the median of 1.5 years before the eye was enucleated after brachytherapy. Primarily-enucleated eyes tended to be performed in younger patients than irradiated ones ($P=0.077$ Wilcoxon signed rank test). Matching the irradiated and non-irradiated eyes balanced the tumor height ($P=0.43$) and LBD ($P=0.48$), indirectly. In 38% of the matched pairs, the tumors extended to the ciliary body. Of the irradiated eyes, 18 (53%) were enucleated because of tumor regrowth or nonresponse, and 16 (47%) because of treatment complications.

Some matching variables, such as cell type and grade of pigmentation, were possible to determine only after treatment. After brachytherapy, uveal melanomas typically seem to become more pigmented, but this is possibly a consequence of tumor height reduction rather than an increase in pigment content within the tumor. Both heavy pigmentation and presence of epithelioid cells are in primarily-enucleated uveal melanomas strongly associated with a high number of macrophages.\textsuperscript{168} Thus, in my opinion, the matching design was a better alternative than matching simply on the basis of pretreatment height and location.

6.1.1.1. Cell type, pigmentation, and necrosis

Of the irradiated and matched, primarily-enucleated tumors, 65% contained epithelioid cells, and 6% of them were essentially necrotic. According to the study definition, pigmentation was weak in 18%, and strong in 47% of the matched pairs.

More extensive necrosis was present in 25 of the 34 irradiated tumors as compared to the matched pairs (median difference, $+9\%, P=0.0012$ Wilcoxon signed rank test).

6.1.2. General characteristics of the patients and the tumors in the progression arm

The median age at enucleation was 55 years, and the median disease-free interval was 4.2 years. The date of the diagnosis of hepatic metastasis was known for the 22 patients; the median time from metastasis to death was 1.9 months. The median height and LBD of the primary tumor were 7 and 14 mm, respectively, and the median largest diameter of the hepatic metastasis available, was 16 mm (II, Table 1, p. 3).
Results and discussion

6.1.2.1 Cell type, pigmentation, and mitotic count
Epithelioid cells existed more often in the hepatic metastasis than in the primary tumor that had spawned it ($P=0.0027$ Stuart-Maxwell test). The grade of pigmentation was lower in the metastasis than in the primary tumor ($P<0.0001$ Stuart-Maxwell test for trend). Epithelioid cells were not lost along metastasizing, whereas 9 of 12 (75%; 95% CI, 43-95) spindle cell melanomas progressed to have epithelioid cells in their hepatic metastases. Comparable number of mitotic figures existed in the primary tumor and in their corresponding specimens of hepatic metastasis ($P=0.40$ Wilcoxon signed-rank test).

6.1.3. Tumor-infiltrating macrophages in the regression arm
It was possible to estimate the CD68 epitope in 97% of the matched pairs. The number of CD68-positive cells was comparable between the irradiated and the primarily-enucleated melanomas ($P=0.67$, Fig. 6A), despite more extensive necrosis in the former. This may reflect destruction of microvessels caused by brachytherapy leading to a decrease in leukocyte density in the tumor. Additionally, irradiation could reduce chemotactic signaling causing diminished recruitment of macrophages to the melanoma. The type of the macrophages did not alter along the regression caused by brachytherapy ($P=0.95$ Stuart-Maxwell test, Fig. 6B).

Necrotic macrophages that had lost intermediate filaments after irradiation could still be immunopositive for CD68 antigen (I, Fig. 2, p. 242), which may indicate that along with melanoma cells, irradiation also have sterilised resident macrophages.

6.1.4. Tumor-infiltrating macrophages in the progression arm
The number of CD68-immunopositive cells in the primary tumors that metastasized was graded few in 4% and many in 46%. In an unselected population of 167 primary choroidal and ciliary body melanomas, the corresponding figures were 17% and 32%, respectively. The number of CD68-positive cells was comparable between the corresponding hepatic metastasis and the primary tumor ($P=0.16$ Stuart-Maxwell test for trend, Fig. 6C).

My study could not demonstrate that progression to metastasis was accompanied by an increase in the number of tumor-infiltrating macrophages in the metastatic tumor. One reason for this may be that metastases were mainly weakly to moderately pigmented, and in primary tumors weak pigmentation was also associated with a smaller than average number of infiltrating CD68-immunopositive cells. Additionally, the hepatic metastasis specimen encompassed often only a small part of the metastasis, which may also explain the results.

The type of CD68-immunopositive cells in the primary tumors that metastasized was predominantly round in 15%, intermediate in 71%, and dendritic in 15%, as compared to 19%, 59%, and 22% in the unselected population of uveal melanomas. Even though the intermediate type of macrophages formed the majority in both groups, there was an increase in the proportion of dendritic macrophages in the metastases ($P=0.0031$ Stuart-Maxwell test, Fig. 6D).

Progression to hepatic metastasis was associated with more dendritic than round type of CD68-positive cells, which may in part be a consequence of the hepatic metastases being significantly less pigmented than the corresponding primary tumors. In primary uveal melanomas, weak pigmentation is strongly associated with the predominance of the dendritic type of tumor-infiltrating macrophages.
The cohort of paired primary uveal melanomas and their metastases is so far unique in its size and also because all of the primary tumors were *primarily*-enucleated. Therefore, there was no possibility of treatments prior to enucleation influencing the inflammatory status of the tumor or its microcirculation. Indeed, it would be interesting to study in greater detail the type of macrophages with different immunohistochemical stainings in this paired material.

The morphological subtype of macrophages is an interesting issue. Macrophages may act as cancer-inhibiting or promoting cells depending on their inflammatory or non-inflammatory status. It has been proposed that depending on the action and phenotype of macrophages, they can be divided to M1 and M2 macrophages.\(^{221}\) Classical, or M1 macrophages, are highly antigen-presenting with effective cytotoxic activities against foreign antibodies and tumor cells. M2 macrophages in contrast are poor antigen presenters, but are believed to enhance tumor progression and invasion by promoting angiogenesis and tissue remodelling. While M1 macrophages activate type I T-cell responses, M2 cells suppress the immune responses.\(^ {221-223}\) Tumor-infiltrating macrophages in uveal melanoma are believed to be mainly of M2 type,\(^ {188}\) but a thorough knowledge of the inflammatory status of macrophages, and of cascades switching on and off different immunological states of macrophages, is lacking to date.

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*Fig. 6.* Scatterplots of number and type of macrophages (i.e. CD68-positive cells) in the regression arm (A, B) and in the progression arm (C, D). When the open squares cluster above and below the diagonal, lower and higher categories predominate, respectively, in irradiated tumors (A and B) and in primary tumors (C and D).
The term “inflammatory phenotype” was proposed by Jager et al\textsuperscript{35,188} to describe a certain profile of inflammatory cells within tumor tissue. Inflammatory phenotype in aggressive tumors was characterized by high numbers of macrophages and lymphocytes, and a high level of HLA I and II expression. These tumors were associated with a poor prognosis, and their “inflammatory phenotype” was associated with the presence of epithelioid cells and a high MVD.

Would it be possible to switch the phenotype from a tumor-promoting inflammatory to a tumor-inhibiting one? What would be the best way to target tumor-related inflammation? If macrophages would have a central role in modulating the process of tumor regression or progression, we should try to understand their multifactorial roles and distinct types in more detail. In any case, combination therapies which could target both tumor cells and the inflammatory cells within the tumor microenvironment, might give us a more efficient and a long-lasting adaptive immunity against the tumor.

6.2. MICROCIRCULATION IN REGRESSED AND PROGRESSED CHOROIDAL AND CILIARY BODY MELANOMAS

My results suggested that regression after irradiation modulates the features of tumor microcirculation. Following brachytherapy, a lower MVD, a tendency toward less frequent EVM networks, and more necrosis were present. Considering that irradiated melanomas may contain proliferating tumor cells\textsuperscript{258,259} a clinically-relevant consequence of my study would be reassurance that the risk for metastasis likely is reduced, given that low MVD and infrequent EVM networks in untreated uveal melanomas indicate a favorable prognosis.\textsuperscript{95,147,149,165,166,168,179} Such a difference in the rate of metastasis after treatment is, however, likely to be small compared with the postulated high rate of micrometastasis already before treatment.\textsuperscript{9,10} Progression of the primary tumor to metastasis was associated with a higher MVD in the metastatic uveal melanomas. Thus, in addition to the clinical parameters now available, determining MVD of biopsied hepatic metastases might be a supplementary mean of estimating the prognosis of patients with metastatic uveal melanoma.

6.2.1. Extravascular matrix loops and networks in the regression arm

It was possible to evaluate EVM loops and networks in 88\% of the matched pairs. A tendency towards decreased numbers of EVM loops and networks was observed after brachytherapy ($P=0.077$ Stuart-Maxwell test for trend, Fig 7A). In 13 of 15 pairs in which the matched, non-irradiated tumor had EVM networks, the irradiated tumor did not have this pattern, although 10 uveal melanomas had EVM loops.

6.2.2. Extravascular matrix loops and networks in the progression arm

In unselected primary uveal melanomas, EVM loops and networks were present in 60\% and 35\%, respectively.\textsuperscript{95} The corresponding figures were 67\% and 50\%, respectively for the 42 matched primary tumors from which a metastatic tissue sample at least 4 mm by largest diameter was available. In hepatic metastases, these percentages were 74\% and 60\%, respectively. Progression to metastasis did not bring along shift toward EVM networks when compared with the primary tumor ($P= 0.38$ Stuart-Maxwell test for trend, Fig. 7B). The largest diameter of the metastatic tissue sample was not associated with the presence of EVM loops and networks ($\rho = –0.073$, $P=0.65$ Spearman’s rank correlation). Only one of the 6 tissue samples that were less than 4 mm in diameter, contained EVM loops or networks.
Results and discussion

When primary melanomas and hepatic metastases were compared in an unpaired manner, more EVM networks existed in the metastases than in the primary tumors. This observation is consistent with a previous report of primary and metastatic tumors from 19 patients, in which all except one metastasis had EVM networks and all had EVM loops. However, in my series some metastases lacked EVM loops and networks, which are two of several matrix patterns associated with metastatic death. Analysis of EVM loops and networks included only those metastatic tissue samples that were at least 4 mm in diameter, because EVM networks may be present only in foci. Despite this, in paired analysis, my study could not prove that progression to hepatic metastasis would increase the presence of EVM networks. This increase may be difficult to detect also because more frequent EVM networks are present in primary uveal melanomas that metastasize than in those in general. Consequently, I believe that it is still premature to conclude that EVM loops and networks are not associated with the progression of uveal melanoma to metastatic disease.

Fig. 7. Scatterplots of loops and networks and MVD in the regression arm (A, C) and in the progression arm (B, D). When the open squares cluster above and below the diagonal, lower and higher categories predominate, respectively, in irradiated tumors (A and C) and in primary tumors (B and D).
6.2.3. Microvascular density in the regression arm

It was possible to evaluate MVD in all but two matched pairs. The irradiated tumors had a lower MVD as compared to the matched, primarily-enucleated melanomas (median difference, −10 counts/0.313mm$^2$; range, −80 to 42, $P=0.011$, Fig. 7C).

It is important to bear in mind that MVD does not measure the total vascularity of the tumor nor does it reflect the maximum density of tumor vessels. This is because aggressive, genetically deregulated tumor cells, in contrast to non-aggressive tumor cells, may also label antibodies to the CD34 epitope. However, MVD is a prognostic factor, especially when it is determined from areas of densest immunoreactivity.\textsuperscript{166;179;180} Why is MVD lower after brachytherapy? The most straightforward explanation for this would be that irradiation destroys microvessels\textsuperscript{176-178} and deregulated tumor cells. Alternatively, irradiation may decrease angiogenesis by reducing angiogenic and vasculogenic factors secreted by tumor cells and macrophages.\textsuperscript{261}

Whether irradiation and reduced MVD might be causally unrelated cannot be excluded by my cross-sectional study. More malignant tumors with high MVD\textsuperscript{166;179} might react more effectively to irradiation and regress without complications, whereas tumors with low MVD might be more resistant to irradiation resulting in a larger residual mass, which would increase risk for complications and recurrence. If so, tumors with originally low MVD would be the ones requiring enucleation, thus ending up to this study. The fact that epithelioid cells are more frequent in tumors enucleated after irradiation than in non-irradiated ones does not support this theory.

6.2.4. Microvascular density in the progression arm

MVD was significantly lower in the 47 primary melanomas than in the tissue samples from their corresponding hepatic metastases (median MVD 41.5 counts/0.313mm$^2$ versus 57 counts/0.313mm$^2$, respectively; median difference, 15 counts more/0.313mm$^2$; range, 38 counts less to 79 counts more; $P=0.0003$ Wilcoxon signed rank test, Fig. 7D). In 31 patients, MVD was higher in the hepatic metastasis was than that in the primary tumor (66%; 95% CI, 51–79).

The largest diameter of the metastatic tissue sample was not associated with MVD ($\rho = –0.064$, $P=0.69$ Spearman’s rank correlation). The range of MVD was comparable regardless of the size of the metastatic specimen.

The present analysis identifies MVD as a variable that is associated with progression of choroidal and ciliary body melanoma from primary tumor to metastasis. As mentioned above (3.7.2.6.), immunopositivity to the CD34 epitope may also be present in some aggressive tumor cells.\textsuperscript{180} Consequently, MVD in uveal melanoma might reflect both tumor angiogenesis and aggressiveness. The presence of more aggressive cell type, i.e. epithelioid cells, is in fact associated with high MVD in primary uveal melanoma.\textsuperscript{166} The metastases in this study had also more frequent epithelioid cells than did the corresponding primary tumors. Hence, my study cannot prove without doubt that microvascularity increases independently with tumor progression. On the other hand, MVD measured from six metastatic tissue samples that were biopsies less than 4 mm by largest diameter, represents more random than “hot spot” sampling, which, at least in primary uveal melanomas, has blurred the association between MVD and prognosis.\textsuperscript{166;182} Thus, the MVD in primary as compared to metastatic tumors could have been even greater if these six metastatic specimens had been larger.
I had significant numbers of metastases available only from the liver. So, whether different tumor microenvironments contribute to the immunopositivity (i.e. whether the MVD could differ between hepatic and non-hepatic uveal melanoma metastases), remains to be studied.

6.3. INTERRELATIONSHIP BETWEEN MACROPHAGES AND MICROCIRCULATION FEATURES

6.3.1. Irradiated tumors in the regression arm
In the 34 irradiated tumors, the number of tumor-infiltrating macrophages was unrelated with area of necrosis ($P=0.34$, Spearman), with the predominant type of macrophages ($P=0.87$, Kruskal-Wallis test), with EVM loops and networks ($P=0.87$, Jonckheere-Terpstra test), and with MVD ($P=0.30$, nonparametric test for trend). The presence of epithelioid cells was associated with the higher number of macrophages ($P=0.017$, Kruskal-Wallis test). No interrelationship was found between any of these variables and the predominant morphologic type of macrophages. MVD was unassociated with EVM loops and networks ($P=0.89$, test for trend) and epithelioid cells ($P=0.25$, Kruskal-Wallis test).

In non-irradiated melanomas, the presence of epithelioid cells, EVM loops and networks, and high numbers of macrophages are associated with high MVD but these interrelationships did not apply after brachytherapy. Probably, extracellular matrix, which is suggested to form the frame of EVM loops and networks, remains as a skeleton for a time after the cells, supposedly tumor cells, which secreted it, have died after irradiation. Thus, EVM loops and networks are possibly remodeled more slowly after irradiation than vascular and, possibly, tumor cells, which contribute to MVD, are.

6.3.2. Non-irradiated tumors in the regression arm
Among the matched, non-irradiated tumors, no association existed between the number of macrophages and epithelioid cells ($P=0.61$, Kruskal-Wallis test). The greater the number of macrophages, the greater the MVD ($P<0.001$, test for trend) in non-irradiated uveal melanomas.

Cell type at the time of enucleation was one of the four matching variables. Hence, the matching should have controlled for any effect of cell type on MVD, on EVM loops and networks, and on the number of macrophages, because they all are associated with presence of epithelioid cells in primary tumors.

6.4. DISEASE-FREE INTERVAL AND SURVIVAL IN PROGRESSED CHOROIDAL AND CILIARY BODY MELANOMAS

In my paired study of primary choroidal and ciliary body melanomas and their corresponding liver metastases, the disease-free interval was unassociated with presence of EVM loops and networks ($P=0.50$ Mann-Whitney $U$-test, II, Fig. 2A) and with MVD in the hepatic metastases ($P=0.42$, II, Fig. 2B).

It was impossible to analyze reliably whether the presence or absence of EVM loops and networks in the metastasis would contribute to the survival, because the date of diagnosis of metastases was known for only two patients without EVM loops in the metastasis (II, Fig. 2C). The same reasoning applies also for the presence of epithelioid cells, because only two patients did not have them in the hepatic metastasis.
A tendency for shorter overall survival existed among patients with higher MVD in the hepatic metastases as compared to those with a lower MVD (1.4 versus 3.2 months, \( P=0.11 \) log-rank test; II, Fig. 2D). A trend was even observed when Cox regression was used to adjust for differences in the size of the hepatic sections available (HR 1.02 for each unit increase in MVD, \( P=0.098 \)).

Several studies have shown that a high MVD is associated with a poorer prognosis than low MVD in primary uveal melanoma.\(^\text{166;179;180}\) Progression of primary tumor to hepatic metastasis seems to retain this association because a high MVD in hepatic metastasis tended to be associated with shorter survival after diagnosis of metastasis in my study.

Estimating the prognosis of patients with metastatic uveal melanoma is important from patient’s point of view, if we can offer different treatment modalities depending on the predicted prognosis. In the future, we hopefully can prolong the survival of patients with metastasized uveal melanoma. In addition to the clinical parameters now available – Karnofsky index, largest diameter of the largest metastasis, and serum alkaline phosphatase\(^\text{12}\) - MVD of biopsied hepatic metastases might be a supplementary aid in estimating the prognosis of patients with metastatic uveal melanoma, and thus in helping us to give the most appropriate treatment for our patients.

6.5. MIGRATING MACROPHAGES IN CHOROIDAL AND CILIARY BODY MELANOMAS

After irradiation, the majority of treated eyes have been \textit{clinically} observed to have pigmented episcleral deposits. In my cross-sectional follow-up study, these deposits were related to transsclerally migrating macrophages and debris they contained within them. An association existed between the number of deposits and the plaque size and the isotope used in brachytherapy. During shorter follow-up, the grade of tumor pigmentation and tumor size seemed to be unassociated with the number of deposits. In the long term, the absolute grade of tumor reduction additionally influenced on the presence of pigmented deposits.

In the paired, cross-sectional study of irradiated and non-irradiated choroidal and ciliary body melanomas, clinically-visible episcleral deposits and migrating macrophages in other extratumoral tissues were studied \textit{histopathologically}. Irradiation increased both the number of CD68-immunopositive macrophages in the sclera beneath the tumor and the number of clinically-observed ipsi- and contralateral episcleral aggregates of macrophages near the limbus. Resident macrophages were present in extratumoral tissues in eyes with both irradiated and non-irradiated uveal melanoma. Brachytherapy seemed to alter the route of migration of macrophages.

Clinically-observed pigmented episcleral deposits have sometimes been suspected to represent extrascleral growth of the uveal melanoma, necessitating an enucleation of the irradiated eye. HUCH still receives patients in consultation for the same reason even years after brachytherapy. Therefore, I believe that the ophthalmologists should know about the harmless nature of these macrophage-related deposits to differentiate them from true relapses.

6.5.1. General characteristics

In the clinical study, of the 211 enrolled patients (108 males, 103 females), the median age at diagnosis was 61 years (range, 14-88 years), while in the histopathological study the median age given was the age at \textit{enucleation} (65 versus 53 years in irradiated and non-irradiated patients, respectively). In the brachytherapy group, patients were first followed after
irradiation and later enucleated because of suspected regrowth or treatment complications, which explains the older age in this group. At diagnosis, the median tumor height and LBD were 5.5 mm (range, 1.5-16.8 mm) and 12 mm (range, 3-26 mm), respectively, in the clinical study. In the histopathological study, these tumor characteristics were given at primary treatment and were 7.6 mm (range, 1.8-16.8 mm) versus 7.0 mm (range, 2.0-20.0 mm) for height and 13.5 mm (6.0-21.0 mm) versus 13.5 mm (range, 6.0-25.0) for LBD in irradiated versus non-irradiated tumors. The clinically-determined calculated tumor volume was 442 mm³ (range, 9-4026 mm³). Tumor pigmentation in clinically-determined eyes was weak in 32 (16%), moderate in 85 (42%), variable in 35 (17%), and strong in 49 (24%) tumors, while in the matched pairs it was amelanotic to weak in 6 (18%), moderate in 12 (35%), and strong in 16 (47%) tumors. In 49 (23%) patients studied clinically, uveal melanoma extended to the ciliary body, and in 100 (49%) eyes, the tumor had grown through the Bruch’s membrane. The median IOP of the tumor eye was 15 mmHg (range, 8-42 mmHg) at diagnosis. In the histopathological study, ciliary body involvement was present in 13 (38%) of 34 matched pairs of irradiated and non-irradiated uveal melanomas.

In the clinical study, brachytherapy was given with ruthenium plaques in 111 (53%) treatments with the median dose of 475 Gy (interquartile range, 350-666 Gy) to tumor base. Iodine plaques, with the median dose of 392 Gy (interquartile range, 304-480 Gy) to tumor base, were used in 100 (47%) eyes. By 6 months after irradiation, the median reduction of tumor height was 22% (range, 67% increase to 100% decrease). With follow-up for 2 and 5 years after brachytherapy, the median reduction increased to 42% and 49% (range, 29% increase to 100% decrease), respectively (III, Fig 3B, p. 869). The patients were followed-up a median of 4.3 years (range, 0.7-17.2 years) after irradiation. In the histopathological study, the median dose to tumor base was 713 Gy (range, 213-2403 Gy) including 4 (12%) cobalt, 21 (62%) ruthenium, and 9 (26%) iodine treatments. Re-treatment was needed in seven eyes.

6.5.2. Description of clinically-visible episcleral deposits after brachytherapy

The size of the pigmented episcleral deposits ranged from 8 µm (which is the diameter of red blood cells observable by the slit lamp) to 3 mm. The appearance of the deposits varied considerably: some of them were black or brownish spots, some had a shiny metallic surface or glistened like cholesterol crystals, and some resembled small or even slightly thickened patches (III, Fig 2A-E). Frequently, small vessels existed adjacent to the deposits while the main perforating ones were usually unassociated with the deposits. The deposits were located either in the conjunctiva, within episclera or within superficial sclera.

One irradiated eye with numerous deposits was available for immunohistochemical stainings. The clinically-identifiable deposits were positive for the CD68-epitope for macrophages but negative for the HBM-45 antigen for melanoma cells (III, Fig 2F-H). After this study was completed, additional samples of superficial deposits have been taken to confirm the origin of the deposits in the clinical setting. All of these have proven to be macrophage-related so far.

After brachytherapy, it took between 1 and 6 months for the deposits to appear and their median number increased until 7 years (III, Fig 3A). Eighty-seven percent (95% CI, 78-94) of the patients had at least one clinically-observable deposit at 6 months after irradiation, and the corresponding percentages for 1, 3, and 5 years were 85% (95% CI, 77-93), 88% (95% CI, 78-95), and 100% (95% CI, 89-100), respectively.
6.5.3. Number of clinically-visible episcleral deposits in relation to tumor location

As frequently as the deposits appeared over the tumor base (III, Fig 2F), they were also found in the anterior episclera even when the tumor was situated in the posterior pole (III, Fig 2B, 2C, and 2E). Anterior deposits were mostly located 1 to 4 mm from the corneoscleral limbus but could sometimes be found deep in the fornix.

The sectors that were next to the tumor center had the highest mean number of deposits one year after brachytherapy (III, Fig 4A). With increasing distance from the tumor center, the number of the deposits consistently decreased. Two years after irradiation, the sector that corresponded to the tumor center contained most deposits (III, Fig 4B).

The sectors coinciding with the tumor center and its neighboring sectors had most episcleral deposits. Why did the centermost sector contain fewer deposits than the adjacent sectors one year after brachytherapy? Even after my histopathologic study, the data are insufficient to answer this question yet.

6.5.4. Number of histopathologically confirmed macrophages in extratumoral tissues - pairwise comparison of non-irradiated and irradiated eyes

In the sclera beneath the tumor, significantly more CD68-immunopositive macrophages were present in irradiated eyes than in non-irradiated eyes (\(P=0.0001\) Wilcoxon signed rank test; IV, Figs 2A-B, 3A). No significant difference between the matched pairs existed in the number of macrophages in the adjacent normal choroid and ciliary body (\(P=0.41\) and \(P=0.17\), respectively; IV, Figs 2C-F, and Figs 3B-C).

In the episclera near the limbus, CD68-immunopositive aggregates of macrophages were possible to evaluate ipsilaterally in 25 (74%) and 22 (65%) irradiated and non-irradiated eyes, and contralaterally in 22 (65%) and 17 (50%) of the 34 eyes, respectively. Of the irradiated and non-irradiated eyes, 21 (84%) and 9 (41%) contained ipsilateral aggregates, while contralateral aggregates were found in 15 (68%) and 5 (29%) eyes, respectively. In the given matched pairs, it was possible to count the aggregates ipsilaterally of the tumor in 44% and contralaterally in 32%. The irradiated eyes contained significantly more aggregates than non-irradiated ones both ipsilaterally (\(P=0.0034\), IV, Figs 2G-H, Fig 3D) and contralaterally (\(P=0.014\), IV, Figs 2I-J, Fig 3E). No difference between the number of ipsi- and contralateral deposits was found between the matched pairs (\(P=0.17\), IV, Fig 3F). In the irradiated eyes, the median number of ipsilateral deposits was higher than that of the contralateral ones (median 132 versus 79).

The fact that irradiated eyes had the higher number of episcleral macrophages near the limbus than non-irradiated eyes supports my clinical observations of clinically-visible macrophage-related deposits. The clinically-visible deposits were more numerous ipsilateral to the tumor and least abundant in the opposite sector, but histopathologically this difference in distribution could not be statistically confirmed even though there were, numerically, more aggregates in the ipsilateral limbus. One explanation for this can be that in my clinical study I could count all deposits in every clock hour whereas histopathologically, the aggregates were counted just in one plane which represents two opposite clock hours. When the deposits are observed clinically, they seem to be unevenly distributed and thus, any section studied histopathologically, represents a random sample of them. Why then was the histopathologically detected number of macrophage-related aggregates much higher than the number of clinically-found deposits? A ready explanation to this question is that some of the histopathologically detected CD68-immunopositive deposits could have been amelanotic and
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thus clinically-undetectable. Secondly, despite the grade of pigmentation, the smallest deposits of approximately 8 µm in size could also remain clinically-invisible.

Why was the distribution of macrophages different in irradiated eyes as compared to non-irradiated eyes? It has shown that irradiation causes vascular changes such as thickening of basement membranes and sclerosis and thrombosis of tumor vessels.\textsuperscript{176-178} Additionally, my first study showed that while EVM loops and networks tended to become less frequent, the MVD significantly decreased after brachytherapy. Closure of tumor microvessels identifiable with anti-CD34 antibodies could potentially change the routes of migrating macrophages in irradiated eyes.

6.5.5. Interrelationship between histopathologically confirmed migrating macrophages in extratumoral tissues and tumor characteristics

It was possible to count the CD68-immunopositive elements in the sclera, choroid, and ciliary body in 71%, 59%, and 44% of the matched pairs, respectively. Of the 34 matched tumors, 13 (38%) extended to the ciliary body and in these cases only the contralateral ciliary body was available for counting of macrophages.

6.5.5.1. Non-irradiated eyes

In primarily-enucleated eyes, no significant interrelationships existed between the number of macrophages in extratumoral tissues and tumor characteristics.

6.5.5.2. Irradiated eyes

A higher number of macrophages within sclera under the tumor base was associated with a larger area of necrosis within the tumor tissue ($P<0.001$, nonparametric test for trend and $P=0.003$, Spearman) and with a trend toward a smaller number of contralateral episcleral deposits ($P=0.11$ and $P=0.046$). Increasing tumor pigmentation tended to be associated with a higher number of macrophages within the sclera and choroid ($P=0.11$ and $P=0.098$, respectively, nonparametric test for trend). In tumors with larger LBD, a trend toward more contralateral episcleral deposits existed ($P=0.090$, nonparametric test for trend; $P=0.11$, Spearman). No other associations were found between the size variables of the tumor and the number of macrophages in any extratumoral tissue.

Increasing extent of necrosis increased the number of intrascleral macrophages beneath the tumor. One reason for this may be that plaque radiotherapy may modify the integrity of the sclera leading to an increased migration of macrophages via this route. Even in eyes that have not been irradiated, the sclera underneath a uveal melanoma may differ from normal.\textsuperscript{194} Irradiation may also occlude vessels within the tumor and in the surrounding choroid which in turn may contribute to the preferential migration of macrophages through the sclera. Migration to this direction might also be enhanced by later scarring around the regressing tumor. However, even though these scars are more pronounced after irradiation with ruthenium plaques, no association was found between the number of scleral macrophages and the isotope used in the treatment.

6.5.6. Interrelationship between histopathologically confirmed migrating macrophages in extratumoral tissues and tumor-infiltrating macrophages

The CD68-immunopositivity could be evaluated in all but one of the matched pairs.
6.5.6.1. Non-irradiated eyes
In primarily-enucleated eyes, the greater the number of CD68-immunopositive macrophages in the choroid and ciliary body, the greater the number of tumor-infiltrating macrophages \( (P=0.044 \) and \( P=0.003 \), respectively).

6.5.6.2. Irradiated eyes
An association was found between the larger number of tumor-infiltrating macrophages and the larger number of CD68-immunopositive macrophages within the sclera under the tumor base \( (P=0.010 \) nonparametric test for trend).

The interrelationship between extratumoral and tumor-infiltrating macrophages seemed to change with the regression caused by irradiation. Why in primarily-enucleated eyes did a larger number of tumor-infiltrating macrophages associate with larger numbers of macrophages in the adjacent choroid and ciliary body? Either the macrophages represent tumor spillover, or they indeed migrate preferentially toward and, perhaps, from the uveal melanoma through the uveal tract in the non-irradiated eyes, because of reasons mentioned earlier (6.5.4. and 6.5.5.2.). The situation may be reversed after irradiation because it seems that macrophages then migrate from the tumor and, perhaps, in to it preferentially through the sclera. One additional possibility is that they are recruited from the adjacent choroid in irradiated eyes.

This histopathological study is the first to study macrophages in normal extratumoral tissues in uveal melanoma eyes. Resident macrophages are also present within the normal eyes, mainly in the uvea and cornea.\(^{215,263}\) In this study, it would have been interesting to further compare the distribution of macrophages between non-irradiated and irradiated uveal melanoma eyes to healthy eyes but this was not possible because of lack of suitable specimens.

In genetically modified animals, migration of macrophages and other cells has been studied during the last decade.\(^{264,265}\) Perhaps in the future, we can examine the dormant and migrating macrophages \textit{in vivo} both in healthy and pathological environments, and thus understand the functions of macrophages more deeply.

6.5.7. Univariate analysis of clinically-visible episcleral deposits in relation to tumor characteristics
No association was found between patient characteristics at diagnosis and the number of clinically-visible episcleral deposits 1 and 3 years after brachytherapy in univariate logistic regression.

At 1 year after brachytherapy, no association was found between the number of deposits and tumor height, LBD, volume, grade of pigmentation, and plaque isotope. However, with the increasing number of sectors involved by the tumor (odds ratio [OR] 1.16 for each sector increase; \( P=0.065 \)), a tendency toward a higher than median number of deposits was observed. A strong association existed between plaque size and the presence of deposits (OR 3.83 for each 5 mm change in diameter, OR 1.15 for each 25 mm² change in area, \( P=0.001 \) for both variables).

At 3 years after brachytherapy, the number of sectors involved by the tumor \( (P=0.16) \) and plaque area \( (P=0.048) \) had lost significance, while LBD \( (P=0.14) \), plaque diameter \( (P=0.006) \), and tumor reduction \( (P=0.14) \) gained significance. Otherwise, similar associations than at 1 year existed.
The larger the plaque used in brachytherapy, the greater the number of clinically-visible episcleral deposits. Interestingly, no statistically significant association existed between the grade of tumor pigmentation and the deposits. Phagocytized pigment in macrophage-related deposits seems thus mostly to be derived from the retinal pigment epithelium (RPE) rather than from tumor cells. This theory is also supported by the fact that the deposit count was more strongly related to plaque size than to tumor dimensions or volume. It is also possible that the amount of tumor necrosis, which may be more extensive in large tumors, contributes to the number of deposits. Finally, only pigmented deposits were visible but perhaps also non-pigmented deposits, which could not be detected in the present clinical study, exist in episclera (discussed further in 6.5.4.).

Histopathologically, I could not confirm an association between the number of episcleral deposits and the plaque size. This inconsistency may be a consequence of histopathological samples representing only two instead of the twelve clinically-observed clock hours or presence of more lightly pigmented deposits among the immunopositive ones.

### 6.5.8. Multivariate analysis of clinically-visible episcleral deposits in relation to tumor characteristics

Altogether six bivariate models were tested. The best of them included plaque diameter (OR, 6.35 and 5.05 for each 5-mm increase; \( P < 0.001 \) and \( P = 0.002 \) at 1 and 3 years) and isotope (OR 0.20 and 0.40; \( P = 0.006 \) and \( P = 0.13 \), respectively). Ruthenium plaques caused more deposits than iodine plaques. The results remained unchanged even though tumor pigmentation was added to this model as a confounding variable.

When tumor reduction in millimeters (OR 1.59 for each 1-mm change; \( P = 0.05 \)) was added into the model, all three variables were independently associated with the number of pigmented deposits at 3 years. In this trivariate model, the effect of plaque isotope gained significance. The number of observations was too small to allow analysis of a multivariate model with all four variables mentioned.

Ruthenium brachytherapy causes a white scar with disappearance of the retinal pigment epithelium while iodine irradiation induces only mild changes in it in most cases. Hence, the finding that ruthenium plaques cause more deposits than iodine plaques supports the theory that the pigment carried by the migrating macrophages originates largely from the RPE.

By 3 years after brachytherapy, the number of pigmented episcleral deposits had increased with the increasing diminution of tumor height. It seems thus that the amount of tumor tissue lost or destroyed after brachytherapy is associated with the number of the deposits. This finding is of interest because of the concept of anterior chamber-associated immune deviation. Are the macrophages on their way from the tumor to a non-ocular location in the body, potentially participating in immune tolerance, or do they represent dormant scavenger macrophages? Alternatively, the deposits might include various types of macrophages with different tasks. Where the macrophages found in the episcleral deposits are heading to, and what kind of immunological message, if any, they mediate, remains to be investigated in subsequent studies. Knowledge of the role of macrophages in uveal melanoma regression is potentially important, because future vaccines against cancer may be designed to act via the cells within the microenvironment, including the macrophages.

### 6.5.9. Survival in relation to the number of clinically-visible macrophage-deposits

Conditional that the patient had survived 1 and 2 years after brachytherapy, the number of pigmented deposits was associated with a 0.20 difference in subsequent melanoma-related
mortality by Kaplan-Meier analysis. The power of this study was insufficient to identify a difference of this size as statistically significant (log-rank test, \( P=0.80 \) and \( P=0.31 \), respectively, III, Fig 5A-B).

6.6. LIMITATIONS

6.6.1. Limitations in the regression arm (I and IV)
The main limitations of these studies are their cross-sectional design, which provides a snapshot of the moment of enucleation and does not necessarily reflect the cumulative numbers of macrophages over time, and the limited number of specimens available in spite of the study centre being a national referral center. As many pairs of eyes with uveal melanoma as could be found were retrieved (n = 34). Because this number was quite small and previous data was not available for power calculation, this was more an explorative study based on a convenience sample. However, my series is comparable to or larger than any previous histopathologic studies on irradiated and secondarily-enucleated globes. Further, it is unique in that it is a case-control design.

Another limitation is the unavoidable fact that the largest group of irradiated tumors managed successfully without complications and recurrence, was not available for analysis.

Moreover, the number of extratumoral macrophages in the ciliary body was possible to assess in less than one half of the matched pairs because of technical reasons.

Non-invasive methods, such as confocal imaging, are needed to clinically identify microcirculation attributes\(^{94,267}\) and macrophages, and to confirm the sequence of events by longitudinal analysis in tumor regression after brachytherapy suggested herein. At the moment, such methods have not yet been developed to the reliability levels required.

6.6.2. Limitations in the progression arm (II)
This study could not conclusively prove or reject the hypothesis that progression of uveal melanoma to metastases would increase the number of CD68-immunopositive cells and that EVM patterns would not be associated with progression, mainly because of the limited number of paired primary and metastatic tumors. More than half of the metastatic tissue samples were obtained at autopsy. Even though only specimens in which immunoreaction was present were included in the analysis, post-mortem autolysis may have had a quantitative influence on the epitopes studied and, thus, an effect on the immunoreaction.

Another limitation is that the number of events in survival analysis was small, which may explain why high MVD in hepatic metastasis only tended to be associated with shorter survival. A larger, preferably independent material is needed to confirm these findings.

6.6.3. Limitations in the clinical study (III)
The main limitation is that a proportion of irradiated patients were followed up elsewhere in Finland and others had died when the systematic registration of the pigmented deposits started. Particularly the patients, who had died, may have caused bias in this study, because mortality is associated with many tumor characteristics. Thus, my study cannot definitely exclude the possibility that clinically-visible episcleral deposits are associated with survival, especially because of the limited number of events (i.e. deaths) in my data set.

Another limitation in observing the deposits clinically is that it is not possible to see non-pigmented macrophage deposits, which contributes to their smaller number when compared to histopathologically counted deposits.
6.7. CONCLUSIONS AND FUTURE DIRECTIONS

Uveal melanomas enucleated both primarily and secondarily after brachytherapy, as well as their hepatic metastases, contain tumor-infiltrating macrophages.

Regarding the first aim of this thesis, my study provides preliminary evidence that, when a uveal melanoma regresses after brachytherapy, EVM patterns become reorganised and MVD decreases (Fig. 8A and B). The number and type of macrophages within the primary tumor seems to be unassociated with regression of uveal melanoma caused by brachytherapy (Fig. 9A and B). The presence of epithelioid cells is associated with higher number of macrophages in irradiated tumors but not in their matched non-irradiated pairs. While in non-irradiated melanomas, high MVD is related to greater macrophage density; in irradiated tumors, such an interrelationship does not seem to exist.

The second aim was to study microcirculation and tumor-infiltrating macrophages during progression from the primary uveal melanoma to hepatic metastasis. In my study II, MVD (Fig. 8B), as well as presence of epithelioid cells, increased during this progression to metastatic disease. These two tumor characteristics may be interrelated, and high MVD may help to predict survival after detection of hepatic metastases. Progression to metastasis also seems to alter the inflammatory status within the tumor, because the type of macrophages seems to be more intermediate and dendritic in metastases than in the primary tumors that have resulted in metastatic disease (Fig. 9B). On the other hand, we do not know the status of macrophages and microcirculation features within the primary tumor at the time when seeding of tumor cells occurred. This means that the primary tumor and its microenvironment available for the study may have changed, perhaps considerably, from the moment of seeding to the time of enucleation. This fact makes direct comparisons even between the matched pairs difficult.
Fig. 8. Extravascular matrix loops and networks (A) and microvascular density (B) in irradiated uveal melanomas secondarily-enucleated because of treatment complications (a) or recurrence (b) or any reason (c, all), in matched (d and f) and all (e) primarily-enucleated melanomas, and in their corresponding hepatic metastases (g). NET, networks; LOOP, loops; NONE, no loops or networks.
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After studying macrophages within uveal melanomas and their metastases, I concentrated on macrophages in extratumoral tissues in uveal melanoma eyes. As a response to the aim 3, study III showed that pigmented macrophage-related episcleral deposits are found clinically in most eyes with uveal melanoma after brachytherapy, and that these deposits likely are related to transsclerally migrating macrophages and the debris they carry. Their number cannot be predicted solely by tumor characteristics; however, plaque size and the isotope are important.

Concerning the aim 4, the number of events (deaths) in my study turned out to be too small to allow reliable assessment of whether the number of deposits is related to mortality.
Nevertheless, knowledge of the existence and the benign nature of the deposits is important and may save patients from unnecessary enucleation. In borderline cases, where extrascleral tumor relapse cannot be ruled out, it would be advisable to take a small superficial biopsy of the deposit, and thus confirm the nature of the deposit histomorphologically, rather than unnecessarily enucleate the eye. On the other hand, where are these cells heading to? What is their role in immunological responses against micrometastases of uveal melanoma, if any? Might it be informative to take superficial biopsies of these deposits and investigate the inflammatory phenotype and status of the macrophages?

My last aim of this thesis was to study migrating macrophages in extratumoral tissues in irradiated and non-irradiated uveal melanomas to assess their routes of migration after irradiation. I also wanted to confirm histopathologically the presence of clinically-visible episcleral deposits seen after irradiation. My study IV showed that resident macrophages are present in different extratumoral tissues in eyes with irradiated and primarily-enucleated uveal melanoma. Brachytherapy may alter the route of migration of macrophages and change local inflammatory responses reflected in distribution of macrophages: irradiation seems to increase their number in the sclera and episclera relative to the number in choroid. To test the hypothesis of preferential migration through the choroid before irradiation and through the sclera after irradiation, and to understand the influence of these routes on tumor progression and regression, one would need to be able to label and track activated macrophages in vivo. In animal models, such studies are becoming possible, and hopefully similar methods suitable for clinical research will be developed as well.

In the future, to confirm the sequence of events in tumor regression after brachytherapy by longitudinal analysis, non-invasive methods such as confocal imaging will be needed to identify clinically microcirculation features and macrophage distribution and number. In progressive uveal melanoma, we could estimate clinically the prognosis of patients with metastatic disease by investigating MVD, and perhaps also macrophage subtype and density, of biopsied hepatic metastases. This could be helpful, especially if we will find new ways of prolonging the survival after metastasis.

Better general knowledge of macrophages within the microenvironment of uveal melanoma might help us to develop biological tools to shift the macrophage status from “bad” to “good”, and thus to become more effective in the battle against progressive uveal melanoma, which is still unfortunately associated with a poor prognosis in too many of our patients.
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