Conjunctival melanoma: New insights in tumour genetics and immunology, leading to new therapeutic options

Niels J. Brouwer a, Robert M. Verdijk a,b,c, Steffen Heegaard d,e, Marina Marinkovic a, Bita Esmaeli f, Martine J. Jager a,*

a Department of Ophthalmology, Leiden University Medical Center, Leiden, the Netherlands
b Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands
c Department of Pathology, Erasmus University Medical Center, Rotterdam, the Netherlands
d Department of Ophthalmology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark
e Department of Pathology, Eye Pathology Section, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark
f Department of Plastic Surgery, Orbital Oncology and Ophthalmic Plastic Surgery, M.D. Anderson Cancer Center, Houston, TX, USA

ARTICLE INFO

Keywords:
Eye disease
Conjunctival melanoma
Ocular oncology
Oncogenetics
Targeted therapy
Immunotherapy

ABSTRACT

Recent developments in oncology have led to a better molecular and cellular understanding of cancer, and the introduction of novel therapies. Conjunctival melanoma (CoM) is a rare but potentially devastating disease. A better understanding of CoM, leading to the development of novel therapies, is urgently needed.

CoM is characterized by mutations that have also been identified in cutaneous melanoma, e.g. in BRAF, NRAS and TERT. These mutations are distinct from the mutations found in uveal melanoma (UM), affecting genes such as GNAQ, GNA11, and BAP1. Targeted therapies that are successful in cutaneous melanoma may therefore be useful in CoM.

A recent breakthrough in the treatment of patients with metastatic cutaneous melanoma was the development of immunotherapy. While immunotherapy is currently sparsely effective in intraocular tumours such as UM, the similarities between CoM and cutaneous melanoma (including in their immunological tumour microenvironment) provide hope for the application of immunotherapy in CoM, and preliminary clinical data are indeed emerging to support this use.

This review aims to provide a comprehensive overview of the current knowledge regarding CoM, with a focus on the genetic and immunologic understanding. We elaborate on the distinct position of CoM in contrast to other types of melanoma, and explain how new insights in the pathophysiology of this disease guide the development of new, personalized, treatments.

1. Introduction

Conjunctival melanoma (CoM) is a rare but potentially devastating disease. With an incidence of 0.3–0.8/million in Caucasian adults (Isager et al., 2005; Triay et al., 2009; Tuomaala et al., 2002; Yu et al., 2003), it accounts for about 5% of ocular melanoma cases (Isager et al., 2005; McLaughlin et al., 2005). The estimated number of new cases per year is 130 in the USA, and 320 in Europe. CoM originates from melanocytes in the basal layers of the conjunctiva, and can develop in an area of primary acquired melanosis (PAM), in a nevus, or de novo (Shields et al., 2011). The role of ultraviolet (UV) radiation in CoM development is under discussion, following long-standing epidemiological and more recent genetic work.

Originating from the conjunctiva, CoM is a mucosal melanoma with much resemblance to melanoma of the skin (Mikkelsen et al., 2016; Rodrigues et al., 2019). This may be no surprise when looking at the histological and functional similarities of these epithelial tissues. CoM is a very different entity compared to uveal melanoma (UM), which affects the choroid, ciliary body or iris (Jager et al., 2020), and CoM and UM have distinct aetiologies and genetic backgrounds (Smit et al., 2019).

In clinical practice, CoM most typically presents as a pigmented lesion near the limbus of the eye. Any part of the conjunctiva can be...
affected, however, and lesions may range from amelanotic to deeply pigmented or even black (Fig. 1). Localised disease is commonly treated by surgical excision and adjuvant therapy (such as cryotherapy, radiotherapy or topical chemotherapy), while widespread disease on the ocular surface or palpebral conjunctiva may need more extensive therapy such as orbital exenteration (Wong et al., 2014). Despite treatment, up to 66% of CoM patients may develop local recurrences (Missotten et al., 2005), and up to 38% will die due to the disease within 10 years of primary treatment (Brouwer et al., 2018; Tuomaala et al., 2002) (Fig. 2). Risk factors for metastases formation include a greater tumour thickness, non-bulbar location, low tumour pigmentation, histologic ulceration, and local invasion (Brouwer et al., 2019b; Esmaeli et al., 2019; Larsen et al., 2015; Shields et al., 2011). A better understanding of CoM, leading to novel therapies, is therefore urgently needed.

Recent developments in the field of oncology have led to a better understanding of cancer and the introduction of novel therapies. These therapies include ‘targeted therapy’, aiming at specific cellular pathways and genetic mutations of cancer cells, and ‘immunotherapy’, that activates the patient’s own immune system to block tumour growth. Knowledge of the genetic and immunologic environment of CoM may expedite the introduction of these therapies in CoM.

The genetic background of CoM is characterized by mutations in genes such as BRAF, NRAS, and TERT (Griewank et al., 2013a, 2013b). These mutations are common in cutaneous melanoma as well, and distinct from the mutations that occur in UM, affecting e.g. GNAQ, GNA11, and BAP1. Targeted therapies have recently been introduced successfully in the treatment of cutaneous melanoma: the use of vemurafenib (targeting the BRAF mutation) resulted in a better overall survival of cutaneous melanoma patients (Chapman et al., 2011). New insights regarding the development of treatment resistance led to the combined therapy of BRAF and MEK (i.e. Mitogen-activated ERK kinase) inhibitors, with even better results (Ascierto et al., 2016). Because of the molecular resemblance of CoM and cutaneous melanoma, these drug developments may be introduced to treat CoM. Some promising case studies of targeted therapy in CoM have been published recently (Esmaeli and Sagiv, 2019), and further (pre-clinical) studies are being performed.

The tumour micro-environment of different types of melanoma has been studied for many decades, but is under increasing interest following the discovery of immunotherapy that enhances the body’s own immune system to attack tumour cells. Examples of this breakthrough in the treatment of metastatic melanoma are checkpoint-inhibitor therapies with ipilimumab (targeting CTLA-4) and nivolumab (targeting PD-1), which via different routes activate a CD8+ T cell response. Early studies showed improved survival in patients with unresectable metastatic cutaneous melanoma who were treated with ipilimumab, compared to gp100 vaccination (Hodi et al., 2010). Later studies found improved survival for nivolumab treatment compared to dacarbazine chemotherapy (Robert et al., 2015b). While the success of immunotherapy is as yet limited in intraocular UM (Heppt et al., 2017b) (possibly because of the immune privilege of the eye where tumour escape mechanisms hamper immune surveillance (Niederkorn, 2009)), the similarities in tumour micro environment between extra-ocular CoM and cutaneous melanoma led to the belief that immunotherapy should also be applied to CoM. Promising data on small scale use of immunotherapy in CoM have been reported (Hong et al., 2020; Sagiv et al., 2019), and the evaluation of the newest therapies is awaited.

This review aims to provide an overview of the current knowledge regarding the genetic and immunologic understanding of CoM, and the implications for treatment. We touch upon similarities and differences between CoM and other types of melanoma, and explain how new insights in the pathophysiology of this disease guide the development of new therapies.

2. Epidemiology and etiology

2.1. General incidence

The incidence of CoM ranges between 0.3 and 0.8/million in Caucasian adults (McLaughlin et al., 2005; Missotten et al., 2005; Triay et al., 2009; Tuomaala et al., 2002; Virgili et al., 2020). It is the second most prevalent malignancy of the conjunctiva, after squamous cell carcinoma (also known as ‘ocular surface squamous neoplasia, OSSN’) (Shields et al., 2017). CoM accounts for approximately 5% of all primary ocular melanoma, being overshadowed by the far more prevalent UM (Isager et al., 2005; McLaughlin et al., 2005). The incidence of CoM increases with age: CoM mainly affects patients from the fifth/sixth decade of life onwards (Isager et al., 2005; Larsen et al., 2016a; Triay et al., 2009; Tuomaala et al., 2002; Yu et al., 2003), and is rare in children and adolescents (Balzer et al., 2019). The incidence can be considered equal between men and women, although some studies report a slightly higher incidence amongst males (with a male-to-female...
The number of reports on the incidence of CoM is limited. In national registries, CoM is often classified together with other types of ocular melanoma, limiting the ability to obtain tumour-specific data (Virgili et al., 2020; Yu et al., 2003). Current data mainly originate from North America or Europe, limiting data on population groups other than Caucasians.

2.2. Geographical and racial differences

The incidence of CoM varies between geographical areas as well as between population groups with a different racial background. This may point towards a genetic (or population-related) predisposition, as well as a role for environmental factors (such as UV-radiation) in development of CoM.

CoM is typically considered a disease of people with (northern) European ancestry, occurring most frequently in the Nordic countries and parts of North America (Fig. 3); however, it can impact people of any descent. A significantly higher incidence has been observed among Non-Hispanic Whites (0.49/million) compared to Hispanics (0.33/million), Blacks (0.18/million), American Indians (0.17/million), and Asians (0.15/million) in a large American study on CoM and race (Hu et al., 2008). Recent work from Canada identified a higher incidence of CoM in the eastern Canadian provinces, with presumably many inhabitants of European descent (Ghazawi et al., 2019a), corresponding with elevated incidences of cutaneous melanoma. In this study, the incidence of CoM was somewhat lower in Canada compared to the USA. This was attributed to the South-to-North gradient, with a lower occurrence of CoM in Canada due to less UV-radiation at the higher latitude (Yu et al., 2006). This Canadian study demonstrated that effects from both latitude (comparing Canada and the USA) as well as ethnic background (within Canada itself) are important factors in CoM development. A recent population-based study from Europe found a CoM incidence of 0.28/million in Southern Europe, and up to 0.90/million in Northern Europe (Virgili et al., 2020). The highest incidences were found in Norway, The Netherlands and Switzerland, which were also the
countries with the highest incidence of cutaneous melanoma. This study did not identify a significant association between the incidence of CoM and the latitude of the reported countries, which may be due to an analysis that did not stratify for racial background, levelling out an effect of latitude.

Epidemiologic observations from CoM in the US partially parallel those of UM, a disease that is most prevalent in non-Hispanic Whites of latitude.

Abbreviations: NA, not applicable; NS, not significant. M, Male; F, Female.

2.3. Time trends

The incidence of CoM has been rising during the last few decades (Larsen et al., 2016a; Triay et al., 2009; Tuomaala et al., 2002; Yu et al., 2003) (Table 1). Some studies report stable incidences, but this may be due to limitations in study size or a short studied time span (Ghazawi et al., 2019a; Isager et al., 2005; Norregaard et al., 1996; Park et al., 2015; Seregard and Kock, 1992). Between 1960 and 2005, the age-standardized incidence in Sweden has risen from 0.08 per million to 0.56 per million, with a more frequent occurrence on the bulbar parts of the conjunctiva (Triay et al., 2009). A similar pattern was observed in Denmark, with a peak incidence of 0.87 per million in 2000–2009, and an increase in bulbar lesions between 1960 and 2012 (Larsen et al., 2016a). As will be discussed later on, there may be an etiologic and genetic difference between bulbar and non-bulbar CoM. In the USA, data from the SEER database showed an overall increase in the incidence of CoM between 1973 and 1999, age-adjusted from 0.22 to 0.46 per million (Yu et al., 2003). Stratified for gender, however, a clear increase was seen in the incidence amongst men, but not amongst women. The authors hypothesized that this gender difference may be caused by differences in sunlight protection and outdoor activities. A recent large

Table 1
Epidemiologic studies on the incidence of CoM.

| Study                     | Origin      | Cases | Years          | Overall incidence (Cases/million) | Current incidence (Cases/million) | Change over time | Race (Cases/million) |
|---------------------------|-------------|-------|----------------|-----------------------------------|-----------------------------------|------------------|----------------------|
| Yu et al. (2003)          | USA         | 206   | 1973–1999      | All: 0.46                         | M: 0.63; F: 0.32                  | Increase         | Whites: 0.48, Blacks: 0.19, Others: 0.26 |
| Vajdic et al. (2003b)     | Australia   | 37    | 1996–1998      | All: 0.6                          | Not reported                      | Not reported     | Not reported          |
| Isager et al. (2005)      | Denmark     | 120   | 1943–1997      | All: 0.4; NS                       | M: 0.3; F: 0.7; NS                | Stable           | Not reported          |
| Hu et al. (2008)          | USA         | 168   | 1992–2003      | All: 0.41                        | Not reported                      | Not reported     | Non-Hispanic Whites: 0.49, Blacks: 0.18, American Indians: 0.17, Asians: 0.15, Others: 0.26 |
| Triay et al. (2009)       | Sweden      | 170   | 1960–2005      | All: 0.56                         | M: 0.74; F: 0.45                  | Increase         | Not reported          |
| Park et al. (2015)        | South Korea | 90    | 1999–2011      | All: 0.12                         | All: 0.12                         | Stable           | (Asian population)    |
| Larsen et al. (2016a)     | Denmark     | 138   | 1960–2012      | All: 0.50; NS                     | M: 0.53; F: 0.48; NS              | Increase         | Not reported          |
| Ghazawi et al. (2019a)    | Canada      | 190   | 1992–2010      | All: 0.32; M: 0.35; F: 0.29        | All: 0.32; M: 0.35; F: 0.29        | Stable           | Differences based on population background |
| Virgili et al. (2020)     | Europe      | 714   | 1995–2007      | All: 0.46, (crude)                | All: 0.46, (age adj.)             | Stable           | Not reported          |

Abbreviations: NA, not applicable; NS, not significant. M, Male; F, Female.

a Higher rates of CoM were seen in the eastern Canadian provinces (with many people of Caucasian/European descent), lower rates were seen in the provinces Ontario and Quebec (with higher numbers of self-identified minorities).

b Overall calculation is based on the presented data per race, weighted for number of cases.
European study showed no significant overall change in incidence for 1995–1998 to 2003–2007 (incidence of 0.40–0.43 per million) but stratified by gender, there was a significant increase for men (0.41–0.53 per million) and a stable incidence for women (0.39–0.34 per million) (Virgili et al., 2020).

The increasing incidence of CoM follows observations from cutaneous melanoma, with increasing numbers in the last decades in the USA (McLaughlin et al., 2005) and Europe (Isager et al., 2005; Mansson-Brahme et al., 2002; Tuomaala et al., 2002). Similar to CoM, the increased cutaneous melanoma incidence is believed to be due to increased UV radiation exposure (specifically intermittent exposure) (de Vries et al., 2003), and is most pronounced amongst males (Bianconi et al., 2020).

Time trends that are observed in CoM are in contrast to observations from UM. Large population-based studies from the USA and Europe demonstrate no significant alterations in UM incidence for the last three decades, with overall values of 2–8/million in different regions (Aronow et al., 2018; Virgili et al., 2007). Specific analyses show minor increases in UM, however, e.g. in the white population of the US (Aronow et al., 2018), and in Canadians over the last two decades (Ghazawi et al., 2019b).

A complicating factor in the comparison of incidence rates of CoM (as well as other melanomas) between geographical areas and time periods are changing populations due to migration. As mentioned, genetic background is related to the risk for melanoma development. This may partially explain the differences between overall time-dependent rates of melanoma and numbers per subgroup in the literature. Data on race or ethnic background are not always available, calling for cautious interpretation of crude results.

2.4. Incidence adjusted to tissue size

The absolute rarity of CoM suggests that the conjunctiva as a tissue is unlikely to develop melanoma. An interesting figure emerges, however, when the incidence of CoM is related to the small surface of the conjunctiva, as compared to cutaneous melanoma and the much larger surface size of the skin. In a large study from the USA, the incidence of CoM was estimated at 0.4/million persons per year, and that of cutaneous melanoma at 153.5/million persons per year (McLaughlin et al., 2005). With an approximate skin area of 1.7 m² for a human adult (Marks and Motley, 2019), and a conjunctival surface area per eye of 17.6 cm² (Watsky et al., 1988), the incidence of cutaneous melanoma can be estimated at 90 per million m² skin per year, and the CoM incidence at 113 per million m² conjunctiva per year. These figures are now well within a comparable range, which is not surprising due to the similarities between skin and conjunctiva, and their respective melanomas (Rodrigues et al., 2019).

To illustrate the difference with melanoma of the choroid, with a choroidal area approximating the retinal area of 1100 mm² (Algaeed and Kozak, 2018), and a choroidal melanoma incidence of 4.3/million persons per year (McLaughlin et al., 2005), the incidence of choroidal melanoma can be estimated at 1955 per million m² choroid per year; this is a remarkable 20-fold higher per area unit compared to melanoma of the conjunctiva or skin. We conclude that CoM is rare in absolute numbers, but we put the rarity of CoM in perspective considering the conjunctival size. This calculation stresses the differences between intraocular and extraocular melanoma, with supposedly a different role for genetic and environmental factors (Fig. 4).

2.5. Precursor lesions of CoM

Melanocytic diseases of the conjunctiva comprise a wide range of entities, based on the number and characteristics of melanocytes, and aberrations in the production of melanin. An illustrative overview of melanocytic disease was recently provided by Jakobiec (2016). Examples of conjunctival melanocytic disease include conjunctival nevi, primary acquired melanosis (PAM), complexion-associated melanosis (previously known as ‘racial pigmentation’) and CoM. By definition, CoM has invaded beyond the basement membrane into deeper tissues, but it may develop from other (non-invasive) disease. Many of the precursor lesions are far more prevalent than CoM, as was reported in an American study with population-based incidences of conjunctival nevi (50 cases per million), PAM (44 cases per million) and CoM (1.5 cases per million) (Dalvin et al., 2018a). As with CoM, the prevalence of the precursor lesions differs between various races.

Most CoM (42–74% of cases) are believed to develop from PAM (Larsen et al., 2015; Paridaens et al., 1994; Shields et al., 2011) (Table 2). PAM is clinically described as a unilateral, flat, light pigmentation of the conjunctiva (resembling ‘cinnamon dust’), with a variable presence (i.e. ‘waxing and waning’). The likelihood of PAM to develop into melanoma depends on histological characteristics: while PAM ‘with atypia’ develops into CoM in 13% of cases, PAM ‘without atypia’ is considered an indolent condition that rarely ever leads to malignancy (Shields et al., 2008).

The histological classification of PAM is under continuous debate (Jakobiec, 2016). Grading PAM into ‘with’ or ‘without’ atypia was proposed (Folberg et al., 1985b) following systems that may have led to overrating by clinicians (using the term ‘precancerous melanosis’, by Reese) or to underestimation (using the term ‘benign acquired melanosis’, by Zimmerman). Issues remained for grouping PAM without
Table 2

| Study                | Study size | PAM (%) | Nevus (%) | PAM and Nevus (%) | De Novo (%) | Unknown (%) |
|---------------------|------------|---------|-----------|-------------------|-------------|-------------|
| Shields et al. (2011) | 382        | 74      | 7         | 19                |             |             |
| Paridaens et al. (1994) | 256        | 57      | 18        | 22                | 2           |             |
| Missotten et al. (2005) | 194        | 57      | 2         | 4                 | 26          | 11          |
| Larsen et al. (2015) | 139        | 62      | 33        | 2                 | 11          |             |
| Tuomaala et al. (2002) | 85         | 61      | 30        | 8                 |             |             |
| Anastassiou et al. (2002) | 69         | 42      | 39        | 16                | 3           |             |
| De Potter et al. (1993) | 68         | 56      | 26        | 18                |             |             |
| Norregaard et al. (1996) | 42         | 19      | 21        | 60                |             |             |

Abbreviations: PAM, primary acquired melanosis.
* These categories were not reported in all studies.

atypia, however, and the lack of ‘melanoma-in-situ’ terminology. It can be advocated that PAM is the conjunctival equivalent of lentigo maligna of the skin, and that PAM with atypia is melanoma-in-situ, but the terminology from dermatopathology is not directly translatable. A newer system introduced ‘conjunctival melanocytic intraepithelial neoplasia’ (CMIN) on a 1–10 point score (Damato and Coupland, 2008). This score is increasingly being implemented. A consensus meeting for the most recent 4th ed WHO classification of tumours of the eye proposed a simplified scheme of the aforementioned PAM and CMIN terminology, using ‘low-grade conjunctival melanocytic intraepithelial lesions (CMIL), ‘high-grade CMIN’, and ‘melanoma-in-situ’ (Grossniklaus et al., 2018). All three scoring systems were deemed suitable and comparable in sensitivity, specificity, and accuracy; however, grading of low-risk lesions may remain difficult (Milman et al., 2020). While the debate on histological grading may continue, it should be noted that ‘PAM remains a suitable term for the clinical description of lesions, however, without information on atypia.

About 7% of CoM is believed to develop from a nevus (Shields et al., 2011) (Table 2). A range between 2 and 39% has been reported, which may be due to difficulties in histologic examination (Anastassiou et al., 2002; Larsen et al., 2015; Missotten et al., 2005; Paridaens et al., 1994). Conjunctival nevi are quite common (Dalvin et al., 2018a; Shields et al., 2016), and only rarely develop into melanoma: a large study from the USA found that 3 out of 149 conjunctival nevi (2%) underwent malignant transformation (Shields et al., 2004). Compared to CoM, nevi are seen more often in patients with a younger age (first/second decade) and often present with cysts; nevertheless, clinical differentiation can be challenging (Shields and Shields, 2019).

In about 11–26% of CoM cases, no precursor lesion can be identified; these CoM are considered to have developed ‘de novo’ (Anastassiou et al., 2002; Larsen et al., 2015; Missotten et al., 2005; Paridaens et al., 1994; Shields et al., 2011) (Table 2).

In spite of these reports, determination of the origin of CoM is controversial and imposes some difficulties. Clinical and histological findings may seem contradictory, and potential precursors may be overlooked or be impossible to determine (Damato and Coupland, 2009). In her 1990 thesis, De Wolff-Rouendaal noted that 16 of 33 CoM that were clinically graded as ‘de novo’, showed acquired melanosis on histopathological examination, questioning the origin (De Wolff-Rouendaal, 1990). As such, a co-occurring component of intra-epithelial melanocytes may be either pre-existing PAM or lateral spread of melanoma. Similarly difficult is the coexistence of potential precursors (such as PAM and nevi) making it difficult, if not impossible, to attribute melanoma outgrowth. While ‘de novo’ lesions were found to have a more unfavourable outcome compared to lesions from PAM or nevi (Shields et al., 2011), we hypothesize that this observation is biased, and that this (in part) can be due to rapid melanoma growth, clinically lacking an obvious precursor lesion. We advocate a thorough clinicopathological correlation, combining data from clinical and histopathological observations, ideally with mapping biopsies. In the absence of these data, we suggest cautious use of the ‘de novo’ terminology and think that a ‘de novo origin’ should be regarded as ‘uncertain origin’.

2.6. UV radiation and CoM

UV radiation is a well-established risk factor for the development of cutaneous melanoma, but has been debated in the development of CoM (Kastelan et al., 2018). Several epidemiological and genetic studies indicate that UV-mediated mechanisms are involved, but the number of studies is small. Since CoM may develop at sites that are not exposed to sunlight, direct UV exposure may not be a necessity, but may be a risk factor.

2.6.1. Mechanisms of UV-mediated damage

Sunlight includes three classes of UV radiation: UVA (320–400 nm), UVB (290–320 nm), and UVC (100–280 nm). UVA (95%) is more abundant than UVB (5%), while UVC is filtered by the atmosphere and hardly reaches the earth’s surface. UVA and UVB have a different capacity to enter tissues, and differentially effect melanoma formation (Budden and Bowden, 2013).

UVB has a direct damaging effect on DNA: photochemical reactions cause the production of cyclobutane pyrimidine dimers (CPD’s) and pyrimidine pyrimidone photoproducts (PP’s) (Shah and He, 2015) at locations where the pyrimidine bases (i.e. cytosine (C) or thymine (T)) are adjacent on the DNA (in sequences of CC, CT, TT, or TC). The presence of a dimer interferes with base pairing during DNA replication, leading to mutations. Both CPD’s and PP’s can be repaired by the nucleotide excision repair (NER) pathway, and dysregulation of NER therefore increases the risk for (cutaneous) melanoma development (Budden and Bowden, 2013).

UVA causes production of reactive oxygen species (ROS), and to a lesser extent of CPD’s (de Grijil, 2002). Recently it was found that melanin in UVA-mediated damage: reactive oxygen and nitrogen species excite electrons in melanin, their energy is transferred to DNA and induces CPD’s, hours after the initial UV exposure (Premi et al., 2015).

In the skin, UVB is predominantly absorbed in the epidermis, while UVA can reach the dermal stroma. The uvea is relatively protected from UV radiation by filtering in the cornea, lens, and other structures: only up to 1% of UV reaches the retina, most of which is UVA (Slimey, 2002). While the bulbar conjunctiva is sun-exposed, the tarsal and fornical conjunctiva are not (Fig. 4).

Based on the mechanism of action, an abundance of C > T and CC > TT mutations is typical for UV-mediated damage; this is called the UV ‘signature’ or ‘footprint’ in cancer development (Plesance et al., 2010). Additional effects of UV radiation on melanoma formation act via the immune system, as UV causes recruitment of macrophages and neutrophils in skin lesions (Bald et al., 2014; Zaidi et al., 2011). The role of these immune cells in melanoma are discussed further in the sections on tumour immunology (chapter 4).

2.6.2. Epidemiological studies

An association between UV radiation and CoM can be derived from epidemiological studies, as was mentioned in section 2.2 (on...
geographical incidence) and 2.3 (on time trends). In short, areas with a lower latitude (i.e. more towards the equator) are related to higher incidences of CoM (Yu et al., 2006), and increased numbers of CoM in recent years are particularly due to lesions of the bulbar (sun-exposed) conjunctiva (Larsen et al., 2016a; Tray et al., 2009). Though vitamin D synthesis (following sun exposure) has been proposed as a protecting factor for cancer (Feldman et al., 2014), in CoM this may be overshadowed by DNA-damaging effects of UV. An Australian study on sun exposure was inconclusive regarding CoM due to low numbers (with only 19 cases reported), but CoM was related to a self-reported history of cutaneous melanoma, which could suggest either a role for sun exposure or a shared genetic susceptibility (Vajdic et al., 2002).

In cutaneous melanoma, intermittent exposure and sunburn are of particular importance for tumorigenesis, but cumulative exposure infers a risk as well (Tucker and Goldstein, 2005); the relation between patterns of sunlight exposure and CoM development is not known.

### 2.6.3. Genetic studies

Early work regarding the role of UV on genetic changes in CoM was limited by the techniques to detect mutations. One of these early studies (targeting mutations in the NRAS gene), found no aberrations in six cases of CoM and concluded on a minor role for UV (El-Shabrawi et al., 1999). The authors recognized, however, that UV may affect the immune system to create an environment that is more prone to melanoma development. A decade later, identification of a UV signature in DNA damage (Plesance et al., 2010) showed direct evidence for UV-mediated mechanisms in CoM. Griewank found TERT promoter mutations in 12/38 (32%) CoM samples, all with the typical UV-related C > T and CC > TT changes (Griewank et al., 2013a). Later work confirmed the presence of this typical UV signature in a genome-wide sequencing study of two CoM (Rivolta et al., 2016) and five CoM (Swaminathan et al., 2017) which were all from bulbar, (i.e. sun exposed) sites analysed by whole-exome sequencing. A recent extension to the work by Rivolta et al. found that in 12/14 (86%) CoM more than 70% of the mutational load consisted of C > T changes, and the three studied CoM with the least C > T changes were tarsal and not bulbar (Cisarova et al., 2020); these tarsal lesions had significantly lower amounts of single nucleotide variants than bulbar lesions. Other work, however, noticed no differences in gene expression of 161 oncology-related genes between 6 sun-exposed and 6 non-exposed CoM, suggesting less influence of UV on genetic profile (Mikelsen et al., 2020). Interestingly, a recent study found a UV signature in (sun-exposed) iris melanoma but not in posterior UM (Johansson et al., 2020), suggesting a spectrum of influence of UV rather than a strict distinction between CoM and UM; this warrants further studies in genetic similarities between CoM and iris melanoma.

The position of BRAF mutations in UV-mediated damage is not well understood, particularly because the most-common BRAF mutation lacks a UV signature (Craig et al., 2018). However, intermittent sun exposure of the skin (compared to either chronically or non-exposed sites) has been related to BRAF mutations (Curtin et al., 2005). Similarly, CoM at sun-exposed bulbar sites more often have BRAF mutations than CoM at non-bulbar sites (Larsen et al., 2016a). The increased frequency of BRAF mutations in cutaneous and conjunctival lesions may therefore be due to UV, with bulbar sites being intermittently sun-exposed; it was suggested that skin melanoma at chronically-exposed sites develops (partially) by other pathways (Curtin et al., 2005). A link between UV, BRAF mutations and melanoma is further observed since a greater exposure to UV radiation during childhood is related to the presence of more acquired nevi of the skin, carrying BRAF mutations, which then constitutes a risk for development of melanoma of the skin (Craig et al., 2018). Similarly, BRAF mutations are more frequently found in CoM lesions that originate from conjunctival nevi (Larsen et al., 2016a), which harbour BRAF mutations as well (section 3.2.1).

In cutaneous melanoma, patients with a higher mutational burden (as seen following UV damage) may be better candidates for immunotherapy (Craig et al., 2018); this should be studied in CoM as well, as the use of immunotherapy is increasing [chapter 5].

### 2.6.4. Sun protection and CoM

A question that is relevant population-wide, is whether the eye needs to be protected from sunlight to diminish the risk for melanoma. Sun-glasses have been suggested to prevent CoM (Rivolta et al., 2016), but it will be hard to study the effects on CoM on a large scale by the rarity of the disease. Even more, as much of the UV that reaches the eye is through reflection, this may hamper good blockade (Slaney, 2002). Eye protection (e.g. with sunglasses) should certainly not be discouraged as it serves several purposes, but the protective effects regarding CoM should not be exaggerated in the absence of evidence.

### 2.7. Melanocytes and melanin

Melanin pigments have a role in the development and behaviour of different types of melanoma. This follows epidemiological data on skin and iris colour in cutaneous melanoma and UM, and is supported by the understanding of UV-mediated and UV-independent mechanisms of melanoma formation. While reports on melanin in the conjunctiva are rare, recent work suggests that tumour pigmentation is related to the behaviour of CoM (Brouwer et al., 2019b), warranting further investigation.

Two main types of melanin pigment are reported in the eye: dark-coloured eumelanin, and light-coloured pheomelanin. The amount and ratio of these pigments determine visible traits, as a low total melanin and relative abundance of pheomelanin cause light skin colour (De Leeuw et al., 2001) and blue iris colour (Wakamatsu et al., 2008), while a high total melanin and abundance of eumelanin causes dark skin colour and brown iris colour. Eumelanin has protective effects on melanoma formation, by shielding against UV radiation (De Leeuw et al., 2001) and scavenging reactive oxygen species (ROS) and free radicals (Brenner and Hearing, 2008); pheomelanin can be involved in DNA damage via UVA (Premi et al., 2015) and by itself via independent ROS formation (Mitra et al., 2012). It has been suggested that melanin also is linked to the efficacy of the immune system, partially via aspects of ROS production, as ROS inhibits CD8 T cell function (Kusmartsev et al., 2004) and stimulates differentiation of macrophages into an M2 type (Zhang et al., 2013), the implications of which are discussed in the chapter on immunology [chapter 4].

Conjunctival, uveal (including iridal), and cutaneous melanocytes are derived from the same embryonic (neural crest) cells (Hu et al., 2007), though they migrate in different waves. Conjunctival and cutaneous melanocytes migrate to the surface ectoderm-derived epithelium and have functional similarities such as being able to transfer melanin to other cells (Hu et al., 2007; Iwamoto et al., 2002); this is in contrast with uveal melanocytes that migrate into deeper mesoderm-derived tissues and do not transfer melanin. Cutaneous melanoma (Olsen et al., 2010) and UM (Houtzagers et al., 2020; Schmidt-Pokrzywińska et al., 2009; Vajdic et al., 2003; Weis et al., 2006) are known to occur more frequently in patients with fair skin and blue irises. No such population-based assessments exist for CoM, but as conjunctival melanocytes of light-iris eyes contain less total melanin and relatively more pheomelanin (Hu et al., 2007), it can be hypothesized that melanocytes in the conjunctiva of patients with light-coloured eyes are more prone to CoM development (Brouwer et al., 2019b). Indeed, as CoM typically occur in countries with an abundance of people with light-coloured eyes, the role of melanin warrants further research.

CoM themselves can present as amelanotic to darkly-pigmented (Fig. 1). Light tumour pigmentation in CoM is related to a worse clinical outcome compared to darker lesions, with a hazard ratio for melanoma-related death of 2.42 (p = 0.020, studied in 444 CoM patients) (Brouwer et al., 2019b), corresponding to observations from cutaneous melanoma. This could be due to direct melanocyte-related
factors (such as the genotoxic/phototoxic effects of pheomelanin, and the absence of UV protection), or due to indirect effects such as late identification, insufficient treatment due to hard-to-detect tumour margins and late observation of recurrences (Fig. 5) (Brouwer et al., 2019b). CoM recurrences are more often lightly pigmented compared to their primary lesion, which could be due to more aggressive melanocytes (lacking pigment production) or treatment-related factors as clinicians may be more meticulous in assessment of melanoma-proven patients (Brouwer et al., 2019c). In contrast to what was seen with primary lesions, degree of pigmentation of recurrences is not related to outcome, however (Fig. 5).

As yet, no relation has been observed for iris colour and clinical outcome in CoM (Brouwer et al., 2019b), nor for skin type and prognosis (Shields et al., 2020).

2.8. Conclusions (Epidemiology and Etiology)

CoM is a rare disease that accounts for 5% of all ocular melanoma. It is most prevalent in Caucasians, and is showing a rising incidence in recent decades. Adding to epidemiological studies, recent genetic work identified UV signatures in CoM, supporting the role of UV in CoM development, and suggesting different aetiologies for tarsal versus bulbar lesions. This is similar to what is seen in cutaneous melanoma. We calculated that, adjusted for tissue size, the incidence of CoM is very similar to that of cutaneous melanoma, and very different from that of UM.

It is promising that awareness of CoM increases worldwide, as improved recognition may cause earlier detection. More extensive knowledge about CoM may help clinicians to apply the appropriate treatment. Further studies are needed to determine whether CoM behaves similarly in all populations, as most current studies originate from North-America and Europe, and the genetic profile of CoM may differ between populations.

Melanin pigments (as a visible trait of melanocytes) have been related to melanoma development in the skin and uvea. A lower metastasis-free survival in CoM lacking visible pigment was observed, suggesting that the presence of pheomelanin, and absence of eumelanin, are unfavourable. Further molecular studies with quantification of melanin are warranted to understand its exact role in CoM biology.

The traditional theory of precursor lesions for CoM (i.e. being derived from PAM, nevi, or de novo) may need revision, as it is often impossible to determine a precursor, and both internal factors (such as genetics, pre-existing lesions, and melanin pigments) and external factors (such as UV radiation) are involved in melanoma development. We advocate to thoroughly study clinical data, histological data, and perform mapping biopsies to determine the origin of a lesion. It may be necessary to be cautious with ‘de novo’ terminology and we urge the use of ‘tumour of unknown origin’ in the appropriate cases.

3. Genetics

3.1. Tumour genetics

The development of cancer is a multistep process that has been portrayed by Hanahan and Weinberg in the ‘hallmarks of cancer’ (Hanahan and Weinberg, 2011). Many of these hallmarks relate to genetics, such as sustained proliferative signalling, evasion of growth suppressors, and resistance to cell death. Simplified, cancer develops from the accumulation of genetic mutations, in addition to several epigenetic processes and interactions with the tumour micro-environment (TME). The TME is discussed in the chapter on tumour immunology (chapter 4); here we elaborate on the genetic background of CoM, the comparison with other types of melanoma, and the implications for newly-developed targeted therapies.

An important concept in tumour genetics is that of proto-oncogenes and tumour suppressor genes. Proto-oncogenes are essentially normal genes, that, when overactive due to a mutation, contribute to malignancy. Tumour suppressor genes have an opposite role: they suppress malignancy in the normal situation, but contribute to it in case of decreased expression or mutational loss. Examples of proto-oncogenes in melanoma biology are B RAF , N RAS , and G N A Q /11; examples of tumour suppressor genes are N F I and P T E N .

Tumour genetics - as well as normal cellular processes - act via pathways: multistep cascades of proteins, enzymes (kinases), and other cellular components that result in a certain function or effect. Two important pathways in (conjunctival) melanoma biology are the ‘MAPK’ (mitogen-activated protein kinase, also known as ‘RAS-RAF-MEK-ERK’) pathway and the ‘PI3K-AKT’ (also known as ‘PI3K-AKT-mTOR’) pathway. Overactivity of these pathways causes cell survival and proliferation. The pathways are highly complex and intertwined, but can be simplified to explain the etiology of CoM, and the mechanisms of targeted therapy (Fig. 6). Via these pathways, we will discuss how the genetic signature of CoM has several similarities to cutaneous and mucosal melanoma, while there are many differences with UM. In chapter 5.2, we will elaborate on newly-developed targeted therapies and discuss the first clinical observations of their application in CoM.

Fig. 5. Kaplan-Meier analysis of metastasis-free survival based on tumour pigmentation. A combined set of Dutch and American CoM patients, of whom data on tumour pigmentation was known, was studied. (a) Patients were categorised by pigmentation of the primary lesion. A significantly worse outcome is shown for patients with low tumour pigmentation (n = 41) compared to high pigmentation (n = 64, p = 0.028). (b) The same group of patients is depicted, but is now categorised by the pigmentation of their recurrences. Outcome is not significantly different for those with recurrences with always high pigmentation (n = 46) compared to those with always low (or variable) pigmentation (n = 71, p = 0.151). (Figure re-used with permission from (Brouwer et al., 2019c)).
3.1. The MAPK pathway

The MAPK pathway consists of the cascade of RAS, RAF, MEK, and ERK (McCain, 2013). RAS is a small G protein, that can be activated by receptor tyrosine kinases (RTKs, a transmembrane protein) following binding by a ligand. RAF activates the cascade of protein kinases RAF, MEK, and ERK. Activated ERK (also known as MAPK) then enters the nucleus to cause expression of several proliferative genes. Mutations can occur throughout the MAPK pathway. Three different RAS genes (NRAS, KRAS, and HRAS) can harbour a mutation, resulting in an activated state. Among the RAF genes, a mutation in BRAF is the most common, resulting in increased kinase activity (Garnett and Marais, 2004). In the most common BRAF mutation, a glutamic acid (presented by ‘E’) substitutes valine (presented by ‘V’) at the 600th amino acid, explaining the mutation terminology V600E (Garnett and Marais, 2004). Valine can be substituted by lysine (presented by ‘K’) as well, resulting in V600K; even more rare substitutions like V600M have been described.

3.1.2. The PI3K-AKT and other pathways

The PI3K-AKT pathway consists of phosphatidylinositol 3-kinase (PI3K), AKT, and mTOR (Davies, 2012). PI3K can become activated by RTKs or RAS, providing a link between the MAPK and PI3K-AKT pathways. PI3K activates the protein kinase AKT, which then activates the kinase mTOR (mammalian target of rapamycin). This is a regulator of cell proliferation and survival. PTEN is a natural inhibitor of PI3K, as it antagonizes its activity. Mutations or loss of PTEN can therefore upregulate PI3K activity.

Two actors that are proximal to the MAPK and PI3K-AKT pathway are NF1 and the RTKs. NF1 is a gene that encodes the neurofibromin 1 protein, an inhibitor of RAS that works through GTPase activity (Kitu and Busam, 2017). Most mutations that occur in NF1 are loss-of-function, causing upregulation of RAS. One of the transmembrane RTKs is KIT (also known as c-KIT). Binding of the KIT ligand stem cell factor causes activation of several downstream pathways including MAPK and PI3K-AKT signalling. KIT has an important role in the function of melanocytes (Meng and Carvajal, 2019). Activating KIT mutations cause increased downstream signalling.

Apart from the MAPK and PI3K-AKT pathway, important actors in melanoma proliferation are the telomeres. Telomeres are end caps at chromosomes, that shorten with cell division to cause a limit in replication (Sugarman et al., 2019). The telomerase reverse transcriptase (TERT) gene encodes a catalytic subunit of the telomerase complex that is involved in preservation of these telomeres. TERT promoter mutations increase TERT expression, allowing for survival (‘immortality’) of malignant cells (van Ipenburg et al., 2020b).

3.2. Mutations in CoM

Most work on genetic mutations in melanoma has been performed on cutaneous melanoma, which is not surprising due to their abundance over other melanoma types. Cutaneous melanoma are often classified according to their mutational status, resulting in groups of BRAF-mutated, NRAS-mutated, and PI3K-AKT and triple-WT (wild type) melanoma (Cancer-Genome-Atlas-Network, 2015). Several studies analysed the presence of mutations in CoM (Table 3), resulting in the idea that the same categorization may apply (Scholz et al., 2018). Recent work suggests that the NF1-mutated group is most frequent in CoM, while the BRAF-mutated group is the largest in cutaneous melanoma, pointing out that differences may exist between the two tumour types (Cisarova et al., 2020).

3.2.1. BRAF mutations

BRAF mutations were first detected in cutaneous melanoma, where they occur in more than half of the cases (Greaves et al., 2013). The most common BRAF mutation in cutaneous melanoma is V600E (73%), followed by V600K (19%) and some sporadic types (<5%), such as V600R, V600M or V600G (Menzies et al., 2012). The frequency of BRAF mutations differs between types of cutaneous melanoma, as they are seen more often in lesions without chronic sun damage (i.e. no or intermittent sun exposure) compared to chronic sun exposure (Curtin et al., 2005).

Mutations in BRAF are observed in about a third of CoM (Griewank et al., 2013b; Larsen et al., 2016a), and similar to cutaneous lesions, the most common BRAF mutation in cutaneous melanoma is V600E (in approximately 80%), followed by V600K (in approximately 20%) (Cao et al., 2016; Griewank et al., 2013b; Larsen et al., 2016a). There may be racial differences in the occurrence of BRAF mutations, as these were less frequently observed in Asians (8%) compared to Caucasians (Sheng et al., 2015), similar to what is observed for cutaneous melanoma (Kim et al., 2015).

While several authors looked for possible associations between BRAF mutations and clinical parameters in CoM, only a few significant relations were found, possibly due to small sample sizes. The largest series of CoM (111 cases with a known BRAF mutation status) showed that BRAF mutations (univaritately) are more common in younger patients, males, lesions with an epibulbar location (compared to non-epibulbar), with absent or mixed pigmentation (compared to dark pigmentation), and lesions with a lower tumour-node-metastasis (TNM) stage (Larsen et al., 2016a). Additionally, BRAF mutations are more often seen in CoM that originate from a nevus than those that originate from PAM (Griewank et al., 2013b; Larsen et al., 2016a).

As no relation has been observed with recurrences, metastasis or survival, the presence of a BRAF mutation is of limited use for prognosis in CoM (Griewank et al., 2013b; Larsen et al., 2016a).

During the time period 1960 to 2012, the percentage of CoM with a BRAF mutation has not increased, while this had been expected due to an increase in the number of bulbar CoM (Larsen et al., 2016a); however, it may be that both demographic changes as well as an increased exposure to sunlight influenced the number of CoM, resulting in more
gesting that other factors are involved in pathway activation (e.g., status was not the sole predictor of MEK, ERK and AKT signalling in CoM tissue (Cao et al., 2016; El Zaoui et al., 2019), implying that the exposure and presented in more detail in section 2.6.3. Importantly, the

Any mutation in RAS genes was observed in 12/71 (17%) of cases; NRAS mutations comprised 8/71 (11%), KRAS mutations 4/71 (6%) (Cosgarea et al., 2017).

Note the marked difference in KIT frequency between CSD and non-CSD lesions (Curtin et al., 2006).

In contrast to many other studies, this work includes an Asian population (Sheng et al., 2015).

Any mutation in GNA11, consisting of Q209 in 73/163 (44.8%), and R183 in 4/145 (2.8%) (Van Raamsdonk et al., 2010).

Any GNA11 mutation in 34%, consisting of Q209 in 52/163 (31.9%), and R183 in 3/145 (2.1%) (Van Raamsdonk et al., 2010).

The abundance of BRAF mutations in conjunctival nevi parallels that of nevi of the skin, where a

Any mutation in NF1 was observed in 13/33 (39%) (Koopmann et al., 2014b) (El Zaoui et al., 2019).

The abundance of BRAF mutations in conjunctival nevi parallels that of nevi of the skin, where a

Any mutation in NF1 was observed in 13/33 (39%) (Koopmann et al., 2014b) (El Zaoui et al., 2019).

The abundance of BRAF mutations in conjunctival nevi parallels that of nevi of the skin, where a study reported these in 82% (Pollock et al., 2003).

BRAF mutations are rare in PAM, either with or without atypia, and most studies observed no BRAF mutations in PAM at all (Cao et al., 2016; Goldenberg-Cohen et al., 2005) (Table 4). One report noted a BRAF mutation in 2/8 PAM lesions (with atypia), but these PAM were selected for later outgrowth of CoM, which may indicate that these have represented melanoma in situ (Larsen et al., 2016a).

Most often a pre-malignant lesion has the same BRAF status as its CoM outgrowth: in 19 out of 20 pairs (12 nevi, 8 PAM) the BRAF status concurred (Larsen et al., 2016a). However, heterogenous lesions can
occur, as one nevus (BRAF mutated) later recurred into a melanoma without BRAF mutation, possibly indicating outgrowth of a specific strain of cells. These sequential differences have also been reported for CoM lesions and their recurrences, occurring years later (Gear et al., 2004).

BRAF mutations are less common in mucosal melanoma (other than CoM), with a likely frequency of 4–8% (Cosgarea et al., 2017; Zebary et al., 2013). BRAF mutations are not seen in UM of the choroid and ciliary body (Greaves et al., 2013; Spendlove et al., 2004; Van Raamsdonk et al., 2009), although they have been described recently in iris melanoma in 1/30 cases (3%) (van Poppelen et al., 2018).

3.2.2. NRAS mutations

In cutaneous melanoma, mutations in BRAF and NRAS are generally mutually exclusive, with fewer than 1% carrying both (Krauthammer et al., 2015). NRAS mutations occur less frequently than those in BRAF, with a frequency in cutaneous melanoma of 12–27% (Beadling et al., 2008; Heppt et al., 2017a; Houben et al., 2004; Krauthammer et al., 2015).

In CoM, NRAS mutations are also mutually exclusive with BRAF (Griewank et al., 2013b), and occur with a frequency of 0–18% (Beadling et al., 2008; Griewank et al., 2013b). NRAS makes up almost all mutations in RAS genes in CoM; activating mutations in KRAS are rare, those in HRAS (with unknown consequences) are reported hardly at all (Scholz et al., 2018). Though NRAS mutations are common in conjunctival nevi, with 39% (Francis et al., 2018), their occurrence in PAM is unknown. There is no clear association between tumour origin and CoM NRAS status (Scholz et al., 2018).

NRAS mutations are seen in 11–24% of mucosal melanoma other than CoM (Beadling et al., 2008; Cosgarea et al., 2017; Zebary et al., 2013). NRAS mutations have not been reported in posterior UM (Cruz et al., 2003; Van Raamsdonk et al., 2009), although (similar to what is seen for BRAF) they may be encountered in iris melanoma (reported by one study in 3/10 cases (van Poppelen et al., 2018)).

3.2.3. NF1 mutations

In cutaneous melanoma, NF1 mutations are common (occurring in 12–14%) being the third most frequent mutation after BRAF and NRAS; often NF1 co-occurs with either of these (Cancer-Genome-Atlas-Network, 2015; Krauthammer et al., 2015). NF1 mutations in the skin are associated with UV exposure (Krauthammer et al., 2015) and NF1-mutated cutaneous melanoma have a high mutational load (compared to BRAF/RAS/triple-WT) (Cireniajwis et al., 2017), which may imply that they are more sensitive to immunotherapy (as was shown for anti-PD-1 therapy in cutaneous melanoma patients (Johnson et al., 2016)).

In cutaneous melanoma, patients with NF1-mutated lesions have a worse survival than those with BRAF/RAS mutations (Cireniajwis et al., 2017). All of this makes NF1 an interesting gene.

NF1 mutations are indeed frequently observed in CoM and occur in 33% (Scholz et al., 2018), however (possibly due the smaller numbers studied) they are not associated with clinical characteristics or prognosis (Scholz et al., 2018). To our knowledge, NF1 status is unreported in precursors of CoM.

In mucosal melanoma, NF1 mutations are readily observed with a frequency of 18–37% (Cosgarea et al., 2017; Hintzschke et al., 2017). NF1 mutations are commonly absent in UM, but deletion of the NF1 locus was reported in one tumour in a study on 38 cases of UM (3%) (Foster et al., 2005).

3.2.4. KIT mutations

KIT mutations are rare in cutaneous melanoma: the incidence varies for anatomic location however, as total absence is reported in non-chronic sun-damaged (CSD) melanoma, and up to 28% in CSD melanoma (Curtin et al., 2006). Commonly, KIT mutations are mutually-exclusive with BRAF and NRAS.

In a composite of four studies on CoM, only 1 in 68 cases (1%) demonstrated a KIT mutation (Alessandrini et al., 2013; Beadling et al., 2008; Griewank et al., 2013b; Wallander et al., 2011), demonstrating similar rarity. Immunohistochemical staining of KIT occurs in about 50% of CoM, but this showed no correlation to mutational status (Alessandrini et al., 2013). KIT mutations are seen more often in CoM in Asians (11%) compared to Caucasians (Sheng et al., 2015), which concurs with the finding of a lower BRAF frequency. KIT mutations are absent in conjunctival nevi, and rarely seen in PAM with atypia (Alessandrini et al., 2013), however little data are available.

KIT mutations are more common in acral and mucosal melanoma (23 and 16%, resp. (Beadling et al., 2008), and some studies even report up to 40%. While they were initially unreported in UM (Beadling et al., 2008), later reports found up to 9% in choroidal lesions (Wallander et al., 2011), and 7% in iris melanoma (van Poppelen et al., 2018).

3.2.5. PTEN mutations (PI3K/akt)

An important mediator of the PI3K/akt/mTOR pathway is PTEN, that acts as an inhibitor. PTEN’s function is partially determined by its location in the cell: nuclear (instead of cytoplasmic) PTEN has a tumour suppressive role; loss of PTEN therefore stimulates tumour formation. PTEN loss is often mutually exclusive with NRAS mutations (and thus concurrent with BRAF mutations).

PTEN loss is commonly observed in cutaneous melanoma (65%) (Tsao et al., 2003), and recent work showed that it was associated with
worse survival, possibly by helping immune evasion (Cabrita et al., 2020). PTEN loss has been described in CoM as well, but apart from a relation with more pigmentation, it was not related to other characteristics or prognosis in 70 lesions (Westekemper et al., 2011). Several other mTOR-related proteins in CoM were associated with a high mitotic rate and thicker lesions, however (Populo et al., 2010). Nuclear PTEN loss was observed more frequently in CoM than in conjunctival nevi (Westekemper et al., 2011), suggesting an important role in melanoma development. This was similarly seen in cutaneous melanoma versus nevi (Tsoa et al., 2003).

A comparative study between CoM and UM showed that mTOR effectors were higher in CoM, and that UM showed a higher PTEN expression (Populo et al., 2010). PTEN loss in UM (of unreported anatomical location) was reported in 12/75 cases (16%) (Abdel-Rahman et al., 2006), and PTEN mutations were observed in 3 out of 30 (10%) iris melanoma (van Poppelen et al., 2019).

3.2.6. TERT promoter mutations

TERT promoter mutations (further referred to as ‘TERT mutations’) are found in approximately 30% of primary cutaneous melanoma lesions (Horn et al., 2013; Vinagre et al., 2013). TERT mutations are reported more often in older patients, and their frequency may vary based on tumour location, resulting in studies reporting up to 68% in primary cutaneous melanoma (Hugdahl et al., 2018). Increased TERT activity relates to worse prognosis in cutaneous melanoma, but the role of either TERT mutations or TERT expression (not necessarily coinciding) is unclear (Hugdahl et al., 2018).

Very similar to the observed rates in the skin, TERT mutations are present in 32–43% of primary CoM (Griewank et al., 2013a; Koopmans et al., 2014a; van Ipenburg et al., 2020b). In two studies, analysing 38 and 39 CoM lesions, no relation was found between TERT mutation status and clinical parameters or outcome (including patient age, tumour size, location, recurrences, survival) (Griewank et al., 2013a; Koopmans et al., 2014a). The absence of a relation between TERT mutations and tumour location is remarkable, as TERT mutations in CoM show C > T or CC > TT nucleotide changes (Koopmans et al., 2014a), demonstrating a UV signature (Pleasance et al., 2010), and one would expect these to occur mainly in bulbar conjunctiva. A recent, and larger, study with data of 47 CoM showed that presence of a TERT mutation correlated with metastatic disease, emphasizing an importance for therapeutic decision making (van Ipenburg et al., 2020b).

TERT mutations are not found in conjunctival nevi or PAM without atypia (Koopmans et al., 2014a). A small number, 2/25 (8%), of PAM with atypia carried the mutation, however (Koopmans et al., 2014a). This may indicate an important distinction between benign and malignant lesions, equally to what is observed in cutaneous melanoma where melanoma and melanoma in situ have TERT mutations, but benign precursors not. A change in TERT may be an important early step in melanoma transformation, occurring, however later than the BRAF mutation (Shain et al., 2015).

TERT mutations are rare in mucosal melanoma, with a range of 6–8% (Cosgarea et al., 2017; Jangard et al., 2015). Presumably, this relates to the tumour locations, often lacking exposure to UV. Similarly it may be no surprise that TERT mutations are very rare in UM: several studies report on a total absence (Griewank et al., 2013a; Vinagre et al., 2013), but they are found sporadically (in 1/102 cases (Koopmans et al., 2014a), and 1/50 cases (Dono et al., 2014)).

3.2.7. Other mutations: GNAQ/11 and BAP1

An analysis of genetics of CoM shows its distinction from the most common ocular melanoma: UM. Important genes in UM biology are GNAQ/11, BAP1, SF3B1 and EIF1AX (extensively reviewed by Smit et al. (2019)). Knowledge of these genes is relevant for CoM to understand a link with possible melanoma predisposition syndromes, and to identify the origin of unknown (secondary) conjunctival lesions based on tumour genetics, e.g. differentiating CoM from intraocular melanoma that has perforated the sclera.

GNAQ/11 signalling activates several pathways in cancer including MAPK (Van Raamsdonk et al., 2009), and Yap1 (Feng et al., 2014; Yu et al., 2014). The GNAQ/11 gene is mutated in nearly all UM (Van Raamsdonk et al., 2009, 2010) and mutations are seen already in uveal nevi (Vader et al., 2017). GNAQ/11 mutations are absent in CoM (Griewank et al., 2013b), other mucosal melanoma (Mikkelsen et al., 2020), conjunctival nevi and PAM (Dravtivman-Storobinski et al., 2010; Francis et al., 2018). A GNAQ mutation was reported in two cases of conjunctival blue nevi, however, indicating a different cellular origin than the common epithelial nevus (Francis et al., 2018). GNAQ/11 mutations are absent in cutaneous melanoma, but individual cases have been reported in chronically sun-damaged skin (Van Raamsdonk et al., 2010).

Other mutations in UM that are important for tumour progression occur mainly in three genes: BAP1, SF3B1, and EIF1AX, which were reported in 43%, 26%, and 21% of primary UM, respectively (Smit et al., 2019). Mutations in BAP1 are related to the worst prognosis and these tumours often metastasize within a few years (Yavuzyigitoglu et al., 2016). BAP1 immunohistochemistry (IHC) is nowadays commonly applied to assess BAP1, as a prognostic factor in UM (Koopmans et al., 2014b).

BAP1 (located on chromosome 3) was discovered a decade ago (Harbour et al., 2010), with the BAP1 protein as a deubiquitinating hydrolase with several functions such as protein deubiquitination, cell cycle regulation, DNA damage repair, and regulation of gene expression (Carbone et al., 2013); loss of BAP1 expression has been linked to increased inflammation (Gezgin et al., 2017) and angiogenesis in UM (Brouwer et al., 2019a), but much about its function remains to be unveiled.

Different from what is seen in posterior UM, BAP1 mutations sporadically occur in iris melanoma (3%) (van Poppelen et al., 2018) and are uncommon in melanomas other than UM. They are practically absent in acral, mucosal, and cutaneous melanoma, though they may occur in cutaneous melanoma lacking chronic solar damage (Wiesner et al., 2011). Remarkably however, only few studies exist on sporadic BAP1 mutations in cutaneous melanoma. Recent work on TCGA data showed that the prognostic effect of BAP1 mRNA expression was opposite for UM and cutaneous melanoma, suggesting differential roles (Liu-Smith and Lu, 2020). Even so, BAP1 loss is not observed in conjunctival lesions, and its status is not commonly assessed (Swaminnathan et al., 2017).

3.3. Chromosomal aberrations

Chromosomal copy number alterations (CNAs) are relevant to tumourigenesis as they influence the function of locally encoded genes. Though few reports exist on the topic, a plethora of CNAs has been reported for CoM, indicating complex karyotypes. Gains have been reported in chromosomes 1q, 3p, 6p, 7, 8q, 10q, 11p, 12p, 13q, 14p, and 17q, and losses in chromosomes 1p, 3q, 4q, 6p, 8q, 9, 10, 11q, 12q, 13, 15p, 16, 17p, 19, and 22 (Cisarova et al., 2020; Griewank et al., 2013b; Kenawy et al., 2019; Lake et al., 2011; Swaminathan et al., 2017; Vajdic et al., 2003a). The most frequently reported CNA is 6p amplification, which has been reported in up to 61% of CoM (Kenawy et al., 2019).

The observed CNAs in CoM resemble those in cutaneous melanoma (Curtin et al., 2005). And similar to what is seen in cutaneous melanoma (Gust et al., 2010), CNAs in CoM were observed more frequently in BRAF/NRAS-wildtype tumours (Griewank et al., 2013b). CNAs in CoM are distinct from observations in UM (Griewank et al., 2013b) where loss of chromosome 3 (which includes the BAP1 gene) occurs frequently and is related to the development of metastases (Harbour et al., 2010). Other alterations that are frequently observed occur in chromosomes 8q and 6 reviewed by (Jager et al., 2020).

Most of the reported CNAs in CoM have no relation with clinical
parameters or prognosis. A recent study identified that deletion of chromosome 10q was related to the presence of \textit{BRAF} mutations, increased tumour thickness, metastasis development, and lymph invasion (Kenawy et al., 2019). Genes encoded by the 10q region are \textit{SUFU}, \textit{NEU1L1}, \textit{PDCD4}, and \textit{C10orf90} (all of which are tumour suppressor genes). The work by Kenawy et al., studying 59 lesions, shows the relevance of CNAs in CoM when assessing a relatively large cohort (Kenawy et al., 2019); multicentre projects such as these are therefore essential to obtain sufficient numbers.

### 3.4. Predisposition syndromes

Several genetic disorders or syndromes exist that predispose to the development of cancer and melanoma, e.g. the Familial atypical multiple mole melanoma (FAMMM) syndrome and the \textit{BAP1}-tumour predisposition syndrome. To our knowledge, no such syndromes have been identified for CoM, possibly due to the rarity of this disease. It is likely however, from melanocyte biology, that certain syndromes that are associated with cutaneous melanoma, relate to development of CoM as well. Further studies are warranted, to identify patients at risk of CoM, and to optimize guidelines for screening.

A well-known melanoma syndrome is the FAMMM syndrome, also known as ‘dysplastic nevus syndrome’, which is associated with an increased risk for dysplastic nevi and cutaneous melanoma (Soura et al., 2016). Despite suggestions from earlier reports (Bataille et al., 1993), more recent insights show that individuals with FAMMM do not have an increased risk for developing conjunctival pigmented disease (including melanoma) compared to others in the population (Seregard et al., 1995). Small numbers limit the strength of conclusions however.

Neurofibromatosis type I (von Recklinghausen disease) is a genetic disorder, caused by a loss-of-function mutation in the (tumour suppressor) \textit{NF1} gene (Seminog and Goldacre, 2013). Abnormal function of the neurofibromin protein increases RAS activity, resulting in development of several benign and malignant tumours. From this biology, it is no surprise that an increased risk for cutaneous melanoma has been reported (Seminog and Goldacre, 2013), although others state that the risk is not above chance (Guillot et al., 2004), and that sampling bias is a major concern. Several reports exist of CoM in patients with NF1-disease, but rarity of both conditions limits a conclusion on chance (Medina Mendez and Singh, 2014; Rubinstein et al., 2015; Stacy et al., 2010; To et al., 1989). It remains controversial whether NF1-disease predisposes to UM (Honavar et al., 2000).

The nevus of Ota (oculodermal melanocytosis) is a congenital pigmented condition of the periorbital area, which includes the skin, sclera, uvea and orbit. It was estimated that the lifetime risk for UM development in this condition was 1:400 (Singh et al., 1998), which is clearly above chance alone, and once UM develops, these patients have an increased risk for metastases (Shields et al., 2013). Several cases of cutaneous melanoma have been reported in relation to a nevus of Ota (Patel et al., 1998), including detrimental melanoma with orbital invasion (Radhadevi et al., 2013), but it has not been concluded that there is a true increased risk. Despite an apparent involvement of the ocular surface, we are not aware of reports on CoM associated with an ocular nevus of Ota. Pigmentation in nevi of Ota is not conjunctival however, and mutations in \textit{GNAQ} can be found (Van Raamsdonk et al., 2009), explaining a relation to blue nevi and UM rather than to cutaneous melanoma or CoM.

The \textit{BAP1} tumour predisposition syndrome leads to increased risks for several malignancies, including UM (Abdel-Rahman et al., 2011) an to a lesser extent cutaneous melanoma (Wiesner et al., 2011). There has been a report on a patient with the \textit{BAP1} tumour predisposition syndrome and a conjunctival melanoma, who later developed a cutaneous melanoma; unfortunately no molecular testing or \textit{BAP1} staining was performed on the conjunctival lesion, questioning its true origin as primary conjunctival or metastatic lesion (Chau et al., 2019).

### 3.5. miRNA

Micro-RNA (miRNA) is a class of small non-coding RNA that can regulate gene expression post-transcriptionally (Gajos-Michniewicz and Czyz, 2019). Conceptually, they can affect the expression of proto-oncogenes and tumour suppressor genes, and can serve as potentially diagnostic and prognostic markers, and as targets for therapy. Often, miRNA have multiple targets, making it difficult to study individual effects. A plethora of miRNA have been identified in cutaneous melanoma, emerging as a new field in oncology (Gajos-Michniewicz and Czyz, 2019).

The first analysis of miRNA in CoM was presented by Larsen et al. (2016a). Studying 37 lesions, they found 25 miRNA that were differentially expressed between CoM and normal conjunctiva; several were concordant with miRNA known from cutaneous melanoma, while none were observed that had previously been associated with UM. Clustering based on seven miRNA showed that low, intermediate and high expression related in ascending order to increased CoM tumour thickness, but a true prognostic value was limited as only two miRNA related to recurrences, and none to development of metastasis.

Later work from the same group by Mikkel森 et al. analysed 13 CoM with paired metastatic lesions, and 25 CoM lesions that did not develop metastasis during a follow up of at least 5 years (Mikkel森 et al., 2019). MiRNA were identified that showed a differential expression between non-metastatic and metastatic CoM, between CoM and its coupled metastasis, and between CoM and normal conjunctiva. Interestingly, pathway analysis of the involved miRNA showed that the hippo pathway and p53 were involved in the differentiation of normal conjunctiva to CoM. Unfortunately, there was a poor correlation between the array data and qPCR validation, implying that results need to be confirmed.

Ipenburg et al. studied 20 CoM and 6 conjunctival nevi, and validated the results in 19 CoM and 13 conjunctival nevi from another institution (van Ipenburg et al., 2020a). They identified five miRNA’s (out of the 377 studied) that showed increased levels in CoM versus conjunctival nevi, and found that the homeobox gene clusters constituted a possibly shared pathway. No differences were found between lesions with or without metastases and no relation with clinical characteristics was reported. As an advantage of miRNA analysis, Ipenburg noted that miRNA testing may be used in cases with only very little available tissue, making it into a potential classifier to differentiate between a nevus and a melanoma.

### 3.6. Conclusions (Genetics)

Genetic mutations in CoM follow the same pattern as cutaneous and other mucosal melanoma. Frequently-observed mutations are those in \textit{BRAF}, \textit{NRAS}, \textit{NF1}, and \textit{TERT}. \textit{KIT} mutations are rare but may relate to subgroups in non-Caucasians. Mutations in CoM combine the patterns from skin lesion with chronic sun damage (CSD) as well as non-CSD, being most like intermittently sun-exposed cutaneous melanoma. Although both are ocular tumours, CoM are genetically very different from UM, with mutations usually occurring in \textit{GNAQ}/11 or \textit{BAP1}.

Little is known about tumour predisposition syndromes and CoM development. It may be expected that the FAMMM syndrome and Neurofibromatosis type 1 are related to CoM development, but the rarity of both limits the chance of finding a CoM in a patient with NF.

The diagnostic value of tumour genetics is currently confined to specific cases. The mutational profile may differentiate primary CoM or cutaneous melanoma from UM (and its metastases); differentiating a primary CoM from a lesion with a cutaneous origin is genetically difficult. Recent studies on miRNA show that expression profiles may help to differentiate benign from malignant conjunctival lesions, but this requires further validation.

The value of genetics for prognostic purposes in CoM is currently limited. Recently, \textit{TERT} mutation presence was identified as an important factor (van Ipenburg et al., 2020b), as was loss of chromosome 10q.
inhibitor therapy, for selected patients. Although BRAF status is currently not predictive of outcome in CoM, it may become a prognostic factor in the future now that patients with BRAF mutations can receive targeted treatment.

4. Immunology

4.1. Tumour immunology

The human immune system is of paramount importance for tumour growth and control. Inflammation is therefore considered one of the hallmarks of cancer (Colotta et al., 2009; Hanahan and Weinberg, 2011). The immune system may inhibit tumour growth by killing tumour cells, but may also provide cytokines and chemokines that stimulate growth and tumour spreading. Tumours, on their turn, may use mechanisms to prevent attack by the immune system.

An important part of the specific immune response against tumour cells is played by Cytotoxic T cells (CTLs, also referred to as ‘effector T cells’). These cells kill tumour cells when they recognize a specific antigen, presented via HLA Class I on the tumour cell membrane (Antohe et al., 2019). Tumours may learn to escape the killing effects of these T cells by loss of expression of HLA molecules, causing decreased recognition. Another escape mechanism acts via immune checkpoints, i.e. molecules that naturally help to diminish the activity of CTLs, preventing auto-immunity. It was recently discovered that these checkpoints can be blocked, leading to a new class of drugs (i.e. immune checkpoint inhibitors). For instance, the immune checkpoint inhibitor ipilimumab, an IgG monoclonal antibody that blocks cytotoxic T lymphocyte-associated protein 4 (CTLA4) (Hodi et al., 2010). Later, in 2014, monoclonal antibodies against Programmed cell death protein 1 (PD-1) followed, when nivolumab and pembrolizumab were approved by the FDA (Robert et al., 2015b, 2015c). The 2018 Nobel Prize in Physiology or Medicine was awarded to Dr James P. Allison and Dr Tasuku Honjo for their discovery of CTLA4 and PD-1, respectively.

In this section, we discuss the current knowledge on infiltrating immune cells and expression of immunologic markers in conjunctival melanoma (CoM), and relate it to relevant observations from cutaneous and uveal melanoma (UM). In chapter 5.3, we elaborate on newly developed checkpoint inhibitor therapies and discuss the first clinical observations of their application in CoM.

4.2. Infiltrating lymphocytes and macrophages

4.2.1. Cell types of tumour infiltrate

The tumour micro-environment may contain a wide range of immune cells which play a role in the innate (non-specific) and the adaptive (specific) immune responses. Two main types of cells can be identified: histiocytes (i.e. macrophages and dendritic cells) and lymphocytes (i.e. B cells, T cells, and NK cells).

Macrophages are monocytes that originate in the bone marrow and circulate in the blood, until they are recruited to specific sites by chemokines. They have a role in protection against infections and in wound healing, through the production of various growth factors and cytokines (Coussens and Werb, 2002). Macrophages can be of an M1 or M2 subtype (though this should be considered as a spectrum), with different receptors, effector functions and chemokines (Mantovani et al., 2002). M1-type macrophages target infectious diseases and can kill bacteria; they express high levels of pro-inflammatory cytokines, such as IL-12 and tumour necrosis factor (TNF). M2-type macrophages have an anti-inflammatory, pro-angiogenic, tissue remodelling role (and produce IL-10). Differentiation follows in response to microbial agents or exposure to cytokines such as interferon (IFN)-gamma (Mantovani et al., 2002). While the total number of macrophages can be determined through marker CD68, the M2 type macrophages are commonly identified by double staining with monoclonal antibodies against CD68 as well as CD163. Tumour-associated macrophages (TAM’s) are mainly of the M2 type (Mantovani et al., 2002), which was demonstrated in cutaneous melanoma, CoM (Cao et al., 2017), as well as in UM (Bronkhorst et al., 2011). M2 type macrophages are notably linked to increased tumour angiogenesis in several melanomas (Bronkhorst et al., 2011; Makite et al., 1999; Storr et al., 2012), where vessels serve to supply nutrients as well as provide a dissemination route for metastases.

Similar to macrophages, dendritic cells (DCs) are part of the antigen-presenting cell family; they play a major role in the initiation and regulation of immunological processes. They induce a distinct response by cross-presenting antigens to both CD8+ and CD4+ cells, and can activate NK cells. DCs go through a maturation process (Antohe et al., 2019), but this will not be further discussed in this chapter.

Lymphocytes comprise a group of cells with various functions. B cells are known to produce antibodies, which help protect against infections (LeBien and Tedder, 2008). Antibodies have a great target specificity, and B cells may support immune responses of other cells, such as T cells. Several different T cell types can be identified, such as the already mentioned CTLs, which express marker CD8. These CD8+ T cells can kill tumour cells and inhibit tumour growth by releasing IFN-gamma and TNF-alfa. Another important group of T cells is made up of T helper cells (Th) that express CD4. This group consists of several subtypes: Th1 and Th2 help the anti-tumour response by stimulating CD8+ T cells via the production of IFN-gamma, Transforming Growth Factor (TGF)-beta and IL-2 (Zhu and Paul, 2008). Th1 cells can activate macrophages and help with the maturation of dendritic cells (Antohe et al., 2019). T regulatory cells (Tregs) are a specific subclass of Th cells that suppress immune responses. They express the protein forkhead box P3 (FoxP3). Normally they have a role in maintaining immunologic self-tolerance and preventing autoimmunity. In cancer, they may inhibit the anti-tumour action of other T cells (Corhoy, 2009). A cell type with a broader reactivity is the Natural Killer (NK) cell. NK cells are able to kill cells that lack HLA Class I expression, or that express NK-activating ligands (Ljunggren and Karre, 1990). They are the effector cells of the innate immune system, but also interact with adaptive responses of the T and B cells (Antohe et al., 2019).

4.2.2. Tumour infiltrate in CoM

The first reports on inflammation in CoM date back to the second half of the 20th century (Crawford, 1980; Jay, 1965). Using regular histopathological examination, the presence of inflammatory cells was studied. Inflammatory cells could be divided into lymphocytes (small cells with a large nucleus) and macrophages, which have a large nucleus and ample cytoplasm, and often contain pigment (i.e. melanophages). It was soon recognized that infiltrate could be analysed for its prognostic value, analogous to findings from cutaneous melanoma (Clark et al., 1985); Folberg et al., 1985a) (Table 5).

The amount of infiltrate demonstrated quite some variability. In early studies, absence of infiltrate was reported in 0–51%, and several subjective grading scales were used to describe the infiltrating cells. Jay reported on a considerable set of 73 cases of CoM, noting no infiltrate in 13 cases (18%), few cells in 17 cases (23%), moderate numbers in 26 cases (36%) and numerous cells in 17 cases (23%) (Jay, 1965).

Several studies found no relation between the presence of infiltrate in CoM and prognosis (Jay, 1965; Jeffrey et al., 1986; McGhee et al., 1982). Others did find such an association (Crawford, 1980), including Folberg, who studied the thickest known lesion of each patient (which could
Anastassiou et al. observed variable amounts of infiltrating CD3+ T cells in the same era, used an IHC approach in a large set of 98 CoM (Anastassiou et al., 2004). CD68+ macrophages were present in almost all cases (in 33/34 (97%) of evaluable CoM (Anastassiou et al., 2004). CD68+ macrophages were lower; the cause of this is unknown. Just as in UM and other studies on UM, CoM contains higher densities of CD4+/CD8- lymphocytes (Jay, 1965) (Cao et al., 2017) (Fig. 7). All samples demonstrated infiltrate, again in varying amounts. Epibulbar (or cT1) lesions showed higher numbers of CD3+ cells in non-bulbar location than UM, but densities of CD8+ T cells and macrophages were lower; the cause of this is unknown. Just as in UM and cutaneous melanoma, the majority of macrophages in CoM were of the M2 type (Cao et al., 2017).

The number of infiltrating cells was not related to gender, age, thickness, location, and extent of the tumour or its growth pattern (Crawford, 1980). The number of infiltrating cells was not related to gender, age, thickness, location, and extent of the tumour or its growth pattern (Crawford, 1980). However, the number of infiltrating cells was related to the number of infiltrating cells, again in varying amounts. Epibulbar (or cT1) lesions showed higher numbers of CD3+ cells in non-bulbar location than UM, but densities of CD8+ T cells and macrophages were lower; the cause of this is unknown. Just as in UM and cutaneous melanoma, the majority of macrophages in CoM were of the M2 type (Cao et al., 2017).

Some studies report on the importance of ratios between infiltrating cells: a high CTL/Treg and M1/M2 ratio was related to improved survival in cervical cancer and cutaneous melanoma (Jordanova et al., 2008). A decade later, Cao et al. continued on this work and studied the infiltrate of T cells and macrophages in 27 CoM using immunofluore-scence (Cao et al., 2017) (Fig. 7). All samples demonstrated infiltrate, again in varying amounts. Epibulbar (or cT1) lesions showed higher numbers of CD3+CD8+ (i.e. Th) cells compared to non-bulbar (or cT2) CoM. The number of infiltrating cells was not related to gender, age, recurrence, metastasis or survival. There was an inverse relation between lesion thickness and numbers of CTLs (CD3+CD8+) and M2 macrophages (CD68+CD163+), which corresponded to the findings of Tuomaala. Cao additionally observed an inverse relation between largest basal diameter (LBD) and all types of lymphocytes. It was hypothesized that in the absence of infiltrate, including CTL’s, CoM can grow unrestrained.

Cao noted that, using the same antibodies and techniques as in prior studies on UM, CoM contains higher densities of CD4+ overall, CD4+ Th, and CD4+ Treg cells than UM, but densities of CD8+ T cells and macrophages were lower; the cause of this is unknown. Just as in UM and cutaneous melanoma, the majority of macrophages in CoM were of the M2 type (Cao et al., 2017).

### Table 5

Studies reporting on immune infiltrate in CoM. The separation between early and later work is based on the development of immunohistochemical staining techniques.

| Study | n | Infiltrate (cell type and technique) | Presence of infiltrate | Relation with Characteristics | Relation with Survival |
|-------|---|-------------------------------------|------------------------|-------------------------------|------------------------|
| **“early work”** | | | | | |
| Jay (1965) | 73 | (no methods or cell types specified) | none n = 13 (18%), few n = 17 (23%), moderate n = 26 (36%), numerous n = 17 (23%) | No data reported | No sign. relation with survival |
| Crawford (1980) | 19 | (H&E) “most were lymphocytes” | no cells at base: 0 (0%), few at base: 6 (32%), moderate at part of base: 4 (21%), moderate at whole base: 6 (32%), intense at whole base: 3 (16%) | No data reported | Presence has better survival, |
| McGhee et al. (1982) | 28 | (no methods or cell types specified) | mild: 19 (68%), moderate: 7 (25%), severe: 2 (7%) | No data reported | No relation with survival |
| Folberg et al. (1985a) | 98 | (no methods or cell types specified) | [First lesion] lack = 47%, present = 53% | No data reported | No sign. relation with survival |
| Jeffrey et al. (1986) | 37 | (H&E) | Not reported | No data reported | Presence has better survival, |
| Bobic-Radovanovic et al. (1998) | 61 | (no methods or cell types specified) | Not reported | No data reported | No relation with survival |
| **“later work”** | | | | | |
| Anastassiou et al. (2004) | 32 | (IHC) CD3+ | No: 6 (19%), few: 14 (44%) mod: 10 (31%), strong: 2 (6%) | No data reported | No relation with survival |
| Tuomaala et al. (2007) | 60 | (H&E) Lymphocytes | No: 1 (3%), few: 12 (35%) mod: 15 (44%), strong: 6 (18%) | No data reported | No relation with survival |
| Cao et al. (2017) | 27 | Immuno-fluorescence, (median [range]) | CD3+ CD8+: 68 [26–335] | No relation with rec./survival | No relation with rec. & survival |
| Lassalle et al. (2020) | 60 | IHC CD8+ | No: 6 (10%) | More in non-epithelioid cell types, non-bulbar location | No relation with rec. & survival |

Abbreviations: Rec, recurrences; M2, M2-type macrophages; H&E, haematoxylin and eosin; IHC, immunohistochemistry.
In Cao’s work on CoM, however, these ratios did not relate significantly to survival or recurrences.

Major historical work on infiltrate in cutaneous melanoma was performed by Clark et al. (1969), who introduced the term tumour-infiltrating lymphocytes (TILs) and proposed the traditional classification system of TILs as being ‘absent’ (absent or not infiltrating), ‘non-brisk’ (i.e. focal presence) or ‘brisk’ (i.e. present at the base of lesions, or diffuse intratumourally) (Clark et al., 1989). [An illustrative review on the history of TIL research was presented by Mihm et al. (Mihm and Mule, 2015)].

Clark noted that the absence of TILs was related to a worse survival in cutaneous melanoma (Clark et al., 1989). Later studies were contradictory: immune infiltrate was often related to unfavourable tumour characteristics, but not uniformly to worse survival (Azimi et al., 2012; Taylor et al., 2007).

Possibly, these discrepancies can be explained by characteristics of...
study groups. It has long been reported that TILs have impact in the vertical growth phase, but not in the (earlier) radial growth phase (Clark et al., 1989), limiting conclusions in studies that include smaller, radial growth phase, lesions. Even so, the impact of TILs may be more clear in thicker lesions, despite thicker lesions having lower TIL numbers in general (Taylor et al., 2007).

When looking at Tregs specifically, it was observed that a high FoxP3 expression was associated with worse survival in 185 primary cutaneous melanoma patients, independent of lesion thickness. This points towards a suppressive action of Tregs in this malignancy (Gerber et al., 2014).

The role of macrophages in cutaneous melanoma is less well understood. In 202 samples, high counts of CD68+ cells were related to unfavourable features such as a greater Breslow thickness, ulceration, a higher mitotic rate and a high microvascular density (of both blood vessels and lymphatic vessels) (Storr et al., 2012). However, in this study no relation with relapse-free or overall survival was noticed. This finding that both blood vessels and lymphatic vessels were increased in lesions with many macrophages is interesting, as cutaneous melanoma (as well as CoM) is known to disseminate via both routes, with an especially important role for the lymphatic route.

UV radiation was found to influence the immune infiltrate in cutaneous melanoma lesions, by recruiting macrophages and neutrophils (Bald et al., 2014; Zaidi et al., 2011). This is relevant for CoM as parts of the conjunctiva are sun-exposed. Macrophages have pro-angiogenic effects that are relevant for tumour dissemination. These effects include an increased IFN-gamma signalling, with upregulation of CCL8 (Zaidi et al., 2011). Even so, neutrophils stimulate angiogenesis and promote migration of melanoma cells towards blood vessels (Bald et al., 2014).

Many concepts from tumour immunology have been contrasted between extraocular melanoma and intraocular UM. In the latter, the presence of inflammatory cells is known to be unfavourable for many decades (Davidsorf and Lang, 1977; de la Cruz et al., 1990). By this remarkable position, UM provides an example of immunologic failure to destroy tumour cells, elucidating mechanisms of tumour escape that are relevant to other tumours as well. As such, alterations in HLA and PD-L1 expression are very relevant to CoM and are discussed in sections 4.3 and 4.4.

Similarities between extra-ocular melanoma and UM are seen in the role of (pro-inflammatory) macrophages and (immune suppressing) Tregs. The presence of both CD68+ and CD163+ cells is related to unfavourable features in UM as monosomy 3, ciliary body involvement, greater LBD, and worse survival (Bronkhorst et al., 2012; Maat et al., 2008; Makitie et al., 2001). Even so TAMs are related to a higher vascular density (Bronkhorst et al., 2011; Brouwer et al., 2019a; Makitie et al., 2001). FoxP3+ cells (Tregs) were identified relatively recently in UM (Bronkhorst et al., 2012), and while the prognostic significance needs further study, the presence of intratumoural Tregs has been related to a poorer clinical outcome (Mougiasakos et al., 2010) similar to what is seen in cutaneous melanoma.

Recent work shows that UM with increased numbers of CD8+ cells, increasingly express immune checkpoint inhibitors 'Indolamine 2,3-Dioxygenase 1' (IDO1) and 'T cell immunoreceptor with Ig and ITIM domains' (TIGIT) (which limit the efficacy of CTLs to kill tumour cells) (Robertson et al., 2017). This may explain the opposite effects of TIL presence on survival in UM and other melanomas. The expression of these checkpoints in CoM is unknown, but may be relevant in cases that fail to respond against immunotherapy. Even so, Fas Ligand is a suppressor of immune activity and being expressed in conjunctival epithelium (Brignole et al., 1998), may contribute to CoM resistance against new therapies.

4.2.3. Discussing the infiltrate in CoM

Despite a limited number of studies on CoM, several conclusions can be drawn regarding the role of the immune infiltrate (Table 6). As such, immune cells appear to be favourable: in early reports on CoM an association was established between the presence of immune cells and a favourable prognosis (Bobic-Radovanovic et al., 1998; Crawford, 1980; Folberg et al., 1985a). The specific roles for subtypes of the infiltrate need to be elucidated however, as later studies, examining the specific presence of T cells or macrophages, failed to confirm an association with survival (Anastassiou et al., 2004; Cao et al., 2017; Tuomaala et al., 2007). Two recent works (by Tuomaala and Cao) identified an inverse relation between lymphocytes and tumour thickness (a known unfavourable factor), but did not see the same relation with macrophages. These findings in CoM largely mirror those of cutaneous melanoma where infiltrating lymphocytes correspond with a favourable prognosis (and thin lesions). Interestingly, an immune infiltrate in cutaneous melanoma has especially prognostic significance in vertical growth phase and thick lesions. CoM apparently behaves more as a vertical-growth phase cutaneous melanoma than as a radial growth phase tumour. Though little data is available for other mucosal melanoma, findings are similar to those in CoM, as in oral mucosal melanoma the absence of TILs was related to more metastases (but not to worse survival) (Song et al., 2015).

The role of TILs in CoM, and cutaneous melanoma, is clearly different from the role in intraocular UM, where their presence is associated with a worse prognosis. Interestingly, the presence of TILs is often positively correlated with the presence of TAMs: this has been reported for CoM (Cao et al., 2017), cutaneous melanoma (Storr et al., 2012), and UM (Bronkhorst et al., 2012). In UM, the presence of both cell types is prognostically unfavourable, while in cutaneous melanoma they appear to have opposing effects. The mild or absent relations between infiltrate and prognosis in cutaneous melanoma and CoM may therefore be explained, as the different cell types counteract each other. Another important issue may be that inhibitory forces (from Tregs or immune checkpoints) are more pronounced in intra-ocular than extra-ocular melanoma, explaining why the prognostic role of infiltrate is less in cutaneous melanoma and CoM.

Future projects could analyse the infiltrate in CoM with larger sample sizes, to compare findings from cutaneous melanoma with more statistical power. The role of macrophages should be unveiled, together with its influence on angiogenesis and development of lymphatic vessels (which is of major importance for metastases). Translating important lessons from UM research, the inhibitory aspects of checkpoint inhibition, expression of mechanisms as IDO and TIGIT, and the role of Tregs deserve attention. The presence of immune cells cannot be considered a binary event, with varying and even opposing roles for various cell types, and understanding this is a requirement before clinical steps may be undertaken, as to select patients for T cell-based immunotherapies.

4.3. HLA expression

4.3.1. The HLA system

The human leukocyte antigen system (HLA, the human counterpart of

Table 6

| Presence of | Conjunctival Melanoma | Cutaneous Melanoma | Uveal Melanoma |
|-------------|-----------------------|--------------------|---------------|
| TILs        | Good                  | Good/NS           | Bad           |
| TAMs        | NS                    | NS                 | Bad/NS        |

Abbreviations: TIL, tumour infiltrating lymphocyte; TAM, tumour associated macrophage; NS, not significant.
the major histocompatibility complex (MHC)) comprises a class of molecules with a major role in immunology. Two main types of HLA are identified: HLA Class I and HLA Class II. The HLA Class I molecule consists of two polypeptide chains: the non-polymorphic light b2-microglobulin (B2M) chain (encoded on chromosome 15), and the highly polymorphic heavy alpha chain (encoded by the HLA gene on chromosome 6p21) (Natarajan et al., 1999). Different types of the HLA Class I molecules are HLA-A, HLA-B and HLA-C. HLA Class I proteins are expressed on (almost) all nucleated cells. Their function is to present peptides from intracellular proteins and invasive viruses to the T cell receptor of CD8+ killer T cells. Also, HLA Class I inhibits NK cell activity (Mosaad, 2015). HLA Class II molecules have a different structure with an alpha and beta chain (both encoded on chromosome 6). Major variants are HLA-DM/DO/DP/DQ/DR. HLA Class II is mainly present on immune cells, such as B cells, some T cells, and antigen-presenting cells (APCs). It can be upregulated on other cells during inflammation. Its function is to present peptides from outside the cell, and to interact with CD4+ T helper cells (Mosaad, 2015). Some HLA alleles, as HLA-B27, are strongly related to the development of specific diseases (Howell, 2014).

In cancer research, HLA Class I has received much attention for its role in mediating interactions between tumour cells and T cells. Loss of HLA Class I (which can either be reversible (“soft”) or irreversible (“hard”) (Garrido et al., 2010)) is a mechanism to escape immune surveillance, and has been associated with worse survival in many malignancies, including cutaneous melanoma (Kageshita et al., 1999). IFN-gamma can upregulate HLA Class I expression and therefore may restore the susceptibility of tumour cells to be lysed by T cells. However, counterbalancing mechanisms exist as NK cells on their turn are being activated by the absence of HLA Class I (Ljunggren and Karre, 1990).

4.3.2. Expression of HLA class I on CoM

HLA Class I expression in CoM was studied by Cao et al. in 23 samples using immunofluorescence (Cao et al., 2018a). A marked positive expression was observed for HLA-A, HLA-B/C or B2M in a third of lesions, which is less than seen in cutaneous melanoma (Campoli and Ferrone, 2008).

The level of expression of HLA Class I in CoM was not related to the tumour’s basal diameter, development of recurrences or metastases, or survival. There was a correlation with prognostic factors however, as epibulbar/T1 CoM had a higher HLA Class I expression, and thicker CoM had a lower expression of HLA Class I. An increased expression of HLA Class I was associated with a higher number of CD68+ TAP1 and 2 is associated with decreased lesion thickness (Brocker et al., 1985; D’Alessandro et al., 1987; Kageshita et al., 1999), and a longer time to disease progression and longer survival (Kageshita et al., 1999). Even so, metastases have a lower HLA Class I expression compared to primary lesions (D’Alessandro et al., 1987; Ferrone and Marincola, 1995; Kageshita et al., 1999) and HLA expression is favourably associated with tumour regression (Carretero et al., 2008).

HLA expression may be of a different phenotype in metastases compared to primary lesions due to selective outgrowth, and the expression on metastases may become resistant to upregulation due to mutations in the IFN pathway (Garrido and Aptsiauri, 2019).

Recent work shows that HLA I expression relates to survival after checkpoint inhibition in advanced melanoma patients: homozygosity of at least one allele and LOH are related to worse survival (with less variation to present tumour antigens), and while the presence of an HLA-B44 allele was related to significantly better survival after checkpoint inhibition, presence of an HLA-B62 allele was related to reduced survival. This can have implications for the design of future trials (Chowell et al., 2018), including for potential studies on CoM.

The role of tumour infiltrate on HLA expression can be demonstrated by a comparison between CoM and UM. In UM, a high HLA Class I expression is similarly associated with the presence of both leukocytes and macrophages (CD3, CD4, CD8, CD11b, CD68) (de Waard-Siebinga et al., 1996; Robertson et al., 2017; van Essen et al., 2016), but it is associated with unfavourable tumour characteristics, such as monosomy 3 and decreased prognosis (Maat et al., 2008; van Essen et al., 2016). This led to the hypothesis that NK cells are specifically important for tumour surveillance in UM (Blom et al., 1997; Ericsson et al., 2001), while CTLs are more important in cutaneous and conjunctival melanoma. This is supported by findings that NK cells are more effective in killing HLA-negative cells in the blood compared to in lymphoid vessels (Garrido and Aptsiauri, 2019), which fits the metastasis pattern of both UM and CoM.

4.3.3. Discussing HLA expression in CoM

Few projects focused on HLA expression in CoM, but the available data suggest that HLA expression is associated with favourable tumour traits, and that expression is increased in the presence of an immune infiltrate. These concepts are in line with work from cutaneous melanoma, and to some extent with UM. Understanding HLA expression in CoM is important as it underlies the efficacy of T cell-mediated therapy. Downregulation of HLA Class I can limit this efficacy, and HLA expression may therefore be a selection criterion for therapy in patients, as advised by Cao (Cao et al., 2018a). Even so, upregulation of HLA may be a part of future therapies to enhance the efficacy of immunotherapy. It should be studied whether subtypes of HLA respond differently to T cell-mediated therapy in CoM, as can be expected from work on cutaneous melanoma.

4.4. PD-1/PD-L1 expression

4.4.1. Immune checkpoints

The interaction between CTLs and tumour cells is influenced by various stimuli, including the checkpoint pathways. One of the major checkpoint pathways acts via PD-1/PD-L1. PD-1 is a glycoprotein that is expressed on activated T cells and that can bind to its ligand PD-L1 on the surface of tumour cells and macrophages. The PD1/PD-L1 interaction results in several inhibitory events within T cells, including inhibition of cytokine and enzyme production, and inducing stagnation of cell cycle or even apoptosis. This prevents T cells from targeting the tumour cell (Okazaki and Honjo, 2007) (Fig. 8). Other ligands for PD-1 exist, such as PD-L2, but these are beyond the scope of this manuscript.

Blockade of the PD-1/PD-L1 interaction is the underlying mechanism of anti-PD-1/PD-L1 therapies. In advanced cutaneous melanoma, anti PD-1 therapy has proven successful, with nivolubabatubation showing a better overall survival (OS) and progression-free survival (PFS) than chemotherapy (dacarbazine) (Robert et al., 2015b). Both nivolubabatubation (Larkin et al., 2015) and pembrolizumab (Robert et al., 2015c) monotherapy (both against PD-1) provide a better response (with a higher overall survival and progression-free survival) compared to the original checkpoint inhibitor ipilimumab (anti-CTLA4).

The CTLA-4 protein is another negative regulator of T cells (Egen et al., 2004).
The function is based on the fact that T cells require more than one stimulatory signal to be activated. CTLA4 is expressed on T cells, and competes with CD28 molecules to bind B7, which is their shared ligand. CTLA4 has a greater affinity for the ligand however, and while a CD28-to-B7 binding would lead to increased T cell activity, a CTLA4-to-B7 binding does not cause activation and may even cause inhibition of the cell. The full mechanism is not fully understood however, and is likely more complex, involving for instance CTLA4-mediated activation of Tregs. To explain checkpoint inhibition in CoM, we will focus on the PD-1/PD-L1 interaction.

4.4.2. Predictive value of PD-L1 expression for treatment response

Tumour cells can express variable levels of PD-L1. One would expect that the level of PD-L1 expression on tumour cells is a marker to predict response against specific (invasive) checkpoint inhibitors, since it is the blocking of this exact PD-1/PD-L1 axis that underlies the mechanism of action. Various studies on advanced cutaneous melanoma indeed observed better response rates to checkpoint inhibitors and longer survival in patients with PD-L1 positive tumour cells compared to PD-L1 negative lesions: this was reported for pembrolizumab (anti-PD-1) (Daud et al., 2016), nivolumab (anti-PD1) (Robert et al., 2015b), ipilimumab (anti-CTLA4) and nivolumab + ipilimumab combination therapy (Hodi et al., 2018). Even so, a melanoma type with little PD-L1 expression (UM) (Javed et al., 2017; Qin et al., 2017) shows little response to checkpoint inhibitor therapy (Hepp et al., 2017b).

However, in cutaneous melanoma many patients with PD-L1 negative lesions also responded favourably to these treatments: while 53% of patients with PD-L1 positive lesions had an objective response to nivolumab treatment, also 33% of patients with PD-L1 negative lesions had a response, providing survival benefit compared to chemotherapy for PD-L1 negative lesions as well (Robert et al., 2015b). It has been suggested that not only PD-L1 expression on tumour cells is relevant to therapy, but also the expression on cells in the tumour microenvironment (Kleinovink et al., 2017b).

Adding to the debate on the usefulness of PD-L1 assessment, there is concern about the sensitivity of specific immunohistochemistry staining tests for PD-L1, with different scoring systems and cut-off levels being used between studies (Patel and Kurzrock, 2015). In addition, PD-L1 expression may vary over time (Mahoney et al., 2015).

4.4.3. PD-L1 expression in CoM

Only a few studies analysed expression of checkpoint inhibitors in CoM. An early study on PD-L1 expression in mucosal melanoma of the head and neck, observed positive PD-L1 in 3/23 cases; the three cases of CoM that were included in this set were all negative (Thierauf et al., 2017). All nine samples of cutaneous melanoma that were used as control were PD-L1 positive.

Cao et al. studied PD-L1 expression in 27 cases of CoM (Cao et al., 2017) (Fig. 9). Using a cut-off of 5% of cells, PD-L1 was expressed on tumour cells in 5 (19%) cases, and on stromal cells in 16 (59%) cases (Fig. 9). Stromal expression mainly involved M2 macrophages. PD-L1 expression on tumour cells was associated with more metastasis and disease-specific death, studied in a cohort with a median follow up time of 46 months.

Recent work on 65 CoM confirmed that PD-L1 is expressed more often on immune cells (58%) than on tumour cells (10%), and that PD-L1 expression on CoM is associated with worse survival at a median follow-up time of 29 months; however, expression results varied between two applied IHC antibodies (Lassalle et al., 2020).

The reported PD-L1 expression on CoM tumour cells (up to 19%) (Cao et al., 2017; Lassalle et al., 2020) is somewhat less than reported in cutaneous melanoma (30–35%) (Robert et al., 2015b; Taube et al., 2012). A large study on cutaneous melanoma reported a somewhat lower level of PD-L1 positive expression (24% of cases), but in a fairly large number of cases (11%), no status could be determined so the actual expression may be different (Jarkin et al., 2015). PD-L1 expression on CoM is higher than on other mucosal melanoma (sino-nasal, vaginal, rectal) (Mikkelsen et al., 2020). Importantly, the cut-off for deeming a sample positive has a major influence on the reported numbers: one study reported PD-L1 expression in 76% of cutaneous melanoma samples, but used a cut-off of only 1% of cells expressing PD-L1 (Daud et al., 2016).

PD-L1 expression has not been identified on CoM tumour cells, but is expressed on T cells in 17 (63%) (Cao et al., 2017) and 15 (23%) (Lassalle et al., 2020) of cases. In both studies, no significant relation with patient outcome was established, but Cao reported a trend between PD-L1 expression and a higher number of recurrences.

IFN-gamma is known to enhance PD-L1/PD-1 expression in cutaneous melanoma (Taube et al., 2012). In vitro analysis by flow cytometry of three CoM cell lines (CRMM1, CRMM2, CM2005.1) demonstrated no background expression of PD-L1, while one cell line (CRMM2) showed expression of PD-1. Addition of IFN-gamma induced upregulation of HLA Class I (used as control) in all three cell lines, with two lines showing an increase in the expression of PD-L1 (CRMM2 and CM2005.1), and one of PD-1 (CRMM2). These findings show that IFN-gamma, produced by tumour-infiltrating lymphocytes (TILs), may be responsible for enhancing PD-L1 expression in CoM (Cao et al., 2017). This is similar to findings in cutaneous melanoma (Taube et al., 2012).

A limitation to studies regarding PD-L1 in CoM is the small size of tissue samples. PD-L1 expression can be quite heterogenous within samples (McLaughlin et al., 2016) and it is not known how a small sample represents the PD-L1 status of the tumour as a whole (Cao et al., 2017). Since cutaneous melanoma patients with PD-L1 positive as well as negative tumours may respond favourably to anti-PD-1 treatment, this may not so much be a selection criteria for treatment per se (and negative staining should not prohibit CoM patients from entering trials.
(Daud et al., 2016). Even so, combining checkpoint inhibition with other interventions such as radiotherapy may be beneficial, as is currently attempted in UM (Jager et al., 2020).

### 4.4.4. Discussing PD-L1 expression in CoM

The expression of PD-L1 in CoM seems to mirror the findings of cutaneous melanoma, though somewhat lower percentages of expression have been reported. Whether PD-L1 expression has prognostic value or is a therapeutic indicator in CoM has not yet been established, but it can be expected that larger studies would identify this, as is seen in cutaneous melanoma. The relevance of this predictive effect may be similarly limited however, as PD-L1 negative CoM may still respond to immunotherapy. Prior to initiation of larger translational studies in CoM, it may be needed to overcome technical issues such as the difficulty of obtaining representative PD-L1 expression results from small tissue samples.

Novel approaches regarding checkpoint inhibition in cutaneous melanoma include the combination with radiotherapy (Sato et al., 2020) or photodynamic therapy in preclinical models (Kleinovink et al., 2017a). As radiotherapy has been well-established in CoM, this may be readily-transferable to CoM as well.

### 4.5. Conclusions (Immunology)

Similar to the situation in cutaneous melanoma, the presence of an immune infiltrate is favourable in CoM. Questions remain on the exact role of all cell types of the immune infiltrate, and regarding the role of inhibitory forces such as those of T-regs. It is as yet unknown how infiltrating macrophages relate to (lymph and blood) vessel development in CoM, which may provide a possible dissemination route for metastases. Even more, expression of IDO and TIGIT (relevant in intraocular melanoma as an escape from the immune system) may have relevance in the conjunctiva, but this is currently unknown.

HLA expression is common in CoM, and as expected by its role in interaction with immune cells, has a relation with favourable traits. Downregulation may limit the efficacy of T cell-based therapies, while upregulation may enhance the susceptibility to immunological clearance. Screening for HLA expression, or the presence of specific alleles, may become part of patient workup prior to immunotherapy in CoM.

Like cutaneous melanoma, CoM is known to frequently express PD-L1. The prognostic and predictive value is limited however, as in cutaneous melanoma even negative lesions could respond to anti-PD-L1 therapy. It is a promising target for therapy, possibly in combination with other treatments such as photodynamic therapy or radiotherapy.

### 5. Targeted therapy and checkpoint inhibitor therapy in CoM

#### 5.1. Current therapy of localised and metastatic CoM

Tumour location and extent are currently the main determinants for therapy in CoM. Localised disease is commonly treated by surgical excision using a ‘no touch technique’ and adjuvant therapy (Wong et al., 2014). Adjuvant therapy includes cryotherapy (with a “double freeze-thaw” technique (Shields et al., 1997)), topical chemotherapy (such as mitomycin-c drops (Finger et al., 1998) or interferon-alfa (Herold and Hintschich, 2010)), and/or radiotherapy. For radiotherapy several techniques exist of external radiotherapy or brachytherapy using plaque or handheld applicators (Brouwer et al., 2020; Westekemper et al., 2006). Treatment of palpebral lesions may be more complex, as this site is more difficult to approach, and adjuvant brachytherapy can only be delivered via adapted ‘outward’ applicators (Shields et al., 2003).

Widespread lesions require a more extensive approach, with extensive surgery, radiotherapy (Wuestemeyer et al., 2006), and ultimately even orbital exenteration (Wong et al., 2014).

The approach to adjuvant therapy varies between institutions, as no data supports superiority of either, and availability differs (Cohen and...
O’Day, 2019; Glossmann et al., 2018; Wong et al., 2014). Most authors include cryotherapy to conjunctival margins by default, as part of the surgical procedure presented by Shields et al. (1997); for corneal involvement, alcohol epithelioectomy is performed. Additional adjuvant therapy may be reserved for cases with incomplete resection, with concurrent PAM, or for recurrences, but others advise to use it for all (Lim et al., 2013). Radiotherapy is well-accepted for incomplete margins, topical chemotherapy can be applied for concurrent (and widespread) PAM (Brouwer et al., 2018; Cohen and O’Day, 2019).

Importantly, patients have a better prognosis if initial treatment is delivered in a centre with expertise in ocular oncology (preventing delay and possible inappropriate or incomplete resection), calling for general ophthalmologists to swiftly refer patients with a suspicious conjunctival lesion (Brouwer et al., 2018; Damato and Coupland, 2009).

CoM may disseminate to lymph nodes (regional) as well as systemic sites (distant). Most often dissemination involves the parotid (pre-auricular), cervical and submandibular lymph nodes, and the lungs, liver, brain and skin, respectively (Esmaili et al., 2001; Missotten et al., 2005). In CoM, the lymph nodes are often believed to be the first site of metastasis; systemic metastases may develop independently as well (Brouwer et al., 2018; Esmaili et al., 2001). While lymph metastases are important in CoM and cutaneous melanoma, this differs from UM since the uvea lacks lymphatic drainage. The sentinel lymph node biopsy (SLNB) is a technique to detect micro-metastases in the first node(s) that are theoretically reached by disseminating tumour cells. If detected, adjuvant therapy can be administered, and a lymphadenectomy can be performed, preventing further spread (Pfeiffer et al., 2017). There is debate on the position of SLNB in CoM management (Mor et al., 2019), however with new therapeutic options for metastatic disease, the clinical relevance is rising and the SLNB is performed more frequently.

There is currently no standard therapy for metastatic CoM (Grimes et al., 2020). Most often, guidelines from cutaneous melanoma are followed, e.g. as presented in the European guideline for melanoma (Garbe et al., 2020). In patients with few metastases, or those at specific accessible locations, selective surgical metastactectomy or radiotherapy can be applied. In widely disseminated disease, systemic agents are required. Up till a few years ago, cytotoxic chemotherapy was the only available option, which was associated with poor response rates and survival benefit. In the management of cutaneous melanoma, newly-developed immunotherapy and targeted therapy (as will be discussed later on) have substituted these agents as first line therapy, leaving conventional chemotherapy as a last-resort option after failure, or in those cases where (more expensive) other treatments are not available.

5.2. Targeted therapy in CoM

‘Targeted therapy’ or ‘small molecule inhibitors’ involves drugs that target genetic mutations and (upregulated) pathways that are related to malignancies, and that are absent in healthy tissues. It has been suggested to use cutaneous melanoma therapies for CoM. Since 2013, the use of targeted therapies has been reported for a small number of CoM. We are not aware of clinical trials or large cohorts that are formally studying these drugs in CoM, and therefore resort to small series, single case reports and preliminary in vitro work. The aim for systemic targeted therapy in CoM is mainly 1) to treat widespread local disease, that is too large for excision, or as an alternative for orbital exenteration, or 2) to treat regional and distant metastases.

In this section, we will discuss patient-related outcomes of several MAPK-pathway-inhibitors in CoM, and present several new drugs with a suggested potential based on preclinical work or similarities to cutaneous melanoma.

5.2.1. BRAF and MEK inhibitors

The MAPK pathway can be inhibited by drugs targeting BRAF and MEK. Inhibitors of BRAF are the most well-established targeted therapy drug type in cutaneous melanoma (Table 7). First reports on single-agent therapy demonstrated survival benefit for vemurafenib (trade name: Zelboraf, Hoffmann-La Roche (FDA, 2011)) versus dacarbazine chemotherapy in metastatic disease in previously untreated cutaneous melanoma patients with a BRAF V600E mutation (Chapman et al., 2011). Later, other inhibitors were introduced as well (i.e. dabrafenib (trade name: Tafinlar, Novartis (FDA, 2013b)), and recently encorafenib (trade name: Braftovi, Array Biopharma (FDA, 2018a)).

A common issue with BRAF inhibition is the development of treatment resistance, that often occurs within a year. This can be due to several mechanisms such as upregulation of NRAS (Nazarian et al., 2010), NF1 (Whittaker et al., 2013), or ERK (Wagle et al., 2014), and downregulation of PTEN (Catalanotti et al., 2017); it has recently been linked to upregulation of other pathways such as YAP1 (Kim et al., 2016; Lin et al., 2015) and even PD-L1 (Kim et al., 2018), providing escape from immune cells. Combining BRAF and MEK inhibition is a solution to overcome this resistance, and combination therapy demonstrated prolonged survival over BRAF monotherapy in cutaneous melanoma: vemurafenib (BRAF inhibitor) and cobimetinib (MEK inhibitor; trade name: Cotellic, Genentech (FDA, 2020a)) were superior versus vemurafenib alone (Larkin et al., 2014). A similar result was reported when dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor; trade name: Mekinist, Novartis (FDA, 2013a)) were combined versus dabrafenib alone (Long et al., 2014) or versus vemurafenib alone (Robert et al., 2015a). Recently, binimetinib (trade name: Mektovi, Array Biopharma (FDA, 2018b)) has been introduced as well.

While clinical outcomes regarding BRAF and MEK inhibition in CoM have been reported (section 5.2.2.), preclinical work continues to optimize treatment by e.g. analysing combined pathway inhibition. In vitro work on three CoM cell lines by Cao et al. tested the mutation-specific effect of two BRAF inhibitors and a MEK inhibitor (Cao et al., 2016). The two BRAF inhibitors (vemurafenib and dabrafenib) inhibited growth of cell lines CRM11 and CM2005 (both harbouring a BRAF mutation) although not with the same sensitivity. This could not be explained by PTEN loss (which was not found in any of the cell lines). Both drugs caused paradoxical activation of the MAPK pathway in a third cell line, CRM22 (with an NRAS mutation, no BRAF mutation); an effect that was later confirmed by El Zaoui et al. (El Zaoui et al., 2019). Cao et al. showed that MEK inhibition had an inhibitory effect on all three cell lines, and that combined inhibition of MEK and AKT even showed synergistic effects (Cao et al., 2016).

5.2.2. Reported cases of targeted therapy in CoM

BRAF and MEK inhibitors are (similar to the situation in cutaneous melanoma) the most well-studied small molecule inhibitors in CoM. We

![Table 7](https://example.com/table7.png)

| BRAF inhibitors | MEK inhibitors |
|-----------------|----------------|
| dabrafenib | trametinib |
| vemurafenib | cobimetinib |
| encorafenib | binimetinib |

Abbreviations: FDA, Food and Drug Administration.

a Dosing as indicated for (metastatic) melanoma, for other indications please read product information.
b Encorafenib and binimetinib are indicated for combined use.
have been able to find the reports on seven CoM patients who received treatment with a BRAF inhibitor (Table 8). It is likely that more patients have been treated, however, not reported. In one case, treatment of the conjunctival lesion was the sole aim, in a patient who was free of lymph- or distant metastases (Pahlitzsch et al., 2014). This patient had a recurrence of CoM that would otherwise have been treated with orbital exenteration. There was a good response to vemurafenib, with tumour decrease and a stable situation for 3 years. The six other reported patients with targeted therapy had metastatic disease. As a response to the landmark paper by Griewank et al. on the prevalence of BRAF mutations in CoM (Griewank et al., 2013b), Weber et al. noticed that a patient with a BRAF V600E mutation and several distant metastases, developed only a mild response to vemurafenib monotherapy (with progression after 2 months) (Weber et al., 2013). They suggested that BRAF inhibitors may not be successful in CoM as in cutaneous melanoma, possibly due to frequent PTEN loss in CoM, which may have contributed to treatment resistance. Griewank replied by reporting a patient who was treated with dabrafenib for metastases and who had a partial response with a significant 62% tumour reduction; this patient, however, developed new lesions after 6 months (Griewank et al., 2013c). A similar case was reported by Maleka et al.: a patient with metastatic CoM was treated with vemurafenib, and initially had a good response with metastases reduction; however, after 4 months, re-appearance of lesions occurred, with death shortly thereafter (Maleka et al., 2016). Notably, this patient had been treated with several other therapies, including a gene trial with AdCD40L, cyclophosphamide and radiotherapy for brain metastases, questioning the role of each individual therapy to the overall response. A promising report was delivered by Pinto Torres et al. on a CoM patient with metastatic lesions who received vemurafenib and had a complete response during the 3 years of follow up (Pinto Torres et al., 2017).

Similar to what is seen in cutaneous melanoma, BRAF inhibitors have been combined with MEK inhibitors to overcome the issue of resistance in CoM. Two cases of treatment for lymph node metastases have been reported; however, there are limitations to the interpretation. One patient received dabrafenib and trametinib with a good response, being alive 1 year later (Rossi et al., 2019). Though promising, a longer follow up time would be preferred to assess the effect of the combination therapy. The other patient received dabrafenib and trametinib, with good response, but needed a switch to vemurafenib monotherapy after 1.5 months due to the development of adverse events (i.e. nausea and vomiting) (Dagi Glass et al., 2017). Progressive disease then caused a further switch to pembrolizumab (an immune checkpoint inhibitor), and vemurafenib again. Addition of cobimetinib to the vemurafenib was required to obtain a good response. Though this case resulted eventually in disease control, therapy was complex and adverse events were a major issue.

5.2.3. Adverse events following BRAF/MEK inhibitors

Adverse events (AE) following targeted therapy proved common in cutaneous melanoma patients, with the occurrence of any AE in over 90% of all patients following BRAF inhibitors or combined BRAF/MEK inhibitor treatment (Long et al., 2014) (Table 9).

Table 8

Overview of reported cases on targeted therapy in CoM.

| Study                        | Patient | Type of CoM                                      | Type and Dosage Immunotherapy | Other Treatments | Clinical Outcome | Adverse Events |
|------------------------------|---------|-------------------------------------------------|--------------------------------|------------------|------------------|----------------|
| Pahlitzsch et al. (2014)     | M, 36y  | Metastatic CoM (subcutaneous, lung, bone)       | Indic: unresectable metastases | Prior CoM: resection | Good response | Weight loss, nausea, vomiting, headache |
|                              |         | BRAF muta (v600E)                               | R: vemurafenib 960 mg 2dd     | Prior Mets: none  | Initially good response | Not reported |
|                              |         |                                                  | Later: resection              |                  | After 2 months progression. | |
|                              |         |                                                  |                               |                  | [death not mentioned] | |
| Weber et al. (2013)          | M, 45y  | Metastatic CoM (intramuscular, lung, brain)     | Indic: unresectable metastases | Prior: cx (incomplete), Plaque brachytherapy Later: resection | Good response, tumour decrease, stable for 3 yr. Then deterioration general health. | Not reported |
|                              |         | BRAF muta (v600E)                               | R: dabrafenib [no dose]       |                  |                  | |
|                              |         |                                                  | Later: radiotherapy           |                  |                  | |
| Griewank et al. (2013c)      | M, 43y  | Metastatic CoM (intramuscular, lung, brain)     | Indic: unresectable metastases | Prior: cx (incomplete), Plaque brachytherapy Later: resection | Good response, tumour decrease, stable for 3 yr. Then deterioration general health. | Not reported |
|                              |         | BRAF muta (v600E)                               | R: dabrafenib [no dose]       |                  |                  | |
|                              |         |                                                  | Later: radiation              |                  |                  | |
| Maleka et al. (2016)         | F, 53y  | Metastatic CoM (orbit, parotid gland, lung, brain) | Indic: unresectable metastases | Prior: cx (incomplete), Plaque brachytherapy Later: resection | Good response, tumour decrease, stable for 3 yr. Then deterioration general health. | Not reported |
|                              |         | BRAF muta (v600E)                               | R: vemurafenib 960 mg 2dd; later 720 mg 2dd due to AE. |                  |                  | |
| Pinto Torres et al. (2017)   | F, 59y  | Metastatic CoM (Oropharyngeal wall)             | Indic: unresectable metastases | Prior CoM: exc, Prior Mets: radiotherapy 20GY/5 fractions | CR. No recur in 3yr later. | Arthralgia, diarrhoea, skin rash |
|                              |         | BRAF muta (v600E)                               | R: vemurafenib 960 mg 2dd; later 480 mg 2dd due to AE. |                  |                  | |
| Combined therapy             |         |                                                  |                               |                  |                  | |
| Dagi Glass et al. (2017)     | F, 61y  | Recurrent CoM Lymph metastases.                  | Indic: alternative to extensive surgery R: 1) dabrafenib + trametinib; 2) vemurafenib (due to AE); 3) pembrolizumab (due to progression); 4) vemurafenib + cobimetinib (due to progression) | Prior CoM: excision, cryo Prior Lynch mets: parotidectomy and neck dissection 1: good for 1.5 months, then AE. 2:3: mixed, not complete. 4: eventually good response. [death not mentioned] | Good response, 1yr later. | Arthralgia, diarrhoea, skin rash |
|                              |         | BRAF muta (v600E)                               |                              |                  |                  | |
| Rossi et al. (2019)          | M, 70y  | Metastatic CoM (lymph)                          | Indic: unresectable metastases | Prior CoM: excision, cryo Prior Lynch mets: parotidectomy and neck dissection | Good response, 1yr later. | Fever, Hyper-transaminesaemia |
|                              |         | BRAF muta (v600E)                               | R: dabrafenib 150 mg 2dd + trametinib 2 mg 1dd |                  |                  | |

Abbreviations: CoM, conjunctival melanoma; pembro, pembrolizumab; ipi, ipilimumab; nivo, nivolumab; PR, partial response; CR, complete response; extnt, orbital exenteration; cx, excision; cryo, cryotherapy; mmc, mytomycin-c; SLNB, sentinel lymph node biopsy; M, male; F, female; AE, adverse event; met, metastasis; muta, mutation; Indic., indication; R, drug prescription.
Table 9
Occurrence of most common adverse events (AE) following targeted therapy in cutaneous melanoma patients. (Note: less-frequently occurring AEs were omitted in this table, see the original reports for full details).

|                | Vemurafenib<sup>a</sup> | Vemurafenib + Cobimetinib<sup>b</sup> | Dabrafenib<sup>c</sup> | Dabrafenib + Trametinib<sup>b</sup> |
|----------------|------------------------|--------------------------------------|------------------------|--------------------------------------|
| Any AE         | Any grade (%)          | ≥3 (%)                               | Any grade (%)          | ≥3 (%)                               |
| Diarrhea       | 33                     | 1                                    | 61                     | 7                                    | 96                   | 34                   | 95                   | 32                     |
| Fatigue        | 33                     | 3                                    | 37                     | 5                                    | 14                   | 1                  | 24                   | 1                      |
| Rash           | 68                     | 16                                   | 73                     | 17                                   | 22                   | 1                  | 23                   | 0                      |
| Nausea         | 26                     | 1                                    | 43                     | 1                                    | 26                   | 1                  | 30                   | 0                      |
| Vomiting       | 42                     | 5                                    | 38                     | 3                                    | 27                   | 1                  | 24                   | 1                      |
| Arthralgia     | 24                     | 0                                    | 29                     | 1                                    | 28                   | 2                  | 51                   | 6                      |
| Pyrexia        | 33                     | 1                                    | 17                     | 1                                    | 26                   | 0                  | 7                    | 0                      |
| Alopecia       | 9                      | 9                                    | 2                      | 1                                    | 29                   | 1                  | 30                   | 1                      |
| Headache       |                        |                                      |                        |                                      |                      |                    |                      |                         |
| Cutaneous SCC  | 13                     | 13                                   | 4                      | 4                                    | 9                    | 4                 | 2                    | 2<sup>c</sup>            |
| Keratoacanthoma| 9                      | 9                                    | 2                      | 1                                    |                      |                    |                      |                         |

Abbreviations: AE, Adverse event. SCC, squamous cell carcinoma.

<sup>a</sup> Phase III trial, study group vemurafenib n = 246, cobimetinib + vemurafenib n = 247 (Ascierto et al., 2016).
<sup>b</sup> Phase III trial, study group dabrafenib n = 211, dabrafenib + trametinib n = 209 (Long et al., 2014).
<sup>c</sup> SCC and keratoacanthoma combined.

AEs of BRAF inhibition in cutaneous melanoma patients were most commonly arthralgia, rash, photosensitivity, alopecia, fatigue, or diarrhea (Ascierto et al., 2016; Chapman et al., 2011; Long et al., 2014). AEs are comparable between vemurafenib and dabrafenib monotherapy, but some differences are observed, as photosensitivity is seen more often with vemurafenib but pyrexia and chills are more common with dabrafenib. Addition of a MEK inhibitor to a BRAF inhibitor slightly alters the AE profile. Most AEs occur more frequently following combination therapy.

A noticeable AE that raised concern following introduction of vemurafenib was the development of cutaneous squamous cell carcinoma (SCC) (Chapman et al., 2011). The proposed mechanism is that BRAF inhibitors accelerate the progression of subclinical cancerous lesions; addition of a MEK inhibitor reduces this effect, resulting in the observation that patients with combined BRAF/MEK inhibitor treatment less often develop SCC than those on BRAF inhibitor alone.

The management of most adverse events requires dose reduction or switch to another drug type, however, in advanced grades additional topical or systemic therapy is needed (Welsh and Corrie, 2015).

An ocular complication that may occur following BRAF/MEK inhibition is serous retinopathy (including edema and retinal detachment). This has been reported in 4% of vemurafenib, and 27% of vemurafenib + cobimetinib combination patients (Ascierto et al., 2016).

The observed AEs in the sparse reports of targeted therapy in CoM are in line with the earlier reports on cutaneous melanoma. Development of rash urged a vemurafenib dose reduction in one patient (Maleka et al., 2016), and development of arthralgia, diarrhea, and rash caused vemurafenib dose reduction in another (Pinto Torres et al., 2016). One patient who was on dabrafenib plus trametinib developed nausea and vomiting that urged a switch to another drug (Dagi Glass et al., 2017). Of note, since similar dosing schemes of targeted therapy are used for CoM as well for cutaneous melanoma, similar adverse events would be expected.

5.2.4. Preclinical targets

Apart from the relatively well-established BRAF and MEK inhibitors that have been implemented already on a small scale in the treatment of CoM, several newer drugs can be suggested for CoM, based on small scale patient-related studies from cutaneous melanoma, and preclinical assessment using CoM models. Development of these drugs is important to overcome the issue of treatment resistance, and to properly target all tumours regardless of their mutational background.

Preclinical assessment of drugs is often performed using cell lines. To our knowledge, only a few CoM cell lines exist, harbouring either a BRAF or NRAS mutation (Table 10). To examine the potential efficacy of drugs in the full range of CoM, it would be interesting to develop cell lines with various combinations of BRAF/NRAS and other mutations such as PTEN, NF1, and TERT.

5.2.4.1. c-KIT inhibition. KIT inhibition is currently best known for the treatment of gastrointestinal stromal tumours (GIST) using imatinib. In GIST, about 75% of lesions harbour a KIT mutation, explaining the sensitivity to this drug (Joensuu and DeMatteo, 2012). The c-KIT inhibitor imatinib showed a response rate of 16–29% in phase II studies of metastatic (cutaneous) melanoma harbouring KIT alterations (Carvajal et al., 2011; Guo et al., 2011; Hodi et al., 2013). The alteration type is very relevant, as metastatic patients with a KIT mutation showed a response rate of 54%, while those with KIT amplification showed a response rate of 0% (Hodi et al., 2013). Since KIT mutations are rare in CoM, imatinib (or other drugs with KIT inhibitory effects, such as sunitinib, dasatinib and nilotinib that are currently all in phase 2 studies in melanoma (Meng and Carvajal, 2019)) will likely not be suitable for large scale use in CoM. With proper screening for mutation status, however, they can be part of a successful personalized treatment.

5.2.4.2. ERK1/2 inhibition. ERK (consisting of the kinases ERK1 and ERK2) is a distal actor in the MAPK pathway (Fig. 6).

Reactivation can be seen with resistance of upstream MAPK inhibition, and as such it is an important target to overcome BRAF-inhibitor resistance (Wagle et al., 2014). ERK1/2 can be inhibited by ulixertinib (BVD-523), a drug that showed potential in preclinical melanoma models (Germann et al., 2017), and that showed an acceptable safety profile and evidence of activity in solid tumours including melanoma in a phase I study (Sullivan et al., 2018). In that work, 9/19 patients with BRAF-mutated melanoma had a partial response (PR) or stable disease

Table 10
Conjunctival melanoma cell lines.

| Cell line | BRAF mutation | NRAS mutation | Other mutations | Reference |
|-----------|---------------|---------------|----------------|----------|
| CRMM1     | V600E         | WT            |                | Narveyek et al. (2005) |
| CRMM2     | WT            | Q61L          |                | Narveyek et al. (2005) |
| CM2005.1  | V600E         | WT            |                | Reijer et al. (2007) |
| T1527A    | G466E         | WT            | HRAS Q61R      | El Zouei et al. (2019) |

Abbreviations: WT, wildtype.
following failed BRAF inhibition; 9/17 patients with NRAS-mutated melanoma had PR or stable disease as well. To our knowledge, ulti-
eratinib has not yet been evaluated in CoM. It is promising however that both BRAF and NRAS mutated lesions seem to be responding, suggesting a role as a rescue medication.

5.2.4.3. PI3K/AKT/mTOR. The PI3K/AKT/mTOR pathway has an important role in melanoma, acting alongside MAPK (Fig. 6). Targeting can be of specific benefit in BRAF-WT or BRAF-inhibitor-resistant melanoma. The therapeutic potential follows increased activity in CoM compared to conjunctival nevi (El Zouei et al., 2019), and also in CoM compared to UM (Popolo et al., 2010).

Cell proliferation of three CoM cell lines (harbouring either a BRAF or NRAS mutation) could be inhibited by AKT inhibition using MK2206 (Cao et al., 2016). Using cell lines, the most promising effect was obtained by combining MK2206 (AKT inhibitor) with MEK162 (MEK in-
hibitor), that caused a stronger cell cycle arrest compared to single-agent treatment.

Another study using the same cell lines found that the PI3K inhibitor picitilisib was more effective than the dual PI3K/mTOR inhibitor dac-
tolisib (El Zouei et al., 2019). The genetic background of the cell lines was important however: both picitilisib and dactolisib were active against CRMM1 (BRCA mutation), picitilisib alone was effective against CRMM2 (NRAS mutation), and none were effective against T1527A, a cell line lacking BRAF V600E and NRAS mutations (Table 10).

The results from CoM are in line with earlier reports on cutaneous melanoma cell lines: in NRAS-mutated cell lines, cells were more sen-
tive to MEK inhibition than to PI3K/mTOR inhibition alone, but combined inhibition was superior (Posch et al., 2013). However, there are some troublesome reports of PI3K/AKT inhibition in melanoma patients: trametinib (MEK inhibitor) and GSK2141795 (AKT inhibitor) combined had no response in 10 patients: trametinib (MEK inhibitor) and GSK2141795 (AKT inhibitor) were tested in a phase II trial in locally advanced melanoma patients (including 3 UM) and therapy was not well toler-
ated (Algazi et al., 2018).

5.2.4.4. TERT. The abundance of TERT mutations in CoM poses an opportunity for treatment. Reverse transcriptase inhibitors, e.g. azido-
thymidine, can target TERT mutated tumours by targeting reverse transcriptase activity (Humr et al., 2008). Other approaches include a telomerase inhibitor, such as Imetelstat (GRN163L) (Sugarman et al., 2019). These drugs have not been studied in CoM however, but show promising results in vitro and in early stage patient studies of several cancers.

5.2.4.5. EZH2. Epigenetics concern processes that alter gene expres-
sion and regulation, without involving changes in the DNA sequence itself. The polycomb repressive complex 2 (PRC2) is involved in many of these epigenetic processes, with the ‘enhancer of zeste homolog 2’ (EZH2) as a core subunit of PRC2, that is overexpressed in several cancers (Bachmann et al., 2006). EZH2 causes silencing of (tumour suppressor) genes, and is frequently overexpressed in cutaneous melanoma, but not in cutaneous nevi, indicating a role in tumour progression with potential as a therapeutic target (Zingg et al., 2015).

Cao et al. found that EZH2 is not expressed on melanocytes of normal conjunctiva or PAM, but was expressed in 13/26 CoM lesions and 7/8 lymph node metastases (Cao et al., 2018b). Just as in skin lesions, this implies a role for EZH2 in malignant transformation. EZH2 expression in CoM was significantly related to older age, larger tumour thickness and worse overall survival. Using two EZH2 inhibitors, GSK503 and UNC1999, cell growth of three CoM cell lines (CRMM1, CRMM2 and CM2005.1) as well as tumour growth in a zebrafish model could be repressed. While, as far as we know, EZH2 inhibition has not been studied in CoM patients, there may be a potential therapeutic benefit.

5.3. Checkpoint inhibitor therapy in CoM

5.3.1. Checkpoint inhibitors

The first approved checkpoint inhibitor for advanced cutaneous melanoma was ipilimumab, a monoclonal antibody against CTLA4 (Yervoy, Bristol-Myers Squibb) (Table 11). Ipilimumab showed improved survival compared to treatment with gp100 in unresectable stage III or IV cutaneous melanoma patients (overall survival 10.1 months versus 6.4 months) (Hodi et al., 2010). Ipilimumab, combined with dacarbazine chemotherapy, also provided a significantly longer overall survival compared to dacarbazine with placebo (overall survival 11.2 months versus 9.1 months) (Robert et al., 2011). In 2014, two new drugs targeting PD1 were introduced: nivolumab (Opdivo, Bristol-Myers Squibb) and pembrolizumab (Keytruda, Merck Sharp & Dohme).

Pembrolizumab (administered at both two or three week intervals) gave a better survival compared to ipilimumab, with a 6-month PFS of approximately 47% for pembrolizumab, and 27% for ipilimumab (Robert et al., 2015c). In advanced cutaneous melanoma patients, who had progressed after ipilimumab or ipilimumab plus a BRAF-inhibitor, nivolumab demonstrated a better response than chemotherapy (32% versus 11%) (Weber et al., 2015). Nivolumab had also a better overall survival and progression-free survival than dacarbazine in untreated advanced melanoma patients lacking a BRAF mutation; with nivolumab, the 1-year overall survival went from 42.1 to 72.9%, the median progression-free survival from 2.2 months to 5.1 months (Robert et al., 2015b).

Since CTLA4 and PD-1 act on T cells via different mechanisms (Fig. 8), there is a rationale to combine blockade therapy. Indeed, combining nivolumab and ipilimumab led to better survival (progression-free survival of 11.5 months) than either of the therapies alone (6.9 months for nivolumab, 2.9 months for ipilimumab) in advanced cuta-
neous melanoma patients (Larkin et al., 2015).

Checkpoint inhibitors have been used to treat a limited number of patients with locally advanced or metastatic CoM. In the absence of formal trials, the current literature consists of single-case reports and small case-series (Table 12). First treatments were administered around 2013 (Finger and Pavlick, 2019; Sagiv et al., 2018), but the first reports on checkpoint inhibitors in CoM appeared in the literature from 2017 onwards (Ford et al., 2017; Kini et al., 2017; Pinto Torres et al., 2017). The use of checkpoint inhibitors in CoM follows the same basic princi-
ples and similar dosing schemes as in locally-advanced or metastatic cutaneous melanoma, as described in the FDA reports on ipilimumab (trade name: Yervoy) (FDA, 2020d), pembrolizumab (trade name: Keytruda) (FDA, 2020b), and nivolumab (trade name: Opdivo) (FDA, 2020c). Drugs targeting PD-1 (pembrolizumab, nivolumab) as well as drugs targeting CTLA4 (ipilimumab) have been used in CoM, as single-agent as well as in various combinations. Due to different aims of therapy, the findings for patients who were treated for a primary CoM or for metastatic disease, will be discussed separately.

5.3.2. Primary CoM

In several cases, immune checkpoint inhibitors have been used as treatment for primary CoM (Table 12, cases 1–5). In four cases, immu-
notherapy was offered as alternative to orbital exenteration for patients who refused eye-removing therapy; in one case, treatment was for an extensive lesion with an insufficient response to prior local therapy.

The first reported patient (M, 60) received pembrolizumab single-
agent therapy and had an immediate good response, with flattening of a nodular recurrence at 6 months (Kini et al., 2017). A similar favourable response to pembrolizumab single therapy was seen for an in-situ lesion (F, 53) (Hong et al., 2020).

The third patient (F, 94), reported initial progression with pem-
broliumab single-agent therapy, but had a partial response with pem-
broliumab + ipilimumab combination therapy. This patient died 5 months later from an unrelated cause (Finger and Pavlick, 2019).

In two patients, a successful response was reported for the third
attempted scheme of therapy, which included addition of topical IFN-alfa. One of these patients (M, 76) had no response to ipilimumab single-agent therapy, a minimal response to pembrolizumab single-agent therapy, but a complete response following pembrolizumab + topical IFN-alfa drops (Finger and Pavlick, 2019). The second patient (F, 84) had minimal success after pembrolizumab single-agent therapy, showed progression with pembrolizumab + ipilimumab, but stable disease with pembrolizumab + ipilimumab + IFN-alfa topical treatment (Finger and Pavlick, 2019).

Finger noted that local IFN-alfa seemed to synergize the effect of PD-L1 inhibition (Finger and Pavlick, 2019). IFN-alfa on itself has been longer to treat malignancies [with a small survival benefit in cutaneous melanoma patients (Mocellin et al., 2013)], and also ocular malignancies, including ocular surface squamous neoplasia (OSSN) (Karp et al., 2010; Vann and Karp, 1999) and CoM (Benage et al., 2019; Finger et al., 2008). IFN-alfa is known to stimulate immune reactions, with upregulation of HLA molecules, promotion of NK cell activity and activation of CD8+ T cells, making it a candidate for co-treatment with anti-PD-1 and anti-CTLA4 in cancer (Baron et al., 1991; Curtsinger et al., 2005; Rafique et al., 2015). In a one-armed phase 1b/2 study on pegylated–IFN–alfa and pembrolizumab in 43 mucosal and cutaneous melanoma, an improved response rate compared to the expected rate was found for pembrolizumab alone (Davar et al., 2018); however, this study needs further evaluation. As Cao showed, PD-L1 and PD1 could be upregulated on CoM cell lines through IFN-gamma (Cao et al., 2017). It may well be that the same happens under the influence of IFN-alfa, providing a good target for checkpoint inhibition.

5.3.3. Metastatic CoM

In twelve reported cases, immunotherapy has been administered to CoM patients with metastatic disease (Table 12, cases 6–17). Eight patients had systemic metastases, one patient had regional (lymph node) metastases, and three patients had both. In three patients, treatment of metastases as well as local tumour control was attempted at the same time.

Five CoM patients received nivolumab single-agent treatment (Kiyohara et al., 2020; Sagiv et al., 2018). All had a good response, although in one case (M, 71; systemic metastases; BRAF V600E mutated) this treatment was supplemented with dabrafenib, trametinib and radiotherapy, hampering the conclusions on the individual effect of nivolumab (Kiyohara et al., 2020). One patient, with systemic and lymph node metastases, received pembrolizumab single-agent therapy, with a near to complete resolution (Pinto Torres et al., 2017). Another patient, with multifocal CoM and lymph node metastases, received ipilimumab as a single therapy (after tumour debulking and brachytherapy, and lymph node dissection) and had excellent local control and no new lymphatic or systemic metastases (Chaves et al., 2018).

Four patients received more than one type of checkpoint inhibitor (Chang et al., 2019; Finger and Pavlick, 2019; Hong et al., 2020; Sagiv et al., 2018). One patient, with systemic and lymph node metastases, received both ipilimumab and nivolumab. There was a reduction of the systemic tumour burden, and the patient survived at least 3 years (Finger and Pavlick, 2019).

In three patients, there was a switch to other drugs due to treatment failure, or development of adverse events. In the first patient with systemic metastases, ipilimumab + nivolumab combination therapy induced hepatitis, causing a switch to nivolumab alone. This treatment with nivolumab, however, induced an infusion reaction, necessitating a switch to pembrolizumab, which was followed by a favourable response with stable disease for 2 years (Chang et al., 2019). Interestingly, the response to this anti-PD-1 drug was favourable, while PD-L1 expression of the primary CoM was negative.

The second patient, who initially had stable disease for 6 months with pembrolizumab, eventually showed progression. Ipilimumab (with dacarbazine) caused a partial response, but had to be discontinued due to the development of hepatotoxicity (Sagiv et al., 2018).

In the third patient, who was initially treated with ipilimumab single-agent therapy, a new lymph node metastasis developed. After another round of ipilimumab, the patient later developed skin metastases. A third treatment with pembrolizumab was started, and no new developments were reported in the 2 years thereafter (Finger and Pavlick, 2019).

5.3.4. Discussing the cases on checkpoint inhibitor therapy in CoM

All of the 17 currently-reported cases (both with primary as well as metastatic CoM) eventually developed a favourable response to checkpoint inhibition. Unfortunately, little is known about the reasons for initial treatment failure in some of the cases. In only one patient, the PD-L1 status of the tumour was known, which was negative, and this patient experienced a good response to PD-1 blockade (Chang et al., 2019). More studies into the patient characteristics are needed to learn about these mechanisms and the predictive value of checkpoint expression. With the small numbers, and various different treatment regimens, it is impossible to conclude on the superiority of any of the checkpoint inhibitors in CoM. It may be expected however, as in cutaneous melanoma, that PD-1 blockade is more effective than blockade of CTLA4, and that a combined blockade of PD-1 and CTLA4 may yield even better results (Hodi et al., 2018; Larkin et al., 2015).

While the current reports are promising, it may be that other (unsuccessful) cases did not end up in the literature, skewing the results for CoM to a favourable outcome. We concur with the statement of Sagiv et al. of the group of Dr. Esmaeil that “our observations are so far cautiously optimistic” (Sagiv et al., 2018). Immunotherapy can be considered promising for CoM patients who need additional treatment to local therapy, or who develop metastatic disease, and is well-justified in those needy cases.

5.3.5. Adverse events following checkpoint inhibitor therapy

Checkpoint inhibitors allow T cells to respond against tumour cells, but can equally cause an increased T cell-response against normal tissues. These unwanted events are known as immune-related adverse effects (irAEs) and can be severe. Part of the pathophysiology can be an increased number of activated CD4+ and CD8+ T cells, as was detected in peripheral blood of cutaneous melanoma patients following ipilimumab treatment (Weber et al., 2012).

Adverse events due to checkpoint inhibitors can affect any organ, but commonly are directed against the skin (pruritus, rash) or gastrointestinal system (diarrhea, colitis), and less frequently the liver or endocrine system (including general fatigue) (Hodi et al., 2010, 2018; Robert et al., 2015; Weber et al., 2015, 2016). The profile and frequency of irAEs is similar for nivolumab and pembrolizumab (both being anti-PD-1 drugs), while somewhat more common in ipilimumab (as an anti-CTLA4 drug),
Table 12
Overview of reported cases on checkpoint inhibitor therapy in CoM.

| Study | Patient | Type of CoM | Type and Dosage of Immunotherapy | Other Treatments | Clinical Outcome | Adverse Events |
|-------|---------|-------------|----------------------------------|------------------|------------------|----------------|
| **Indicated for primary CoM** | | | | | | |
| Kini et al. (2017) | M, 60s | Recurrence, Fornix, anterior orbit, limbus. | Indic: Preferred non-exenteration (other eye low vision). R: pembro 150 mg iv every 3wks, 1 y. | Prior: exc and cryo Later: exc and cryo | Good response. At 6 m, nodule was flat. [survival not reported] | No AE. |
| Finger and Pavlick (2019) | F, 94 | First, Bulbar to eyelid. No metastasis. | Indic: Age, comorbidity, refused ext. R: First: pembro. Second: pembro + ipi6. | None | Pembro: progression Pembro + ipi: PR. Died after 5 m, unrelated.4 | No related AE. |
| Finger and Pavlick (2019) | M, 76 | Recurrence, Cornea to eyelid. No metastasis. | Indic: Progression despite chemo R: First: ipi. Second: pembro. Third: pembro + IFN-alpha drops. | Prior: multiple local treatments incl IFN-alpha drops. | Ip: no response Pembro: minimal response, but complete with IFN-alpha. After 36 m still CR. | Ipi: adrenal insuff > ipi stop + steroids. Pembro: dermatitis > steroids, antihist |
| Finger and Pavlick (2019) | F, 84 | Recurrence, Cornea to eyelids. No metastasis. | Indic: Preferred non-exenteration R: First: pembro. Second: pembro + ipi. Third: pembro + ipi + IFN-alpha. | Prior: multiple local therapies incl exc, cryo, mmc, brachytherapy | Pembro: minimal success Pembro + ipi: progression Alive 1,5yr after first pembro | No AE reported |
| Hong et al. (2020) | F, 53 | First, Bulbar to tarsal. No metastasis. | Indic: Preferred non-exenteration R: pembro | Prior: none Later: mmc | CR, after 12 m near complete reduction of pigment. Disease free 12 m of follow up. | Cutaneous pruritus |
| **Indicated for metastatic disease** | | | | | | |
| Sagiv et al. (2018) | F, 68 | Recurrent CoM. 2yr after last: syst mets to lung. BRAFneg. | Indic: systemic metastases R: First: pembro. Second: ipi + dacarbD | Prior CoMc: exc, mmc, ext. 30Gy orbit radiotherapy, SLNB, parotidectomy. | Pembro: Stable at 6 m, then progression. Ipi + dacarb: PR Alive at time of writing. | Pembro: No reported. Ipi + dacarb: hepatotoxic > stop. |
| Pinto Torres et al. (2017) | M, 51 | Recurrent CoM. BRAFwt. mets to lymph + skin | Indic: systemic and lymph node mets R: pembro | Prior CoMc exc. Lymphadenectomy Antiviral therapy for HIV. | Near to CR after 3rd cycle. Survival at least 2yr after diagnosis of mets. | No AE: “good tolerance” |
| Sagiv et al. (2018) | F, 58 | CoM recurrence to orbit Mets to lung, liver | Indic: orbital and systemic metastases R: nivo | Prior CoMc excisions, parotidectomy, extant | CR of orbital and meta lesions. Alive at time of writing | Elevated liver enzymes at 3 m > nivo stopped. |
| Sagiv et al., (2018)4 | F, 28 | CoM recurrence After 5yr: mets in breast, lung, bone | Indic: systemic metastases Rnivo | Prior CoMc: Exc + cryo + mmc | PR of systemic mets, later CR. Disease free 3yrs after nivo | No AE reported. |
| Sagiv et al. (2018) | F, 47 | Recurrent CoM 6.5yr after last CoM: mets to lung. | Indic: systemic metastases R: nivo | Prior CoMc exc, cryo, plaque, parotidectomy, lymphadenectomy, adjiv topical IFN, mmc | Resolution of lung lesions. 7 m after nivo stop free from disease | Diarrhoea (AI colitis) > nivo stopped, prednison, infliximab |
| Sagiv et al. (2018) | M, 74 | Recurrent CoM 2 m after last: lung mets. | Indic: systemic metastases R: nivo | Prior CoMc excisions. (declined extant) | Decrease in tumour size Disease free 1 m after nivo stop | After 11 m: colitis > nivo stopped, prednison e |
| Kiyohara et al. (2020) | M, 71 | Recurrent CoM BRAF v600e pos mets bone + liver | Indic: systemic metastases R: nivo | Prior CoMc exc, cryo, enucle, vemurafenib Currently: dabrafenib, trametinib, radiotherapy | Died 24 m after combi therapy | No AE reported |
| Chaves et al. (2018) | M, 72 | First CoM, bulbar to tarsal Lymph nodes pos. | Indic: multifocal CoM + lymph mets R: ipi | This CoM: debulking, brachytherapy, (refused extant). SLNB > dissection | Response, excellent local tumour control. Okay 16 m post radioth. | Mild fatigue, ceased after last cycle ipi. |
| Finger and Pavlick (2019) | F, 72 | CoM: Epibulbar BRAF v600 k mut 9y later: metastases in lung, liver, bone, skin, lymph nodes | Indic: Systemic and lymph metastases R: ipi + nivo 1 | CoM: exc + topical chemo. Metas: no other tx | Resolution of the subcutaneous nodules. Reduction of systemic tumour burden. 3yrs survival at least. | Hepatotoxicity > treatment delay Colitis > fluids, steroids, infliximab |

(continued on next page)
Occurrence of most common adverse events (AE) following immunotherapy in cutaneous melanoma.

| Study | Patient Type of CoM | Type and Dosage of Immunotherapy | Other Treatments | Clinical Outcome | Adverse Events |
|-------|---------------------|----------------------------------|------------------|-----------------|----------------|
| Chang et al. (2019) | F, 60 | CoM recurrence NRAStm, BRAFwt, KITwt, PD-L1+ | Indic: primary CoM + syst metastasis. R: First: ipi + nivo. Second: nivo. Third: pembro | Prior CoM: Excisions, cryo, SLNBneg (declined orbital radiotherapy) | Ipi/nivo: unknown Pembro: CoM and mets response, stable at 2yr. | Pneumonitis > steroids, inhalers |
| Finger and Pavlick (2019) | F, 76 | First CoM. NRAStm Mult. Lymph mets Skin metastasis | Indic: Lymph and systemic metastasis. R: First: ipi. Second: ipi. Third: pembro | CoM: exc, cryo, mmc LN: Paradoxicotomy, surgery, radiotherapy. Skin Mets exc, radiation | Ipi: (1.5yr) New lymph mets Ipi: (3yr) skin meta Pembro: alive after 2yr | Ipi: no AE No other AE reported |
| Hong et al. (2020) | M, 66 | First CoM. Fornix and orbit. Syst mets in lung and liver. | Indic: primary CoM + syst metastasis. R:ipi + nivo | None reported | Resolution of the conj lesion, nice response of syst mets. | Pituitary failure > replacement hydroxy cortisolone and thyroxine. |

Abbreviations: CoM, conjunctival melanoma; pembro, pembrolizumab; ipi, ipilimumab; nivo, nivolumab; PR, partial response; CR, complete response; exent, orbital exenteration; exc, excision; cryo, cryotherapy; mmc, mytomycin-c; SLNB, sentinel lymph node biopsy; M, male; F, female; AE, adverse event; met, metastasis; Indic, indication; R, drug prescription.

Footnotes:
- Unless otherwise reported, standard regimens for immunotherapy were: Pembrolizumab: 2 mg/kg every 3wks, up to 200 mg. Ipilimumab: 3 mg/kg every 3wks (for unresectable/metastatic melanoma) Nivolumab: 240 mg every 2wks (which equals 3 mg/kg).
- Added dose of ipilimumab: 1 mg/kg.
- The patient died due to congestive heart failure.
- Dose of IFN-alfa drops: 1 million units/cc, 4 times daily.
- Intraleisional IFN-alfa, 3 million units per eyelid, monthly interval.
- The reported diagnosis was ‘conjunctival melanoma in situ’.
- Dose of dacarbazine: 800-1000mg/m2, every 3wks.
- Other (likely unrelated) AE were: partial lash loss, dry eye, peripheral corneal vascularization, secondary glaucoma and radiation-induced posterior cataract.
- Dose of nivolumab: 1 mg/kg every 3wks.
- Dose of nivolumab: 240 mg every 2 weeks for 2 cycles and 480 mg every 4 weeks for 1 cycle.
- This case was presented earlier as well in a report by Ford et al. (2017).

particularly when combined with nivolumab (Weber et al., 2016). Grade 1-2 adverse events of diarrhea, fatigue, pruritus and rash have each been reported in about 20–40% of patients receiving checkpoint inhibition, demonstrating their relatively common occurrence (Table 13). Adverse events of grade 3 or higher were reported in 10% of pembrolizumab-treated patients, 22% of nivolumab, 28% of ipilimumab, and 59% of nivolumab + ipilimumab treated cases with cutaneous melanoma (Hodi et al., 2018; Robert et al., 2015c).

Some of the adverse events resemble manifestations of auto-immune disease. Checkpoint inhibitors were reported to cause a decrease in pigmentation of the skin (i.e. vitiligo) (Byrne and Fisher, 2017) and even of choroidal nevi in the fundus (Sophie et al., 2019).

As there is only a limited number of reports on checkpoint inhibition in CoM, there are very few reports on AE’s in these patients (Table 12). In 6 out of 17 reports, no AE’s were reported at all. In the CoM patients who did develop AE’s, similar events were noted as in patients receiving immunotherapy for cutaneous melanoma (Hodi et al., 2018; Robert et al., 2015c). For ipilimumab single-agent therapy, adrenal insufficiency (Finger and Pavlick, 2019), hepatotoxicity (Sagiv et al., 2018), and fatigue (Chaves et al., 2018) have been reported. With nivolumab single-agent therapy, elevated liver enzymes (Sagiv et al., 2018), diarrhea (Sagiv et al., 2018), colitis (Sagiv et al., 2018), and an infusion reaction (Chang et al., 2019) have been reported. Pembrolizumab single-agent therapy may have been the best-tolerated drug, with only one out of 8 patients reporting dermatitis (Finger and Pavlick, 2019). The combination of ipilimumab + nivolumab led to hepatotoxicity, colitis and pneumonitis (Finger and Pavlick, 2019), and pituitary failure (Hong et al., 2020). The development of reported AE’s in CoM patients required discontinuation of therapy, switch to another type of immunotherapy, or additional specific treatments, e.g. with corticosteroids or antihistamines.

Of specific note are the ‘ocular irAEs’, i.e. adverse events in the ocular region after checkpoint inhibitor treatment for melanoma at any site. Such ocular irAEs have been reported after anti-CTLA4 treatment in 1.3% of patients (Tarhini, 2013) and after anti-PD1 treatment in 1.6% of patients (Zimmer et al., 2016). Ocular irAEs mostly include uveitis, orbital inflammation, dry eyes, and blurred vision (Papavasileiou et al., 2016; Tarhini, 2013; Zimmer et al., 2016), as recently reviewed by Dalvin et al. (2018b). Rare events include Vogt-Koyanagi-Harada (VKH) syndrome with serous retinal detachment (Wong et al., 2012), or ocular rosacea (Brouwer et al., 2017). Most ocular irAEs can be treated with topical corticosteroids, and only rarely systemic therapy is required (Fierz et al., 2016). Notably, immune checkpoint inhibition may be associated with site-specific metastases, as remarkable cases of vitreous
metastases of cutaneous melanoma were reported (Francis et al., 2020; Kellermann et al., 2017). Not only ocul oncologists, but also general ophthalmologists should be aware of these events as immunotherapy is increasingly being applied, and it becomes more common for these irAEs to present in an ophthalmological practice.

5.4. Conclusions (Targeted therapy and checkpoint inhibitor therapy in CoM)

Conventional therapy of localised CoM relies on surgical excision with adjuvant therapy including cryotherapy, topical chemotherapy and/or radiotherapy. Treatment of extensive disease can be more complex, depending on individual cases. Treatment options for disseminated disease are very limited, and no consensus exists on the optimal approach. It is mainly in extensive and disseminated disease that new therapies are urgently needed. Targeted therapy and immunotherapy have been recently introduced successfully for the treatment of (advanced) cutaneous melanoma, and these therapies can be beneficial to CoM patients as well. Inhibitors of BRAF and MEK act in CoM due to the presence of BRAF mutations and activation of the MAPK pathway; a notable benefit is seen in the combination of these two drugs.

Several new targets for therapy are under investigation in genetics, e.g. c-KIT, ERK1/2, PI3K/akt/mTOR, TERT and EZH2. While the value of such targeted therapies has yet to be determined, these may perhaps not be a cure for all, but should be seen as part of a personalized approach after genetic screening. This can e.g. be beneficial for patients with no BRAF mutation (limiting response to current BRAF inhibitors) or when a (rare) KIT mutation is present.

Checkpoint inhibitors emphasize similarities in the tumour microenvironment of CoM and that of cutaneous melanoma, while differing greatly from UM. Promising results from anti-CTLA4 and anti-PD-1/PD-L1 antibodies in small series of CoM show that these have a first-line position in metastatic disease. While testing for expression of checkpoint molecules such as PD-1/PD-L1 could theoretically add to a personalized approach, current predictive values for treatment response are limited and we urge that negative-expressing patients are not excluded.

A secondary effect of the introduction of aforementioned therapies is the renewed interest in the SLNB in CoM. Findings of SLNB can now be followed by a curative intent, and early detection of metastases may improve the benefit of new treatments.

While results of both targeted and checkpoint inhibitor therapy are promising, clinicians should be aware of the specific adverse events and not forget that these can include all organ tracts and can be severe. As in any clinical approach, this should be weighted in the decision for certain treatment.

6. Future directions and conclusions

6.1. Future directions

Recent developments in cancer research show that tumour genetics and immunology are promising fields that not only lead to a better understanding of CoM, but also provide new targets for therapy. Future projects are numerous, and illustrate that genetics and immunology are intertwined. A recurrent theme for CoM is to translate knowledge from studies on more abundant cutaneous melanoma. While this may be beneficial to the treatment of CoM (as a rare disease), it should be stressed however that the eye has several unique features and that conjunctiva-specific characteristics must not be overlooked.

Important new work is to further investigate the genetic background of CoM, and to evaluate the impact of genetics on tumour behaviour. This is facilitated by rapidly-developing sequencing techniques. Important questions that need answering are how to differentiate benign from malignant lesions, how to identify the most ominous lesions (with a risk for recurrence or metastasis, warranting extensive treatment and intensive follow-up) and how to select the most suitable therapy for individual patients. The mutational status (of genes such as BRAF, NRAS, and TERT) has proved to be important, and warrants studies into less common genes such as KIT. Chromosome status and expression of miRNA showed promising results to differentiate and prognosticate lesions, but require confirmation for further use. Genetics suggest that subgroups of CoM exist with distinct driver mutations and pathway activation (Cisarova et al., 2020). While this parallels the principles learned from cutaneous melanoma it is important to look for CoM-specific groups. Following up on the questions on the development of CoM, differences between e.g. sun-exposed and non-exposed lesions need further evaluation, as well as the role of precursor lesions, melanin pigments and the immune system in melanocyte transformation.

The clinical revolution of recent years was the introduction of targeted therapies (BRAF/MEK inhibitors) and checkpoint inhibitors (anti CTLA4/PD-L1) as treatment of locally-advanced or disseminated CoM. This parallels guidelines from cutaneous melanoma and is likely to be implemented even more as drugs become more available and clinicians learn about their use. A first question is to identify patients who may benefit most from these therapies, or reversely, to match a patient to the optimal therapy. Apart from earlier mentioned tumour genetics, immune parameters as expression of HLA and PD-L1, or presence of immune cells, should be evaluated as biomarkers for a therapeutic response. Current studies show that expression of theoretically-important markers (such as PD-L1) is not a prerequisite for a therapeutic response, however, and that much is to be learned. A second question is how to overcome treatment resistance, which is unfortunately a common event in patients who respond well initially. We advocate to study combinations of BRAF inhibitors with not only MEK inhibitors, but also drugs targeting the AKT pathway, and possibly even YAP1 (Brouwer et al., 2021). Furthermore, the combination of PD-1/PDL1 and CTLA4 inhibition should be studied. The addition of immune-stimulating agents such as IFN-alfa may be interesting as promising reports have emerged in the CoM literature; topical IFN-alfa drops are already used for topical treatment of malignant ocular surface disease, and are readily available. Even so, radiotherapy or photodynamic therapy may be added to immunotherapy as an enhancer of the immune system. This may facilitate use of aforementioned drugs in not only metastasized CoM patients, but also local disease.

In addition to earlier mentioned developments, several new drugs and drugable targets are under investigation in preclinical studies for CoM or in cutaneous melanoma. New drugs target cKIT, ERK1/2, PI3K/akt, TERT and EZH2. Some rely on specific (rare) mutations, suggesting that these are suitable for individualized therapy, or as last-resort, but with unknown value for the majority of patients. By the rarity of CoM it is not feasible to evaluate all of these targets in CoM itself, so data may need extrapolation from other tumour types. Screening in CoM models is warranted prior to introduction in clinical studies, however, to prevent the pursuit of inappropriate targets. The plethora of new drugs poses an additional question, however, to determine which combination of (targeted and immuno-) therapy is optimal, and whether simultaneous or sequential treatment should be applied.

For targeted therapy and checkpoint inhibitor therapy, evidence on their potential benefit is solid enough to advise inclusion of (metastatic) CoM patients in trials. The earlier-mentioned lack of proper biomarkers would argue for liberal inclusion criteria. The rarity of CoM calls for international collaboration to obtain sufficient numbers, and to include CoM patients in trials of cutaneous (and when applicable, mucosal) melanoma. A separate registry should be advised however, to prevent loss of CoM specific data.

A concurrent development with the introduction of therapies for metastatic CoM, is the increased use of screening methods for (lymph node) metastases such as with the SLNB. There is debate on its position in CoM (Mor et al., 2019), but the SLNB is being implemented more frequently, and has been suggested as an addition to the current AJCC staging system for its prognostic value (Esmaili et al., 2019). It is likely
that advanced staging becomes routine practice for CoM, in which not only tumours are better characterized, but (lymph node) metastases as well.

6.2. Conclusions

From an ophthalmological perspective, CoM is a remarkable melanoma: it is very different from the far more prevalent UM (often referred to as `ocular melanoma`), and much more resembles cutaneous and mucosal melanoma in its biology. The genetic background of CoM is characterized by mutations in BRAF, NRAS, NF1, and TERT and it has a complex karyotype with various aberrations. Genetic studies confirm that UV radiation contributes to CoM development, suggesting differences between sun-exposed and non-exposed conjunctiva, but little is yet known about the relation between clinical (tumour) characteristics and genetic profile. The relation between CoM and the tumour microenvironment is being unravelled, with a favourable role for the presence of immune cells and expression of HLA molecules, and an unfavourable role for expression of checkpoint molecules such as PD-L1.

New therapies that became available for cutaneous melanoma in recent years, show promising results in CoM. Targeted therapy (such as BRAF and MEK inhibitors) and checkpoint inhibitors (such as anti-CTLA4 and anti-PD1L1 drugs) are an extension to the toolbox of clinicians who currently rely on excision and topical adjuvant therapy for localised disease, while options for treatment of disseminated disease are limited. While the major advancement of new therapies may be in the treatment of metastatic CoM, new therapies can be used to prevent mutilating extensive surgery for advanced primary CoM as well. As a side effect, better staging and screening methods (such as with a SLNB) are gaining popularity, now that they can be followed by therapeutic sides effect, better staging and screening methods (such as with a SLNB) are gaining popularity, now that they can be followed by therapeutic

Acknowledgements

The authors like to thank Dr. Demetrios G. Vavvas (Massachusetts Eye and Ear Infirmary, Boston, USA) for his contribution to the section on melanoma incidence and tissue size (section 2.4).

References

Abdel-Rahman, M.H., Piliarkis, R., Cebulla, C.M., Massengill, J.B., Christopher, B.N., Boru, G., Hovland, P., Davidson, G.F.H., 2011. Germline BAP1 mutation predisposes to uveal melanoma, lung adenocarcinoma, meningioma, and other cancers. J. Med. Genet. 48, 856–859.

Abdel-Rahman, M.H., Yang, Y., Zhou, X., Craig, E.L., Davidson, F.H., Eng, C., 2006. High frequency of submetacentric hemizygous deletion is a major mechanism of loss of expression of PTEF in uveal melanoma. J. Clin. Oncol. 24, 288–295.

Alessandrini, L., Parrozzani, R., Bertorello, R., Valenti, C., Candiotti, G., Giacomelli, L., Mileda, E., Blom, D.J., Luyten, G.P., Mooy, C., Kerkvliet, S., Zwinderman, A.H., Jager, M.J., 1997. Tumor infiltrating lymphocytes: the regulator of melanoma evolution. Oncol Lett 17, 4155–4161.

Aronow, M.E., Topham, A.K., Singh, A.D., 2018. Uveal melanoma: 5-year update on incidence, treatment, and survival (SEER 1973-2013). Ocul Oncol Pathol 4, 145–151.

Ascierto, P.A., McArthur, G.A., Dreno, B., Atkinson, V., Lizziak, G., Di Giacomo, A.M., Mandala, M., Demidov, L., Stroyakovskiy, D., Thomas, L., de la Cruz-Merino, L., Dutriaux, C., Garbe, C., Van, Y., Wangchenko, M., Chang, L., Hsu, J.J., Korsak, D.O., Rooney, L., Ribas, A., Larkin, J., 2016. Cohortinib combined with vemurafenib in advanced BRAF(V600) mutant melanoma (coBIM): updated efficacy results from a randomised, double-blind, phase 3 trial. Lancet Oncol. 17, 1248–1260.

Azim, F., Scolyer, R.A., Rumble, P., Moncrieff, M., Murali, R., McCarthy, S.W., Saw, R.P., Thompson, J.F., 2012. Tumor-infiltrating lymphocyte grade is an independent predictor of sentinel lymph node status and survival in patients with cutaneous melanoma. J. Clin. Oncol. 30, 2678–2683.

Backmann, L.M., Halvorsen, O.J., Gollett, K, Stoffaness, L.M, Straume, O, Haukaas, S.A., Salvesen, H.B., Otte, A.P., Axelsen, L.A., 2006. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J. Clin. Oncol. 24, 268–273.

Bald, T., Quast, P., Berg, J., Rogova, M., Gedde, N., Lopez-Ramos, D., Kohlmeyer, Mierenburg, J., Villeneuve, K., Gaffal, E., Tuting, T., 2014. Ultraviolet-radiation-induced inflammation promotes angiotropism and metastasis in melanoma. Nature 507, 109–113.

Bauer, G., Hovland, P., Davidson, G.F.H., 2011. Germline BAP1 mutation predisposes to uveal melanoma, lung adenocarcinoma, meningioma, and other cancers. J. Med. Genet. 48, 856–859.

Benage, M.J., Morrow, N.C., Janson, B.J., Greiner, M.A., 2019. Evaluation of interferon alpha 2b as adjunctive therapy for conjunctival melanoma. Am J Ophthalmol Case Rep 15, 100467.

Bianconi, A.P., Esteve-Puig, R., Nosrati, A., Hinds, B., Hobbs-Muthukumar, A.

N.J. Brouwer et al.

Progress in Retinal and Eye Research 86 (2022) 100971

No financial disclosure exists for any author.

Contributions

Niels J. Brouwer (45%), Robert M. Verdijk (9%), Steffen Heegaard (7%), Marina Marinkovic (7%), Bita Esmaili (7%), Martine J. Jager (25%)
Griewank, K.G., Westekemper, H., Murali, R., Mach, M., Schilling, B., Livingstone, E., Schimming, T., Hu, D.N., Yu, G.P., McCormick, S.A., Schneider, S., Finger, P.T., 2005. Population-based incidence of uveal melanoma in various races and ethnic groups. Am. J. Ophthalmol. 140, 543–553.

Hodahl, E., Kalvennes, M.B., Mannelqvist, M., Ladstein, R.G., Askel, L.A., 2018. Prognostic impact and concordance of TERT promoter mutation and protein expression in matched primary and metastatic cutaneous melanoma. Br. J. Cancer 119, 105.

Humer, J., Ferko, B., Wallenberger, A., Rappberger, R., Pehamberger, H., Muster, T., 2008. Acidophilin inhibits melanoma cell growth in vitro and in vivo. Melanoma Res. 18, 314–321.

Iagker, P., Osterlin, A., Enholm, G., Heegaard, S., Lindegaard, J., Overgaard, J., Storm, H.H., 2005. Uveal and conjunctival malignant melanomas in Denmark, 1943-97: Incidence and validation study. Ophthalmologic Epidemiol. 12, 232–233.

Karnes, S., Burrows, R.C., Orci, L., Kalina, R.E., Rodig, S.J., Bothwell, M.A., Schmidt, R., 2002. Immune phenotype of conjunctival melanomas: comparisons with uveal and cutaneous melanomas. Arch. Ophthalmol. 120, 1625–1629.

Jager, M.J., Shields, C.L., Cebulka, C.M., Abel-Bahman, M.H., Grossniklaus, H.E., Stern, M.J., Carvajal, R.D., Belfort, R.N., Jia, R., Shields, J.A., Damato, B.E., 2020. Uveal melanoma. Nat Rev Dis Primers 6, 24.

Jakobiec, F.A., 2016. Conjunctival primary acquired melanocytic is it time for a new terminology? Am. J. Ophthalmol. 162, 3–19.

Jangard, M., Zebary, A., Ragnarsson-Olding, B., Hansson, J., 2015. TERT promoter mutations in sinonasal malignant melanoma: a study of 49 cases. Melanoma Res. 25, 185–188.

Javed, A., Arogulio, D., Johnston, C., Gatalica, Z., Terai, M., Weight, R.M., Orloff, M., Mastrangelo, M.J., Sato, T., 2017. PD-L1 expression in tumor metastasis is different between uveal melanoma and cutaneous melanoma. Immunother. 12, 1333–1330.

Jay, B., 1965. Naevi and Melanomas of the conjunctiva. Br. J. Ophthalmol. 49, 69–72.

Jeffrey, I.J., Lucas, D.R., McEwan, C., Lee, W.R., 1986. Malignant melanoma of the conjunctiva. Histopathology 10, 363–378.

Joensuu, H., DeMatteo, R.P., 2012. The management of gastrointestinal stromal tumors: a new era for targeted and multidisciplinary therapy of malignancy. Annu. Rev. Med. 63, 247–285.

Johnson, P.A., Brooks, K., Newell, F., Palmer, J.M., Wilmott, J.S., Pritchard, A., Brow, N., Wood, S., Carlsson, M., Leonard, C., Koufariotis, L., Nathan, V., Beasley, A., Howie, M., Lawson, R., Rizzio, H., Schmidt, C.W., Long, G.V., Hamilton, H., Kilgara, J.F., Isaac, S., Gray, E.S., Rolfe, O.J., Park, J.J., Stark, A., Mann, G.G., Slattery, L.R., Pearson, J.V., van Baren, N., Waddell, N., Klui, W.D., McGrath, L.A., Warrier, S.K., Glisson, B.S., Hayden, W., 2010. Whole genome landscapes of uveal melanoma show an ultraviolet radiation signature in iris tumours. Nat. Commun. 11, 2408.

Johnson, D.B., Freamont, G.M., Riott, M.J., Yasko, E., Yu, Y., Guo, H., Brenner, M., Friedlander, P., Gonzalez, R., Weber, J.S., Gajewski, T.F., O'Neill, L.A., Lu, X., Matkowsky, D., Imagawa, M., Hsu, J., Medeiros, L., Haanen, J., Haas, M., Cooper, C., Li, L., Li, J., 1986. Malignant melanoma of the conjunctiva. Histopathology 10, 363–378.

Kastelan, S., Gverovic Antunica, A., Beketic Oreskovic, L., Salopek Rabatic, J., Kasun, B., 2017. Immune checkpoint blockade for unresectable or metastatic melanoma during treatment with ipilimumab. Ophthalmologe 114, 163–170.

Kim, S.Y., Kim, S.N., Hahn, H.J., Lee, Y.W., Choe, Y.B., Ahn, K.J., 2015. Metaanalysis of melanoma in a patient with neurofibromatosis. Surv. Ophthalmol. 45, 231–238.

Kimmel, B., 2017. The role of lifestyle factors in the epidemiology of melanoma. J. Med. 363, 711–723.

Kenawy, N., Kalirai, H., Sacco, J.J., Lake, S.L., Heegaard, S., Larsen, A.C., Finger, P.T., 2014. Frequent mutation of BAP1 in conjunctival lesions. Graefes Arch. Clin. Exp. Ophthalmol. 248, 111–115.

Lina, V., Eertwegh, A.J., Lutzky, J., Lorigan, P., Vaubel, J.M., Linette, G.P., Hogg, D., 2017. Tumours of the Eye, fourth ed. International Agency for Research on Cancer, Lyon.

Lodish, H.F., Berk, J., Zipursky, S.L., Matsudaira, P., Berget, S.M., Darnell, J.E., 2000. Molecular Cell Biology, 5th ed., W. H. Freeman and Company, New York.

Liu, Y., Wang, J., Wang, M., Li, S., Zhang, N., Chen, Z., Wang, W., Wang, Q., 2019. Phase II, open-label, single-arm trial of imatinib mesylate in patients with metastatic melanoma harboring c-KIT mutation or amplification. J. Clin. Oncol. 29, 2904–2909.

Hodder, H.C., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.

Hodges, L., 2017. TERT promoter mutations in uveal and conjunctival uveal melanoma: a systematic review. Cancer Treat. Rev. 60, 44–52.

Hodges, L.R., Jorritsma, R., 2010. Interferon for the treatment of melanocytic conjunctival lesions. Graefes Arch. Clin. Exp. Ophthalmol. 248, 111–115.

Hofvendahl, K., Acke, E., Acke, B., Acke, J., Acke, H., Acke, V., 2013. The relative risk of developing uveal melanoma. Int. J. Mol. Sci. 21.

Hoffman, W.W., 2014. HLA and disease: guilt by association. Int. J. Immunogenet. 41, 327–336.

Hui, D.N., McCormick, S.A., Seedor, J.A., Ritterband, D.C., Shah, M.K., 2007. Isolation, purification and cultivation of conjunctival melanocytes. Exp. Eye Res. 84, 655–662.

Huang, H.Y., Yu, C., Taminiau, J., Bjoerk, P.T., 2008. Population-based incidence of conjunctival melanomas in various races and ethnic groups and comparison with other amelanomas. Am. J. Ophthalmol. 145, 418–423.
patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol. 16, 375–384.

Weber, J.S., Hamid, O., Chasalow, S.D., Wu, D.Y., Parker, S.M., Galbraith, S., Gnjatic, S., Berman, D., 2012. Ipilimumab increases activated T cells and enhances humoral immunity in patients with advanced melanoma. J. Immunother. 35, 89–97.

Weber, J.S., Postow, M., Lao, C.D., Schadendorf, D., 2016. Management of adverse events following treatment with anti-programmed death-1 agents. Oncol. 21, 1230–1240.

Weis, E., Shah, C.P., Lajous, M., Shields, J.A., Shields, C.L., 2006. The association between host susceptibility factors and uveal melanoma: a meta-analysis. Arch. Ophthalmol. 124, 54–60.

Welsh, S.J., Corrie, P.G., 2015. Management of BRAF and MEK inhibitor toxicities in patients with metastatic melanoma. Ther Adv Med Oncol 7, 122–136.

Westekemper, H., Anastassiou, G., Sauerwein, W., Chauvel, P., Bornfeld, N., Steuhl, K.P., Meller, D., Wong, J.R., Nanji, A.A., Galor, A., Karp, C.L., 2014. Management of conjunctival exenteration in the management of extended conjunctival melanoma. Graefes Arch. Clin. Exp. Ophthalmol. 244, 438–446.

Yavuzigitoglu, S., Koopmans, A.E., Verdijk, R.M., Vaarwater, J., Eusken, B., van Bodegom, A., Paridaens, D., Kilic, E., de Klein, A., Rotterdam Ocular Melanoma Study, G., 2016. Uveal melanomas with SF3B1 mutations: a distinct subclass associated with late-onset metastases. Ophthalmology 123, 1118–1128.

Yu, F.X., Lao, J., Mo, J.S., Lü, G., Kim, Y.C., Meng, Z., Zhao, L., Peyman, G., Ouyang, H., Jiang, W., Zhao, J., Chen, X., Zhang, L., Wang, C.Y., Bastian, R.C., Zhang, K., Guan, K.L., 2014. Mutant Gt(11) promote uveal melanoma tumorigenesis by activating YAP. Canc. Cell 25, 822–830.

Yu, G.P., Hu, D.N., McCormick, S., Finger, P.T., 2003. Conjunctival melanoma: is it increasing in the United States? Am. J. Ophthalmol. 135, 800–806.

Yu, G.P., Hu, D.N., McCormick, S.A., 2006. Latitude and incidence of ocular melanoma. Photochem. Photobiol. 82, 1621–1626.

Zaidi, M.R., Davis, S., Noonan, F.P., Graff-Cherry, C., Hawley, T.S., Walker, R.L., Feigenbaum, L., Fuchs, E., Lyakh, L., Young, H.A., Hornyak, T.J., Arndtner, H., Trinchieri, G., Meltzer, P.S., De Fabo, E.C., Merlino, G., 2011. Interferon-g links ultraviolet radiation to melanomagenesis in mice. Nature 469, 548–553.

Zebary, A., Jangard, M., Osmohl, K., Ragnarsson-Olding, B., Hansson, J., 2013. KIT, NRAS and BRAF mutations in sinonasal mucosal melanoma: a study of 56 cases. Br. J. Canc. 109, 559–564.

Zhang, Y., Choksi, S., Chen, K., Pobezinskaya, Y., Linnoila, I., Liu, Z.G., 2013. ROS play a critical role in the differentiation of alternatively activated macrophages and the occurrence of tumor-associated macrophages. Cell Res. 23, 898–914.

Zhou, C., Wang, Y., Jia, R., Fan, X., 2017. Conjunctival melanoma in Chinese patients: local recurrence, metastasis, mortality, and comparisons with Caucasian patients. Invest. Ophthalmol. Vis. Sci. 58, 5452–5459.

Zhu, J., Paul, W.E., 2008. CD4 T cells: fates, functions, and faults. Blood 112, 1557–1569.

Zimmer, L., Goldinger, S.M., Hofmann, L., Loqui, C., Ugurel, S., Thomas, I., Smidigen, M.I., Gutzmer, R., Uitkai, J.S., Goppner, D., Hassel, J.C., Meier, F., Tieretz, J.K., Forchchner, A., Weishaupt, C., Leverkus, M., Wahl, R., Dietrich, U., Garbe, C., Kirchberger, M.C., Eigentler, T., Berking, C., Gesierich, A., Ackardhardt, A.M., Schadendorf, D., Schulner, G., Dummer, R., Heinzlerling, L.M., 2016. Neurological, respiratory, musculoskeletal, cardiac and ocular side-effects of anti-PD-1 therapy. Eur. J. Canc. 60, 210–225.

Zingg, D., Debbache, J., Schafer, S.M., Tuncer, E., Frommel, S.C., Cheng, P., Arenas-Ramirez, N., Haeusel, J., Zhang, Y., Bonalli, M., McCabe, M.T., Creasy, C.L., Levesque, M.P., Boyman, O., Santoro, R., Shakhova, O., Dummer, R., Sommer, L., 2015. The epigenetic modifier EZH2 controls melanoma growth and metastasis through silencing of distinct tumour suppressors. Nat. Commun. 6, 6051.