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New approaches to imaging and treatment of ocular melanoma

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BACKGROUND

Chapter 1: Background

1. General Introduction and Outline of Thesis



1

General Introduction and Outline of Thesis

GENERAL INTRODUCTION

Ocular Melanoma

Melanoma is a malignancy of melanocytes. Since melanocytes are naturally present in the eye, melanoma may develop as a primary ocular disease. Notable for ophthalmology, it is one of the few conditions that is not only sight-threatening but also life-threatening.

The caption ‘ocular melanoma’ is commonly used as synonym for *uveal melanoma* (UM), which refers to the most prevalent kind of melanoma of the eye.¹ The uvea concerns the intraocular tissues of the choroid, ciliary body and iris, all of which can harbour a primary melanoma. The word ‘uvea’ is derived from the Greek word for grape, following the appearance of the eye when the sclera (outer layer) has been removed. Distinct from the uvea, melanoma may develop in the conjunctiva as well, resulting in *conjunctival melanoma* (CoM). While UM and CoM both affect the eye, they differ significantly in genetic background, behaviour, and the required therapeutic approach, as will be discussed in this thesis.

The first reports of melanoma (of any origin) date back to the ancient Greeks, with presumably Hippocrates of Cos in the 5th century BC describing dark skin lesions.² Around the 18th century AD, reports on several melanoma patients emerged in the European literature. In 1806, Laennec provided the first detailed presentation of skin melanoma, naming it ‘melanosis’.³ In 1838, the word ‘melanoma’ was first introduced by Carswell.⁴ The famous physician Virchow provided a further classification of melanocytic tumours in 1869.⁵ The first report on the natural history of UM was presented in the early 19th century by Scottish surgeons Wardrop and Burns, linking liver lesions to a dark brown intraocular tumour in the same patient.^{6,7} Notably, this resulted from cadaveric work as the invention of the ophthalmoscope by von Helmholtz in 1851 was a prerequisite for in vivo work on intraocular UM.⁸ Some debate exists on the first report of extraocular CoM,^{9,10} but likely this was presented by Travers in 1820.¹¹ Decades later in 1868, Stellwag von Carion identified the pigment-loaded cells that constitute CoM.¹²

In the roughly two centuries that passed since the first descriptions of UM and CoM, much has been learned about melanoma as a disease. Ocular oncology has evolved into a fruitful field of study, collaborating with other fields such as pathology, medical/radiation oncology, immunology, and radiology. Technological advances in general medicine as well as in ophthalmology proper, lead to better diagnostic procedures, staging, and therapeutic possibilities. Unfortunately, both CoM and UM remain malicious diseases, requiring further studies for better patient care.

This chapter provides an introduction to *extraocular* CoM (Part I) as well as *intraocular* UM (Part II). At the end of the chapter, the aims and outline of the thesis will be discussed.

Part I - Conjunctival melanoma

Epidemiology

CoM is a rare ocular tumour that accounts for about 5% of all primary ocular melanoma.^{1,13} The incidence ranges between 0.3 and 0.8 per million adults in Caucasians.¹⁴⁻¹⁷ It is the second most prevalent malignancy of the conjunctiva, after squamous cell carcinoma (also known as ‘ocular surface squamous neoplasia, OSSN’).¹⁸ CoM is typically a disease of people of Caucasian descent,¹⁹ but may occur in any race. The incidence has been rising in the last few decades, which possibly relates to increased ultraviolet (UV)-radiation exposure.¹⁵

Pathophysiology

The conjunctiva is a mucous membrane that covers the bulbar surface of the eye and inner parts of the eyelids, with melanocytes located in its basal layers. The number, characteristics, and pigment production of melanocytes can vary, resulting in a range of melanocytic diseases.²⁰ Benign melanocytic disease includes ‘hypermelanosis’, i.e. increased melanin production without melanocyte alterations, ‘naevus’ with increased clusters of melanocytes without malignancy, and ‘primary acquired melanosis’ (PAM) with a range of melanocyte alterations. When melanocytic growth extends beyond the basement membrane into deeper tissues, a lesion is deemed a ‘melanoma’.

CoM is thought to originate from PAM (in approximately 74%), from a nevus (in 7%) or de novo (i.e. without a known precursor lesion, in 19%).²¹

The genetic background of CoM resembles that of cutaneous melanoma. Mutations are seen in *BRAF*, *NRAS* and *TERT* promotor genes, while mutations that are commonly seen in UM (such as in *GNAQ11* and *BAP1*, as discussed in Part II) are absent.^{22,23} *BRAF* mutations activate the MAPK pathway,²⁴ while *NRAS* mutations activate the MAPK and PI3K/AKT pathway,²⁵ both promoting cell proliferation.

As in cutaneous melanoma,²⁶ the presence of inflammation in CoM appears to be favourable for clinical outcome,^{27,28} suggesting that immune cells have a role in tumour surveillance in this malignancy. However, the individual roles of the plethora of immune cell types and components that can be identified in the tumour micro environment of CoM is not fully understood.

Clinical presentation

CoM typically presents as a thickened, pigmented lesion on the conjunctival surface, with notable ‘feeder’ or ‘sentinel’ vessels (Figure 1). There is a wide range of presentations, however, as any part of the conjunctiva can be affected, with nodular or flat disease, and lesions can range from amelanotic and pink to black. Some lesions are easily discovered; other lesions (with a pale appearance or

located at the tarsal conjunctiva) are difficult to detect, causing delayed presentation. Often, CoM is accompanied by a component of PAM, and as PAM may cause widespread pigmentation of the eye, it may be difficult to delineate the exact border of infiltrative disease.

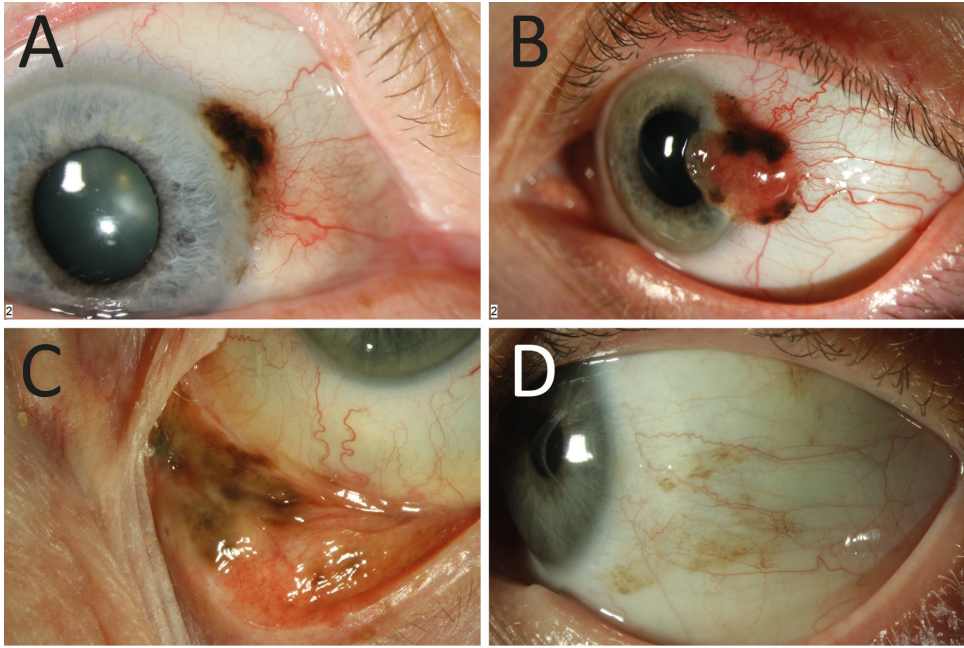


Figure 1. Clinical presentation of CoM. (A) Pigmented lesion near the limbus of the eye. (B) Mixed-pigmented lesions near the limbus of the eye, with growth extending into the cornea. Notice the vessels approaching the lesion. (C) Pigmented lesion at tarsal and forniceal conjunctiva. (D) Faint pigmentation at bulbar conjunctiva, which proved to be CoM in an area of PAM with severe atypia.

Diagnosis

The diagnosis of CoM is based on histology. Tissue can be obtained via several techniques: excisional biopsies are preferred over incisional biopsies, to prevent iatrogenic tumour spread.²⁹ Imaging techniques are not commonly applied to differentiate lesions, but anterior segment OCT or ultrasound investigation may be used to determine invasion into deeper ocular structures.³⁰ It is difficult to properly image thick lesions or those located in the caruncular area or plica, however, and improvements in spatial resolution and tissue penetrance will be needed before imaging can play a larger role in the diagnostic process of conjunctival lesions.

Conjunctival melanomas are currently staged by the 8th ed TNM (tumour-node-metastasis) classification, as presented by the American Joint Committee on Cancer (AJCC).³¹ This scoring

system has been validated by an international collaboration on CoM and proved to predict recurrences and mortality.³² Currently, the most important parameters to predict clinical outcome are tumour basal diameter, thickness, location on the eye (bulbar / non-bulbar) and local invasion;^{17,21,33,34} the value of other parameters such as ulceration and necrosis is unclear. As expected with a multitude of studies on small numbers, various studies have conflicting findings. A recent development is the study of genetic markers (including gene mutations and miRNA expression) for prognostication,³⁵⁻³⁷ but this requires further confirmation.

Treatment

Localized CoM is preferably treated with surgical excision and adjuvant therapy (i.e. cryotherapy, topical chemotherapy, and/or radiotherapy).³⁸ In our institution, plaque brachytherapy is the currently-preferred method for adjuvant radiation of bulbar lesions;³⁹ topical mitomycin-c can be added if PAM is present as well.⁴⁰ Widespread lesions cannot be treated with such an approach, and require extensive surgery (such as orbital exenteration)⁴¹ or external radiotherapy⁴². Treatment options for metastatic disease are limited, and follow developments from cutaneous melanoma. Up till a few years ago, this consisted of conventional systemic chemotherapy with unfortunately poor results. Newly-introduced targeted therapy^{43,44} and immunotherapy^{45,46} are now used more often in CoM, with promising results, as the genetic and immunologic profile of CoM and cutaneous melanoma appear to be much alike.

Up to date, evidence for CoM therapy has been obtained by case-series and case reports. We do not know of (reported) trials dedicated to CoM. Treatment strategies may therefore vary between clinicians, and many topics (such as the use of adjuvant therapy, or sentinel lymph node biopsies⁴⁷) are under debate.

Outcome

Local recurrences of CoM are common: the 5-yr estimate is 36-45%, but the recurrence rate may be as high as 61%.^{14,17,21} Recurrences may be derived from residual cells after earlier treatment, or be new developments from precursor lesions such as PAM.

The conjunctiva has lymphatic drainage and CoM may therefore give rise to lymphatic dissemination. Regional spread of CoM has been reported in 11-52% of patients at 5 years.⁴⁸⁻⁵⁰ The lymph nodes are believed to be the first site of metastasis in many CoM cases,⁴⁸ but distant metastasis without prior lymph node involvement may occur as well.

Systemic metastasis may occur with a 5-yr estimate of 10-16% and a 10-yr estimate of 17-26%.^{49,51} The most frequent sites of distant metastases are the lungs, liver, brain and skin.^{17,48,49,51,52}

The visual outcome of CoM patients is commonly not influenced by the disease itself, but may be affected by local therapy (such as corneal damage due to surgery or limbal stem cell deficiency due to topical chemotherapy) or by last-resort therapy such as removal of the eye.

Part II - Uveal melanoma

Epidemiology

Uveal melanoma (UM) is the most common intraocular primary malignancy in adults. It comprises melanoma of the choroid (90%), ciliary body (6%) and iris (4%).⁵³ The incidence of UM in total ranges from 5.1 to 8.6 per million in Caucasian adults.^{54,55} It is most prevalent in persons of (northern) European ancestry, and has a south-to-north increasing gradient in America as well as Europe.^{55,56} The incidence has been relatively stable over the last few decades,^{55,57} suggesting (unlike what is observed in CoM) no strong relation with UV exposure.

Pathophysiology

Melanocytes are present throughout the uveal tract, and malignant transformation of these underlies the development of UM.⁵⁸ UM is usually initiated by mutations in *GNAQ/11*, which occur in almost 90% of cases.^{59,60} Mutations in *GNAQ/11* are involved in several processes of cell growth and proliferation⁵⁹ including in activation of the YAP1 (“hippo”) pathway^{61,62}. Interestingly, mutations in *GNAQ/11* are already present in choroidal nevi,⁶³ so for malignant transformation, a second mutation is required. Common ‘secondary’ mutations in UM are those in the *BAP1*, *EIF1AX* or *SF3B1* genes.^{58,64}

Important events in UM behaviour are occurrence of chromosomal aberrations (copy number variations).⁶⁵ Frequently observed changes are loss of chromosome 3 or gain of chromosome 8q (both related to worse clinical outcome), and gain of chromosome 6p (related to a favourable outcome).⁶⁶

A decade ago it was discovered that the BAP1 protein (encoded by the *BAP1* gene on chromosome 3) is a major player in UM behaviour.⁶⁷ BAP1 is a deubiquitinating protein which functions in cell cycle regulation, DNA damage repair and regulation of gene expression.⁶⁸ Loss of BAP1 protein expression is related to an unfavourable outcome and is often assessed in patient care to provide information on prognosis.^{69,70}

Tumour micro environment / angiogenesis

In UM, the tumour micro environment involves immune cells and extracellular structures such as blood vessels. Both the immune system and angiogenesis are portrayed as a ‘hallmark of cancer’,⁷¹ which is especially important in UM as the eye is an immune-privileged site,⁷² and UM are highly-vascularized.

The presence of immune cells has long been known to relate to an unfavourable prognosis in UM,⁷³⁻⁷⁵ which is exactly opposite to what is seen in cutaneous (and conjunctival) melanoma. Tumour-infiltrating leukocytes (TILs) produce several pro-inflammatory cytokines, that may stimulate UM growth.⁷⁶ Important players in the tumour microenvironment are macrophages, of which the M2 type is known to stimulate angiogenesis via production of Vascular Endothelial Growth Factor (VEGF). Vessels provide nutrients and oxygen to proliferating cells, and provide a route for hematogenic dissemination. Unsurprisingly, a high vascular density is known to relate to worse survival in UM.^{77,78}

There is a close relation between the immune environment and UM genetic make-up: monosomy 3 is related to an increased presence of TILs.⁷⁸ BAP1 loss and gain of chromosome 8q are related to increased inflammation.⁷⁹

Clinical presentation

The clinical presentation of UM depends on the originating site in either the anterior or posterior segment of the eye. *Iris lesions* can often be readily observed as a pigmented nodule, or by deformation of the pupillary margin (Figure 2). Despite rarely causing other symptoms, they are often diagnosed early by their presentation. *Ciliary body and choroidal lesions* are usually not visible from the outside and are detected by coincidence during ophthalmological inspection (in one third of cases), or following the development of secondary symptoms.⁸⁰ These symptoms include decreased visual acuity, metamorphopsia, or increased floaters; this is due to subretinal fluid (SRF), retinal detachment, haemorrhage or the physical presence of a nodule in the eye. The common presentation of choroidal melanoma is that of an (un)pigmented lesion that is seen by fundoscopy (Figure 3).

Clinically, it may be challenging to differentiate a melanoma from a nevus. Choroidal nevi are a common finding, seen in approximately 5% of Caucasians,⁸¹ and they may transform into melanoma in about 1:9000 cases per year.⁸² A set of clinical parameters has been defined to identify choroidal nevi with increased risk for transformation into melanoma.⁸³ These factors are Thickness (>2mm), Subretinal Fluid, Symptoms, Orange pigment, Margin near the optic nerve, Ultrasonographic Hallowness, Halo absent, Drusen absent; together they form the mnemonic '*To Find Small Ocular Melanoma Using Helpful Hints Daily*'.

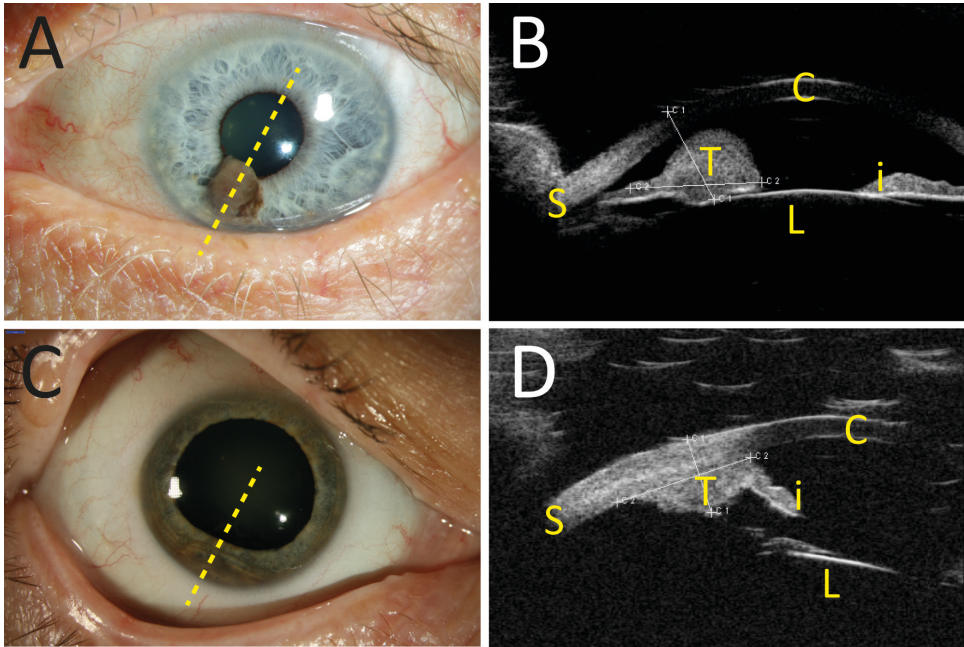


Figure 2. Clinical presentation of iris melanoma and ciliary body melanoma. (A) Pigmented lesion of the iris. The dotted line indicates the cross section as presented by ultrasonography in panel B. (B) Ultrasound image of the same patient as in A. note the nodular configuration, limited to iris tissue. (C) Pigmented lesion of the ciliary body, inferior. The dotted line indicates the cross section as presented by ultrasonography in panel D. (D) Ultrasound image of the same patient as in B. Note that the lesion originates from ciliary body tissue, and is located behind the iris.

Abbreviations: C=cornea, S=sclera, i=iris, L=lens, T=tumour.

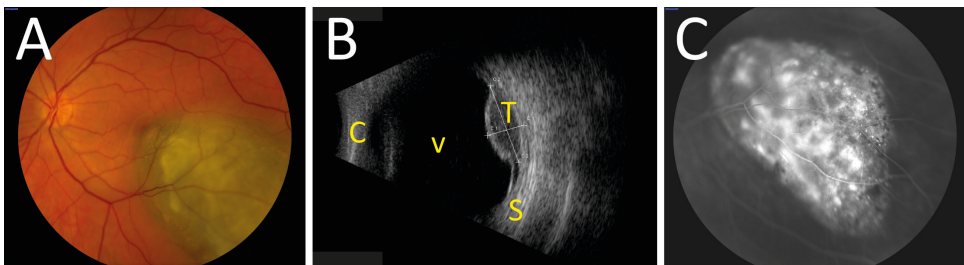


Figure 3. Clinical presentation of choroidal melanoma. (A) Pigmented lesion of the choroid, located in the posterior pole. (B) Ultrasound image of the same patient as in A. Note the dome-shaped configuration and internal 'dark' or 'low' reflectivity of the tumour lesion. (C) Fluorescein angiography of the same patient as in A. Note the vascular pattern and 'pinpoint leakage' of the lesion, which is indicative for melanoma.

Abbreviations: C=cornea, S=sclera, V=vitreous, T=tumour

Diagnosis

The diagnosis of UM is usually based on clinical characteristics (as obtained with fundoscopy), and auxiliary tests such as fluorescein angiography (FA) and ultrasound imaging. Using FA, vascular patterns and leakage are assessed that differentiate between various choroidal lesions.⁸⁴ Ultrasound imaging provides information on lesion size, extent, and internal structure (Figure 3). Some centers apply tissue biopsies as a routine investigation.^{85,86} While advantageous for diagnostic and prognostic purposes, this is an invasive procedure and it is not routinely practiced in The Netherlands.

UM's are staged by the AJCC TNM staging system for choroidal/ciliary body or iris lesions,⁸⁷ based on tumour dimensions and anatomical extent. When tissue material is obtained (after biopsy or enucleation), the prognosis can be refined using the tumour's status of chromosome 3 and 8q,⁸⁸ its gene expression profile (GEP; class 1 and 2),⁸⁹ or immunohistochemical staining for the BAP1 protein.⁷⁰ A schematic approach to categorize UM based on their genetic background, presence of inflammation, and prognostic outcome results into A-, B-, C- and D-type tumours (Table 1).⁹⁰

Table 1. Uveal Melanoma categories and corresponding chromosome aberrations and outcome. [Adapted from Jager et al 2018.⁹⁰]

	A	B	C	D
Metastases risk	Low	Intermediate	High	High
mRNA GEP	Class 1	Class 1	Class 2	Class 2
Chromosome 3	Disomy	Disomy	Monosomy	Monosomy
Chromosome 8q	Normal	Partial gain	Partial gain	Multiple gain
Chromosome 6p	Partial/total gain	Gain	No change	No change
Inflammation	None	None	Some	Much

Abbreviations: GEP= gene expression profile

Treatment

The most common treatments of UM are radiotherapy or enucleation. Less common approaches are local resection, transpupillary thermo therapy, photodynamic therapy, or the recently-introduced nanoparticle AU-011.⁵⁸

Radiotherapy can be administered as brachytherapy (using plaques with an I-125, Ru-106, Pd-103 or other isotope sutured to the eye) or as external radiotherapy (using electron or proton beam devices). A benefit from this approach is that the eye is preserved. Depending on the location of the tumour and radiation source, however, several adverse events may occur. Common events are radiation retinopathy, cataract, or neovascular glaucoma. The underlying mechanism of several events

is vascular damage,⁹¹ which may occur directly via DNA damage,⁹² or indirectly via production of free radicals and inflammatory cytokines.⁹³⁻⁹⁵ Anti-VEGF medication is used as therapy for several adverse events, the value of preventive use for retinal damage is under investigation.^{96,97}

Removal of the eye is the indicated procedure in cases where the tumour is too large to irradiate, when earlier treatment failed, or when severe adverse events of other therapies have occurred or are to be expected.

Unfortunately, there is currently no successful treatment for metastatic UM. Several therapies that are successful for cutaneous melanoma, such as targeted therapy and checkpoint inhibitors, showed no benefit for UM.⁹⁸ Possibly this is due to differences in the immune environment.⁹⁹ Conventional chemotherapy is similarly of little benefit.⁵⁸ Individual patients with limited metastatic disease to the liver may benefit from regional approaches such as surgical resection or intra-arterial chemotherapy. These procedures, as well as several other therapies based on checkpoint inhibition, reduction of angiogenesis or T cell therapies are under investigation.⁵⁸

Outcome

The primary outcome measure for UM patients is development of metastases. Lacking proper treatment this is closely related to survival. A well-reported figure is that up to 50% of UM patients die from metastases,¹⁰⁰ and this has not changed over the last five decades.¹⁰¹ There is a considerable spread of metastatic potential based upon tumour dimensions and genetic profile. Based on the TNM staging criteria for choroidal tumours, the 5y/10y risk for metastasis development is 8/15% in T1 lesions, 14/25% in T2 lesions, 31/49% in T3 lesions, and 51/63% in T4 lesions.¹⁰² The prognosis of iris melanoma is more favourable with a 5y/10y metastasis risk for T1 lesions of 2/5%, for T2 lesions of 9/14%, and for T4 lesions of 33%/unknown.¹⁰³

A secondary outcome measure for UM patients is visual outcome. Visual outcome can be severely threatened in UM; e.g. by the direct position of the tumour affecting the visual axis, and by adverse events of therapy (including radiation retinopathy or loss of the eye with enucleation).

THESIS AIMS AND OUTLINE

This thesis aims to evaluate new imaging techniques to diagnose ocular melanoma lesions, and to identify new treatment targets for ocular melanoma in a preclinical phase. A central theme is angiogenesis, which is studied at a basal level with genetics and histology, as well as at a clinical level with vascular imaging techniques. The first part of this thesis focusses on the understanding of CoM, and continues with potential new therapies. The second part of this thesis focusses on the understanding of UM, and continues with the clinical evaluation of vascular imaging techniques. Some projects of this thesis address both CoM and UM; these are discussed in the part that fits their content best (Figure 4).

Part I – Conjunctival melanoma

The need for better therapies in CoM follows the substantial rates of recurrences and metastases in these patients. Only a few large series with long-term follow-up data on CoM have been reported, as the disease is rare and follow-up in many countries is scattered over local hospitals. We evaluated the current treatment of CoM patients in our institution, benefitting from our position as a national referral center with systematic follow-up (**chapter 2.1**). Triggered by the various clinical presentations of CoM, and the knowledge that melanin pigment has a role in melanoma development on a genetic level (see chapter 3.1), we determined whether clinical pigment characteristics are related to CoM behaviour of the primary tumour (**chapter 2.2**), and its recurrences (**chapter 2.3**).

Recent developments in oncology led to the introduction of two new classes of drugs: ‘targeted therapy’ aimed at genetic mutations, and ‘immunotherapy’ aimed at the interaction between the host’s immune system and tumour cells. These two drugs revolutionized the therapy of cutaneous melanoma patients. In 2018, the Nobel Prize in Physiology or Medicine was awarded for the *‘Discovery of cancer therapy by inhibition of negative immune regulation’*. Several studies identified similarities in the genetic background and immune environment between cutaneous and conjunctival melanoma, prompting the question whether the new drugs are useful to treat CoM. In **chapter 3.1**, we summarize the current knowledge of the genetic background and immunologic microenvironment of CoM, and discuss the first observations from targeted therapy and immunotherapy in patients with CoM. One type of immunotherapy is based on inhibition of the PD-1/PD-L1 pathway; we set out to study this pathway in CoM tissue and performed in vitro tests to determine the feasibility of this new therapeutic approach (**chapter 3.2**).

Part II – Uveal melanoma

Uveal melanoma has a distinct position compared to cutaneous and conjunctival melanoma by having a different genetic background and interaction with the immune system (see chapter 3.1). The earlier mentioned targeted and immunotherapies have – unfortunately – as yet not been

successful in UM, leading to an urgent need for better therapies. Most UM carry a mutation in *GNAQ11*, which is known to activate the YAP1 pathway. The YAP1 pathway is a regulator of cell growth and was found to stimulate tumour growth of various cancers. Interestingly, the readily-available ophthalmic drug verteporfin can inhibit YAP1 activity. We studied the significance of the YAP1 pathway in UM, and tested whether verteporfin would inhibit the growth of UM and CoM cell lines in vitro; we analyzed the role of the genetic background in the treatment response (**chapter 4.1**).

An important parameter in the development and behaviour of UM is angiogenesis: vessels are needed to provide nutrients and oxygen to a proliferating tumour, and vessels provide a route for tumour cells to disseminate. Several drugs can target vessel growth and new drugs have been developed to target specific parts of angiogenesis such as by ischemic mediator HIF1 α . Angiogenesis is stimulated by the tumour micro environment, as immune cells can produce pro-inflammatory and pro-angiogenic cytokines. Recent work showed a relation between the genetic evolution of UM and the presence of different immune cells.⁷⁹ We hypothesized that the genetic status of UM relates to angiogenesis as well, and compared the vascular density in UM tissue and the expression of several angiogenesis-related genes (**chapter 4.2**).

Blood vessels are not only important for ocular melanoma on a microscopic scale, but translate into clinical practice for diagnostic purposes and evaluation of therapy. Tumour vessels, as a differentiating feature between malignant and benign choroidal lesions, are commonly assessed with fluorescein angiography. We wondered whether not only the presence of vessels, but also the oxygen content of vessels can be used diagnostically, as this may provide information on the metabolism of (tumour) cells. We hypothesized that the oxygen metabolism in melanoma eyes is different from that in eyes with a nevus, and therefore studied oximetry in eyes with choroidal lesions (**chapter 5.1**).

The role for vascular imaging to diagnose and differentiate lesions of the iris and conjunctiva is currently limited. Fluorescein angiography has been used to study iris lesions, but the diagnostic value of many parameters remained unclear; conjunctival tumour vessels have been studied even less with this technique as dye easily leaks out of conjunctival vessels. A new imaging technique to study ocular vessels is OCT-angiography (OCTA), with the beneficial properties of being non-invasive and non-dye dependent. We tested the feasibility of this technique to study iris and conjunctiva lesions, with the ultimate aim to differentiate between benign and malignant tumours (**chapter 5.2**).

In summary, this thesis reports on several studies investigating the genetic, immunologic and vascular characteristics of CoM and UM, and the application of new imaging techniques to differentiate between benign and malignant lesions.

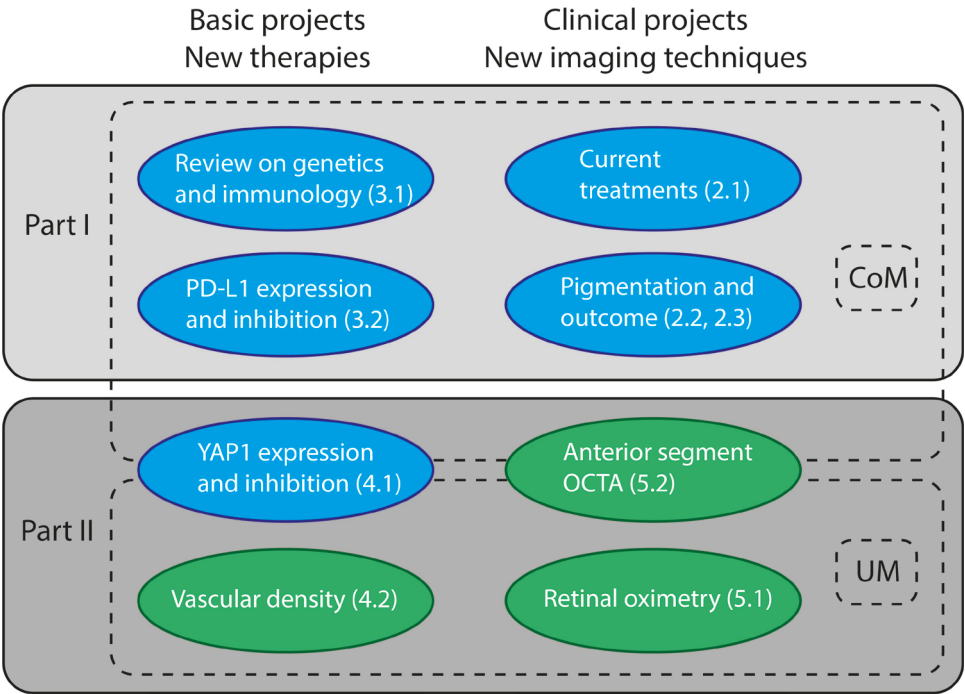


Figure 4. Projects of this Thesis. The outline of this thesis in part I and II is presented, together with the partially overlapping division in CoM and UM. Each oval shape represents a project, numbers refer to the chapters of this thesis. Projects related to angiogenesis are depicted in green, projects related to treatment of patients are depicted in blue.

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