Interconverted Phenotype: a new prognostic factor for uveal melanoma

Claudia P. Correia

Department of Pathology
McGill University, Montreal
March, 2001

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science

Acknowledgements

I would like to take this opportunity to express my deepest gratitude to some of the people who touched my life in the past two years. I would like to thank my research advisors, Dr Jean Deschênes and Dr Jerry Prud'Homme for their guidance and help. Dr Zorychta, the director of the Graduate Studies Program in the Pathology Department, whom always had a smile that made my days easier. Dr Ian W McLean from the Armed Forces Institute of Pathology, for his help providing specimens for this thesis.

I started thinking of how I could acknowledge my supervisor, Dr Burnier, or Miguel as I usually call him for the way he has always treated me. He is the most generous and kind person I have met in my life, besides being the best teacher, always patient, amazingly bright and honest. He not only received me in his laboratory, trusted, taught me, but he received me at his home and has let me share so many laughs and fun times with his family: Carolina, Eugênio, and Miguel, thank you so very much for receiving me with all the love and friendship you did; a special thanks to Julia who read and corrected this final version. So many times, I would go home, late at night from the lab, and there was a pot of warm carrot soup waiting for me... I will never, ever forget that Miss Nora ... I love you all very, very much. You will always be in my heart.

During the past two years, I came to meet a lot of staff members both from the Department of Ophthalmology and Pathology. Margaret Jackson, Dr Burnier's secretary and certainly one of the most competent and reliable

persons I ever met, who still had time to be a nice friend, who I trust and love. Mira Hoffman, the Graduate Studies secretary who helped me all the way, from the registration to the handing of the final version of this thesis. Carmen of the immunohistochemistry lab who helped me with the staining for my thesis as well as all the clinical work, always with a smile. Cecile and her team at the histology lab who supplied me with specimen slides for this project. Josina of our lab who took the wonderful pictures you can see throughout this thesis, and always supported and cared for me. To the fellows and graduate students in our lab: Andresson Figueiredo and Juliana his wife, who always had nice words of encouragement and friendship, and were always there for me, even in the most difficult days; Amanda Caissie, who always had a smile, no matter how bad the news; Abelardo Rodrigues, who taught me a lot, with so much patience and care, and Karine Oliver, a medical student at Toronto University, who extensively revised this thesis several times, but most of all, made me fell like her own sister.

I would like to thank my family in Brazil, my father Wilson who taught me that I could be strong and my mother Marli who taught me that I could be strong while still being sweet. To my brothers Wilmar and Paulo Cristhiano I also thank you for always being there for me, no matter what.

At last I would like to thank and to dedicate this thesis to the love of my life and the reason why I smile every day: my beloved husband André. I am a better person because of you.

Abstract

Intermediate filaments are highly regulated and conserved during cell transformation and tumour development. Interconverted phenotype, defined as the co-expression of vimentin and cytokeratin 8,18, has been shown to be related to recurrence and metastasis in both cutaneous and conjunctival melanomas. The results show that primary uveal melanomas as well as liver metastasis do express the interconverted phenotype. In addition this thesis indicates that the presence of the interconverted phenotype in three fourths of patients with metastatic disease is a reliable prognostic factor for uveal melanoma.

Résumé

Les filaments intermédiaires sont sujet à une importante régulation et sont conservés durant la transformation cellulaire et le dévelopement tumoral. Le phénotype interconvertie est défini comme étant l'expression simultanée de la vimentine et cytokératine 8, 18. Cette dernière a démontré sa relation au récurrence et métastase dans le cas des mélanomes cutanées et de la conjonctive. Les résultats démontrent que les melanomes primaires de l'uvée ainsi que les métastases au foie expriment le phenotype interconvertie. De plus, cette thèse démontre que la présence du phénotype interconvertie dans ¾ des patients avec métastases s'avère être un indice de pronostique significatif dans les cas des mélanomes de l'uvée.

Table of Contents

List of tables

List of Plates

Introduction

Justification

Uveal melanoma is the most common primary intraocular tumour among Caucasian adults, affecting approximately 7 out of 1 million people per year [1,2,224]. In comparison with other malignant tumours, which may occur throughout the body, uveal melanoma is an uncommon tumour with a high incidence of metastasis [1,2]. It is the second malignant primary intraocular tumour in frequency [3], and represents 5,2% of all human melanomas [194].

A greater understanding of the biological aspects of uveal melanoma over the last few decades has lead to new treatment modalities, such as radiotherapy[204], thermotheraphy [203,204] and immunotherapy [177]. Unfortunately, there has been no real improvement in patient survival rates [1]. The 5-year mortality rates are 16% in patients with small tumours, 32% for those with medium tumours, and 53% for those with a large choroidal melanoma at the time of diagnosis [5,216,220]. These percentages show that individuals with a small choroidal melanoma are at a 1.3 times greater risk of death within 5 years in comparison to the general population of similar age and gender [5,6].

Strong evidence to suggests that the melanoma may have already metastasized by the time of diagnosis [159,191,195,201], therefore specific prognostic markers for metastatic potential need to be identified. The metastatic spread of uveal melanoma is associated with an extremely poor prognosis [1,5]. Although the highest rate of metastasis occurs, on average, at an interval of 6.5 years after enucleation, [7,78] recurrences up to 42 years following treatment have been reported [8]. Furthermore, there is no metastatic staging for uveal melanoma because the tumour metastasizes

hematogeneously, and lymph node involvement occurs secondary to hepatic metastasis. Because it remains impossible to prevent tumours from developing, future research could be focused on devising a way to confine uveal melanoma to the eye before it metastasizes. This would prevent death due to such tumours.

Metastasis is the greatest cause of death for cancer patients[194, 195,201]. Treatments such as surgery, chemotherapy, and radiotherapy can now cure more than 50 % of patients who develop a malignant tumour elsewhere in the body. The majority of the patients in the treatment failure group succumb to either the direct effect of the metastasis, or to the complications associated with the treatment of metastasis. The dispersed anatomic location of the metastasis and their heterogeneous composition prevent surgical removal and limit the patient's response to systemic anticancer agents. Consequently, remains a major challenge for cancer scientists is the development of improved methods to predict the potential for metastasis of a patient's individual tumour, to prevent local invasion, and to identify and clinically treat silent micro metastasis [195,201].

The goal of studies such as this one are to understand the process of migration, changes to the cells themselves, and changes to the surrounding tissue, which allows the cells to travel throughout the body and lethally implant in other locations. Further understanding of these steps may lead to the development of methods of containing the cells in the site of the primary

tumour, and to further kill or extirpate the tumour, thus freeing the patient of the disease.

In 1978 Zimmerman and co-workers [7] challenged the effectiveness of enucleation in preventing metastatic disease in uveal melanoma. They hypothesized that enucleation may in fact promote metastasis. This hypothesis has switch towards less invasive forms of treatment over the past 20 years. As a result, it is now possible to diagnose patients in the early stages and more adequately, through fundoscopy, ultrasound, and CT scans. The advent of radiation therapy has also provided hope in preventing enucleation [205].

However, progress to free the patient of this tumour remains unchanged and the following fundamental questions remain unanswered:

- Why does metastasis occur that late after the removal of the primary tumour?
- How is it possible for uveal melanoma cells to remain in the body without harming it for many years?
- What caused them to suddenly begin growing and invading? What are the mechanisms involved?

It is well known that cell type is the most important prognostic factor in determining the outcome of uveal melanoma. It is also known that metastases found in the liver of patients with metastatic uveal melanoma are mostly epithelioid, regardless of the cell type of the primary tumour.

The expression of IFs is highly regulated during embryonic development and cellular differentiation. Immature embryonic cells may co-express keratin 8, 18 and vimentin, all of which are lost at the later stage of cellular differentiation[129, 190]. The co-expression of IFs by tumour cells may be attributed to the reversion of such cells to an embryonic pattern, and thus be in accordance with the theory of "dedifferentiation" proposed for such malignancies. Otherwise, cells that co express both keratins and vimentin are regarded as "interconverted" in that they display both mesenchymal and epithelial phenotype markers [184]. Studies have shown that skin melanoma cells co-expressing vimentin, a mesenchymal marker, and cytokeratin, an epithelial marker, are up to 6 fold more invasive in the extra cellular matrix than the cells without this co-expression [10, 11, 12,13]. This "dedifferentiated" state is called Interconverted Phenotype (IP).

In order to better understand the connection between these different phenotypes in malignant cells and in their invasiveness, following questions have been raised:

- Does the presence of specific intermediate filament proteins predict the metastatic phenotype and disease outcome?
- How is the coordinate expression of the differentiation of the IF phenotype regulated?
- Is the expression of cytokeratin in a mesenchymal cell a step that the cell undergoes in order to then disseminate throughout the body?

- Are the cells changing their own cytoskeleton to thus be better capable of causing metastasis?

Hypothesis

In vieu of the previous concepts, the following hypothesis is formed:

It is hypothesized that the uveal melanoma cells express the interconverted phenotype, and that this expression leads to a more invasive pattern of disease, therefore showing that tumours with the interconverted phenotype metastasize more frequently.

The characterization by immunohistochemistry of the interconverted phenotype may predict the patients with ocular melanoma who have more chance of metastatic disease.

Objectives

To confirm the hypothesis, the following objectives were established:

1. In order to demonstrate that the interconverted phenotype is present in uveal melanomas, the co expression of vimentin and cytokeratin will be investigated.
2. In order to demonstrate that the interconverted phenotype is present in liver metastasis of patients with histopathologically confirmed primary uveal melanomas, the co expression of vimentin and cytokeratin will be investigated.
3. In order to determine whether the interconverted phenotype in uveal melanomas correlates with metastatic disease, an association between the interconverted phenotype and clinical information regarding patient outcome, as well as known prognostic factors will be investigated.

Anatomic characteristics of the uveal tract

The uveal tract is the middle, or vascular tunic of the eye which may be divided into three anatomical sites: the iris, the ciliary body, and the choroid. The uveal tract is a highly vascular structure with loose connective tissue. This vascularity, which is greatest in the choroid, permits hematogenous dissemination of tumour cells to and from the uveal tract. The uveal tract contains no lymphatic channels; hence, lymphatic dissemination of tumour cells does not occur. Branching pigmented cells, known as uveal melanocytes, are located throughout the uveal stroma. These cells are believed to arise from the neural crest and to migrate to the uvea during embryonic development [212]. The uveal melanocytes are believed to be the cells of origin of malignant melanoma of the uveal tract [22].

The **iris** is situated in front of the crystalline lens and separates the anterior part of the eye, which contains the aqueous humour, into the anterior and posterior chambers. The iris consists of four layers: the anterior limiting or border layer, the stroma, the dilator muscle, and the posterior epithelium. The anterior border layer is formed by an accumulation of stromal cells and melanocytes. It varies considerably in thickness and pigmentation of different eyes and of different portions of the same iris[22]. There are also many places where the layer is not present and where no cell processes cover the underlying stroma, especially in the iris crypts that have large gaps in the anterior border layer. The second layer, the stroma contains blood vessels,

nerves, melanocytes, clump cells, and the sphincter muscle of the pupil embedded in a loose connective tissue that contains an abundance of hyaluronidase-sensitive acid mucopolysacharide. Many branching, pigment-bearing melanocytes are scattered through the stroma. The epithelium of the iris consists of two layers of densely pigmented cells, distinguishable as separate layers throughout bleaching and electron microscopy. The anterior layer contains the nuclei of the dilator muscle fibres. The posterior layer, the iris pigment epithelium, forms the posterior surface of the iris and provides a lining for the anterior part of the posterior chamber. The melanin pigment granules in these cells are fairly uniform in amount and distribution. At the pupillary margin, the two layers of epithelium are continuous at the point where they form the pigment seam[22].

The **ciliary body** is approximately 6.0 to 6.5 mm wide and extends from the base of the iris and becomes continuous with the choroid at the ora serrata. On a sagittal section appears as a right triangle, the short side or face forming the lateral boundary of the posterior chamber. It is composed of two parts. The first, the corona ciliaris (pars plicata), forms the anterior 2 mm of the ciliary body and contains the ciliary processes, which are irregular radial ridges 2 mm long, 0.8 mm high, and about 70 in number. The second part, the orbiculus ciliaris (pars plana), is the flat posterior part of the ciliary body and measures 4.0 to 4.5 mm in length. Posteriorly, the stroma of the pars plana merges with the choroid, and the ciliary epithelium abruptly meets with the retina[22]. The ciliary body consists of seven layers: (1) the outermost

lamina fusca, or suprachoroidal tissue plane; (2) the ciliary muscles; (3) the layer of vessels; (4) the basement membrane of the pigmented ciliary epithelium (external basement membrane, lamina vitrea); (5) the pigmented ciliary epithelium; (6) the nonpigmented ciliary epithelium; and (7) the basement membrane of the nonpigmented ciliary epithelium (internal basement membrane). The lamina fusca, or suprachoroidal tissue plane, is a potential space between the sclera and the ciliary body. The connective tissue stroma of the ciliary muscle layer contains blood vessels, nerves, and melanocytes[22].

The **choroid** is the principal vascular and pigmented tissue that forms the middle coat of the posterior part of the eye and extends from the ora serrata to the optic nerve. It is attached to the sclera by connective tissue strands that run in a tangential fashion anteriorly and in a perpendicular, direct fashion posteriorly. The numerous blood vessels and nerves that enter the choroid from the sclera attach the choroid. It varies in thickness from 0.1 mm anteriorly to 0.22 mm posteriorly. Small amounts of choroidal tissue, including melanocytes, extend into the scleral canals, through which the ciliary vessels and nerves enter the eye[22]. In more darkly pigmented individuals, melanocytes may also be present in the inner layers of the sclera and in the lamina cribrosa.

The choroid has four principal layers: (1) the lamina fusca, (2) the stroma, (3) the choriocapillaris, and (4) Bruch's membrane. The lamina fusca (suprachoroid layer, suprachoroidea) consists of collagenous and elastic

fibres and fibrocytes and has a prominent number of melanocytes. The stroma contains arteries and, predominantly, veins. The arteries decrease in calibre as they approach the choriocapillaris. The melanocytes are located in the stroma of the choroid[22].

Embryological characteristic of choroidal melanocytes

The origin of melanocytes, the pigment producing cells in the basal layer of the epidermis and in the uveal tract is still subject of discussion and controversial interpretations in literature. Their morphology may vary from an epithelioid dendritic shape to a fibroblast like appearance. Presently the most commonly accepted theory is that uveal melanocytes originate from the neural crest. This theory is substantiated by the fact that melanomas contain S-100 protein [214], a specific protein for nervous tissue, and the ancestral cells that form the melanocytes may have a dendritic shape and can be stained specifically for Dopa [44,80,81,211,212]. The other melanin-containing lineage of cells in the eye is retinal pigment epithelium (RPE) that derives from the outer neuroectodermal sheath of the eyecup. As previously mentioned the tumours arising from these cells are adenomas or carcinomas, and sometimes are confused with melanomas[211].

Uveal Melanoma

As previously mentioned, uveal melanoma is a rare tumour with an incidence of 6 – 7 cases per million people every year [2]. Although rare, it remains the most common primary intraocular malignancy seen in Caucasian adults. The tumour arises from melanocytes located in the iris (3%), choroid (93%), and ciliary body (4%), all of which are the structures of the uveal tract [2,109]. It is important to emphasize that the eye contains two types of melanocytic cells; the previously mentioned uveal melanocytes, and pigment epithelial cells of the retina, ciliary body, and iris. Nevi and malignant melanomas arise from the uveal melanocytes, whereas tumours of the pigment epithelium are referred to as adenomas and carcinomas [1].

Iris melanomas are uncommon tumours. They were observed in only 0.9 to 1.3% of surgically enucleated eyes, accounted for 49% to 72.4% of iris tumours or lesions treated surgically, and accounted for 3.3% to 16.6% of all uveal melanomas. Iris melanomas may be locally aggressive but do not ordinarily metastasize; it has been observed, however, that death can occur from metastatic tumour. The incidence of metastases of iris melanomas has been variously reported varying from 2.4% to 3.5% [22].

Ciliary body melanomas account for up to 9% of all uveal melanomas. The cytologic features of ciliary body melanomas are similar to those of the choroid and are factors in the discussion of choroidal melanoma. Because of their location, ciliary body melanomas have some unique features, including extension anteriorly through the iris, which gives the appearance of a primary iris tumour. Like iris melanomas and nevi, anterior chamber angle involvement may lead to secondary open-angle glaucoma[22].

Choroidal melanomas are the most common of the intraocular melanomas, and the series studied in this thesis comprise only cases of choroidal melanoma.

Risk Factors

Although the etiology of uveal melanomas remains to be elucidated there are several risk factors known to be associated with the disease. These include age, race, gender, genetics, geographic factors, predisposing lesions, viruses, pregnancy and smoking.

Age. A compilation of the cases on file at the Registry of Ophthalmic Pathology at the Armed Forces Institute of Pathology (AFIP) determined that

the incidence of uveal melanoma increases as one ages, with the median age at presentation being 53 years [4]. In order to measure accurately the impact of age, the cause-specific survival rate should be estimated by classifying patients who die of causes other than uveal melanoma as withdrawn alive at the time of death. Extrapolating the cause-specific survival rate to infinite time provides an estimate of the cure rate. When cause-specific survival rates are compared for older and younger patients, both curves appear to have nearly the same cure rate (50%). Older patients however, die more quickly from metastatic melanoma, while the disease is rarely seen in younger patients. Shields [13] reports that 0,8% of uveal melanoma patients at present under the age of 20 years.

Race. Race is an important risk factor due to the inverse relationship between racial pigmentation and incidence of uveal melanoma. Uveal melanoma is more frequently seen in Caucasians, particularly in blue-eyed, blonde-haired individuals. The incidence for this population is approximately 8.5 times greater than in African Americans [14]. Margo and McLean [39] observed that only 1% of African Americans develop this tumour while African natives have an even lower incidence. The prevalence of uveal melanoma in black Africans is significantly less that the prevalence of retinoblastoma, suggesting that uveal melanoma is less common in black Africans than it is in black Americans. Orientals have an incidence of uveal melanoma that is intermediate between that of white and black Americans. At

the time of enucleation, Blacks tend to have larger, more heavily pigmented and more necrotic tumours than those of Caucasians. Despite these differences, African Americans have essentially the same tumour-related survival as their Caucasian counterparts [39].

Gender. Uveal melanoma commonly affects more men than women. A review of 4,995 cases at the AFIP Registry of Pathology determined that male patients made up 55% of all uveal melanoma cases, while 45 % were female[4]. Epidemiological studies performed in Canada [15] and the United States [3, 194] support this finding. The reason for the higher incidence rate in men remains unknown.

Geographic Variations. The geographic variations in the incidence of uveal melanoma around the world reflect the different racial groups in certain areas rather than actinic exposure. The higher incidence of uveal melanoma in Scandinavian countries as opposed to Africa is evidence of this fact [4]. Therefore, exposure to sunlight is not considered a major risk factor in the etiology of uveal melanoma [40], however it has been proven that sun exposure can be related to the development of the malignancy. The use of sunlamps may also be associated with a two- to four-fold increase in the risk of developing intraocular melanoma [182].

Genetics. The genetics of uveal melanoma are not well defined due to the sporadic nature of most primary uveal melanoma cases. However, in rare cases, uveal melanoma may be familial or associated with a predisposing lesion [16]. Familial uveal melanoma is believed to be inherited through an autosomal dominant mode, however it is rare for many individuals in a given family over different generations to be affected[16]. In fact, familial uveal melanoma comprises only 0.6% of all uveal melanoma patients [17]. Familial uveal melanoma most often (63%) affects first-degree relatives and rarely affects more than two people in the same family. It may be also associated with a generalized inherited predisposition to cancer [17].

Cytogenetic changes in uveal melanoma are characterized by several chromosome abnormalities such as trisomy 8, and monosomy 3 and 6[18]. This may suggest that both the deletion of a tumour suppressor gene on chromosome 3 and 6, and a multiplication of an oncogene may be implicated on chromosome 8.

Molecular genetic studies have shown an absence in the N-ras proto-oncogene [19]. P53 mutations have also been implicated but they are not the same mutations as found in cutaneous melanoma[20]. Cystine to Thyamidine conversions, which are characteristic of ultraviolet light damage and are frequently seen in cutaneous melanoma, are not present in uveal melanoma. These two findings suggest that ultraviolet light may not play a role in the pathogenesis of uveal melanoma. Also the CDKN2/INK4a gene locus at chromosome 9p21-22 has been one of the most frequent targets for cancer

mutation. In particular, the p16^{INK4a} locus has been associated in the genesis of uveal melanoma in 27 % of cases [172, 173].

More resent studies analysing the genome of metastasis reveals an essential role for Rho C. Rho C is a member of the Rho family of small GTP-hydrolysing proteins, several of which are known to regulate cell migration[98]. They have not been found to be mutated in human cancers. They can however, contribute to cell transformation to a cancerous state, motility and invasion, at least under experimental conditions, and also participate in the formation of distinctive patterns of actin organization and the assembly of integrin complexes. When over expressed, Rho C is highly related with metastatic disease and may be due to another function of the Rho-family that is to control cytoskeletal organization in response to extracellular factors[98, 199].

Predisposing Lesions. In 1967, Yanoff and co-workers [21] discovered that choroidal nevi could predispose an individual to uveal melanoma. Although these nevi are composed of atypical melanocytes, they do posses a benign nature. Based on the high prevalence of uveal nevi and the low incidence of uveal melanoma, it can be estimated that the rate of transformation of nevi to melanomas is approximately 1 per 10,000 to 15,000 cases in a year [22].

The presence of congenital oculodermal melanocytosis (Nevus of Ota) and ocular melanocytosis may increase one's risk of developing uveal

melanoma [23].The former lesion is caused by an increase in pigmented melanocytes in the deep dermal tissues of the eyelids, while the latter is due to an excess accumulation of melanocytes in the uveal tract as well as in the sclera and episclera. Congenital oculodermal melanocytosis is more prominent in African Americans and Asians than in Caucasians [22]. Although ocular melanocytosis is seen in people of all races, the transformation of this lesion to uveal melanoma occurs more readily in the Caucasian population.

Familial atypical mole and melanoma syndrome (FAM-M syndrome) was previously called dysplastic nevus syndrome. This is an autosomal dominant syndrome, characterized by the occurrence of a large number of atypical (often more than 50) irregular and multicoloured cutaneous nevi, cutaneous melanoma in one or more first- or second-degree relatives, and presence of certain distinct histologic features such as melanocytic nuclear pleomorphism, hypercromatism, melanocytic hyperplasia, and architectural disorder with asymmetry. A greater than expected prevalence of cutaneous melanoma was observed in a study of patients with ocular melanoma from the United Kingdom. Because two out of five patients had features of FAM-M syndrome, the investigators concluded that FAM-M syndrome predisposes one to both cutaneous and ocular melanoma[185]. Some other studies however, consider this association to be coincidental [186,187].

Viruses. Albert has summarized the available evidence for a viral etiology of uveal melanoma. Of the fifty-seven cases of ocular melanomas in

this study, viral particles were identified in 6 tumours studied by electron microscopy. The viruses were: one case of herpes virus, one of A type oncornavirus, and 4 cases of togaviruses. In the same study the author proved that injection of an RNA tumour virus into anterior chamber of felines resulted in iris and ciliary body melanoma apparently similar to uveal melanoma [176]. These findings should be interpreted with caution since virus particles are often constituents of normal tissues and few cancers have been associated with a viral etiology.

Pregnancy. The possible role of estrogen in the etiology of melanoma has been a longstanding controversy. Furthermore, importance is also given to this topic because anti-estrogen tamoxifen has been shown to be beneficial in treating metastatic cutaneous melanoma [177]. In a study perfoemed in that year viewing the occurrence of uveal melanoma during pregnancy, the authors found no histological difference between uveal melanomas in pregnant or nonpregnant women. When matched by age, the survival rates between these two groups are also identical [178]. Foss and co-workers, [179] studying choroidal and conjunctival melanomas, have concluded that ocular melanomas do not express estrogen or progesterone receptors and that tumour behaviour is not influenced by either endogenous or exogenous estrogens.

Smoking. Smoking is suspected of altering host immunity and may therefore hasten the development of metastases among cancer patients. Low natural killer cell activity may be associated with worse prognosis in patients with cutaneous melanoma. In one study of cutaneous melanoma in patients who smoked, a significantly decreased natural killer cell activity against cultured melanoma cells was observed when compared to non-smoking patients [181]. However tobacco related studies in uveal melanoma have shown that smoking does not increase the rate of metastases when patients with the disease were matched by age, gender and size of the tumour [180].

Clinical Presentation

The clinical symptomatology of uveal melanoma depends on the location of the tumour. Tumours of the posterior pole cause early symptoms, which can be diagnosed by indirect ophthalmoscopy and A and B mode ultrasonography. Evidence of growth can also be found by serial examination with these methods [4,76]. **Plates 1 to 7.**

There are four clinical stages of uveal melanoma [4,22]. These stages correlate with size, as well as the histopathological features of the tumour. Stages I & II represent the early stages of the disease and correspond to small tumours. These two stages consist of asymptomatic tumours as well as cases with symptoms relating to loss of vision. As the disease progresses (stage III), ocular symptoms appear, such as an elevation of intraocular pressure, inflammation, and pain. Stage IV includes symptoms of extra ocular

extension such as proptosis and occasionally the presence of a subconjunctival mass. These later stages are associated with larger tumours [49].

Tumour size is classified according to the largest tumour dimension (LTD). Small tumours have a discoid shape and are less than 10 mm in the LTD. Medium tumours range from 11-15 mm, while any mass over 15 mm is categorized as a large tumour [215]. Both medium and large sized tumours tend to have either a collar button or mushroom-shaped appearance. An exception to this usual growth pattern is the diffuse melanoma [202], which extends itself around the uveal tract without protruding into the centre [49,75].

Additional clinical parameters are the presence of extra scleral growth, tumour margin location anterior to the equator of the eye and tumour induced glaucoma [49,61,62,75,77,224].

Histopathology

Gross Examination. The most important findings on gross examination of uveal melanoma relate to tumour size. Many pathologists record the height as well as length and width of the tumour, but the most useful single measurement is the largest tumour dimension. Choroidal tumours may break through Bruch's membrane, which normally separates the choroid from the subretinal space. In the subretinal space, the tumour

grows more rapidly than in the choroid resulting in a mushroom shape. In this case, the tumour itself, or serous fluid, may cause a secondary retinal detachment. If growth is allowed to continue, all ocular structures may be invaded and destroyed, eventually leading to an extra-ocular extension. Uncommon exceptions to this pattern are diffuse infiltrating melanomas that grow laterally with little elevation. Diffuse lesions are likely to invade the sclera and may produce an orbital extension that is larger than the intraocular component of the tumour. Complete necrosis of a uveal melanoma rarely leads to phthisis (shrinkage and disorganization) of the eye, with no viable tumour evident on histological examination. When considering the site of origin, it has been found that most tumours originate in the choroid, with a small proportion originating in the ciliary body, and finally the iris being the least common site. The sclera generally provides a tough and effective barrier to the extra-ocular spread of uveal melanoma. When scleral invasion does occur, it is usually from large tumours that involve the emissary canals of the vortex veins, ciliary arteries, or ciliary nerves. Uveal melanomas that arise from the choroid adjacent to the optic nerve may invade the anterior substance of the nerve, but seldom progress through the nerve to invade the orbit or central nervous system[109].

Histopathological examination. Callender's [24] histopathological studies of 111 cases of choroidal and ciliary body melanomas was the first to describe the different cell populations in uveal melanoma. McLean and co-workers [25,26]

later modified this classification to the one presently used, known as Callender's Modified Classification. It is the universally accepted classification for Ophthalmic Pathologists. It distinguishes between benign uveal melanocytic lesions, termed nevi, and malignant uveal melanocytic lesions. Furthermore, it classifies uveal melanoma into two main cell types: spindle and epithelioid. There exists, however, malignant melanocytes within a spectrum ranging between these two extremes, as well as tumours containing both categories of cell types which are described as mixed cell tumours. In fact, at least 48% of tumours are diagnosed as mixed cell type [27,75,109,224].

Spindle cells divided in spindle A and spindle B cells. Spindle A cells are small, slender, compact cells containing elongated, flattened nuclei. A dark " chromatin" line often seen extending through the centre of the nucleus is actually a peculiar infolding of the nuclear membrane. The nuclei of spindle A cells do not usually show mitotic activity by microscopy. Spindle B melanoma cells are more oval and plumper than spindle A cells. The nuclei are also more oval and, in contrast to spindle A nuclei, are characterized by a prominent nucleolus. Spindle B are somewhat less compact than spindle A cells, and their nuclei occasionally have mitotic figures. Spindle B cells often give the appearance of forming a syncytium, since their cellular borders as defined by the plasma membrane, cannot de distinguished. Small tumours seen in the early stages of disease are composed of these well-differentiated melanocytes [49].

Epithelioid cells are large polyhedral cells with abundant eosinophilic cytoplasm and contain large nuclei with massive, round nucleoli. They are relatively pleomorphic as compared to their spindle cell counterparts. Also, epithelioid cells are spread out in the extra cellular matrix architecture, demonstrating a lack of cohesion, and thus allow the identification of their distinctive cell borders. Occasionally, multinucleated epithelioid cells may be observed. These poorly differentiated melanocytes are mainly seen in larger tumours, reflecting the tumours more aggressive nature. Mitotic figures are frequently seen [4,75].

Immunohistochemical Profile

Routinely, uveal melanoma is diagnosed through light microscopy. On occasion however, it may be difficult to differentiate melanoma from metastatic carcinomas, schwannomas, and neurofibromas [28,132]. Immunohistochemical techniques are very important in these instances in order to make a definitive diagnosis of melanoma. To date, the immunohistochemical profile of uveal melanoma consists of HMB-45, S-100 protein, and Neuron-specific enolase (NSE). In a comparative study of these immunomarkers, Burnier and co-workers [29] found HMB-45 to be the most specific marker for melanocytic lesions of the uveal tract, including nevus and melanoma.

HMB-45 HMB-45-defined antigen resides in melanosomes before melanin deposition in the cell cytoplasm[108]. HMB-45 is a mouse monoclonal antibody against whole cell extract of a human melanoma[110]. Adema and co-workers [30,31] discovered that HMB-45 recognizes a glycoprotein with a membrano-cytoplasmic intracellular distribution of 100 kDa known as gp 100. However, another study [32] suggests that HMB-45 recognizes a 3-35 kDa melanosome-associated sialated glycoprotein. HMB-45 specificity for melanocytes is demonstrated by immunoelectrom microscopy studies [33-36] which reveal that HMB-45 reactivity is restricted to melanosomes of malignant melanocytes. It was observed that HMB-45 stained early stage (I, II, III) melanosomes more intensely than late stage (IV) melanosomes. These studies demonstrate that HMB-45 specifically stains melanosomes, as opposed to diffuse cytoplasmic antigen described by light microscopic immunohistochemical analysis. Tattjes and co-workers [33] discovered that pre-treatment of sections with neuraminidase caused immunolabelling with HMB-45 to be drastically reduced or, in some cases absent. This supports the idea [33,75] that HMB-45 is partially composed of sialic acid and that sialylation of the antigen is crucial to HMB-45 binding. HMB-45 immunoreactivity is seen in normal fetal and neonatal melanocytes [93] and not in resting melanocytes, whereas it is detectable in junctional nevi and not in intradermal nevi[108]. The staining pattern is intracytoplasmic and often heterogeneous.

Cheng and co-workers [37] suggest that HMB-45 expression is inducible and is closely related to the functional activity of melanocytes. This was determined after irradiated epidermal melanocytes stained positively for HMB-45. Irradiation may lead to morphological changes in the cell, which may imply cellular activation [111], which in turn may lead to melanosome production. In earlier studies, Skelton and co-workers [38] correlated HMB-45 staining with melanosome production.

It is disconcerting that several studies [39,40] found renal angiomyolipomas (RAML) and smooth muscle cell tumours [41] to be immunoreactive for HMB-45. Zimmer and co-workers [42] also found HMB-45 to be positive in gliosarcomas, primitive neuroectodermal tumours, ependymoma, malignant schwannomas, and intracranial hamartomas, while Hancock and co workers [43] found HMB-45 reactivity in some adenocarcinomas. However, when confined to diagnosing tumours of the eye, non-melanocytic ocular tumours revealed no HMB-45 staining [29,34]. Therefore, in distinguishing between melanocytic and non-melanocytic ocular tumours, HMB-45 is an excellent immunomarker with a positivity greater than 95 % [29].

Radiology

A and B mode ultrasonography are the most accurate methods of substantiating the diagnosis and determining tumour size [76]. The A-scan provides accurate data regarding internal reflectivity and the B-scan provides

two-dimensional topographic information. Growth can be followed by evaluatuin tumour height by B-scan ultrasonography and the tumour diameter by serial fluorescein angiographies. Computed tomography and magnetic resonance imaging are employed in diagnostic evaluation ultrasonography remains the most accurate method [76].

Prognostic Factors

Several clinical and histopathological observations aid in determining the prognosis of uveal melanoma. These include tumour size and location, cell type, size of nuclei and nucleoli, lymphocytic infiltration, mitotic figures, architecture of the microcirculation, and extra scleral extension [26,48-50].

Clinical

The most important clinical prognostic factors are tumour size and location because they provide information about tumour aggressivity and serve as good bases upon which to predict possible metastatic spread.

TUMOUR SIZE. Tumour height was initially considered as the best indicator of tumour size, however a study of 3,432 cases by McLean and co-workers [49] concluded that the maximum measurement in any one dimension correlated better with prognosis. These results explain the poor outcome associated with diffuse melanomas. In a study of 4,410 uveal melanoma cases, McLean [50] found that the 20-year survival rate was approximately 78%

for small tumours, 55% for medium tumours and 30% for large tumours. A recent study also showed that small choroidal melanomas presenting certain risk factors have a greater potential for metastasis than previously thought [51,215].

TUMOUR LOCATION. Uveal melanoma arises frequently in the choroid and less commonly in the iris and ciliary body. Iris tumours have a better prognosis because they are visible and can be detected early. Conversely, tumours of the ciliary body carry a worse prognosis due to the highly vascularized nature of the structure [89].

EXTRA OCULAR EXTENSION. The sclera provides a tough barrier against the local invasion of uveal melanoma. As a result, aggressive tumours tend to invade through the vortex veins, as well as through the ciliary arteries and nerves. Uveal melanoma rarely extends through the optic nerve [4]. Extraocular extension is observed in large tumours that are generally composed of epithelioid cells and an intricate microcirculation. Patients presenting with this characteristic tend to have a worst prognosis.

GENETICS. White and co-authors [52] reported that the simultaneous abnormalities on chromosome 3 and 8 are associated with worse prognosis. However, when each abnormality appeared separately, the risk of a poor outcome was the same as if neither abnormality were present. Interestingly, the authors discovered that an abnormality on chromosome 6 had a protective effect because patients with the abnormality faired better than those without it. In addition, this and other reports [53,54] have found that

abnormalities in chromosome 3 and 8 usually occur together and are associated with ciliary body tumours, whereas abnormalities in chromosome 6 occur alone and are associated with choroidal tumours. These reports may support evidence linking tumour location and genetics for prognosis of uveal melanoma.

Other clinical parameters include tumour margin anterior to the equator of the eye, older age, male gender, and tumour-induced glaucoma, all of which are associated with a poor life prognosis [48,89].

Histopathological

CELL TYPE. Cell type correlates with tumour size and is the single most important prognostic factor in uveal melanoma. Smaller tumours composed of spindle cells offer a better prognosis for the patient, resulting in a 22% death rate due to metastasis. Larger epithelioid cell tumours carry a worse prognosis with a higher death rate due to metastasis of 62% [27, 49,89].

MEAN DIAMETER OF THE TEN LARGEST NUCLEOLI (MLN). This is a technique that measures the nucleoli, and the nuclei of the melanoma cells throughout different methods. The modified two-category scheme developed and used at the AFIP has proven reliable as a routine histopathological method for predicting patient outcome [55,56]. Unfortunately, other laboratories have not been able to consistently classify uveal melanomas with the same

predictability. To further enhance reproducibility, some workers have measured individual cytological structures within the cells of uveal melanomas [57, 58]. Pilot studies have shown that nucleolar features have more prognostic value than nuclear features. Furthermore, when using nucleolar size, absolute values (e.g., mean nucleolar area) seem less correlated with mortality than variations in values (e.g., standard deviation of nucleolar area) [55]. Alternatively, fully automated imaging systems have been used to measure silver-stained nucleoli in specially prepared sections [56]. Although these quantitative methods have produced measurements that are highly correlated with the malignant potential of uveal melanomas, none have become an established, widely practiced routine. Several studies [55,56,89,207] found the MLN to be associated with a poorer prognosis, while others [57,58] found the MLN to be an insignificant prognostic indicator.

MITOTIC FIGURES. Mitotic figures are rare in uveal melanomas, and are more frequently seen in epithelioid cell tumours than in spindle cell tumours. McLean and co-workers [51] found that it is best to observe mitotic figures under magnification of 40 high-power fields. Several investigators have found mitotic figures to be of prognostic significance since they indicate cellular proliferation [59-62].

MICROCIRCULATION. Folberg and co-workers [210] demonstrated that uveal melanoma tumours might be composed of a variety of microcirculation patterns. They can contain straight vessels, parallel straight vessels, cross-linking parallel vessels, and vascular arcs, arcs with branching, loops and

was a positive correlation between T-lymphocytic infiltration and death due to metastasis. This conclusion contradicts the findings regarding cutaneous melanoma. This discrepancy however, is not unexpected when the anatomy of the two lesions are considered along with the knowledge of their modes of dissemination. Metastasis from cutaneous melanoma initially spreads via the lymphatic system, while there is no lymphatic drainage in the eye or orbit. Metastatic spread from uveal melanoma is hematogenous. Since the eye is considered an immune-privileged site, it has been suggested that in order to mount a T cell mediated immune response, malignant cells must first disseminate into blood [60,65]. These viable circulating tumour cells would be the antigen source needed for immune activation [60,65]. This evidence accounts for the fact that TILs carry the worst prognosis for patients with uveal melanoma. It should be emphasized however, that once lymphocytes are present within the tumour, they are believed to have a beneficial effect [66].

Intermediate filaments

Virtually all cells contain a cytoplasmic fibrillary meshwork composed of three types of elements, collectively called cytoskeleton. Intermediate filaments have an axial diameter of 8 to 10 nm periodicity of 21 nm[114, 15,116,117,161], and along with 6nm actin microfilaments and 23 nm microtubules, comprise the cytoskeleton architecture of higher eucariotic cells. Although each intermediate filament is assembled from only one or two different subunits, a large multigene family, the members of which are differently

expressed in different tissues, encodes these fibrous peptides. [112,113,114] The structure of IFs is rod-like, and is compromised of trimer sub particles. Early x-ray diffraction studies on wool keratin microfibrils indicated that this structure contains an undetermined number of closely packed protofibrils comprised of several largely α-helical polypeptide strands intertwined in a coiled-coil configuration[218]. Intermediate Filament proteins have a common structural core of 300-330 amino acids, which are flanked by extra amino- and carboxy terminal domains [117]. The common core is composed of a heptad repeating unit containing a greater than average allowance of hydrophobic residues and sequences, which form α-helical conformations. The repeating hydrophobic residues create a coiled-coil dimer. The formation of such a precise structure is central to the nativity and function of all intermediate filament proteins *in vivo* and *in vitro*[117]. The cytoskeleton provides IFs with more than 95% nativity, which allows one to achieve unmatched confidence in obtained results. The coiled-coil region may also be split into three sub domains.

The amino- and carboxy-terminals vary in length and sequence. For example, cytokeratin 19 has less than 10 amino acids at the carboxy-terminal and the lamins have less than 40 amino acids at their amino-terminal. In comparison, NF-H and nestin each have a terminus greater than 500 amino acids [114,117,218].

IF proteins, which include the nuclear lamins, occasionally share as little as 20% sequence homology. The approximately 50 members of the IF

super-family exhibit cell-type-specific and often complex patterns of expression. Immunological and biochemical criteria allow us to distinguish at least 6 different types of IF's[114, 17,133] which include: 1) keratins, which are characteristic of epithelial cells; 2) vimentin filaments which occur in mesenchymally derived cells, astrocytes, sertoli cells, vascular smooth muscle cells, and many cultured cell lines; 3) desmin filaments, which are typical of most types of myogenic cells; 4) neurofilaments, typical of neuronal cells; 5) glial filaments, typical of astrocytes; 6) nestin, which was recently found in melanoma cells. During the cell transformation and tumour development, the cell type specificity of IFs is largely conserved. Classification of tumours by their type of IFs has recently become a valuable tool in clinical histodiagnosis, as long as data indicates that neoplastic cells preserve the cell-type specific expression of the IF characteristic of the parental cell type [91,106,114,118,119,120,121].

Table 1: Types of intermediate filaments (IFs).

Class	Sequence identity	Mol. wt.	Cell type
Type I	Acidic Keratins	40-64	epithelial
Type II	Basic Keratins	52-68	epithelial
Type III	Desmin	53	muscle
	GFAP	51	astroglia
	Peripherin	54	neuronal
	Vimentin	55	mesenchymal, tissue cultured cells.
Type IV	a-Internexin	66	immature neuronal
	b-Internexin	70	neuronal
Type VI	NF-L	68	neuronal
	NF-M	110	neuronal
	NF-H	130	neuronal
	nestin	240	CNS stem cells
Type V	Lamin A	70	most
	Lamin B	64	most
	Lamin C	58	most

Melanomas were thought to express only vimentin type of intermediate filament [81,183,221], but recent studies show that melanomas may express other IFs as well. Methods used in these studies include: 1) the immunohistochemical analysis for detecting IF antigen expression in tumour cells; 2) an in *vitro* invasion assay for examining the invasion potential of tumour cells; 3) a semi-qualitative Western blot analysis for demonstrating IF protein; 4) the Northern blot analysis to indicate the relative amount of IF m RNA expression; and 5) the use of keratin 8, 18 antisense inhibitor in the highly metastatic tumour cell lines, or transfected by keratin 18 c DNA in the lower metastatic tumour cell lines to observe their effects on invasive ability.

Vimentin

Vimentin belongs to type III of IF proteins and is expressed as a homopolymeric intermediate filament [93]. It has a molecular weight of 58,000 daltons and is the predominant intermediate filament present in mesenchymal cells and in a variety of transformed cell lines and tumours[124]. During embryogenesis, mesenchymal cell lines initially appear to express vimentin, followed by the appearance of differentiation-specific IFs, and later by the disappearance of vimentin in a series of steps leading to cellular differentiation, e.g., muscle and glial cells[102, 121]. This IF is widely present in normal tissue and skin and is found in fibroblasts, lipocytes, smooth muscle cells, vascular endothelial cells, peripheral nerve (Schwann) cells, macrophages and myoepithelial cells of sweat glands. Vimentin is also found to be the only IF expressed by normal melanocytes and malignant melanoma

cells [91,94,183]. The immunohistochemical diagnosis of tumours depends upon a few unique antigens, that present themselves in the membrane and cytoplasm of melanocytes, of which vimentin is included. Originally, it was thought that all cutaneous melanomas were vimentin positive and keratin negative. It was latter confirmed that vimentin could serve as the useful differentiation marker in determining the origin of various cell lines and tissues [125,126,127].

Cytokeratin

Keratins are the largest and most complex group of IF proteins. They are further divided into type I keratins, which are basic and include K1-K8 and type II keratins, which are acidic and include K9-K20. Keratins are expressed as heteropolymer pairs consisting of specific type I and type II proteins [218]. There are at least 19 separate keratins ranging in the size from 40 to 68 kd. Their expression is highly regulated during embryonic development and cellular differentiation. For example, keratin 8 and 18 are first expressed in simple epithelial cells during embryogenesis. When these cells differentiate, they stop making the simple epithelial keratins and start expressing other keratins[75,79,121]. This more primitive phenotype is re-expressed during the development of malignant neoplasms in an analogous fashion to the retrograde expression of other oncofetal antigens. The expression of these antigens is referred to as the "dedifferentiated" phenotype. Carcinomas for example, may stop expressing specific keratins presented in the tissue of origin and further, and may produce additional keratins such as keratin 8 and

18 which are normally absent in healthy tissues. The immunohistochemical demonstration of keratins in these so-called "dedifferentiated" neoplasms has become the current standard in the differential diagnosis of carcinomas versus amelanotic melanomas[11,122].

It has been proven that K 18 is normally co-expressed with K8 to form a structural filament bundle. Transfection of a mutant K18 protein into highly invasive cell lines would likely disrupt the normal interaction between K8 and K18. Unfortunately there is no specific antigenic epitope on the mutant K 18 protein that does not also appear on the endogenous K18. Therefore, it is not possible to differentiate the localization of K18 mutant protein from normal K 18 protein in the cells. The carboxi-terminal deletion on K 18 includes the most conserved part of the coil-2 structure, shared with all intermediate filament proteins. This part, and the tail region on the K8 protein, fails to complex with their normal pairing region, resulting in discontinuous filament formation. This conformational change in filament bundling could account for alterations in signal transduction from the extra cellular matrix to the nucleus, thus affecting the tumours' invasivity and metastatic ability [75,102,163, 169,217].

Integrins and Intermediate filaments

Many steps in melanoma metastasis involve cell-cell or cell-matrix adhesive interactions. The surface molecules,which that mediate these processes, therefore plays an important role in regulating melanoma

dissemination and their level of expression may vary during the course of tumour progression. The melanocyte is the normal cell from which melanomas arise and it is derived from the neural crest [80,81]. Cells from this structure migrate extensively during development, invade extra cellular spaces and interact with various embryogenic cell types [85]. It seems possible that the disseminative capacity of melanoma cells may be a recapitulation of the characteristics evinced by these embryonic progenitors [142, 143, 144, 145].

Integrins appear to be the major receptors by which cells attach to extra cellular matrices, of which some also mediate important cell-to-cell adhesion events [84,131]. The cell surface receptor glycoproteins that constitute the members of the integrin family are trans-membrane, non-covalently associated heterodimers composed of a larger α subunit and a smaller β subunit. The α subunit varies in size between 120 to 180 kd and is associated with a β subunit from 90 to 110 kd. Most integrins are expressed on a wide variety of cells, and most cells express several integrins. While integrin molecules mediate interactions with other cells and components of the complement family, they mainly serve to interact with the adhesive proteins of the extra cellular matrix. Often the binding of the integrins to the extra cellular matrix-protein ligand is mediated by the recognition of a tripeptide Arg-Gly-Asp (RDG) [143, 144, 145, 146, 147]. The functions (ligand and adhesive specificity) of individual integrins have been elucidated using cell adhesion assays, monoclonal antibodies and affinity chromatography.

It has been proven that the CD 44 integrin is the principal cell receptor for hyaluronate on melanoma cells. This suggests that this membrane glycoprotein probably plays a greater role in the cellular processes involved in metastatic dissemination, than simply affecting cell-cell adhesion. It has been found that more CD44 is expressed on cutaneous malignant melanoma cells than on normal melanocytes. Moreover, selection of various lines differing in levels of CD 44 expressed from a single parental tumour is associated with profound changes in the *in vivo* behaviour of these cells. The greater expression of CD 44 therefore correlates with enhanced aggression [171].

There is a body of evidence suggesting that transmembrane integrins function as receptors, which interact with IFs and with their linking proteins. Integrins may mediate cell shape, spreading and migration by effecting changes in the cytoskeleton via a variety of signal transduction pathways [86,131,155]. There is also evidence for a direct interaction between a α subunit and the cytoskeleton [143]. Specific interactions between cell surface fibronectin [139] and vimentin IFs have been observed, as well as contacts between α-actin and the β_1 integrin subunit, collectively suggesting the existence of a morphological transduction pathway [116, 118, 120, 145, 146, 149]. In a variety of cell types, occupation of the integrin receptors by their ligands leads to tyrosine phosphorylation and cytoplasmic alkalinization. A reasonable working hypothesis for both of these cytoplasmic events is that integrins serve as receptors for soluble agonists in stimulating these signals [145, 147]. From these

observations, it is also possible to conclude that interruption of the function of cell adhesion receptors, particularly those of the integrin family or those involved in cell-matrix interactions, has a dramatic effect upon the metastatic dissemination of melanomas.

Among cutaneous melanoma studies, it has been found that some integrins like $\alpha_4\beta_1$, $\alpha_V\beta_3$ are related with invasiveness of the ECM [131,142, 145, 146]. In one study comparing the presence of integrins in cutaneous and uveal melanoma, the presence of integrins in uveal melanoma was shown to be different from cutaneous melanoma, and not related to prognosis [174].

Interconverted Phenotype

Interconverted Phenotype is defined as the co-expression of vimentin and cytokeratin in tumours. Most major cell types *in situ* contain only a single IF protein type[115]. The expression of vimentin is traditionally regarded as a marker of cells of mesenchymal origin. On the other hand, keratin is considered to be a marker of cells of epithelial origin. The co-expression of keratin and vimentin appears to be a rare event *in situ,* but may occur in certain instances, like in some cells of the human parotid gland [128]. It has been reported [129] that the co-expression of keratins and vimentin was found *in situ* in the parietal endoderm of the mouse embryo of 8.5-13.5 days old. [142, 146]

Changes in cell shape and cytoskeleton configuration have been observed directly by laser scanning confocal microscopy during

transendothelial migration. The cells underwent drastic modifications in cell shape, but most interestingly, cytoskeleton fibres and stress fibres changed in conformation, location and quantity inside the melanoma cell [100].

Co-expression of vimentin and cytokeratin (the interconverted phenotype) was described in the beginning of the 80's in certain types of carcinomas such as renal adenocarcinomas as well as thyroid and salivary gland carcinomas. This feature was addressed in the literature as a sarcomatous differentiation of those tumours, without any mention about invasiveness [91].

Later, it was observed that the presence or absence of an additional vimentin in metastatic human carcinoma cells present in ascites and pleural fluids may influence mitotic, as well as motile activity of tumour cells [130]. In 1989, a positive relationship between the vimentin expression in infiltrating ductal carcinomas and high rates of tumour growth was observed [102]. It has been postulated that in breast carcinoma [169] the ability to co-express vimentin and keratins confers a selective advantage to breast cancer cells, although the expression of a single IF, either cytokeratin or vimentin, did not relate to increased agressiveness[136]. These results are based on interpretations of signalling cues from the extra cellular matrix, and also relate to other hypothesis: the cells that are more capable to metastasize successfully are those which have the ability to rapidly and reversibly reorganize their cytoskeleton. This hypothesis may explain why some tumours do not express the IF of their tissue of origin in late stages of the disease.

The same situation seems to occur in malignant melanomas. Using indirect immunofluorescence and immunoblot analysis it has been proven that the expression of keratin intermediate filaments are not artefacts nor contamination, but rather represents the existence of this IF in the cytoplasm of melanoma cells [206]. In a study [11] performed in 1990 a one- and two-dimensional western blot analysis, and an immunohistochemical survey of 100 cutaneous melanomas found that anomalous keratin expression occurred only in metastatic or recurrent melanomas. This study raised an interesting question of a possible association with melanoma progression. Other studies[10,12,103,134,135] have shown that the co expression of keratin 8, 18 and vimentin in human cutaneous and uveal melanomas has been associated with increased invasion and metastatic potential. In 1998 [9] was confirmed that human uveal melanoma cells, which predominantly co-express keratin 8, 18 and vimentin, were 6-fold more invasive through the ECM as compared with uveal melanoma cells expressing vimentin only. These cells were also 8- to 13-fold more invasive than normal uveal melanocytes. When the melanoma cells where treated with antisence oligonucleotides to keratin 8 and 18, they were predominantly vimentin-positive and keratin-negative, and showed a significant decrease in migratory ability. These findings provided the justification for additional studies to be performed viewing the association between the co expression of both keratins and vimentin, and the metastasis of uveal melanomas.

Extra cellular matrix and Intermediate filaments

The extra cellular matrix is a dense latticework of collagen and elastin embedded in a viscoelastic ground substance composed of proteoglycans and glycoproteins. It is a supporting scaffold, which isolates tissue compartments, mediates cell attachment, and influences tissue architecture. The matrix also acts as a selective macromolecular filter and plays a role in mitogenesis and differentiation [86]. Interactions between normal cells and the matrix may be altered in neoplasia, which may influence the tumour proliferation and invasion. The vertebrate organism is separated into tissue compartments bordered by the basement membrane and interstitial stroma. The basement membrane is a meshwork of type IV collagen, specific glycoproteins such as laminin and entactin, and heparin sulfate proteoglycans. Type IV collagen is the backbone of the basement membrane. Type V collagen, as well as other types of collagen may exist at the interface between the basement membrane and the stroma. For most tissues, the organ parenchymal cells secrete and assemble the basement membrane [86,160, 166, 167, 168]. Cell-ECM interactions are frequently mediated by members of the integrin family of cell adhesion molecules, the cytoplasm domain of which are thought to be linked to the cytoskeleton via a series of interacting proteins, including talin, vinculin and α-actin [96,99,101,196]. Actin filaments as well as the other IF are linked to the plasma membrane via those interacting proteins. Normal and malignant cells have also been shown to exhibit different patterns of vinculin-containing cell-substratum contacts. These cell-

substratum contacts are necessary to transform force development into the cell into actual movement[96,99,101]. Therefore altered organization of cytoskeletal components will likely account for altered motile activity. Data in *in vitro* invasion assays in melanoma cells support the idea that there is an inverse relationship of the degree of the actin filament organization as well as the number of vinculin plaques with cell invasiveness [96,97,101]. There has been also reported that some skin malignant melanomas that express elevated levels of actin, described as sarcomatoid tumours, are more invasive than their counterparts [157].

The ability to invade or penetrate biological matrices is of primary importance both in the development of malignant melanoma, as well as in the genesis of many kinds of advanced malignancy. This is due to the fact that tumour cells encounter these matrices during intravasation, extravasation, and dissemination. A three step hypothesis describing the sequence of biological events involved in tumour-cell interaction with such extra cellular matrices has been proposed [160]: (a) tumour cell attachment to a matrix substratum, (b) local tumour cell degradation of the matrix by tumour cell secreted-proteases, and (c) tumour cell locomotion into the matrix modified by proteolysis.

Taking into consideration the fact that intermediate filaments can act as signal transducers relaying information from the extra cellular matrix to the nucleus [161] and that the matrix can regulate gene expression [162], one can

speculate that the ability to co express vimentin and keratins - the interconverted phenotype - offers a selective advantage to tumour cells.

Metastasis, Treatment, And Survival

Uveal melanoma differs from most other human neoplasms in that death virtually always occurs from direct hematogenous metastasis. Extra-ocular extension and orbital recurrence have been noted in a small proportion of cases. However, even these patients died from distant metastasis, rather than from extension of the recurrence into the central nervous system. Spread to regional lymph nodes has been documented in only a few cases of uveal melanoma, since the orbit does not contain lymphatic structures. Although extra ocular extension is associated with a poor prognosis, this association primarily reflects the tendency of these tumours to be large with a high proportion of epithelioid cells.

The treatment of choice for uveal melanoma depends upon the size of the tumour. Small tumours tend to be followed and growth is monitored. If no growth is seen, no treatment is undertaken. If these small tumours begin to enlarge, radiation is applied in order to kill the neoplastic cells while still preserving the vision and aesthetics of the patient [76,209]. Unfortunately, if the tumour continues growing, an enucleation is performed. This is also the case for patients who have large tumours at first presentation or diffuse tumours

that carry a substantial risk for metastasis despite its flat appearance[76,202]. Systemic work-up consisting of several liver function tests is performed each year to ensure no metastatic disease. In a study of 2,627 cases from the American Registry of Ophthalmic Pathology (AFIP), Zimmerman and co-workers [67] discovered that the proportion of patients being diagnosed earlier on has increased, while those diagnosed at later stages of disease have decreased. Although this is a promising sign, it was also concluded that despite earlier treatment, there was no significant improvement in survival rates [67,68,76,195,201].

As discussed earlier, metastasis from cutaneous melanoma initially spreads via the lymphatic system. In the eye and orbit however, there is no lymphatic drainage. Therefore, uveal melanoma cells metastasize hematogeneously, with their preferential destination being the liver. Also, unlike cutaneous melanoma, which metastasizes to regional lymph nodes where systemic involvement can be traced, there is no such staging in uveal melanoma. Ophthalmologists therefore rely upon prognostic factors to identify patients who are at greatest risk of developing metastatic diseases it is known that the median survival following diagnosis of hepatic metastasis is only 2 to 4 months [1]. As of yet, there is no prevention.

Metastasis from uveal melanoma can occur anytime up to 20 years after treatment. After this period, it is unlikely that the tumour will metastasize. On average, the first evidence of metastasis is discovered 6.5 years after the removal of the primary tumour [6]. Most often, the metastasis

occurs from two to four years after the eye treatment with 25% occurring within 18 months, 50% within 40 months, and 75% within 96 months. Furthermore, only 1% of uveal melanoma patients show evidence of metastasis at presentation, yet 40-50% of patients will develop hepatic metastasis [7,59,68,69,195,201]. The five, ten and fifteen year survival rates for uveal melanoma are approximately 72%, 59%, and 53% respectively [5, 70]. On autopsy population based study liver is seen as a site of metastatic disease in 100% of the cases, lungs in 55%, kidney 44% and brain 22% [200]. It is possible that many patients already have sub clinical hepatic metastases that are not detectable upon routine systemic examinations [68,195,201]. It has been proven that the only tests specific enough to be useful were imaging by liver ultrasonography. No single blood test is useful, due to a combination of poor specificities and sensitivities [198] but the gamma-glutamyl transpeptidase test has to be considered further as long as it has been proven that it is the first of the liver enzymes to increase before the diagnosis of metastasis can be suspected [208]. It is also possible to improve the specificity combining blood results, but this implies in a higher cost to the patient [191]. When combined liver ultrasonography and blood tests it is possible to diagnose asymptomatic patients in 76% of the cases [197]. Once a metastatic site is detected clinically, the life expectancy is between two to seven months and is fatal in all cases [71]. In studies where only surgical resection was performed, the summarized complete-remission rate was 0%, and the partial-remission rate was less than 9% [188,195]. Surgical resection of the affected hepatic site coupled with chemotherapy is the best method of improving survival [69,72,192,195].

The Collaborative Ocular Melanoma Study (COMS) [73] reports that there is no survival difference between patients who underwent pre-enucleation radiation (74%) or enucleation alone (72%). Gunlap and co-workers [74] also concluded that pre-enucleation irradiation does not improve survival in patients with uveal melanoma. This data suggests that micro metastases occurred before treatment. This also weakens the argument [7] that enucleation causes metastatic dissemination since the patients who underwent radiation before enucleation had the same survival rate.

The treatment of metastatic uveal melanoma with surgery and systemic chemotherapy offer minimal benefits for these patients[92, 192]. Despite this fact, chemoimmunotherapy combined with a four-drug chemotherapy regimen and interferon Alfa has provided response rates of approximately 20% and may contribute to prolonged survival and modify the tumour doubling time of the metastasis[192, 195]. Because intra-arterial fotemustine (quemotherapic agent) also has shown promise in patients with hepatic lesions, interferon-containing treatment combined sequentially with intra-arterial fotemustine in a rotational fashion might be the next developmental step. Besides all those treatment modalities, metastatic uveal melanoma still remains an incurable disease that is treated only palliativelly [192,195].

The use of molecular biology techniques such as PCR, may significantly improve the ability to detect sub clinical metastasis in patients with uveal melanoma. Though it seems likely that the presence of melanoma

cells in the bloodstream increases the probability of metastasis, more definitive studies are needed [83].

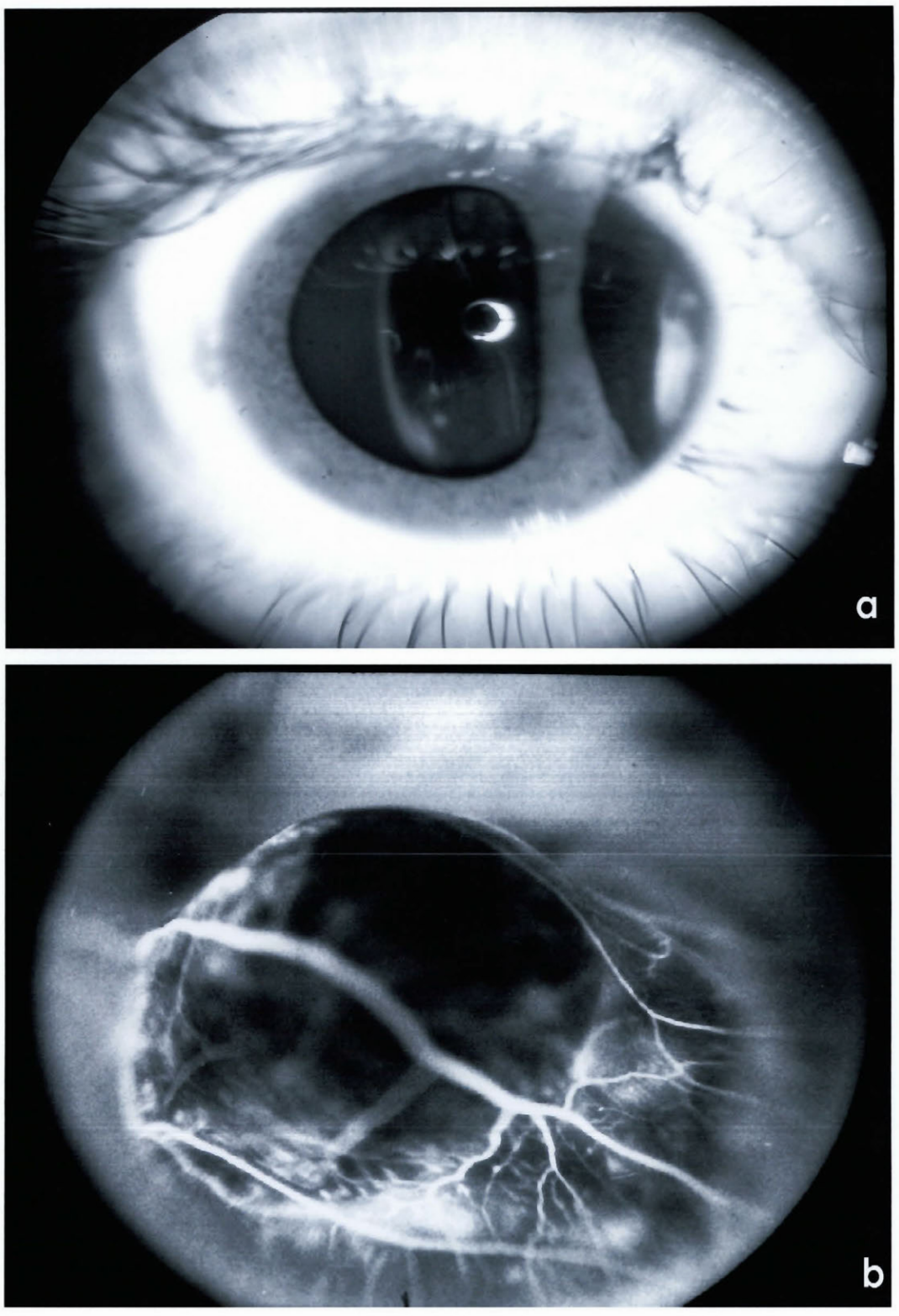


Plate 1.

Figure a) Clinical picture of a patient with a large choroidal melanoma invading the ciliary body . Figure b) Fluorescein angiography of a large, mushroom shaped, choroidal melanoma.

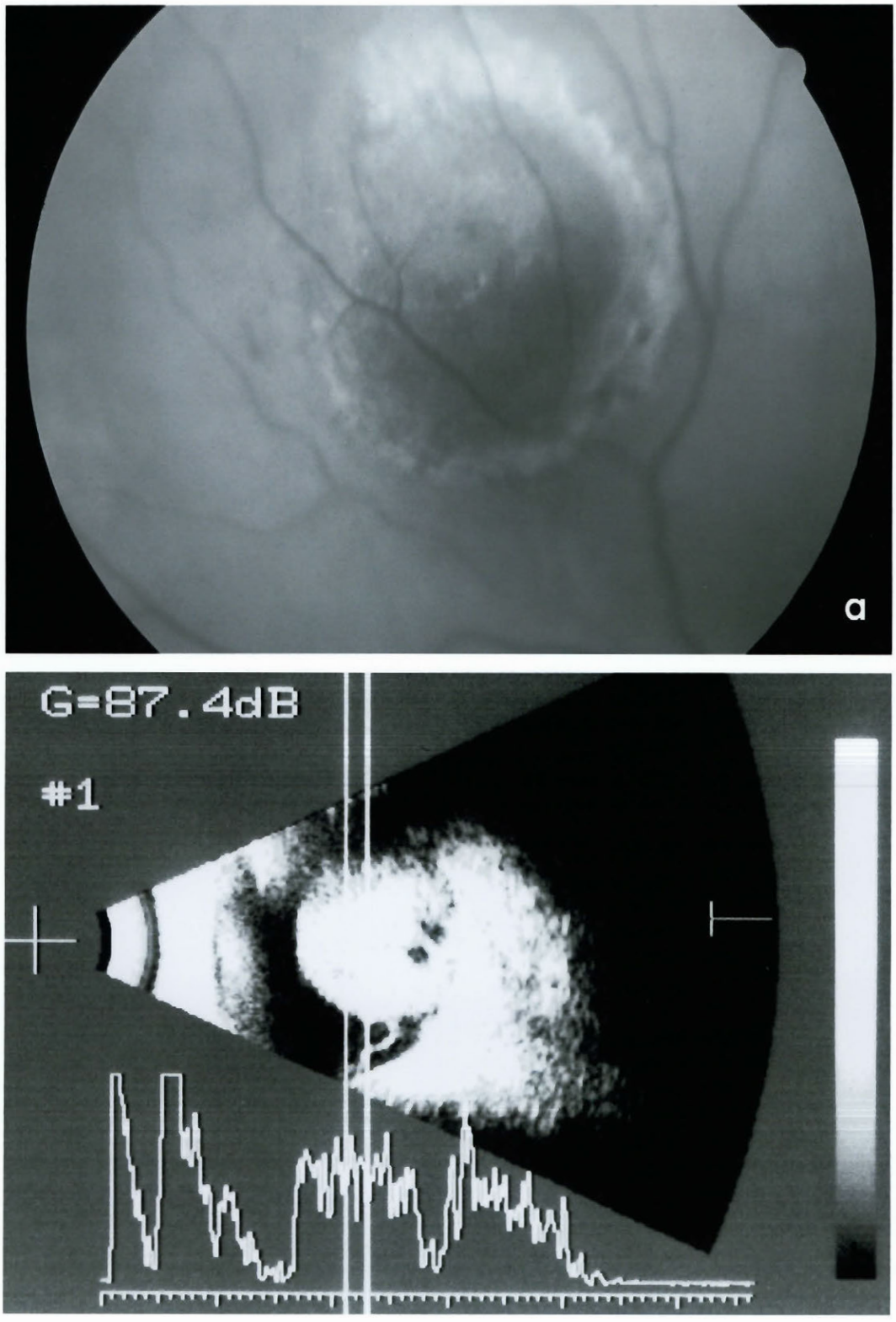


Plate 2.

Figure a) Clinical picture representing the funduscopic finding on a patient with a large choroidal melanoma. Figure b) Ultrasonography of an eye demonstrating a large choroidal mass with medium reflectivity characteristic of a choroidal melanoma.

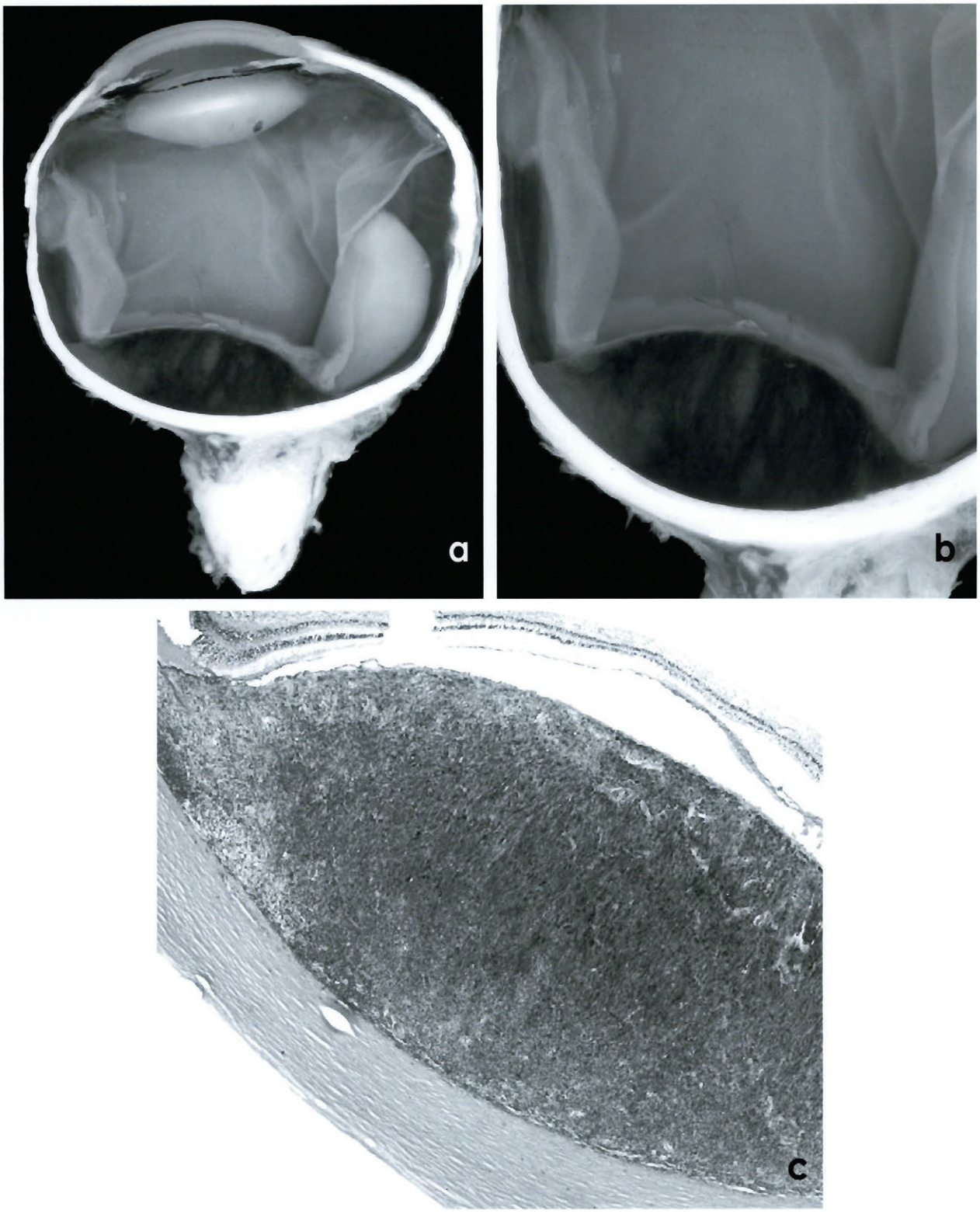


Plate 3.

Figure a) Gross photograph of an enucleated specimen showing a dome shaped,heavily pigmented, choroidal melanoma. Figure b) Higher magnification of the previous picture. Figure c) Histopathological section of the previous case showing a dome shaped choroidal melanoma with a retinal detachment overlying the tumour.(H&E, 125X)

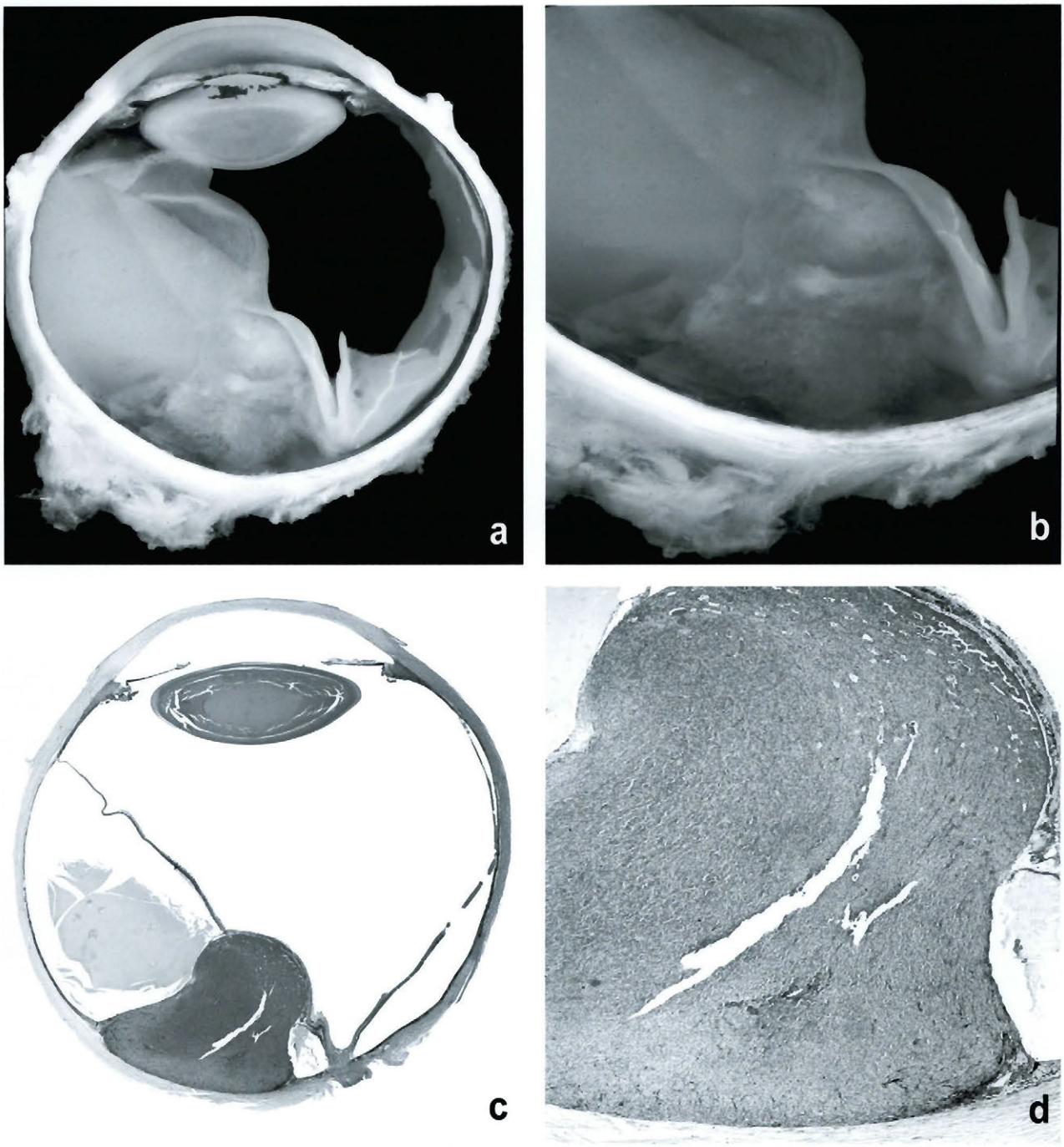


Plate 4.

Figure a) Gross photograph of an enucleated specimen demonstrating a mushroom shaped choroidal melanoma with overlying retinal detachment. Figure b) Higher magnification of previous picture with a close up of the choroidal tumour. Figure c) Histopathological section of the eprevious case(H&E, 1X). Figure d) Histopathological section of a mushroom shaped choroidal melanoma. Higher magnification of the previous picture, diyplaying blood vassels on the apex of the tumour. (H&E , 125X).

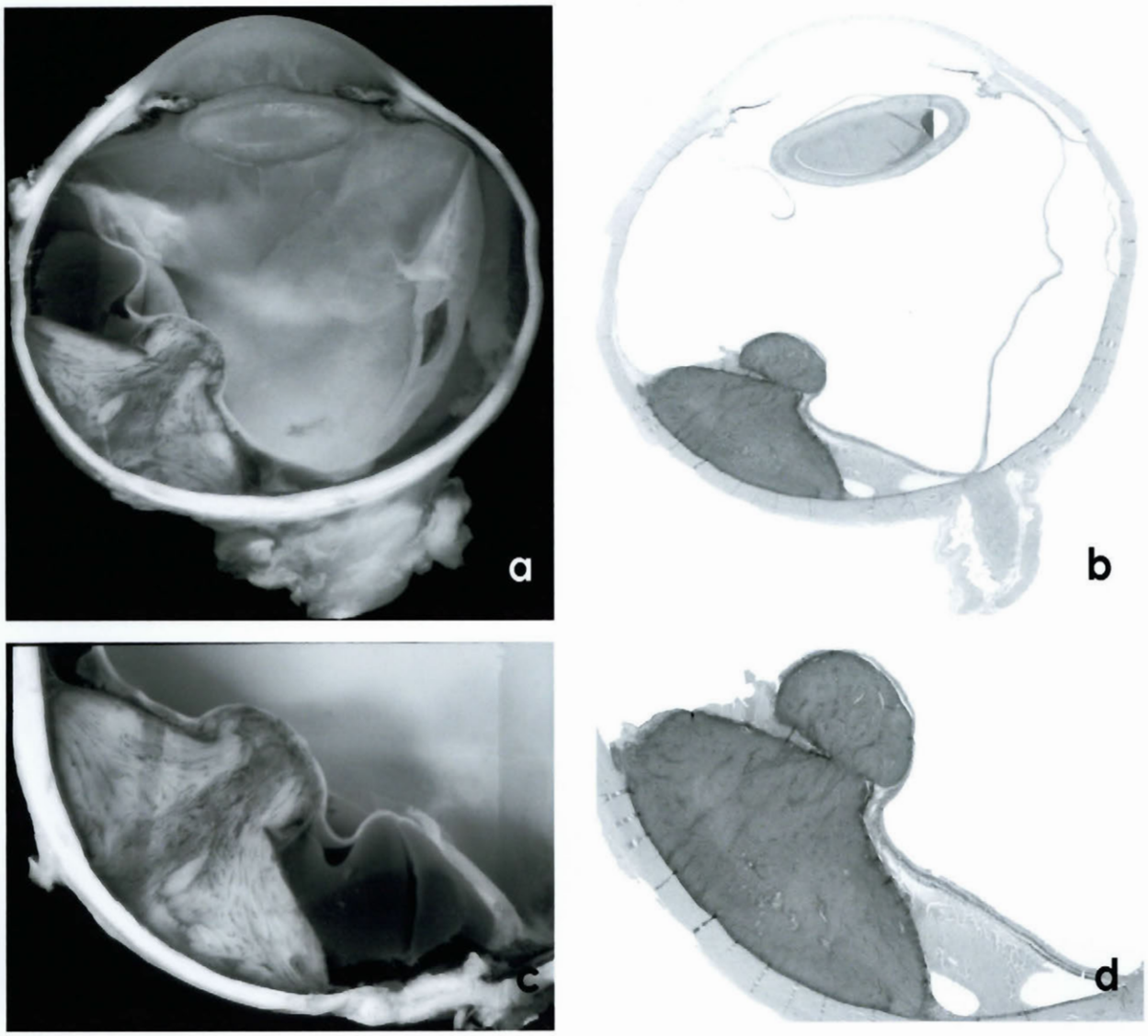


Plate 5.

Figure a) Gross photograph of a mushroom shaped choroidal melanoma. Figure b) Histopathological section of the previous choroidal melanoma (H&E,1X). Figure c) Higher magnification r of specimen in figure a. Figure d) Higher magnification of figure b (H&E, 125X).

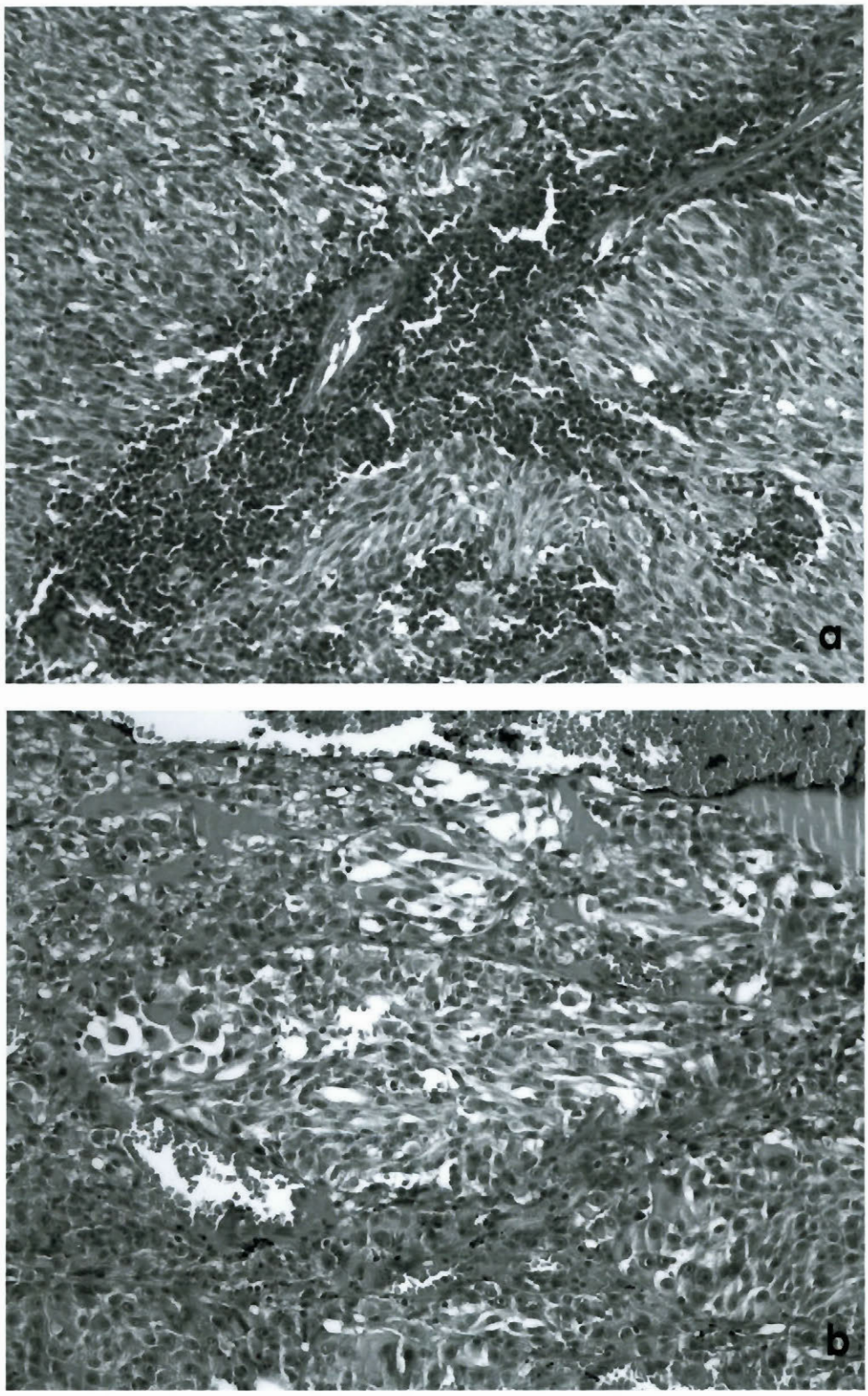


Plate 6.

Figure a) Histopathological section of a choroidal melanoma dysplaying lymphocytic infiltration throughout the tumour (H&E 100X). Figure b) Histopathological section of a choroidal melanoma demonstrating vascular pattern of closed loops containing numerous epithelioid cells. (H&E 100X).

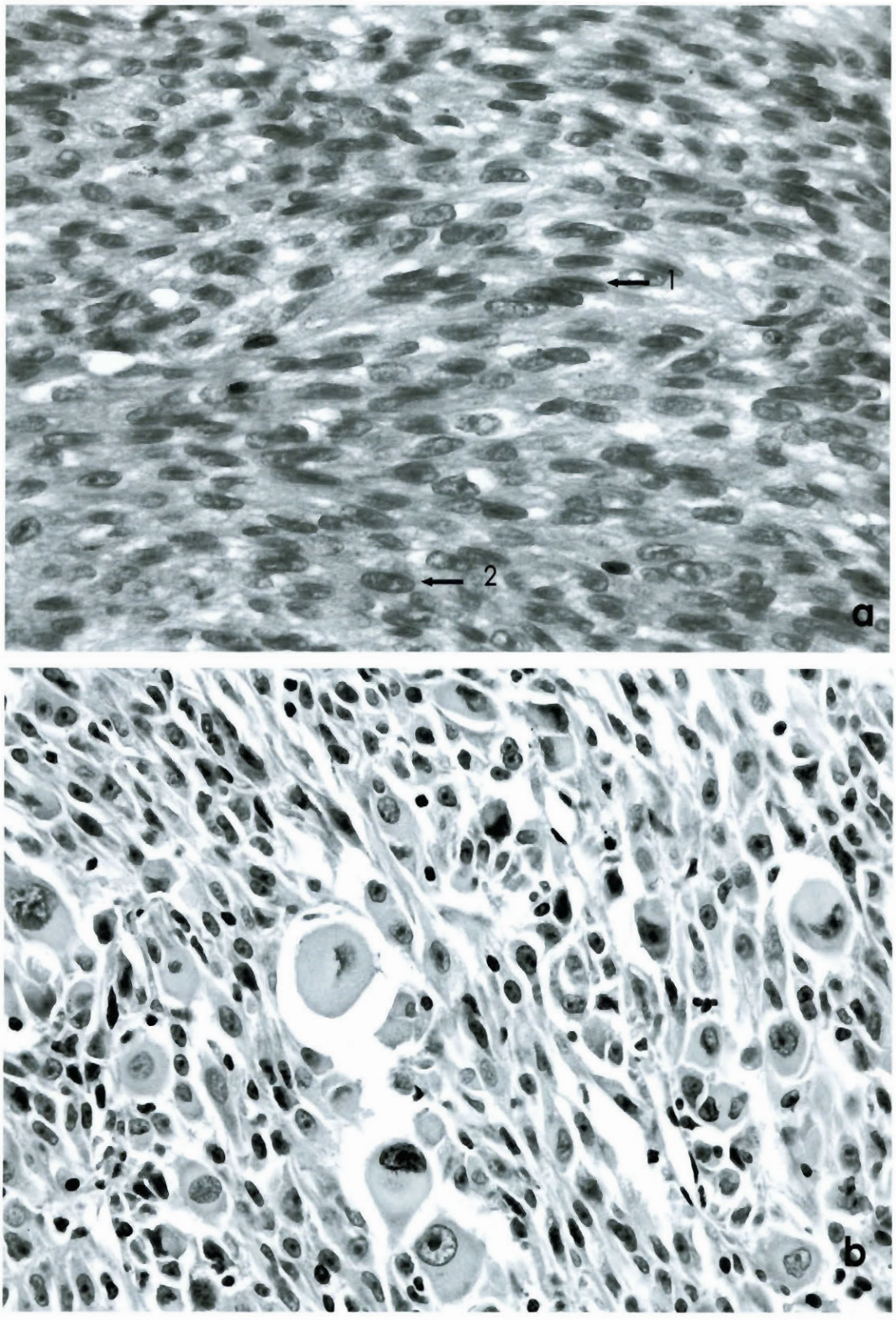


Plate 7.

Figure a) Histopathological section of a primary choroidal melanoma showing spindle A (1) and B (2) cells (H&E 400X). Figure b) Histopathological section of a primary choroidal melanoma showing epithelioid cells (H&E 400X).

MATERIAL AND METHODS

A compilation of 137 histopathological cases represented by 128 enucleated globes and 9 liver biopsies of uveal melanoma patients was used. These specimens were all formalin-fixed and paraffin-embedded. The 137 cases are divided into three groups:

- Group 1: One hundred cases of primary uveal melanoma were retrieved from the American Registry of Ophthalmic Pathology, located at the Armed Forces Institute of Pathology (AFIP), Washington, DC. These intraocular melanomas were classified according to the modified Callender's classification consisting of 3 groups: spindle, epithelioid and mixed cell type.

- Group 2: Nine positive liver biopsies of patients with uveal melanoma liver metastasis were retrieved from the American Registry of Ophthalmic Pathology, located at the Armed Forces Institute of Pathology (AFIP), Washington, DC.

- Group 3 Twenty-eight cases of enucleated globes containing uveal melanomas were selected from The Henry C. Witelson Ophthalmic Pathology Laboratory and Registry, McGill University, Montreal, Canada.

  Those cases were chosen based on availability of clinical follow up information on these patients. The clinical data includes age, gender,

cell type, lymphocytic infiltration, vascular pattern, and presence of metastatic disease.

Primary monoclonal antibodies against intermediate filaments antigen keratin 8,18 and vimentin were used to confirm the hypothesis of this thesis. Their applications are summarized in table 2.

The method used for immunohistochemical staining of the specimens was the Avidin-Biotin method. This method utilizes a high affinity of avidin or strepto-avidin for biotin. It also requires a biotinylated antibody as a link antibody. In resume the sequence of reagent application is primary antibody, biotinylated antibody, performed avidin-biotin complex and substrate solution.

The biotinylated secondary antibodies serve as an efficient link between a primary antibody and a complex substrate labelled with enzymes. According to different primary antibodies, the link antibody should be chosen differently. If the primary antibody was monoclonal from mouse, the link antibody should be biotinylated swine anti-rabbit immunoglobulins. Their applications are summarized in table 3.

Table 2. Primary antibodies applied in immunohistochemical analysis of uveal melanomas

REAGENT	CLONALITY	SOURCE	SUPPLIER	DILUTION
Anti-vimentin (V9)	monoclonal	mouse	DAKO, M725	1:30
Anti-keratin 8, 18 (Cam 5.2)	monoclonal	mouse	Becton-Dickinson, 349205	1:10

Table 3. Biotinylated secondary antibodies applied in immunohistochemical analysis of uveal melanomas

REAGENT	IMMUNOGEN	SOURCE	SUPPLIER	DILUTION
Rabbit anti-mouse	immuoglobulins	mouse	DAKO, E354	1:200

Paraffin sections were cut at 5um, mounted on commercially provided silanized slides (Surgipath, Snowcoat, X-tra), and dried overnight at 37°C, or at 60°C for at least 60 minutes (min).

Haematoxylin and eosin (H & E) staining

Sections of eyes containing uveal melanoma, 28 uveal melanomas that composed Group 3, were cut at 5um thickness from routinely processed paraffin blocks. The sections were then stained with the standard staining procedure of haematoxylin and eosin (H & E).

Immunohistochemistry staining

Immunohistochemistry staining was carried out by the avidin-biotin complex (ABC) method using the Ventana Medical System Immunostainer (Tucson, Arisona), and except for the heat induced antigen retrieval procedure, which was carried out manually [87,150,151,152,153,154]. The ABC method belongs to a modification of indirect enzyme-linked immunohistochemistry resulting from the application of the high affinity of avidin for biotin to link reactants in multistep staining procedures. Avidin, an eggwhite 68-kD glycoprotein, has four binding sites for the vitamin biotin. Generally, biotin has been covalently conjugated with the secondary antibodies as the link antibody. Open sites on avidin, from the avidin-biotin-horseradish peroxidase (ABC) complex, bind to the biotin on the link antibody. Upon oxidation, added chromogens become colored products that express the immune reaction in tissue cells. The strong affinity of avidin for biotin makes the ABC method more sensitive and very efficient. Excellent results can also be achieved using formalin-fixed, paraffin-embedded specimens.

The Ventana detection kit detects a specific mouse IgG, IgM and rabbit IgG antibody bound to an antigen bound in paraffin embedded tissue sections. The antibody is located by a biotin conjugated universal secondary antibody formulation, which recognizes rabbit or mouse immunoglobulins. This step is followed by an avidin enzyme conjugate, which binds to the biotin

present on the secondary antibody. The specific antibody, secondary antibody, avidin enzyme conjugate is then visualized using a precipitating enzyme product. Each step is incubated at 42^{0}C for a predetermined time. At the end of each incubation step, the Ventana instrument washes the sections to stop the reaction and remove unbound material that would hinder the desired reaction in subsequent steps and applies a liquid coverslip, which minimizes the evaporation of the aqueous reagents from the specimen-containing slide. Briefly, slides were deparaffinized in xylene, rehydrated through 100% ethanol, and treated with 0.3% H202 in methanol for 4 min at RT in order to block endogenous peroxidase activity. The heat induced epitope retrieval (HIER) procedure was carried out in 0.75M Tris base (pH 10) in a 1000c water bath for 30 min. Except for vimentin, the other antigens required the use of protease digestion to reveal the specific epitopes. The primary antibodies were diluted with the antibody-diluting buffer from DAKO (CD 200086). Then the following steps were performed by the Ventana Machine, all at 42 ^{0}C: 1) inhibitor removes remaining peroxidase activity for 4 min; 2) primary antibody is applied for 32 min; 3) the secondary antibody is applied for 8 min; 4) the Avidin HRPO – Avidin horseradish peroxidase in protein stabilizer and preservative- is applied for 8 min; 5) the AEC (3-amino-9-ethylcarbazole DAKO, K3469, ready to use) solution is mixed with H2O2 and applied for 8 min; 6) Cooper sulfate solution is applied for 4 min in order to enhance the reaction. The AEC chromogen was used in order to visualize the end product and then counterstained with Gill II haematoxylin (Surgipath), further by mounting them with aqueous mounting medium (DAKO, S3025).

Negative control slides were treated in an identical manner except that the primary antibodies were omitted.

Positive control slides consisted of stock tumours or normal tissues known to contain the determinant of interest. They included ciliary epithelium as internal positive controls for keratin 8 and 18 and blood vessels as internal positive controls for vimentin. The slides were graded as strongly or weakly positive.

Results

As mentioned elsewhere there were three Groups of patients who were studied with different objectives; Group one which is represented by 100 sets of slides, Group 2 who is represented by a set of 9 liver metastasis and Group 3 which are represented by a group of 28 uveal melanoma patients that had an enucleated eye.

The first set of patients named Group one was analyzed to determine the presence and the frequency of interconverted phenotype in uveal melanoma.

The second group of patients was studied to analyse the immune expression of interconverted phenotype in liver metastases of uveal melanoma cells.

The third group was also studied to determine a possible correlation between interconverted phenotype and well-known prognostic factors for uveal melanoma.

Group one: The analysis of 100 cases of uveal melanoma that were stained for cytokeratin and vimentin showed that 44 cases (44%) were positive for cytokeratin, and 86 cases (86%) were positive for vimentin. Fourty three cases were positive for vimentin and negative for cytokeratin. Fourty three cases were positive for both, vimentin and cytokeratin, characterizing the immunohistochemical expressivity of the interconverted phenotype. Of all cases that expressed the interconverted phenotype, five were strongly positive in more that 20 cell per 20 high power fields. The other 38 cases presented with scattered positive cells mainly in the apex of

the tumour and were considered weakly positive. The cases were also evaluated regarding the predominant cell type of the tumour, according to the Callender's modified classification. The majority of the tumours 58 (58%) were mixed cell type, while 22 (22%) were spindle cell tumours and 20 (20%) were epithelioid cell tumours.

The second group, represented by 9 cases of liver metastasis of choroidal uveal melanoma, 6 cases expressed the interconverted phenotype what represents 66,9% of this particular group. There were 2 cases where vimentin was the only immunomarker as well as one case where cytokeratin was the only immunomarker expressed.

The third group, 28 patients that had been enucleated because of malignant melanoma of the choroid, 21 (75%) were mixed cell type, 4 (14.3%) were spindle cell and 3 (10,7%) were epithelioid. Concerning other histopathological prognostic factors vascular pattern with closed loops was seen in 13 (48%) of the patients. Eight tumours (29%) demonstrated lymphocytic infiltration within the neoplastic cells. The immunohistochemical results displayed 3 cases (11%) were negative for vimentin, and only one of those cases was positive for cytokeratin. Eight cases were positive for cytokeratin, and that represents 28,5%. Seven cases of those 28 showed coexpressivity of vimentin and cytokeratin, demonstrating interconverted phenotype. Of those 7 cases, 2 had lymphocytic infiltration (29%), 3 (43%) had vascular pattern of close loops, 2 (29%) were spindle shape cells and 5 (71%)were mixed cell type.

Table 4 - Group 1: Cell type in the 100 cases from AFIP

Cell Type	Number of tumours	Percentage
Epithelioid	20	20%
Mixed	58	58%
Spindle	22	22%

Table 5 - Group 2: Expression of intermediate filaments vimentin and cytokeratin in liver metastasis of patients with uveal melanoma.

Case number	Vimentin	Cytokeratin
1	+	+
2	+	+
3	+	+
4	+	-
5	+	-
6	+	+
7	+	+
8	-	+
9	+	+

Table 6 - Group 2: Absolute number and percentages of expression of intermediate filaments vimentin and cytokeratin and their co expression in liver metastasis of patients with uveal melanoma.

Filaments	% (+/total)
Vimentin	**88.9 (8/9)**
Cytokeratin	**77.8 (7/9)**
Co-expression	**66.7 (6/9)**
None	**0 (0/9)**

Table 7 - Group 3: Cell type in 28 cases of intraocular primary uveal melanoma

Cell Type	Number of tumours	Percentage
Epithelioid	3	10,7%
Mixed	21	75%
Spindle	4	14,3%

Table 8 - Group 3: Correlation between cell type and interconverted phenotype in 7 primary intraocular melanomas.

Cell Type	Number of tumours	Percentage
Epithelioid	0	0%
Mixed	5	71%
Spindle	2	29%

Table 9 - Group3: Vascular pattern in primary intraocular melanomas

Vascular Pattern of closed loops	28 tumours	IP Tumours
Present	14 (50%)	4(57%)
Absent	14 (50%)	3(43%)

IP = Interconverted phenotype

Table 10 - Group3: Co expression of vimentin and cytokeratin in 28 primary intraocular melanomas

	IP	No IP
All cases	7	21
Metastatic cases	5(72%)	4 (19%)

IP = Interconverted phenotype

Table 11 - Group3: Lymphocytic infiltration in 28 cases of primary uveal melanoma.

Lymphocytic infiltration	IP	No IP
Present	5	7
Absent	2	14

IP = Interconverted phenotype

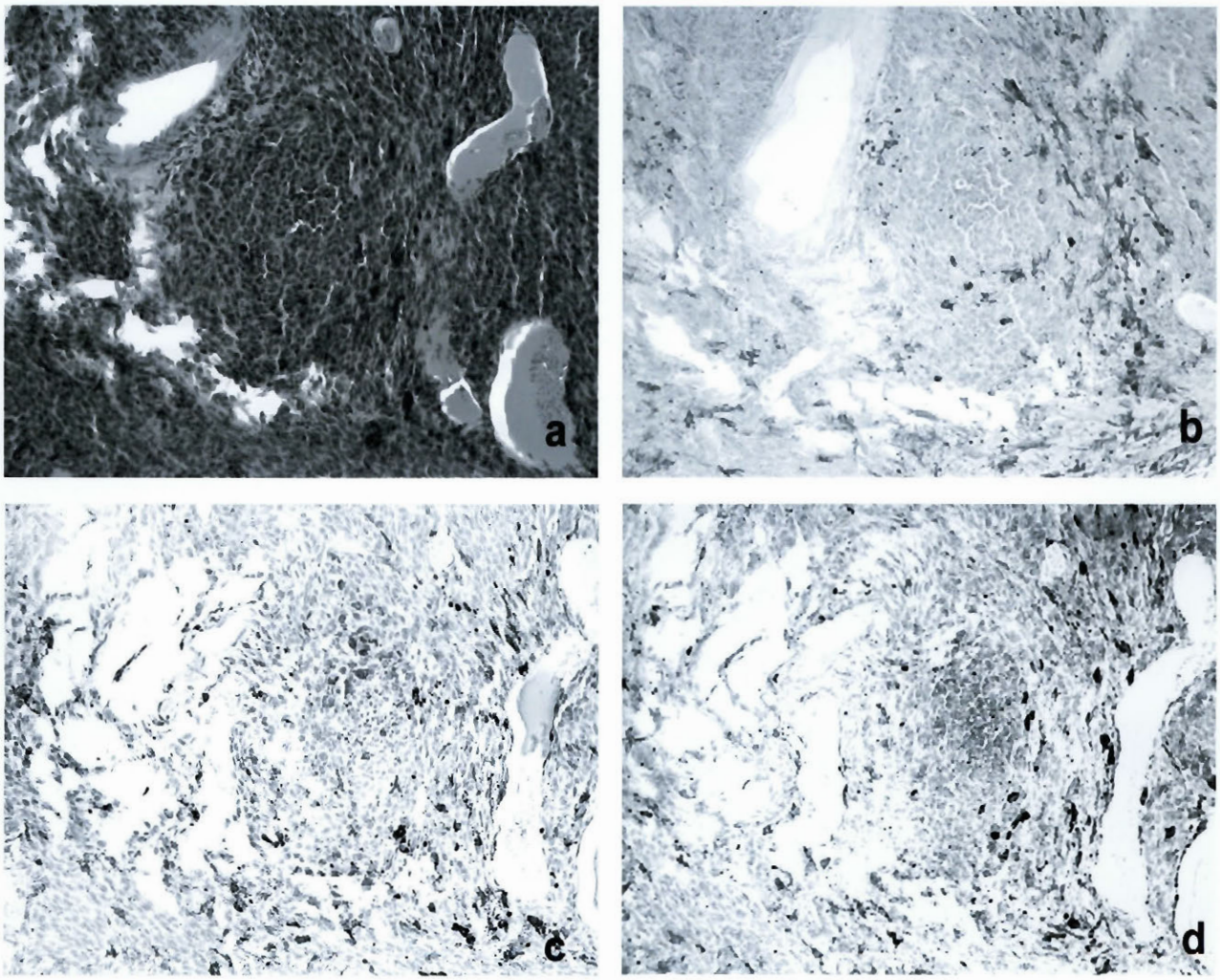


Plate 8.

Figure a) Histopathological section of a primary choroidal melanoma displaying spindle and epithelioid cells (H&E 200X). Figure b) Histopathological section of the same tumour in figure a. Negative control. (ABCI, 200X). Figure c) Histopathological section of the same tumour in previous pictures displaying cytokeratin positive cells (ABC, 200X). Figure d) Histopathological section of the same tumour in previous pictures showing vimentin positive cells (ABC, 200X).

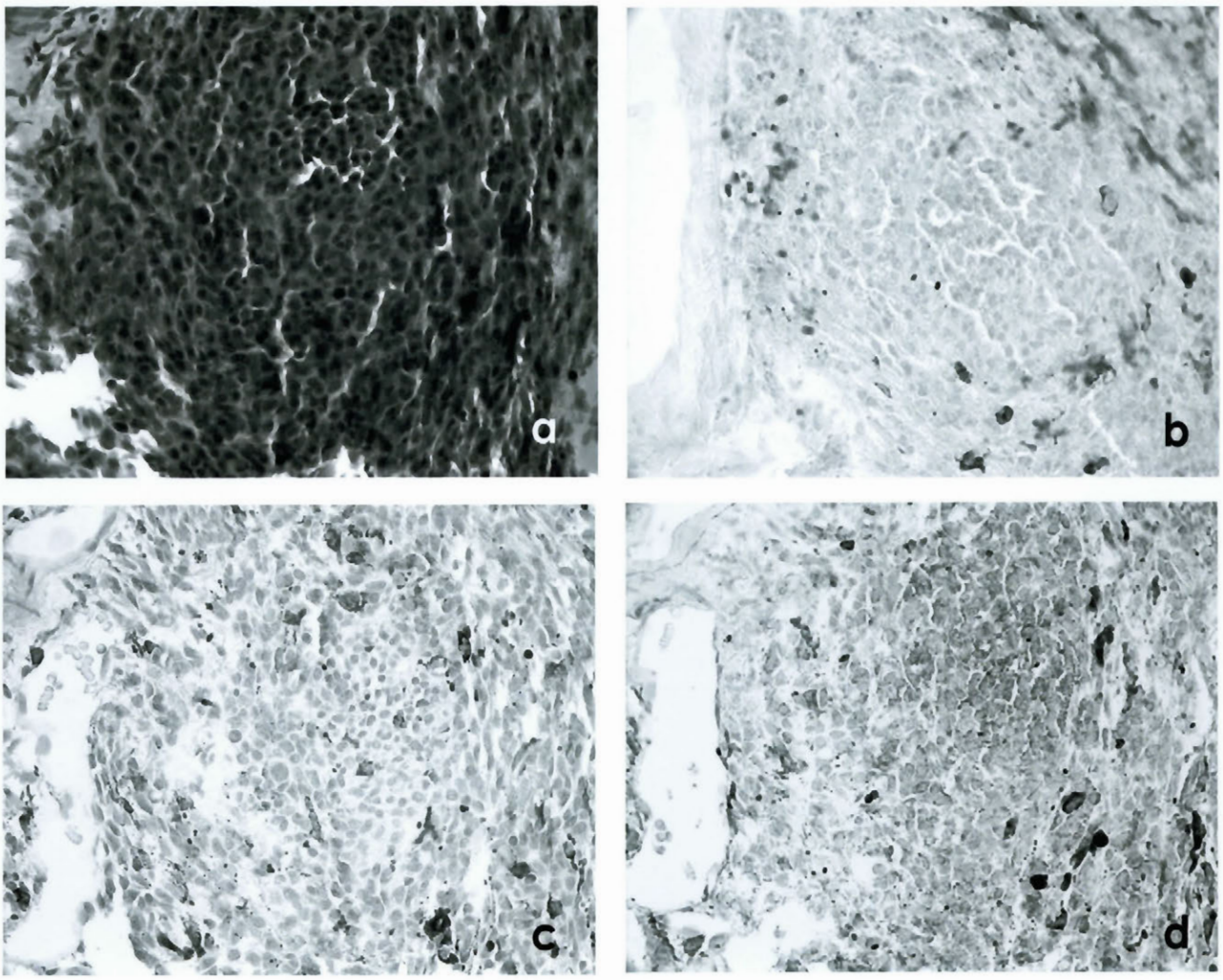
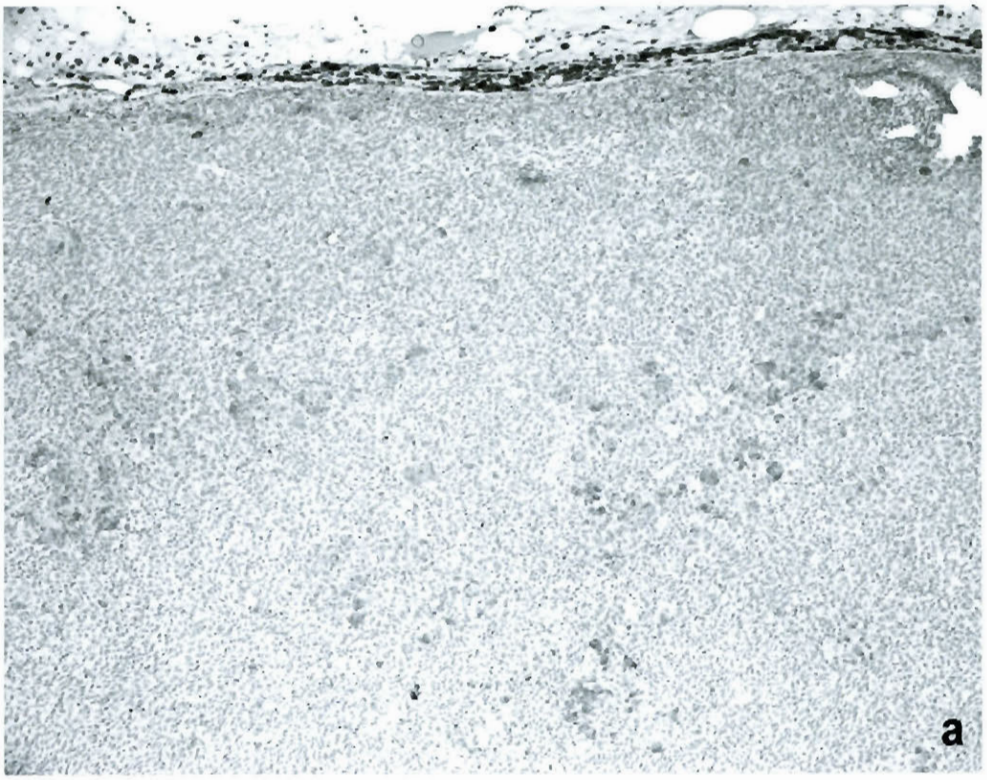


Plate 9.

Figure a) Histopathological section of a primary choroidal melanoma displaying spindle and epithelioid cells (H&E 400X). Figure b) Histopathological section of the same tumour in figure a. Negative control. (ABCI, 400X). Figure c) Histopathological section of the same tumour in previous pictures displaying cytokeratin positive cells (ABC, 400X). Figure d) Histopathological section of the same tumour in previous pictures showing vimentin positive cells (ABC, 400X).



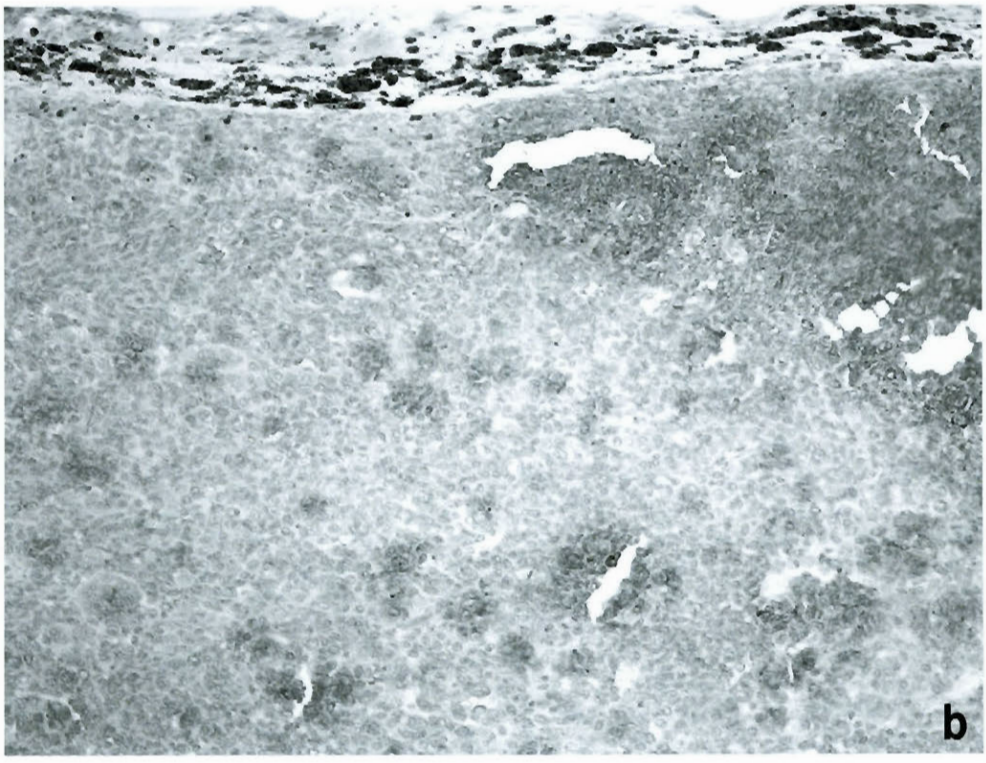


Plate 10.

Figure a) Histopathological section of a choroidal melanoma displaying vimentin positive cells (ABC, 100X). Figure d) Histopathological section of a choroidal melanoma showing cytokeratin positive cells (ABC, 100X).

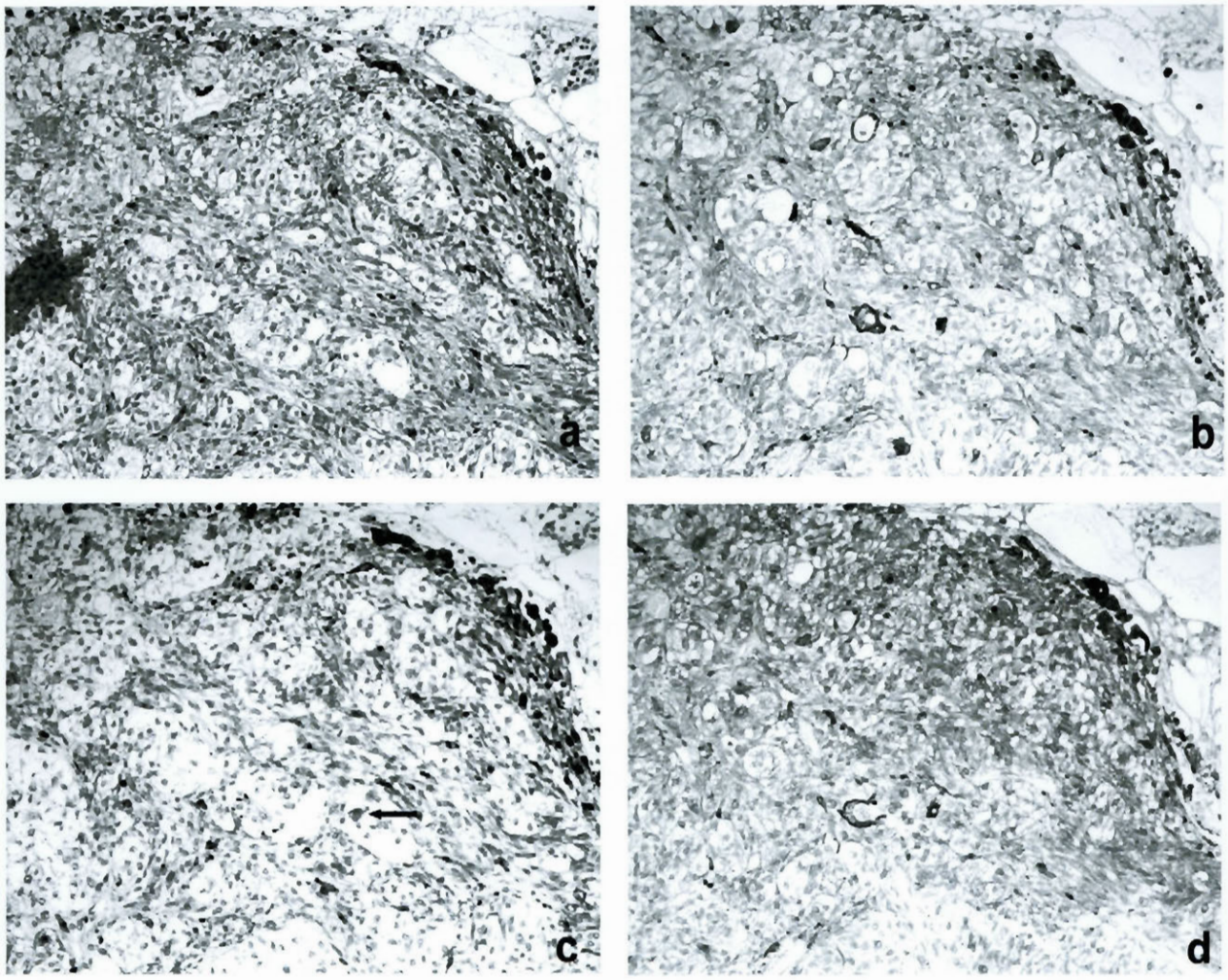


Plate 11.

Figure a) Histopathological section of a primary choroidal melanoma displaying spindle and epithelioid cells (H&E 200X). Figure b) Histopathological section of the same tumour in figure a. Negative control. (ABCI, 200X). Figure c) Histopathological section of the same tumour in previous picture displaying cytokeratin positive cells f(ABC, 200X). Figure d) Histopathological section of the same tumour in previous picture showing vimentin positive cells (ABC, 200X).

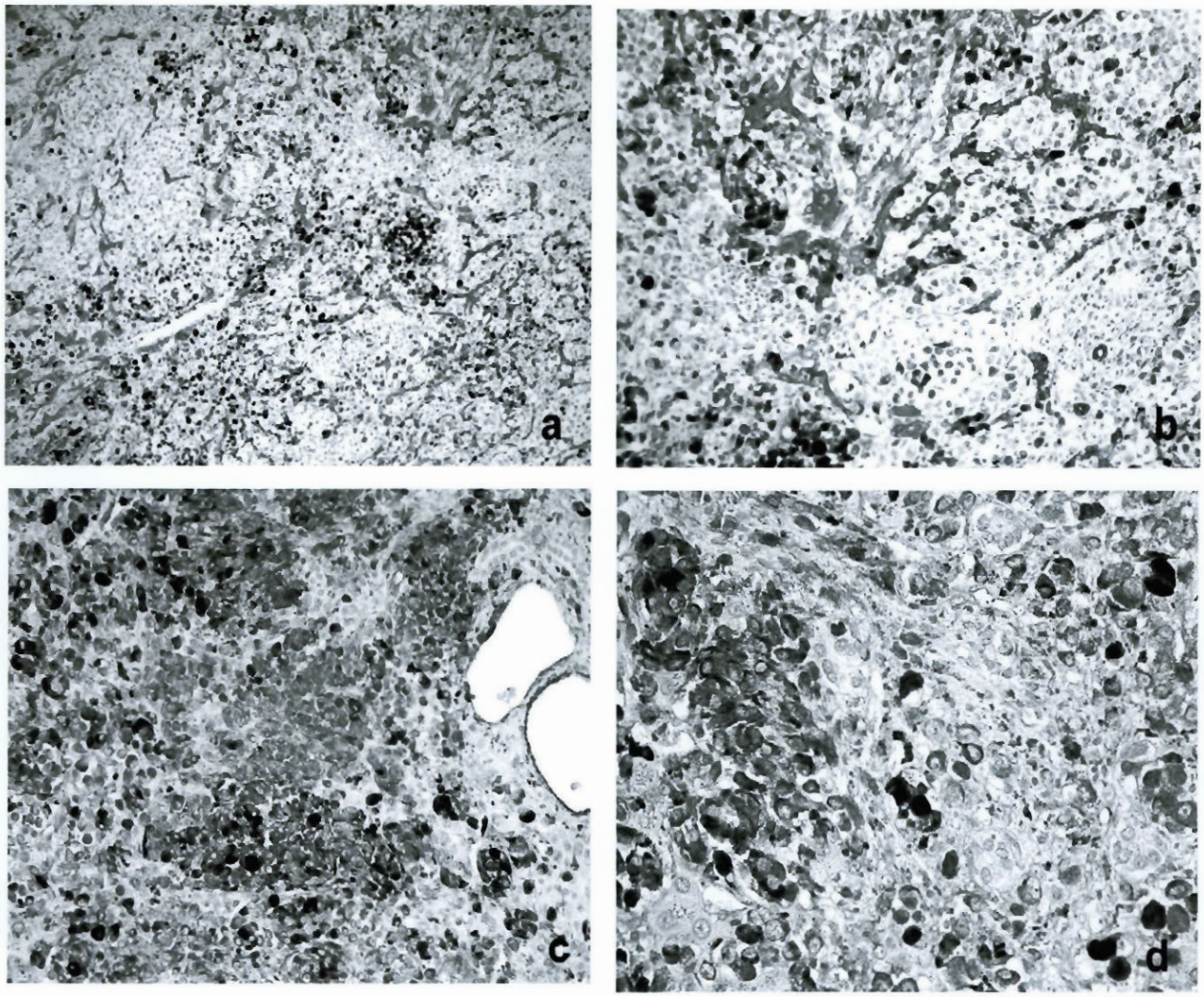


Plate 12.

Figure a) Histopathological section of liver metastasis of choroidal melanoma showing cytokeratin positive cells (ABC, 100X).Figure b) Histopathological section of liver metastasis of choroidal melanoma displaying cytokeratin positive cells (ABC, 200X). Figure c) Histopathological section of a liver metastasis of a choroidal melanoma displaying vimentin positive cells. Note the internal positive contol. (ABC, 100X). Figure d) Histopathological section of liver metastasis ofa choroidal melanoma showing vimentin positive cells (ABC, 100X) Figure d) Histopathological section of liver metastasis of choroidal melanoma displaying vimentin positive cells (ABC,200X).

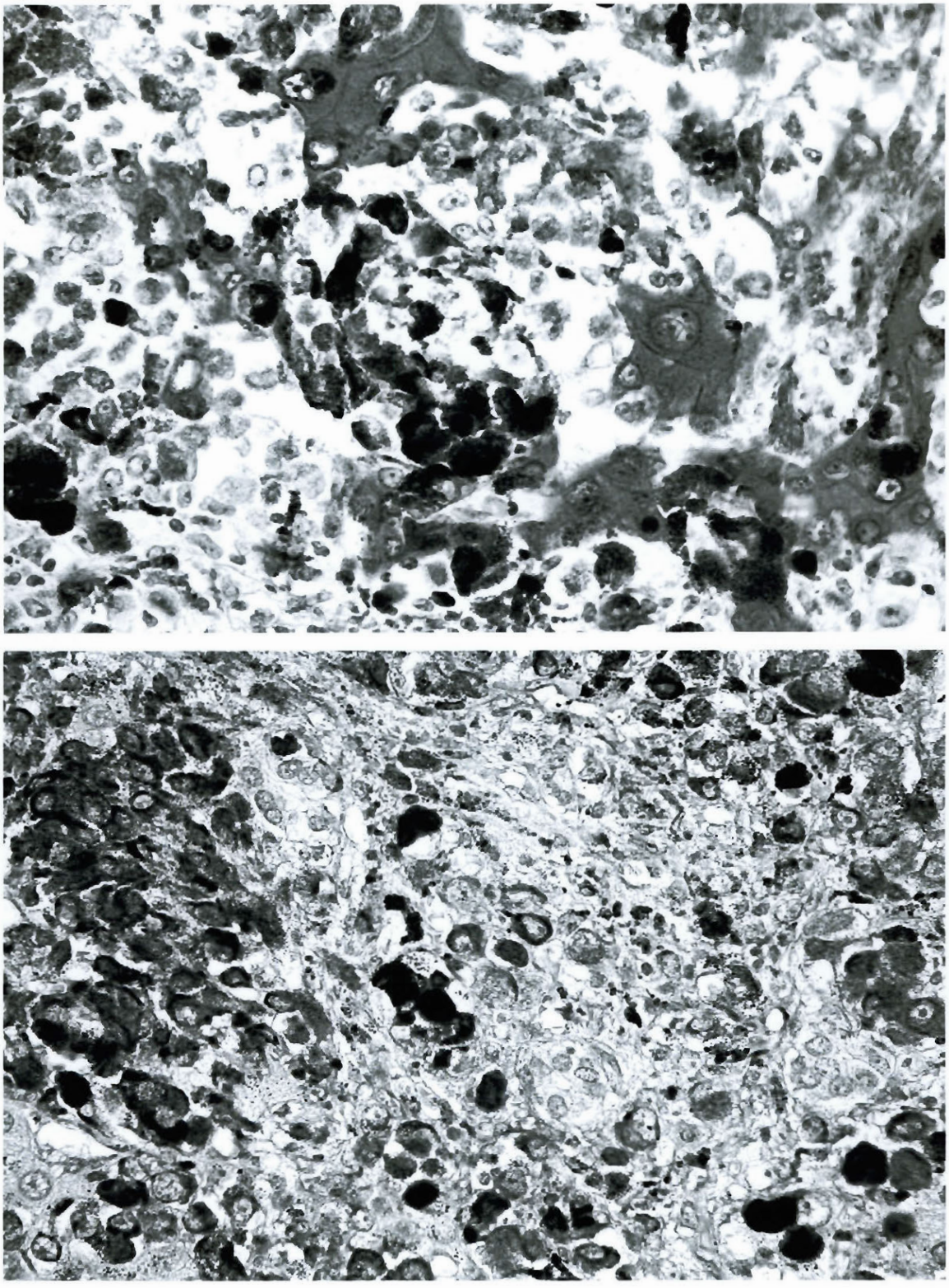


Plate 13.

Figure a) Histopathological section of liver metastasis of choroidal melanoma displaying cytokeratin positive cells (ABC, 400X). Figure b) Histopathological section of liver metastasis of choroidal melanoma showing vimentin positive cells (ABC,200X).

Discussion

Introductory remarks

In trying to postulate the potential role(s) for the expression of keratins in melanomas, it is tempting to correlate the following facts: (a) keratins are the first intermediate filaments to be expressed during embryogenesis. Therefore, epithelial as well as nonepithelial cellular derivatives originate from these precursors [164]. (b) K8 and K18 are complementary and are observed in "simple epithelium" of many embryonic and certain adult tissues. (c) Keratins have been detected in certain sarcomas, astrocytomas, smooth muscle tumours and various other nonepithelial tumours [165]. Collectively, this data reinforces the fact that the expression of keratins by neoplastic cells of neuroectodermal origin represents a reversion to a more primitive embryonic phenotype. This "differentiated" state is compatible with a more uninhibited phenotype capable of adapting to mesenchymal as well as epithelial matrices and thus is able to interpret additional positional cues, which may contribute to a more aggressive behaviour.

The constant expression of keratin 8 and 18 in melanoma cells may reflect the activation of intracellular signalling pathways[107, 136,137,138,139,140]. Genomic changes responsible for cancer progression towards an invasive and metastatic disease take place in the cancer cell itself. Such genomic changes may bring about an immediate phenotypic shift through signalling to the host cells and receiving invasion-promoter responses. The five prominent pathways of activation of latent oncogenes which all lead to uncontrollable

cell division an later to progressive states of malignancy are: 1) point mutations of DNA; 2) large segment rearrangements of DNA and chromosomal translocation that bring latent oncogene under the control of a heterologus enhancer or promoter; 3) translocation of DNA; 4) gene amplifications; 5) inactivation of a constitute suppressor [95]. The momentum for phenotypic changes, given the appropriate genomic status of the cancer cell, may also depend upon undercurrent phenomena, e.g. inflammation, bringing the necessary helper cells into the micro ecosystem of invasion [196]. This cellular transformation results in the altered transcription of a number of cellular genes, some of which are likely to be of importance for tumour growth, progression, invasion and metastasis. Tumourigenic signals, which could come from neighbouring cells or the ECM may stimulate multiple cell surface receptors that include epithelial growth factor receptor (EGFR), neu-oncogene and integrins[107]. These receptors further activate the Ras signalling pathway, which acts to further stimulate the E26 transformation-specific oncogene (Ets) family of transcription factors through phosphorylation. Ets transcription factors influence the production of keratins in two ways, one of which that acts through Jun and Fos. These transcription factors act like a cascade to promote the expression of keratin 8 and 18 [107]. More specifically, it has been learned [9,10,11,12] that tumour cells coexpressing vimentin/cytokeratin Ifs demonstrate: a) an increased ability to migrate and invade extracellular matrices in vitro; b) a change in cell shape, cytoskeletal architecture, and spreading ability; c) a switch in certain integrins; and d) an

increase in tyrosinase phosphorylated proteins colocalized with β_1 integrins on the leading invasive edge.

The co-expression of keratins and vimentin by carcinoma or melanoma cells is advantageous for migratory and invasive functions, due to unique interactions between cell surface receptors, cytoskeletal proteins, and the ECM. Recent studies suggest that IFs influence the invasion and metastasis of tumour cells through the family of integrins [104,105,140,141,142,143,144]. On the other hand, when intermediate filaments are disrupted in the tumour cells they loose both the capability to form colonies, and to induce aggregation of homologous platelets *in vitro* [82].

The integrin-mediated signalling may have important effects on cell adhesion and migration. As previously mentioned, integrins are transmembrane glycoproteins comprised of heterodimeric α and β subunbits which are widely distributed on many cell types, linking the internal cytoskeletal network of cells with the extracellular environment. As with all receptors, ligand occupancy of the active form of an integrin is hypothesized to transduce a signal or signals that results in alterations in second messenger pathways or in cytoskeletal architecture. In turn, these changes regulate downstream targets of adhesion, including cell migration and gene expression [196,217]. Because of the complexity of the integrin family, it has been useful to group integrins according to their cell-binding activity. They can be segregated into one of three groups: those that function as cell-cell adhesion molecules, those that bind primarily to major constituents of the

basement membrane, and those that bind primarily to ECM proteins. They play a critical role in the key step of metastasis: adhesion, migration and invasion[145,146,147].

Integrins have two key functions: 1) the internal regulation of cellular affinity (inside to out); 2) the external modulation of cellular behaviour by the ECM (outside to in). Tail parts of integrins are located in the cytoplasm, connecting with cytoskeletal proteins. In this case, IF proteins not only mediate the cell shape and spreading, but also act as signal transducers that transmit signals from ECM to the cell nucleus through the integrins and further regulate gene expression. IFs also modulate several cellular functions. It has therefore become clear that there is a versatile and complex array of interactions, modulations and signalling events in which integrins play a central role during tumour cell differentiation, invasion and metastasis.

Table 12 . Presence of intermediate filaments, extracellular matrix proteins and integrin profile in benign melanocytes and uveal melanoma in a theoretical scheme about the steps in the malignant transformation of melanocytes.

MELANOCYTES	***EARLY PRIMARY SITE***	***TRANSITION***	***ADVANCED PRIMARY TUMOUR***
Fibronectin $\alpha_5\beta_1$	Fibronectin $\alpha_V\beta_5$ $\alpha_5\beta_1$	Vitronectin Fibrinogen substrates $\alpha_V\beta_3$	Laminin Collagen $\alpha_5\beta_1$ $\alpha_3\beta_1$
Vimentin + Cytokeratin -	Vimentin + Cytokeratin -	**Vimentin +** **Cytokeratin +**	Vimentin ± Cytokeratin ±

Keratins and vimentin are both IFs that are considered principal components of the cytoskeleton of mammalian cells. Although earlier studies have emphasized the use of these cell type-specific markers in tumour differentiation and pathology [75,79], recent studies have confirmed that the co-expression of keratin 8 and 18 and vimentin is associated with recurrence and metastasis in cutaneous and uveal melanomas[9,10,103]. The co-transformation of a poorly invasive melanoma cell line with cDNA's for keratin 8 and 18 resulted in increased cytoskeletal interactions at focal contacts with the ECM. These interactions involved integrin cell signalling events, thus contributing to a more active migratory or invasive behavior[10,190]. The

transient knockout of keratin 8 and 18 in melanoma cells that co-express Ifs, results in a significant decrease in the migratory ability – similar to levels achieved by cells only positive for vimentin[9]. At the same time, Northern blot analysis in cutaneous melanoma shows that lineages of more malignant (more invasive) cells have a 10-fold increase in K8 and K18 mRNA [163]. The expression of keratin 8 and 18 by melanoma cells therefore correlates with an invasive phenotype. In studies with mouse L cells, it was also proven that the co expression of IFs augments the migration of those cells in matrix gel through a special interaction influencing the cell shape [175]. Also it has been proven that actin, although principally found in muscle fibres, is present in uveal melanoma ones. Apart from providing structural support, the contractile protein take part in other mechanicochemical activities of the cell such as cell movement and mitotic division, and also implying that this expression play a possible role in the invasive properties of malignant tumours [90]

The exact function of IFs, especially the significance of the interconverted phenotype observed melanomas, remains enigmatic. IFs appear to connect to the nuclear surface and extend throughout the cytoplasm terminating at the plasma membrane. Such associations provide a continuous link that may have important implications in the organization of the cytoplasm, intercellular or intracellular communication, and perhaps in the transport of information into and out of the nucleus. Many studies have suggested that signals from the ECM are transferred via integrins to the cytoskeletal proteins, which further act as transducers to the cell nucleus.

This mechanism may control cell growth, behaviour and differentiation[184, 190]. This thesis is the first to explore the presence of the interconverted phenotype in uveal melanomas and is also the first to correlate it with prognosis.

Interconverted phenotype in primary uveal melanoma

Of the 100 ocular melanomas stained for vimentin and cytokeratin, 43% of the cases co expressed at the same time vimentin and cytokeratin therefore displaying the interconverted phenotype. Those 100 cases were used solely to prove the expression of the interconverted phenotype in uveal melanoma; they could not be used for correlation with prognosis and metastatic disease because reliable follow up was not available. Fourteen of those 100 cases, even with heat and enzymatic epitope retrieval did not stain for either vimentin nor cytokeratin, demonstrating the loss of antigenicity that can be caused by either age of the specimen itself or the technique used to fix the tissue itself.

Those sections were all formalin-fixed; paraffin embedded which may explain part of those results. Formalin is by far the most commonly used cross-link fixative for immunohistochemistry in ocular pathology [213], however it has a profound damaging effect on primary, secondary and tertiary protein structure. The presence of these protein structures is necessary for

preserving the morphology and for the site-specific immobilization of soluble antigens[156]. Epitope retrieval methods have revolutionized the field of immunohistochemistry. With epitope retrieval, many monoclonal antibodies that were once restricted in their use to fresh-frozen material can now be used on archival paraffin-embedded material. The mechanism of epitope retrieval by either enzymatic digestion or heating is not clearly understood. Proposed mechanisms of heat epitope retrieval include denaturing of binding proteins and breaking of formaldehyde-induced covalent bonds[157]. Enzymatic epitope retrieval is thought to act via the enzymatic digestion of surface binding proteins, thereby exposing the masked antigenic site[123]. The results of immunohistochemistry staining in this study demonstrate the benefits of epitope retrieval using the common anti-IF antibodies. Enzymatic epitope retrieval was only used in anti-keratin antibody, since enzyme treatment can destroy vimentin reactivity Heating retrieval however, was used in both anti-keratin and anti -vimentin antibodies. When compared with ethanol fixed samples, previous studies show that 100% of ethanol fixed tissues maintains their epitopes when even with epitope retrieval, 27% of the formalin fixed samples lose their immunoreactivity [189].

Even though, 43 cases (43%) presented with the IP, co expressing vimentin and cytokeratin which is a result that agrees with previous data in the literature.[12] Other studies in our lab proved similar results[224, 225, 226, 227]. In comparison with conjunctival melanoma, uveal melanomas co express IF in the same proportion of cases[228].

Interconverted phenotype in metastatic uveal melanoma to the liver

As mentioned before, metastasis is a complex, yet well co-ordinated process that can be defined by a series of integrated events: cell attachment to ECM, proteolytic dissolution of the matrix, and movement of cells through the digested barrier[148]. The transformation of melanocytes into malignant melanoma cells result in changes to their repertoire of cell surface markers, most importantly being the increased expression of some integrin subunits [144,146,149], as well as changes in the IF profile of the cell.

Although it have been demonstrated that most of the cells in the metastasis of uveal melanomas are epithelioid, despite the cell type in the primary tumour[71], there exist very few studies in metastasis from patients with uveal melanoma. The lack of studies probably derives from the fact that a liver biopsy is usually unnecessary in diagnosing a patient with metastatic uveal melanoma. One study [12] noted that not all metastases were uniformly positive for vimentin. It was also observed that 7 out of 31 (22,6%) of the metastatic specimens were positive for cytokeratin, which is considerably different from the present finding of 66,7% positivity for cytokeratin. Explanation for this findings may reside in the fact that in the paper by Fuch's et al. epitope retrieval may not been done properly or the fixation was so that epitope retrieval was impossible due to its destruction by the fixating agent. Therefore it is possible to conclude that more than two thirds of the metastatic tumours express the interconverted phenotype when lodged in the

liver. This may prove that, to be capable of extravasion, circulation throughout the body, intravasion and lodgement these clones of cells express, in the majority of the cases, the interconverted phenotype.

Interconverted phenotype in Primary Uveal Melanoma and its correlation with other prognostic factors and metastatic disease

From the 28 cases, which the follow up was available, it has been learned that three fourths of the patients that have the interconverted phenotype had metastatic disease, and died from it. It is a proportion significantly higher than the group that did not express the interconverted phenotype. Whether the interconverted phenotype could be a prognostic marker for malignant for malignant tumour cells still remains in question. Although one group published two studies [104,9] that conclude that the co-expression of IFs in cutaneous and uveal melanomas acted as a predictive marker, other studies[12, 227] report that the interconverted phenotype was useful for determining the prognosis in malignant uveal melanomas, but it did not predict whether or not the tumour would metastasize. The former study also describe that larger tumours had a tendency to contain fewer cells immunopositive for vimentin. It is possible that malignant cells, in an advanced stage of disease, loose all their "regular" intermediate filaments. These cells may keep producing only those IFs transcribed by the oncogenic gene that immunohistochemical techniques are still incapable of detecting.

Another possibility is that even with epitope retrieval, those cases may not be immunogenic enough to permit staining by immunohistochemistry.

In the series of 28 patients, the mean age for patients enucleated from uveal melanoma was 59 years of age what is in agreement with the literature [1,4,13,223]. Gender distribution was 14 males to 14 females, what is also in agreement with the literature [3,4,15,194,223]. From the 28 cases with uveal melanoma 32,2% developed metastasis from the intraocular tumour, in comparison with 72% in the patients which the tumour expressed the interconverted phenotype. The other prognostic factors such as vascular configuration, our series demonstrate that tumours with the interconverted phenotype presented in 57% pattern of vascular closed loops, which is similar to the tumours without the interconverted phenotype, 52% but higher than reported in the literature [55,62]. It was also impossible to verify any difference between the pattern of lymphocytic infiltration between cases that expressed the interconverted phenotype (29%) and cases without the interconverted phenotype (30%). The only prognostic factor that was considerably different between the group with the interconverted phenotype and the group without it was that from all tumours, 71% of them were mixed cell type, 11% were pure epithelioid and 14% were pure spindle, which is consistent with the literature. When analysing the tumours expressing the interconverted phenotype, it was found that 71% were mixed, and 29% were spindle, and there was no single tumour that was purely epithelioid. The finding that 72% of cases with metastatic disease to the liver showed

interconverted phenotype is highly significant. However , statistical analysis was not possible in view of the small number of cases. The reasons for this include the fact that those are 28 patients with a long period follow up information. There is also the question of the timeframe between the diagnosis of the primary uveal melanoma and the occurrence of metastatic disease. Therefore, because the series has only 28 patients, statistical methods either parametreic or non-parametric are not of significance. For instance, to use a T-student test or a QrusQall-Wallis that will indicate a p value is not possible in this particular sample of patients. As previously mentioned cell type is the single most important prognostic factor in uveal melanoma, and epithelioid cells are more aggressive than spindle ones. The fact that 29% of the cases expressing the interconverted phenotype were spindle cell type, and that 100% of those patients died of metastatic disease allows the following conclusion: cells must dedifferentiate from their more benign state, as spindle cells to a intermediate state, where they are more aggressive, and escape from the eyes without even having the time to change their shape inside the eye. It is possible that in the future, the interconverted phenotype, mainly in spindle cells, will predict with more accuracy the patients with most chance of metastatic disease. In our lab it has been proven that uveal melanoma cells as well as skin melanoma cells express the interconverted phenotype, but working with small series was not possible to estimate the precise percentage of tumours that show this phenotype[224, 225, 226, 227].

Location of the positive cells may be suggestive of the fact that melanoma cells with the interconverted phenotype have a more aggressive behaviour, lack cohesiveness with adjacent cells and have less difficulty invading the surrounding normal tissues. Studding the lymph node metastasis of skin melanomas[122] it has been found that they showed a population of keratin-positive-highly-elongated cells with dendrite-like processes. These cells were often quite numerous at the marginal area of metastatic infiltrates. Another study with uveal melanoma cells [9] revealed that the cells that predominantly co-expressed keratin and vimentin were 6-fold more invasive through the ECM as compared with cells expressing vimentin only. These studies have proven that the co-expression of IFs in tumours may influence the motility or invasiveness of tumour cells and may in turn have an effect on the growth rate and aggressiveness of metastatic tumour cells.

Summary of conclusions

Uveal melanoma cells do express the interconverted phenotype in almost half of the cases. Liver metastases of primary uveal melanomas also express the interconverted phenotype in almost three fourths of the cases. The interconverted phenotype proved to be a reliable prognostic factor concerning metastatic disease in uveal melanoma.

Future Implications on Clinical Management of Malignant Melanoma

The fact that 43 % formalin-fixed, paraffin-embedded primary uveal melanomas contained cells positive for simple epithelial-type cytokeratins in the present series may indicate that uveal melanomas express cytokeratins more often that previously thought. For several reasons we may have underestimated the number of positive tumours. Firstly, the loss of antigenicity may have occurred during formalin fixation in spite of the use of heat retrieval and predigestion. Although the antibodies were selected to react well with formalin-fixed, paraffin-embedded material the best way to revert this situation would be to use of frozen sections, where tumour antigens are not destroyed. Secondly, since the immunopositive cells were unevenly distributed, more tumours may have been positive if the specimen had been sectioned through.

It has been shown that the interconverted phenotype presented in the malignant melanoma cells is mainly located in the apical part of the tumour, in marginal areas. Future studies should use immunohistochemical double staining or immuneletrofluorescence to detect whether the vimentin and cytokeratin Ifs really present in the same melanoma cells or not. If is possible to prove this characteristic, it could further provide a target for immunotherapy or genetherapy for uveal melanomas. Techniques to detect

mRNA of the cytokeratin in those malignant cells could also improve the sensitivity of future studies.

Tumour cell phenotype is not autonomously determined by the tumour, but rather reflects the influences of the host's environment [170]. The morphological and biological changes associated with the interconverted phenotype, characteristic of uveal melanoma, suggest a direct link between keratin and vimentin co-expression, changes in integrin profile, and increased invasive, clonogenic and tumorigenic activity. However, the expression of vimentin or keratins alone is not sufficient to confer the complete metastatic phenotype. With this in mind, associated pathways need to be investigated with respect to the underlying molecular pathology of primary and metastatic uveal melanoma to better understand the significance of the interconverted phenotype. These pathways include those related to the formation of these filaments and to the stimuli required to produce more integrins. Following this investigation could be the potential development of a method, which would prevent the occurrence of such cellular transformations. This would enable physicians to restrict the tumours in their primary site, and by doing so, would allow patients to be cured by tumour excision or chemotherapy [158,219].

Interconverted phenotype serves as a prognostic indicator for metastatic disease, and may also represent a useful biological target for therapeutic intervention.

References

1. Egan KM, Seddon JM, Glynn RJ, Gragoudas ES, and Albert DM: Epidemiologic aspects of uveal melanoma. *Surv. Ophthalmol.* 32:239-51; 1988
2. Culter SJ, Young JL. Third National Cancer Survey. Incidence data. NCI Monograph. Vol 41 Bethesda: NHI:1-9; 1975
3. Tumors of the retina. McLean IW, Burnier Jr. MN, Zimmerman LE, Jakobiec FA. In: J. Rosai and L.H.Sobin (eds.), Tumors of the Eye and Ocular Adnexa. Atlas of Tumor Pathology. Washington: Armed Forces Institute of Pathology:101; 1994
4. Malignant Melanoma of the Uveal Tract. McLean IW, Burnier Jr. MN, Zimmerman LE, Jakobiec FA. In: J. Rosai and L.H.Sobin (eds.), Tumors of the Eye and Ocular Adnexa. Atlas of Tumor Pathology. Washington : Armed Forces Institute of Pathology:161-194; 1994
5. Diener-West M, Hawkins B, Markowitz JA, Schachat AP : A review of mortality from choroidal melanoma. A meta-analysis of 5-year mortality rates following enucleation, 1966 through 1988. *Arch Ophthalmol.* 110:245-50; 1992
6. Gamel JW, McLean IW, Rosemberg SH : Proportion cured and mean log survival time as functions of tumour size. *Stat Med.* 9:999-1006; 1990
7. Zimmerman LE, McLean IW : Does enucleation of the eye containing a malignant melanoma prevent or accelerate dissemination of tumor cells? *Br J ophthalmol.* 62:420-5; 1978
8. Shields JA, Ausburger JJ, Donoso LA, Bernardino VB, Portenar M: Hepatic metastasis and orbital recurrence of uveal melanoma after 42 years. *Am J Ophthalmol.* 100:666-8;1985
9. Hendrix JMC , Seftor REB, Gardner LM, Boldt HC, Meyer M, Pe'er J, Folberg R.: Biologic determinants of uveal melanoma metastatic

phenotype: role of intermediate filaments as predictive markers. *Lab Invest.* 78:153-63; 1998

10. Chu YW, Seaftor EA, Romer LH, Hendrix MJC: Experimental coexpression of vimentin and keratin intermediate filaments in uveal melanoma cells augments motility . *Am J Pathol.* 148:63-9; 1996
11. Zarbo RJ, Gown AM, Nagle RB, Vissher DW, Crissman JD. : Anomalous Cytokeratin Expression In Malignant Melanoma: One- and two-dimensional western blot analysis and immunohistochemical survey of 100 melanomas. *Modern Pathology.* 3:494-501; 1990.
12. Fuchs U, Kivela T, Summanen P, Immonen I, Tarkkanen A: An immunohistochemical and prognostic analysis of cytokeratin expression in malignant uveal melanoma. *Am J Pathol.* 141:169-81; 1992
13. Singh AD, Shields CL, Shields JA, Sato T: Uveal melanoma in young patients. *Arch Ophthalmol.* 118:918-23; 2000
14. Scotto J, Fraumeni JF Jr., Lee J A: Melanomas of the eye and other noncutaneous sites: Epidemiologic Aspects. *JNCI.* 56:489-91; 1976
15. Gallagher RP, Elwood JM, Rootman J: Epidemiologic aspects of intraocular malignant melanoma . *Cancer Treat Res.* 43:73-84; 1998
16. Sigh AD, Wang MX, Donoso LA, Shields CL, De Porter P, Shields JA: Genetic aspects of uveal melanoma: a brief review . *Sem in Oncol.* 23:768-72; 1996
17. Sigh AD, Shields CL, De Porter P, Shields JA , Trock B, Carter J, Pastore D: Familial uveal melanoma : clinical observations on 56 patients . *Arch Ophthalmol.* 114:392-99; 1996
18. Sisley K, Cottam DW, Rennie IG, Parsons MA, Potter AM, Potter CW, Rees RC : Non-random abnormalities of chromosomes 3, 6 and 8 associated with posterior uveal melanoma. *Genes Chrom. Cancer.* 5:197-200; 1992

19. Soparker CN, O'Brien JM, Albert DM : Investigation of the role of the Ras-protoncogene point mutation in human uveal melanomas. *Invest Ophthalmol Vis Sci.* 34:2203-9; 1993

20. Ziegler A, Lefell DJ, Kunala S : Mutation hotspots due to sunlight in the P53 gene of nonmelanoma skin cancers. *Proc Natl Acad Sci USA.* 90:4216-20; 1993

21. Yanoff M, Zimmerman LE : Histogenesis of malignant melanomas of the uvea II: relationship of uveal nevi to malignant melanomas. *Cancer.* 20:493-507; 1967

22. Uveal nevi malignant melanomas. Spencer WH, Zimmerman LE, McLean IW, In : Spencer WH. Ed. Ophthalmic Pathology vol III.Philadelphia : WB Saunders. 2121-2168; 1994

23. Singh AD De Potter P, Fijal BA, Shields CL, Shields JA, Elston RC: Lifetime prevalence of uveal melanoma in white patients with oculodermal melanocytosis. *Ophthalmology.* 105: 195-198; 1998

24. Callender GR : Malignant melanotic tumours of the eye : a study of histologic types in 111 cases. *Trans Am Acad. Ophthalmol Otorynolaryngol.* 36:131-142; 1931

25. McLean IW, Zimmerman LE, Evans RM : Reappraisal of Callender's spindle A type of malignant melanoma of the choroid and ciliary body. *Am J Ophthalmol.* 86:557-64; 1978

26. McLean IW, Foster WD, Zimmerman LE, Gamel JW : Modifications of Callender's classification of uveal melanoma at The Armed Forces Institute of pathology. *Am J Ophthalmol.* 96:502-9; 1983

27. Wilder HC, Paul EV : Malignant melanoma of the choroid and ciliary body: A Study Of 2,535 Cases. *Mil Surg* . 109:370-8; 1951

28. Shields JA , Shields CL : Massive orbital extension of posterior uveal melanomas. *Ophthal Plas Reconstr Surg.* 7:238-51; 1991

29. Burnier MN Jr, McLean IW, Gamel JW: Immunohistochemical evaluation of uveal melanocytic tumors. *Cancer.* 68:809-14; 1991

30. Adema GJ, de Boer AJ, van't Hullenaar R, Denijin M, Ruiter DJ, Vogel AM, Figdor CG : Melanocyte lineage-specific antigens recognized by monoclonal antibodies. NKI-beteb, HMB-50, and HMB-45 are encoded by a single cDNA. *Am J Pathol.* 143:1579-1585; 1993
31. Adema GJ , de Boer Aj, Vogel AM, Loenen WA, Figdor CG : Molecular characterization of the melanocyte lineage-specific antigen Gp 100. *J Biol Chem.* 269:10126-20133; 1994
32. Chiamenti AM, Vella F, Bonetti F, Pea M, Ferrari S, Martignoni G, Benedetti A, Suzuki H: Anti-melanoma monoclonal antibody HMB-45 on enhanced chemiluminescence-western blotting recognizes a 30-35 kDa melanosome-associated sialated glycoprotein. *Melanoma Res.* 6:291-98; 1996
33. Taatjes DJ, Arendash-Durand B, von Turkovich M, Trainer TD: HMB-45 antibody demonstrates melanosome specificity by immunoelectron microscopy. *Arch Pathol Lab Med.* 117:264-268; 1993
34. Steuhl KP, Rohrbach JM, Knorr M, Thiel HJ : Significance , specificity, and ultrastructural localization of HMB-45 antigen in pigmented ocular tumors. *Ophthalmol.* 100:208-215; 1993
35. Schaumberg-Lever G, Metzler G, Kaiserling E: Ultrastructural localization of HMB-45 binding sites. *J Cut Pathol.* 18: 432-435; 1991
36. Kikuchi A, Shimizu H, Nishikawa T: Expression and ultrastructural localization of HMB-45. *Br J Dermatol.* 135:400-405; 1996
37. Cheng H, Sun Gj, Thiele B, Wolff HH : The recognition of UV-irradiation melanocyte with HMB-45 monoclonal antibody. *Chinese Med J.* 107:225-229; 1994
38. Skelton HG 3d, Smith KJ, Barret TL, Lupton GP, Graham JH : HMB-45 staining in benign and malignant melanocytic lesions. A reflection of cellular activation. *Am J Dermatopathol.* 13:543-550; 1991
39. Margo CE, McLean IW: Malignant melanoma f the choroid and ciliary body in black patients. *Arch ophthal* 102:77-9;1984

40. Holly EA, Aston DA, Char DH, Kristiansen JJ, Ahn DK: Uveal melanoma in relation to ultraviolet light exposure and host factors. Cancer res. 50:5773-7;1990

41. Pea M, Bonetti F , Zamboni G, Martignoni G, Riva M, Colombari R, Mombelo A, Bonzanini M, scarpa A, ghimenton C, Donati LF: Melanocyte marker HMB-45 is regularly expressed in angiomyilipoma of the kidney. *Pathology* 23:185-88;1991

42. Zimmer C, Gottschalk J, Goebel S, Cervos-Navarro J: Melanoma-associated antigens in tumours of the nervous system: an immunohistochemical study with the monoclonal antibody HMB-45. *Virch Arch.* 420:121-126; 1992

43. Hancock C, Allen BC, Herrera GA: HMB-45 detection in adenocarcinomas. *Arch Pathol Lab Med.* 115:886-890; 1991

44. Hoperskaya OA: Introduction –the main principle of melanocytes in early development. *Differentiation* 20:104-116;1981

45. Lerner AB: Behaviour of pigment cells. *J Invest Dermatol* 75:121;1980

46. Quevedo WC, Fleishamann RD: Developmental biology of mammalian melanocytes. J Invest Dermatol 75:116-120;1980

47. Holme TC: Cancer cell structure: actin changes in tumour cells – possible mechanisms for malignant tumour formation. *Eur J Surg Oncol* 16:161-169;1990

48. Mooy CM, De Jong PTVM : Prognostic parameters in uveal melanoma: a review. *Surv Ophthalmol.* 41:215-228; 1996

49. McLean IW, Foster WD, Zimmermman LE: Uveal melanoma: location, size, cell type, and enucleation as risk factors in metastasis. *Hum Pathol.* 13:123-132; 1982

50. McLean IW: Prognostic features of uveal malignant melanoma . *Ophthalmol Clin N Am.* 8:143-153; 1995

51. Shields CL, Shields JA, Kiratli H: Risk factors for metastasis of small choroidal melanocytic lesions. *Ophthalmol.* 102:1351-1361; 1995

52. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE: Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer*. 83:354-359; 1998

53. Sisley K, Rennie IG, Parsons MA, Jacques R, Hammond DW, Bell SM et al: Abnormalities of chromosome 6 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chrom Cancer*. 19:22-28; 1997

54. Prescher G, Bornfeld N, Hirche H, Horsthemke B, Jockel K-H, Becher R: Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 347:1222-1225; 1996

55. McLean IW, Keefe SK, Burnier Jr. MN: Uveal melanoma: comparsion of the prognostic value of fibrovascular loops, mean of the largest nucleoli, cell type and tumor size. *Ophthalmol*. 104:777-780; 1997

56. McLean IW, Siburg ME, Becker RL, McCurdy JB: The importance of the largest nucleoli in predicting patient outcome- an automated image analysis study. *Cancer*. 79982-988; 1997

57. Pe'er J, Rummelt V, Mawn L, Hwang T, Woolson RF, Folberg R: Mean of the 10 largest nucleoli, microcirculation architecture, and prognosis of cilliochoroidal melanomas. *Ophthalmol*. 101:1227-1235; 1994

58. Coleman K, Baak JPA, Van Diest PJ, Mullaney J: Prognostic value of morphometric features and the Callender classification in uveal melanomas. *Ophthalmol*. 103:1634-1641; 1996

59. McLean IW, Foster WD, Zimmerman LE: Prognostic factors in small malignant melanomas of the choroid and ciliary body. *Arch Ophthalmol*. 95:48-58; 1997

60. De la Cruz PO, Specht CS, McLean IW: Lymphocytic infiltration in uveal malignant melanoma. *Cancer*. 65:112-115; 1990

61. Folberg R, Pe'er J, Gruman LM, Woolson RF, Jeng G, Montague PR, Moninger TO, Yi H, Moore KC: The morphologic characteristics of tumor blood vessels as a marker of tumor progression in primary uveal melanoma : a matched case-control study. *Hum Pathol*. 23:1298-1305; 1992

62. Folberg R, Rummelt V, Parys-Van Ginderdeuren R, Hwang T, Woolson RF, Pe'er J, Gruman LM: The prognostic value of tumor blood vessel morphology in primary uveal melanoma. *Ophthalmol.* 100:1389-1398; 1993

63. Folberg R, Mehaffey M, Gardner LM, Meyer M, Rummelt V, Pe'er J: The microcirculation of choroidal and ciliary body melanomas. *Eye.* 11:227-238; 1997

64. Durie FH, Campbell AM, Lee WR, Damato BE: Analysis of lymphocytic infiltration in uveal melanoma. *Invest Ophthalmol Vis Sci.* 31:2106-2110; 1990

65. Whelchel JC, Farah SE, McLean IW, Burnier Jr MN: Immunohistochemistry of infiltrating lymphocytes in uveal malignant melanoma. *Invest Ophthalmol Vis Sci* . 34:2603-2606; 1993

66. Ksander BR, Rubsamen PE, Olsen KR, Cousins SW, Streilein JW: Studies of tumor-infiltrating lymphocytes in uveal malignant melanoma. *Invest Ophthalmol Vis Sci.* 32:3198-3208; 1991

67. Zimmerman LE, McLean IW: Do growth and onset of symptoms of uveal melanomas indicate subclinical metastasis? *Ophthalmol.* 91:685-691; 1984

68. Shields JA, Shields CL, De Porter P, Singh AD: Diagnosis and treatment of uveal melanoma . *Sem in Oncol.* 23:763:767; 1996

69. Sato T, Babazono A, Shields JA, Shields CL , De Porter P, Mastrangelo MJ: Time to systemic metastasis in patients with posterior uveal melanoma. *Cancer Invest.* 15:98-105; 1997

70. McLean IW: The biology of hematogenous metastasis in human uveal melanoma. *Virchows Arch A Pathol Anat.* 422:433-437; 1993

71. Luyten GP, Mooy CM, Post J, Jensen AO, Luider TM, de Jong PT: Metastatic uveal melanoma. A morphological and immunohistochemical analysis. *Cancer.* 78:1967-1971; 1996

72. Salmon RJ, Levy C, Plancher C, Dorval T, Desjardins L, Leyvraz S, Pouillart P, Schienger P Servois V, Asselain B: Treatment of liver

metastasis from uveal melanoma by combined surgery-chemotherapy. *Eur J Surg Oncol.* 24:127-130; 1998

73. Collaborative Ocular Melanoma Study Group (COMS): The COMS randomized trial of pre-enucleation radiation of large choroidal melanoma II: Initial mortality findings. COMS report N- 10. *Am J Ophthalmol.* 125:779-796; 1988
74. Gunlap I, Batioglu F: Effect of pre enucleation irradiation on the survival of patients with uveal melanoma. *Ophthalmologica.* 212:231-235; 1998
75. Sun TT, Eichner R, Nelson WG, Tseng SCG, Weiss RA, Jarvinen M, Woodcok-Mitchell J: Keratin classes: molecular markers for different types of epithelial differentiation. *J Invest Dermathol.* 81(1):109s-115s;1983
76. Shields JA, Shields CI, Donoso LA: Management of posterior uveal melanoma. Major review. *Surv Ophthalmol.* 36:161-195; 1991
77. McLean IW, Ainbinder DJ, Gamel JW, McCurdy JB : Choroidal-ciliary body melanoma. A multivariative survival analysis of tumor location. *Ophthalmology.* 102:1060-1064; 1995
78. Seddon JM, Albert DM, Lavin PT, Robinson N: A prognostic factor study on disease-free interval and survival following enucleation for uveal melanoma. *Arch Ophthalmol* 101:1894-1899,1983
79. Osborn M: Intermediate filaments as histologic markers: an overview. *J Invest Dermatopathol.* 81(1) 104s-109s;1983
80. O'Rahilly R: The prenatal development of the human eye. Exp. Eye. Res. 21:93-112;1975
81. Ramaekers FCS, Puts JJG, Moesker O, kant A, Vooijs GP, Jap PHK: Intermediate filaments in malignant melanomas. Identification and use as marker in surgical pathology. Clin Invest. 71: 635-643;1983
82. Chopra H, Fligiel SEG, Hatfield JS, Nelson KK, Diglio CA, taylor JD, Honn KV: An in vivo study of the role of the tumour cell cytoskeleton in tumour cell-platlet-endothelial cell interactions. *Cancer research.* 50:7686-7696;1990

83. Foss AJE, Guille MJ, Occleston NL, Hykin PG, Hungerford JL, Lightman S: The detection of melanoma cells in peripheral blood by reverse transcription-polimerase chain reaction. *Br J Cancer* 72:155-159;1995

84. Timar J, Chen YQ, Liu B, Bazaz R, Taylor JD, Honn KV: The lipoxygenase metabolite 12(S) – hete promoters $\alpha_{11b}\beta_3$ integrin-mediated tumour-cell spreading on fibronectin. *Int J Cancer.* 52:594-603;1992

85. Lallier TE: Cell lineage and cell migration in the neural crest. Ann N Y Acad Sci 615:158-71;1991

86. Dogic D, Eckes B, Aumailey M: Extracellular matrix, integrins and focal adhesions. *Current Topics in Pathology* 93:75-85;1999

87. Hsu SM, Raine L, Fanger H: Use of Avidin-Biotin-Peroxidase Complex (ABC) in immunoperoxidase techniques. *J Histochem Cytochem* 29(4): 577-80;1981

88. Ausburger JJ, Gamel JW: Clinical prognostic factors in with posterior uveal malignant melanoma. *Cancer.* 66:1596-1600;1990

89. Gamel JW, McLean IW, McCurdy JB: Biologic distinctions between cure and time to death in 2,892 patients with intraocular melanoma. *Cancer.* 71:2299-305;1993

90. Malaty A, Rahi A, Garner A: Antibodies to skeletal muscle actin and their reactivity with malignant melanoma of the choroid. *Br J Ophthalmol* . 64:181-85;1980

91. Miettinen M, Leehto VP, Virtanen I: Antibodies to intermediate filament proteins in the diagnosis and classification of human tumours. *Ultrastr Pathol.* 7:83-107,1984

92. Cunningham JD, Cirincione E, Ryan A, Canin-Endres J, Brower S: Indications for surgical resection of metastatic ocular melanoma. *Int J Pancreatol.* 24(1) 49-53, 1998

93. Orchard G and Jones EW : Immunocytochemistry in the diagnosis of malignant melanoma. *Br J Biomed Sci.* 51:44-56;1994

94. Miettinen M, Leehto VP, Virtanen I: Presence of fibroblast-type intermediate filaments (vimentin) and absence of neurofilaments in pigmented nevi and malignant melanomas. *J Cut Pathol.* 10:188-192;1983

95. Boley B, Kaiser HE, Golfarb RH: Immunophenotipically varied cell subpopulations in primary and metastatic human melanomas. Monoclonal antibodies for diagnosis, detenction of neoplastic progression and receptor directed immunotherapy. *Anticancer Research.* 16:517-32;1996

96. Helige C, Zellnig, Hofmann-Wellenhof R, Fink-Puches R, Smolle J, Tritthart HA: Interrelation of motility, cytoskeletal organization, and gap junctional communication with invasiveness of melanocytic cells in vitro. *Invas Met* . 17:26-41, 1997

97. Ayala I, Babia T, Baldassarre M, Pompeo A, Fabra A, Kok JW, Luini A, Buccione R, Egea G: Morphological and biochemical analysis of the secretory pathway in melanoma cells with distinct metastatic potential. *FEBS letters* 451:315-320;1999

98. Evers EE, Zondag GCM, marilli A, Price LS, Klooster JP, Kammen RA, Collard JG: Rho family proteins in cell adhesion and cell migration. *Eur J Cancer* 36:1269-74; 2000

99. Boufard D, Duncan L, Howard CA, Mihm MC, Byers HR: Actin-binding protein expression in benign and malignant melanocytic proliferations. *Hum Pathol.* 25: 709-714;1994

100. Voura EB, Sanding M, Kalnins VI, Siu CH: Cell shape changes and cytoskeleton reorganization during transendothelial migration of human melanoma cells. *Cell Tissue Res.* 293:375-387;1998

101. Cunningham CC: actin structural proteins in cell motility. *Cancer and Met Rev.* 11:69-77;1992

102. Raymond WA and Leong AS-Y: Vimentin – a new prognostic parameter in breast carcinoma? *J Pathol.* 158:107-114; 1989

103. Seftor REB, Seftor EA, Gehlsen KR, et al: Role of the avb3 integrin in human melanoma cell invasion. *Proc. Natl. Acad. Sci. USA*. 89:1557-61; 1992

104. Hendrix MJC, Seftor EA, Chu YW, Seftor REB, Nagle RB, McDaniel KM, Leong SPL, Yohem KH, Leibovitz AM, Meyskens FL, Conaway DH, Whelch DR, Liotta LA, Stetler-Stevenson W: Coexpression of vimentin and keratins by human melanoma tumour cells : Correlation with invasive and metastatic potential. *J Natl Cancer Inst*. 84:165-174; 1992

105. Juliano RL and Haskill S: Signal transduction from the extracellular matrix. *J Cell Biol.* 120:577-85; 1993

106. Malik RK and Parsons JT: Integrin-mediated signaling in normal and malignant cells: a role of protein tyrosine kinases. *Biochem Biophys Acta*. 1287:73-76; 1996

107. Oshima RG, Baribault H, Caulin C: Oncogenic regulation and function of keratins 8 and 18. *Cancer and Metastasis Rev*. 15:445-71; 1996

108. Bacchi CE, Bonetti F, Pea M, et al: HMB-45: a review. *Appl Immunohistochem*. 4:73-85; 1996

109. Collaborative Ocular Melanoma Study Group (COMS): Histopathologic characteristics of uveal melanomas in eyes enucleated from the Collaborative Ocular Melanoma Study. COMS report number 6. Am J Ophthalmol. 125(6):745-56;1998

110. Gown AM and Vogel AM: Monoclonal antibodies specific for melanocytic tumours distinguish subpopulations of melanocytes. *Am J Pathol.* 123:195-203; 1986

111. Zimmer C, Gottschim J, Goebel S, Cervos-Navarro J: Melanoma-associated antigens in tumours of the nervous system: an immunohistochemical study withy the monoclonal antibody HMB-45. *Virchows Arch [Pathol Anat]*. 420:121-26; 1991

112. Lazarides E: Intermediate filaments as mechanical integrators of cellular space. *Nature*. 283:249-56; 1980

113. Geiger B: Intermediate filaments: looking for a function. *Nature*. 329:392-93; 1987
114. Steinert PM and Roop DR: Molecular and cellular biology of intermediate filaments. *Ann. Rev. Biochem*. 57:593-625; 1988
115. Osborn M and Weber K: Intermediate filaments: cell-type-specific markers in differentiation and pathology. *Cell*. 31:303-06; 1982
116. Skali O and Goldman RD: Recent insights into the assembly, dynamics, and function of intermediate filament networks. *Cell Motil Cytoskel*. 19:67-79; 1991
117. Moll R, Franke WW and Schiller D: The catalog of human cytokeratins: patterns of expression in normal epithelia, tumours, and cultured cells. *Cell*. 31:11-24; 1982
118. Steinart PM and Liem RKH: Intermediate filament dynamics. *Cell*. 60:521-23; 1990
119. Oshima RG: Intermediate filament molecular biology. *Cur Opin Cell Biol*. 4:110-16; 1992
120. McLean IW and Lane EB: Intermediate filaments in disease. *Cur Opin Cell Biol*. 7:118-25; 1995
121. Klymkowsky MW: Intermediate filaments: new proteins, some answers, more questions. *Cur Opin Cell Biol*. 7:46-54; 1995
122. Miettinen M and Franssila K: Immunohistochemical spectrum of malignant melanoma – the common presence of keratins. *Lab Inves* 61:623-28; 1989
123. Cattoretti G, Pileri S, Parravicini C, et al: Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J Pathol*. 171:83-98; 1993
124. Osborn M and Weber K: Biology of disease: tumour diagnosis by intermediate filament typing: a novel tool for surgical pathology. *Lab Invest*. 48:372-94; 1983

125. Huszar M, Halkin H, Herczeg E, et al: Use of antibodies to intermediate filaments in the diagnosis of metastatic amelanotic malignant melanoma. *Hum Pathol.* 14:1006-08

126. Caselitz J, Jänner M, Breitbart E, et al: Malignant melanomas contain only the vimentin type of intermediate filaments. *Virchows Arch [Pathol Anat].* 400:43-51; 1983

127. Gown AM, Vogel AM: Monoclonal antibodies to human intermediate filament proteins. *Am J Clin Pathol.* 84:413-24; 1985

128. Caselitz J, Osborn M, Seifert G, et al: Use of antibodies to different sized intermediate filament proteins to study the normal parotid gland and parotid gland tumours in humans. *Virchows Arch [Pathol Anat]* 393:273-86; 1981

129. Lane EB, Hogan BLM, Kurikinen M, et al: Co-expression of vimentin and cytokeratins in parietal endoderm cells of early mouse embryo. *Nature.* 303:701-04; 1983

130. Ramaekers FCS, Haag D, Kant A, et al: Coexpression keratin- and vimentin-type intermediate filaments in human metastatic carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 80:2618-22; 1983

131. Cheresh DA: Structural and biologic properties of integrin mediated cell adhesion. Lab Immunol 12(2):217-231;1992

132. Banerjee SS and Harris M: Morphological and immunophenotipic variations in malignant melanoma. Histopathology. 36:387-402;2000

133. Hendrix MJC: Intermediate filaments. *Cancer and Metastasis Ver.* 15:413-16; 1996

134. Bem-Izhak O, Stark P, Levy R, et al: Epithelial markers in malignant melanoma. *Am J Dermatopathol.* 16:241-46; 1994

135. Katagata Y and Kondo S: Keratin expression and its significance in five cultured melanoma cell lines derived from primary, recurrence and metastasized melanomas. *FEBS letters.* 407: 25-31; 1997

136. Thomas PA, Kirschmann DA, Cerhan JR, Folberg R, Seftor EA, Sellers TA, Hendrix MJC: Association between keratin and vimentin

expression, malignant phenotype, and survival in postmenopausal breast cancer patients. *Clin Cancer Res.* 5:2698-2703,1999

137. Wasylyk B, Hahn SL, Giovane A. The Ets family of transcription factors. *Eur J Biochem.* 211:7-18; 1993
138. Werner MH, Clore GM, Fisher CL, et al: The solution structure of the human ETS1-DNA complex reveals a novel mode of binding and true side chain intercalation. *Cell.* 83:761-71; 1995
139. Smith TW, Menter DG, Nicholson GI, McIntire LV: Regulation of melanoma cells adhesion stabilization to fibronectin. Melanoma res 6:351-362;1996
140. Pankov R, Umezawa A, Maki R, et al: Oncogene activation of human keratin 18 transcription via the Ras signal transduction pathway. *Proc. Natl. Acad. Sci. USA.* 91:873-77; 1994
141. Hynes RO: Integrins: versatility, modulation and signaling in cell adhesion. *Cell.* 69:11-25; 1992
142. Albelda SM: Role of integrins and other cell adhesion molecules in tumour prognosis and metastasis. *Lab Inves.* 68:4-17; 1993
143. Qian F, Vaux DL, and Weisman IL: Expression of the integrin a4b1 on the melanoma cells can inhibit the invasive stage of metastasis formation. *Cell.* 77:335-47; 1994
144. Danen EHJ, Marcinkiwicz C, Cornelissen IMHA, et al: The disintegrin eristostatin interferes with integrin α4β1 function and with experimental metastasis of human melanoma cells. *Exp Cell Res.* 28:188-96; 1998
145. Akiyama SK, Nagata K and Yamada K: Cell surface receptors for extracellular matrix components. *Biochem Biophys Acta.* 1031:91-110; 1990
146. Albelda SM, Mette SA, Elder DE, et al: Integrin distribution in malignant melanoma: association of the β3 subunit with tumour progression. *Cancer Res.* 50:6757-64; 1990

147. Ruoslahti E: The Walter Herbert lecture: control of cell motility and tumour invasion by extracellular matrix integrins. *Br J Cancer.* 66:239-42; 1992

148. Liotta LA: Tumour invasion and metastasis: role of the extracellular matrix. *Cancer Res.* 46:1-7; 1986

149. Li X, Chen B, Blystone SD, et al: Differential expression of αv integrins in K1735 melanoma cells. *Invasion Metastasis.* 18:1385-1389; 1998

150. Pinkus GS, O'connor EM, Etheridge CL, et al: Optimal immunoreactivity of keratin proteins in formalin-fixed, paraffin-embedded tissue requires preliminary trypsinization. *J Histochem Cytochem.* 33:465-73; 1994

151. Lan HY, Um W, Nikoklic-Paterson DJ, et al: A novel, simple, reliable and sensitive method for multiple immunoenzyme staining: use of microwave oven and heating to block antibody cross reactivity and retrieve antigens. *J Histochem Cytochem.* 43:97-102; 1995

152. Shi S, Imam SA, Young L, et al: Antigen retrieved immunohistochemistry under the influence of pH using monoclonal antibodies. *J Hiatochem Cytochem.* 43:193-201; 1995

153. Hazelbag HM, Broek LJCM, Dorst EBL, et al: Immunohistostaining of Chain-specific keratins on formalin-fixed, paraffin-embedded tissues: a comparison of various antigen retrieval systems using microwave heating and proteolytic pre-treatments. *J Histochem Cytochem.* 43:429-37; 1995

154. Fan Z, Clark V, and Nagle RB: An evaluation of enzymatic and heat epitope retrieval methods for the immunohistochemical staining of the intermediate filaments. *Appl Immunohistochem.* 5:49-58; 1997

155. Ingber D: Integrins as mechanochemical transducers. *Cur Opin Cell Biol.* 3:841-48; 1991

156. Larsson L-I: Tissue preparation methods for light microscopic immunohistochemistry. *Appl Immunohistochem.* 1:2-16; 1993

157. Riccioni L, Tommaso LD, Collina G: Actin-rich desmoplasic malignant melanoma. Report of three cases. *Am J Dermatopathol.* 21(6):537-541;1999

158. Repesh LA, Drake SR, Warner MC, Downing SW, Jyring R, Seftor EA, Hendrix MJC, McCarthy JB: Adriamycin induced inhibition of melanoma cell invasion is correlated with decreases in tumour cell motility and increases in focal contact formation. *Clin Exp Metastasis.* 11:91-102; 1993

159. Asklen LA, Hove LM, Harveit F: Metastatic distribution in malignant melanoma: a 30 years autopsy study. *Invasion Metastasis.* 7:253-63; 1987

160. Liotta LA, Guirguis R, Stracke M: Biology of melanoma invasion and metastasis. *Pigment Cell Res.*1:5-15; 1987

161. Goldman RD, Goldman AE, Green KJ: Intermediate filament networks: Organization and possible functions of a diverse group of cytoskeletal elements. *J Cell Sci.* 5:69-97; 1986

162. Hay ED: Theory for epithelial-mesenchymal transformation based on the "fixed cortex" cell motility model. *Cell Motil. Cytoskeleton.* 14:455-57; 1989

163. Dewhurst LO, Rennie IG, McNeil S: Positive association between cytoskeletal changes, melanoma cell attachment and invasion in vitro. *Melanoma res.* 8:303-11:1998

164. Franke WW, Grund C, kuhn C, et al: Formation of cytoskeletal elements during mouse embryogenesis. III. Primary mesenchymal cells and the first appearance of vimentin filaments. *Differentiation.* 23:43-49; 1982

165. Franke WW, Jahn L, Knapp AC: Cytokeratins and proteins in certain epithelioid and non-epithelial cells. In Cytoskeletal proteins in tumour diagnosis (Osborn M , Weber K, eds) Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, pp 151-172; 1989

166. Hay ED. Cell Bilogy of extracellular matrix. New York: Plenum Publishing Corporation; 1982
167. Kleinman HK, Klebe, RJ, and Martin GR: Role of colagenous matrices in the adhesion and growth of cells. *J Cell Biol.* 88:473-82; 1981
168. Liotta LA, Rao NC, and Barsky SH: Tumour invasion and the extracellular matrix. *Lab Invest.* 49:636-49; 1983
169. Hendrix MJC, Seftor EA, Seftor RE, Trevor KT: Experimental co-expression of vimentin and keratin intermediate filaments in human breast cancer cells result in phenotypic interconversion and increased invasive behaviour. *Am J Pathol.* 150:483-95; 1997
170. Lunch RG: Differentiation and Cancer. *Proc Natl Acad. USA.* 92:647-8; 1995
171. Hart IR, Birch M, Marshall JF: Cell adhesion receptor expressing during melanoma progression and metastasis. *Cancer and Met Ver.* 10:115-128; 1991
172. Merbs SL, Sidransky D: Analysis of p16 (CDKN2/ MTS- 12/ INK4A) alterations in primary sporadic uveal melanoma. *Invest Ophthalmol Vis Sci.* 40:779-83; 1999
173. Ohta M, Berd D, Shimizu M, et al: detection mapping of chromosome region 9p21-p22 surrounding the CDKN2 locus in melanoma. *Intl J Cancer.* 65:762-7; 1996
174. Berge PJM, Danen EHJ, Muijen GNP, Jager MJ, Ruiter DJ: Integrin expression in uveal melanoma differs from cutaneous melanoma. *Invest Ophthalmol and Vic Sci.* 34:3635-40; 1993
175. Chu YW, Runyan RB, Oshima RG, Hendrix MJC: Expression of complete keratin filaments in mouse L cells augments cell migration and invasion. *Proc Natl Acad Sci USA.* 90: 4261-4265; 1993
176. Albert DM: The association of viruses with uveal melanoma. *Tr Am Ophth Soc.* 77:367-421; 1979

177. McClay EF, Mastrangelo MJ, Sprandio JD, et al: The importance of tamoxifen to a cisplatin-containing regimen in the treatment of metastatic melanoma. *Cancer.* 63:1292-5; 1989

178. Shields CL, Shields JA, Eagle RC, Potter P, Menduke H: Uveal melanoma and pregnancy. *Ophthalmology.* 98:1667-73; 1991

179. Foss AJE, Alexander RA, Phil M, Guille MJ, Hungerford JL, McCartney ACE, Lightman S: Estrogen and progesterone receptor analysis in ocular melanomas. *Ophthalmology.* 102:431-6; 1995

180. Egan KM, Gragoudas ES, Seddon JM, Walsh SM: Smoking and the risk of early metastasis from uveal melanoma. *Ophthalmology.* 99:537-41; 1992

181. Ferson M, Edwards A, Lind A et al: Low natural killer-cell activity and immunoglobulin levels associated with smoking in human subjects. *Int J Cancer.* 23:603-9; 1979

182. Tucker MA, Shields JA, Hartage P, Ausburger J, Hoover RN, Fraumeni JF: Sunlight exposure as risk factor for intraocular malignant melanoma. *N Engl J Med.* 313:789-92; 1985

183. Kivela T and Uusitalo M: Structure, development and function of cytoskeletal elements in non-neuronal cells of the human eye. *Prog Ret Eye Res* 17(3):385-428, 1998

184. Keilholz U, Willhauck M, Rimoldi D, Brasseur F, Dummer W, Rass K, Vries T, blaheta J, voit C, Lethe B, Burchill S: Reliability of reverse transcription-polymerase chain reaction (RT-PCR) – based assays for the detection of circulating tumour cells: a quantitative-assurance initiative of the EORTC melanoma cooperative group. *Eur J Cancer.* 34(5):750-53,1998

185. Bataille V, Pinney E, Hungerford JL: Five cases of coexistent primary ocular and cutaneous melanoma. *Arch Dermatol.* 129:198-210; 1993

186. Greene MH, Sanders RJ, Chu FC: The familial occurrence of cutaneous melanoma, intraocular melanoma and the dysplastic nevus syndrome. *Am J Ophthalmol.* 96:238-45; 1983

187. Taylor MR, Guerry D, Bondi EE: Lack of association between intraocular melanoma and cutaneous dysplastic nevi. *Am J Ophthalmol.* 98:478-82; 1984

188. Kath R, Hayungs J, Bornfeld N, Sauerwein W, Hoffken K, Seeber S: Prognosis and treatment of disseminated uveal melanoma. *Cancer.* 72:2219-23; 1993

189. Azumi N, Battifora H: The distribution of vimentin and keratin in epithelial and nonepithelial neoplasms. *Am J Clin Pathol.* 88:286-96; 1987

190. Knapp AC, Franke WW: Spontaneous losses of control of cytokeratin gene expression in transformed, non-epithelial human cells occurring at different levels of regulation. *Cell.* 59:67-79; 1989

191. Donoso L, Berd D, Ausburger JJ, Mastrangelo MJ, Shields JA: Metastatic uveal melanoma: Pretherapy serum liver enzymes and liver scan abnormalities. *Arch Ophthalmol.* 103:796-8; 1985

192. Pyhŏnen S: Treatment of metastatic Uveal melanoma. *Eur J Cancer.* 34(3):S27-30; 1998

193. Rummelt V, Mehaffey MG, Campbell RJ, Pe'er J, Bentler SE, Woolson RF, Naumann GO, Folberg R: Microcirculation architecture of metastasis from primary ciliary and choroidal melanomas. *Am J Ophthalmol.* 126:303-305; 1998

194. Chang AE, Karnell LH, Menck HR: The national cancer data base report on cutaneous and noncutaneous melanoma. A Summary of 84,836 cases from the past decade. *Cancer.* 83:1664-78; 1998

195. Eskelin S, Pyorkonen S, Summanen P, Hahka-Kemppinen M, Kivela T: Tumour doubling times in metastatic malignant melanoma of the uvea. *Ophthalmology.* 107:1443-1449; 2000

196. Lackie JM, Dunn GA, Jones GE eds. Cell behaviour: control and mechanism of motility. Princeton University Press, 1999

197. Eskelin S, Pyorkonen S, Summanen P, Prause JU, Kivela T: Screening for metastatic malignant melanoma of the uvea revised. *Cancer*. 85:1151-9;1999

198. Hicks C, Foss AJE, Hungerford JL: Predictive power of screening tests for metastasis in uveal melanoma. *Eye*. 12:945-8;1998

199. Clark EA, Golub TR, Lander ES, Hynes RO: Genomic analysis of metastasis reveals an essential role for Rho C. *Nature*. 406:532-5;2000

200. Zakka KA, Foos RY, Omphory CA, Strraatsma BR: Malignant melanoma. Analysis of an autopsy population. *Ophthalmology*. 87: 549-56;1980

201. Seddon JM, Gragoudas ES, Egan KM, Glynn RJ, Howard S, Fante RG, Albert DM: relative survival rates after alternative therapies for uveal melanoma. *Ophthalmology* 97:769-777;1990

202. Shields CL, Shields JA , De Potter P, Cater J, Tardio D, Barrett J: Diffuse choroidal melanoma. Clinical features predictive of metastasis. *Arch Ophthalmol* 114:956-963;1996

203. McCaughan JS: Photodynamic therapy of malignant tumours. *Advances in cancer control*. 163-169;1988

204. Finger PT, Packer S, Paglione RW, Gatz JF, Ho TK, Bosworth JL: Thermotheradiotherapy of choroidal melanoma. *Ophthalmology* 96:1384-8;1989

205. Manschot WA, Strik RV: Is Irradiation a justifiable treatment of choroidal melanoma? Analysis of published results. *Brit J Ophthalmol* 71: 348-52;1987

206. Trejdosiewicz LK, Southgate J, Kemshed JT, Hodges GM: Phenotypic analysis of cultured melanoma cells. Expression of cytokeratin type intermediate filaments by M5 human melanoma cell line. *Exp Cell Res* 164:388-98;1986

207. McCurdy J, Gamel J, McLean IW: A simple, efficient and reproducible method for estimating the malignant potential of uveal melanoma from routine H&E slides. *Path Res Pract* 187:1025-7;1991

208. Felberg NT, Shields JÁ, Maguire J, Piperata S, Amsel J: Gamma-glutamyl transpeptidase in the prognosis of patients with uveal melanoma. *Am J Ophthalmol* 95:467-73;1983

209. Finger PT: Radiation therapy for choroidal melanoma. *Surv Ophthalmol* 42:215-32;1997

210. Rummelt V, Folberg R, Rummelt C, Gruman LM, Hwang T, Woolson RF, Yi H, Naumann GO: Microcirculation architecture of melanocytic nevi and malignant melanomas of the ciliary body and choroid. Ophthalmology 101:718-27;1994

211. Schraermeyer U: The intracellular origin of the melanosome in pigment cells. A review of ultrastructural data. *Histol Histopathol* 11:445-62;1996

212. Douarin NM and Ziller C: Plasticity in neural crest differentiation. *Curr opin Cell Biol.* 5:1036-1043;1993

213. Margo CE and Lee A: Fixation of hole eyes: the role of fixative osmolarity in the production of tissue artifact. *Graefe's arch clin exp Ophthalmol* 233:366-370;1995

214. Cochran AJ and Wen DR: S-100 protein as a marker for melanocytic and other tumours. *Pathology*. 17:340-5;1985

215. Collaborative Ocular Melanoma Study Group (COMS): Factors predictive of growth and treatment of small choroidal melanoma COMS report number 5. *Arch Ophthalmol.*115:1537-44;1997

216. Collaborative Ocular Melanoma Study Group (COMS): Mortality in patients with small choroidal melanoma. COMS report number 4 . *Arch Ophthalmol.* 115:886-893;1997

217. Bershadsky AD. Neoplastic transformations . Possible Unexplored functions of Cytoskeleton. In Cytoskeleton, 1988 Plenum Press, New York. Pp. 267-281

218. Fuchs E, Tyner AL, Giudice GJ, Marchuk D, Chaudhury AR, Rosemberg M: the human keratin genes and their differential expression. *Curr Top Dev Biol.* 22:5-26;1987

219. Malorni W, Meschini S, Matarrese P, Arancia G: The cytoskeleton as a subcellular target for the antineoplastic drug Ionidamine. *Anticancer Res.*12:2037-46;1992

220. Moshfeghi DM, Moshfeghi AA, Finger PT: Enucleation. *Surv Ophthalmol.* 44(4):277-301;2000

221. Shuster S, Huszar M, Geiger B: Immunofluorescent localization of intermediate filament subunits for the differential diagnosis of malignant melanoma. *Am J Dermatopathol .* 7:79-86,1985

222. Moshari A, McLean IW. Uveal melanoma: Mean of the largest nucleoli measured on silver stained sections IOVS 2000; 41:s382

223. McLean IW. Prognostic testing of uveal melanoma. IOVS 1999; 40:s185.

224. Burnier Jr. MN, Correia CP: Tumors of the eye and ocular adnexae In: Diagnostic histopathology of tumors. Second edition. Editor:Christipher D.M. Fletcher, Churchill-Livingstone, Chapter 29 pages 1733-1766; 2000

225. CP Correia, AA Rodrigues, KM Oliver, J Deschenes, MN Burnier Jr. Co expression of vimentin and cytokeratin in uveal melanoma. IOVS 2000; 41(4): s383

226. A Morilla-Grasa, CP Correia, F Pereira, IW McLean, MN Burnier Jr. Coexpression ov vimentin and cytokeratin in liver metastasis of uveal melanoma. IOVS 2000; 41(4): s383

227. KM Oliver, F Pereira, CP Correia, AA Rodrigues, MN Burnier Jr. Cytoskeleton phenotype interconversion and its correlation to the nuclear and nucleolar size. IOVS 2000; 41(4): s383

228. ZY Alhabbab, F Pereira, CP Correia, A Morilla-Grasa, MN Burnier Jr. Correlation between interconverted phenotype and tumour dissemination in uveal melanoma. IOVS 2000; 41(4): s383

229. Zhang Yi. Intermediate filaments: a prognostic marker for conjunctival melanomas. Thesis submitted to the Department of Pathology, McGill University, July 2000.