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Chapter 4

Histopathology of the Ocular Surface

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Abstract

Three integral parts that cover the ocular surface are the conjunctiva, limbus, and cornea. The conjunctiva is a see-through mucous membrane that lines the internal surface of the eyelids and the front surface of the eyeball, ending at the limbus. It is highly vascular with a dense lymphatic network. The limbus forms the boundary between the transparent cornea and the opaque sclera. The cornea is a complex structure that provides a protective function and is responsible for about 75% of the optical power of the eye. Histology of these highly specialized biological materials as well as the ways in which individual components are structurally and functionally related will be discussed in this chapter. Then, we will go over the pathological oncology processes that can affect the ocular surface.

Keywords: histology, histopathology, ocular surface, conjunctiva, limbus, cornea, epithelium, stroma, endothelium, squamous cell carcinoma, melanoma

1. Introduction

The ocular surface is an anatomic entity that is composed of different ocular structures: conjunctiva, limbus, and cornea. A healthy ocular surface should have a healthy tear film overlying it. The maintenance of ocular surface in an optimal and healthy state contributes both esthetic and functional wellness of the eye.

From the anatomic point of view, the ocular surface includes the mucosal epithelium limited by the skin of the free edge of the eyelids. It includes the cornea and the conjunctiva. The interdependence of the structures integrated into this system and their influence on the corneal epithelium and ultimately on the eyeball makes them of great importance to the health of the eye. In addition, the primordial cells of the corneal epithelium are located at the corneoscleral limbus.
Tumors of the conjunctiva can arise from any of the cells that are naturally present and are evident histologically; thus, there is a wide variety of these tumors. They originate from the squamous epithelium, melanocytes, and lymphocytic cells found in the conjunctival stroma. The epithelial and melanocytic origins are more frequent.

This chapter will start by describing the normal histology of the ocular surface with selected correlated functional aspects of the biologic micro-design. Afterward, the most important malignant neoplastic processes affecting the conjunctiva will be reviewed along with the latest updates in the medical literature.

2. Histology of ocular surface

2.1. Conjunctiva

The conjunctiva is a transparent mucous membrane that covers the ocular surface from the limbus to the mucocutaneous junction. It plays an essential role in maintaining a healthy and optically clear cornea. The part covering the sclera is known as the bulbar conjunctiva, while the palpebral conjunctiva lines the posterior surface of the eyelids. The fornical conjunctiva is the portion that connects the bulbar with the palpebral parts. The bulbar conjunctiva is loosely attached to the underlying Tenon’s capsule except at the limbus where they fuse together. The palpebral part is tightly adherent to the tarsus, while the fornical portion is loose and redundant. A crescent-shaped fold of the conjunctiva called the plica semilunaris is found nasally. Medial to the plica lies the caruncle, a pinkish globular nodule that contains sebaceous glands and hair follicles in addition to the adnexal elements of the conjunctival stroma.

The surface layer of the conjunctiva is composed of non-keratinizing stratified squamous epithelium with numerous goblet cells. Melanocytes are normally present in the basal layer of the epithelium. The morphology and the number of layers of the conjunctival epithelial cells change according to the region from which a biopsy was taken. There are approximately six to nine layers of stratified squamous epithelium in the bulbar conjunctiva. The epithelial cells are columnar in the fornical part and cuboidal in the area attached to the tarsus where the epithelial cells are packed in two to five layers. The goblet cells are distributed throughout the bulbar and tarsal conjunctiva and are most concentrated inferonasally and in the region of the caruncle and plica semilunaris.

The conjunctival stroma (substantia propria) is a thin, richly vascularized layer enclosing scattered lacrimal glands (based on the anatomic location), lymphatics, plasma cells, macrophages, and mast cells. Additionally, it comprises numerous elastic fibers that facilitate globe movement in all gazes. Specialized collections of T and B lymphocytes underlying a modified epithelium is known as conjunctiva-associated lymphoid tissue (CALT). It functions to process antigens and provides immunity against pathologic microbes on the ocular surface [1]. Conjunctival stem cells are scattered throughout the bulbar or fornical conjunctiva.

2.2. Corneoscleral limbus

The limbus is the transitional region between the corneal margin and the anterior sclera. It is approximately 1–1.5 mm wide. The limbus contains the corneal stem cells located in the
basal cell layer. There are histological, pathological, and surgical definitions of the limbus. Histologically, the central margin of the limbus is limited by a line connecting the peripheral termination of Bowman’s layer externally and Schwalbe’s line, the peripheral termination of Descemet’s membrane, internally. The peripheral margin of the limbus is bounded by the central margin of the scleral spur. The peripheral margin from pathologist’s point of view is formed by a vertical line that is perpendicular to the scleral spur [1].

Surgically, the limbus is divided into two zones: a central blue-gray zone and a peripheral white zone. The central zone corresponds to the area connecting Bowman’s layer and Descemet’s membrane. The peripheral zone overlies the trabecular meshwork [2].

2.3. Cornea

The cornea is the anterior and transparent portion of the fibrous tunic of the eye globe and is the most powerful refractive element of the optical system of the eye. Its transparency is a function of different factors: avascularity, relative acellularity, relative dehydration, and the remarkable organization of the stromal collagen lamellae. The anterior surface of the cornea measures 11–12 mm horizontally and 10–11 mm vertically. The thickness of the cornea is 0.52 mm centrally and 0.65 mm peripherally. The water content is 78% and is controlled by intact epithelium and endothelium. Its refractive index is 1.376 [1].

In the following sections, the histology of the different layers of the cornea will be described from anterior to posterior: the epithelium, Bowman’s layer, stroma, Descemet’s membrane, and endothelium.

2.3.1. Epithelium

The corneal epithelium is the outermost layer of the cornea and is derived from the embryonic surface ectoderm. It is composed of 4–5 multilayered stratified, non-keratinized squamous epithelium with an underlying single layer of basal cells. It is continuous with the conjunctival epithelium at the limbus. Its overall thickness is approximately 50 μm with a complete turnover in 7–10 days. This multilayered epithelium is distributed in three strata: superficial flattened cells, middle wing cells, and deep basal cells [1].

The basal cell layer is considered the mother of the overlying cells, i.e., wing and the superficial cells. The basal cell density is approximately 6000 cells/mm². They originate from the corneal limbal stem cells. The new cells travel from the limbus in a centripetal manner at a rate of approximately 120 μm/week. The cells communicate with each other through gap junctions and actively secrete their basal lamina (50 nm thick, type IV collagen) where they adhere to it via hemidesmosomes. Alteration of hemidesmosomes can result in recurrent epithelial erosions, as seen in patients with epithelial basement membrane dystrophy (EBMD) [1].

Wing cell layer is composed of 2–3 cell rows that overlie the basal cells. These cells are given this name because they have extensions that make them resemble wings in cross section. They are attached to each other by zonulae occludentes that form a semipermeable membrane, an important factor preventing components of the tear film to get into the corneal stroma. The outermost layer is 2-3 rows of flattened cells that are shed in the tear film to be replaced by other cells. The ultrastructure of their apical surfaces is rough and quite irregular owing to
the presence of countless microplicae and microvilli. All epithelial cells are connected to each other by desmosomes and communicate via gap junctions. Reduction of the number of desmosomes is noted in patients with topical anesthetic abuse [3].

Growth factors secreted by the lacrimal glands and corneal epithelial cells play an essential role in the maintenance of healthy epithelial cells. Among which, insulin-like growth factor (IGF-1) and its receptor IGF-1R alterations have been implicated in cases of hyperglycemia with resultant diabetic keratopathy. This disease is associated with superficial punctate keratitis, recurrent corneal erosions, and persistent epithelial defects in addition to severe neuroepithelial dysfunction. Moreover, the interaction of IGF-1R with its twin insulin receptor (INSR) to form a hybrid receptor (the Hybrid-R) has been reported. The Hybrid-R was detected in the corneal epithelial cell nuclei where it interacts with DNA and is proposed to control gene expression important in maintaining ocular surface biology [4].

2.3.2. Bowman’s layer

Bowman’s layer lies directly underneath the basal lamina. As the name implies, this stratum is a layer and not a true membrane with the absence of staining by periodic acid-Schiff (PAS) stain. It is an acellular layer that cannot regenerate forming a scar after injury. It has a thickness of 8–12 μm. Similar to the corneal stroma, it is composed of type I and type V collagen, but in contrast to the stroma, the ratio of type V to type I is higher. In addition, unlike the stroma, the collagen lamellae are smaller and more randomly arranged. The limbus starts at the peripheral termination of this layer [1].

Bowman’s layer is a key factor in maintaining the corneal biomechanical properties by its stiff and tough nature. Abnormalities of this layer can result in corneal ectatic disorders such as keratoconus due to biomechanical failure [5].

2.3.3. Stroma

The corneal stroma occupies 90–95% of the thickness of the cornea. It is derived from the neural crest cells. In the center, it measures about 500 μm, while it becomes thicker toward the periphery. It is formed by proteoglycans (keratan sulfate and dermatan sulfate) covalently linked to a nucleus of a protein and a large number of collagen fibrils arranged parallel to the surface of the cornea. These corneal stromal collagen lamellae (200–250 lamellae) are composed principally of type I and type V, with lesser amounts of types III and VI. The distance between these lamellae, occupied by proteoglycans, is constant. This property is very important in maintaining the corneal transparency by eliminating any interference with light transmission [1]. A relatively recently introduced layer, the pre-Descemet’s layer (Dua’s layer), is an acellular layer measuring approximately 10 μm and is found in the posterior stroma [6]. Among these collagen fibrils, we find the keratocytes, which are specialized fibroblasts that synthesize collagen and proteoglycans. There are about 2.4 million keratocytes spread within the stroma with higher density being anterior. They are extremely active cells evident by abundant mitochondria, rough endoplasmic reticulum, and Golgi apparatuses. They are flat and evenly distributed. Their plasma membranes are fenestrated. Gap junctions are the conduits through which communication occurs. The cell density decreases with age at a slower rate than the corneal endothelium [1].
The posterior portion of the stroma is typically wetter than the anterior one. This occurs because of the wetting effect of the aqueous humor posteriorly and the drying effect of the atmosphere anteriorly. Corneal stromal edema, as seen in cases of endothelial failure, will result in enlarged spacing between the lamellae and subsequent visual compromise [1].

2.3.4. Descemet’s membrane

Descemet’s membrane corresponds to the basal lamina of the corneal endothelium. It is a true basement membrane that is PAS-positive. Its thickness is variable and linked to the age of the individual as it is continuously secreted by the endothelium. In neonates, it measures 2–4 μm thick and reaches up to 10–12 μm by adulthood [1]. It has two histologically distinct layers: the anterior banded layer produced in utero and the posterior non-banded layer produced throughout life. It is composed of type IV collagen, laminin, and fibronectin [1].

Descemet’s membrane is fundamental in providing support and adhesion to the endothelial cells. In addition, it is resistant to the phagocytic, toxic, and enzymatic insults. However, it is relatively weakly attached to the overlying stroma and, thus, can be surgically dissected as one piece [1].

2.3.5. Endothelium

The corneal endothelium is a monolayer located on the inner side of the cornea and is about 4–6 μm thick. Similar to the stroma, the endothelium comes from neural crest cells. The cells cannot regenerate after birth. The endothelial cell density is about 3000 cells/mm² with 500,000 cells covering the posterior surface of the cornea. This number decreases slowly with age at a rate of 0.6% per year. The most common cell shape is hexagonal. Minimal variation in cell size (polymegathism) and cell shape (pleomorphism) can be seen in normal individuals. The endothelium has two principal functions aiming to maintain the corneal clarity: the barrier and the metabolic pump functions [1]. These cells are joined together by interdigitations that are only visible by an electron microscope. In addition, focal tight junctions can be seen in the apicolateral membranes. They communicate through gap junctions. The endothelial cell layer is relatively semipermeable to allow some nutrients to pass paracellularly to the remaining corneal layers [1].

The high metabolic demand of the endothelial cells is evident by the presence of numerous mitochondria, the prominent smooth and rough endoplasmic reticulum, ribosomes, and Golgi apparatuses. Pinocytic vesicles can be seen in the cytoplasm pumping fluid from the stroma to the anterior chamber [1]. The endothelial pump requires the existence of bicarbonate, the membrane bicarbonate transporters, Na-K ATPase, and carbonic anhydrase activity. Thus, corneas with low or relatively dysfunctional endothelial cells contraindicate the use of topical carbonic anhydrase inhibitors [4].

Peripheral corneal guttae (Hassall-Henle bodies) are minute excrescences that can be observed in the peripheral part of Descemet’s membrane. It is considered a natural aging process. They represent focal thickening of Descemet’s membrane. A histologically similar but pathological in nature is the appearance of central cornea guttae. They are associated with progressive corneal stromal and epithelial edema representing Fuch’s endothelial dystrophy [1].
3. Histopathology of ocular surface

Tumors of the ocular surface are the most frequent of the eye and appendages along with those of the eyelids. They cover a wide spectrum from benign lesions such as papilloma to others that may endanger the visual function and the life of the patient, such as squamous cell carcinoma and melanoma [6, 7]. They can arise from any of the cells that make up the conjunctiva although the most frequent are those of epithelial and melanocytic origins. The tumors of the conjunctiva can be epithelial (non-melanocytic and melanocytic) and stromal (lymphoproliferative, vascular, neural, lipomatous, histiocytic, myogenic, fibrous, and choriostomatous). In addition, tumors of the ocular surface encompass caruncular and metastatic tumors. This chapter will focus on the epithelial tumors.

3.1. Non-melanocytic epithelial tumors

3.1.1. Squamous papilloma

Squamous papilloma appears at any age with variable presentation [8]. Human papilloma viruses (HPV) 6, 11, or 16 result in the development of squamous papillomas in children [9]. Patients can present with pink, single or multiple, sessile, or pedunculated lesion in the inferior fornix and less commonly the bulbar conjunctiva. In older patients, papilloma can result in relation to HPV infection or in patients with compromised immunity [10]. Clinically, it usually presents as unilateral light pink mass at the limbus or the caruncle. It may have the appearance of squamous cell carcinoma (SCC). In addition, squamous papilloma is a premalignant lesion and has been reported to transform to SCC, transitional cell carcinoma, or mucoepidermoid carcinoma particularly in the inverted growth pattern [11–13]. The lesion classically demonstrates many vascularized papillary fronds lined by the acanthotic epithelium with no evidence of pleomorphism or dysplasia (Figure 1).

3.1.2. Conjunctival pseudoepitheliomatous hyperplasia

This lesion is a benign inflammatory lesion manifested by a reactive proliferation of the conjunctival epithelium. It is also called pseudocarcinomatous hyperplasia as it resembles malignant lesions in clinical and histopathological examinations [7]. It is caused by irritation of the conjunctiva by a coexisting or previously existing stromal inflammation, foreign body, vernal keratoconjunctivitis, pterygium, and pinguecula. Clinically, a rapidly progressing elevated pink limbal lesion with leukoplakia and hyperkeratosis is seen. The histopathology shows an extensive acanthosis and hyperkeratosis in addition to parakeratosis of the conjunctival epithelium. There is no cytological atypia.

3.1.3. Keratoacanthoma

It is a rare variant of conjunctival pseudoepitheliomatous hyperplasia. A rapidly progressing hyperkeratotic lesion is seen [8, 14–16]. The documented cases have occurred on the bulbar conjunctiva, within the palpebral aperture, and adjacent to the limbus.
3.1.4. Dacryoadenoma

Dacryoadenoma is an exceedingly rare condition occurring in children and adolescents. It is a benign tumor originating from the conjunctival epithelium and grows into the stroma forming glandular lobules analogous to the lacrimal gland with goblet cells. Clinically, it appears as a translucent fleshy lesion anywhere in the conjunctiva [8, 12, 17].

3.1.5. Conjunctival keratotic plaque and actinic keratosis

These leukoplakic conjunctival lesions cannot be differentiated clinically. They usually arise in the interpalpebral region. Histologically, a conjunctival keratotic plaque is characterized by acanthosis, hyperkeratosis, and parakeratosis. There is no dyskeratosis. Typically, it has no malignant potential.

In actinic keratosis, a gradually progressing flat leukoplakic lesion that may frequently be indistinguishable from conjunctival intra-epithelial neoplasia (CIN) is observed [8, 12, 18]. Positive rose bengal staining of the lesion surface is seen in cases of CIN. Epithelial hyperplasia acanthosis, keratosis, or parakeratosis are found in addition to some atypia. The basement membrane is intact.

3.1.6. Ocular surface squamous neoplasia (OSSN)

Ocular surface squamous neoplasia (OSSN) is a common term that describes a spectrum of benign, premalignant, and malignant epithelial lesions of the conjunctiva and cornea. Thus, OSSN encompasses conjunctival or corneal intraepithelial dysplasia, carcinoma in situ, and invasive squamous cell carcinoma (SCC) [19]. Previously, the terms used to describe the spectrum of OSSN were Bowen’s disease, Bowenoid epithelioma, and intraepithelial epithelioma [20].

CIN approximately accounts for 4% of all conjunctival lesions and 39% of premalignant and malignant lesions of the ocular surface [21]. The incidence of invasive SCC is ranging from...
0.02 to 3.5/100,000 population [22]. Three quarters of cases occur in men and older patients and at the limbus, although any part of the conjunctiva or cornea may be affected mostly within the interpalpebral fissure [12, 21].

Risk factors associated with the development of OSSN are exposure to sunlight, HPV type 16 infections, and immunodeficiency [12, 23]. In addition, xeroderma pigmentosum and Papillon-Lefèvre syndrome are associated with recurrent OSSN, occurring in younger individuals [24]. Rarely, OSSN can be bilateral in immunosuppressed patients. Regional lymph node involvement and infrequently distant metastasis may occur.

Clinically, the lesion may appear fleshy, gelatinous, leukoplakic, or papillomatous. Leukoplakia is most likely due to hyperkeratosis or surface keratinization. Feeder vessels may be prominent, or the lesion may be avascular. As mentioned earlier, rose bengal staining can support the diagnosis and help in the demarcation of the tumor extent. It is essential to examine the tarsal conjunctiva with upper eyelid eversion to look for extension or multifocal involvement. Clinical correlation with histological severity is unpredictable. Intraocular extension occurs in 2–15% of SCC patients for which enucleation and sometimes exenteration, if the orbit is invaded by the malignant process resulting in proptosis, are often needed [22, 25]. The limbal lesion may invade the adjacent corneal epithelium and appear as advancing superficial faint opacity that may be associated with subtle vascularization. Rarely, primary SCC of the cornea can occur. Additionally, there are no reliable clinical measures for characterizing the differences between CIN and invasive SCC. However, leukoplakia raises the suspicion of malignancy and is generally absent or insignificant in CIN. Extensive vascularity and nodularity of the lesion are in favor of SCC. Tumor thickness is not a reliable sign of malignant potential, as there are thick tumors that remain confined within the epithelium. Diffuse conjunctival involvement can masquerade as conjunctivitis-type symptoms and signs [26].

The typical cytological features that are seen in OSSN on impression cytology include pleomorphic cells with hyperchromatic nuclei having an irregular outline and prominent nucleoli. However, the diagnosis of OSSN using this tool is controversial as the previously mentioned features might not be seen in the superficial layers overlying the tumor [27]. Thus, it is less sensitive in regard to the diagnosis of SCC. In addition, it cannot differentiate between CIN and invasive SCC. Hence, biopsy in suspected cases is advisable [28].

The histopathological examination of an incisional or excisional biopsy is of central role in OSSN diagnosis and treatment plan. The submitted conjunctival tissue is flattened with the mucosal surface directed upward using a filter paper with special attention to the proper orientation of the specimen. After being left to dry, it is placed in 10% buffered formalin [19]. Histopathologically, the lesion can show any of the following spectra: conjunctival intraepithelial dysplasia in which dysplastic cells are confined to the basal epithelial layer and/or carcinoma in situ where the full thickness of the epithelium is occupied by dysplastic cells with characteristic abrupt demarcation between the dysplastic epithelium and the normal epithelium (Figures 2 and 3). Invasive squamous cell carcinoma occurs when the underlying basement membrane is violated (Figure 4). The first two are sometimes termed conjunctival/corneal intraepithelial neoplasia (CCIN). The dysplasia is further classified into mild, moderate, and severe grades based on the level of epithelial thickness involvement. Mild dysplasia shows dysplastic cells in the lower one-third of the epithelium, while moderate dysplasia is
involving the lower two-thirds. The histological characteristics of epithelial dysplasia include loss of polarity, increased nuclear-cytoplasmic ratio, increased number of mitotic figures, cellular polymorphism, nuclear hyperchromatism, and enlarged nucleoli. Conjunctival SCC that closely resembles the structure of the normal epithelium with keratinization is described as being well-differentiated. A tumor that resembles the original tissue to a lesser extent is termed poorly differentiated.

Increasing age, large size tumors, involvement of the surgical margins, and high Ki-67 proliferation index are risk factors for recurrence of OSSN [25]. Fortunately, with the invention of new treatment modalities, the prognosis has improved with an overall recurrence rate of approximately 5% and regional lymph node metastasis of less than 2% [19].

SCC is classified depending on the size, tumor location, and the extent of involvement as per the American Joint Committee on Cancer (AJCC) with consideration to the primary tumor

Figure 2. A case of conjunctival squamous cell carcinoma in situ with a transition from normal conjunctival epithelium to the dysplastic epithelium (original magnification ×200 hematoxylin and eosin).

Figure 3. Another case of conjunctival squamous cell carcinoma in situ with intact basement membrane and adjacent area of normal conjunctival epithelium (original magnification ×200 hematoxylin and eosin).
features, lymph node involvement, and metastasis represented using (TNM) classification. Highly malignant variants include spindle cell squamous carcinoma (Figure 5), mucoepidermoid carcinoma, and adenoid SCC [8, 12, 23, 29, 30, 31].

The concept of molecular genetics in cases of OSSN and ocular oncology has expanded in the recent years. It studies the interaction between genes and protein with attention to the activity patterns in different neoplastic cells. Alterations on chromosome 8 with 8p11.22 amplifications have been described in 75% of the tumors. This region encompasses a group of genes that code for ADAM proteins. One of the genes in this group is involved in oral SCC. In addition, Collagen type I alpha1 (COL1A1) is also found in ocular cases and was identified to be upregulated in oral squamous cell carcinoma [32, 33].
3.2. Melanocytic epithelial tumors

Melanocytic conjunctival lesions have a wide spectrum of disorders. They range from benign to highly malignant fatal tumors. In the following subsections, the most common differential diagnoses of melanocytic conjunctival lesions will be discussed.

3.2.1. Conjunctival nevus

Conjunctival nevi are the most common melanocytic conjunctival tumors. Conjunctival nevi usually start to appear in children or adolescents as a group of pigmented cells in the basal layer of the conjunctival epithelium. Conjunctival nevi are more prevalent in Caucasians (89%) with Africans (6%) and Asians (5%) being less commonly affected [33]. Conjunctival nevi are typically pigmented, but approximately 16% can be amelanotic or partially pigmented [12, 34]. Juxta-limbal location is the most common location occurring in more than two-thirds of patients. Other locations include the caruncle, plica semilunaris, fornix, tarsus, and cornea [34, 35].

There are three types of conjunctival nevi based on their histological location: compound, subepithelial, and junctional nevi being the least common. Compound nevi are characterized by the presence of melanocytic cells at the epithelial-subepithelial junction and within the stroma (Figure 6). Subepithelial lesions are located solely in the subepithelial area. They are often associated with epithelial inclusions cysts and goblet cells (Figure 7). Junctional nevi consist of nests of nevus cells at the epithelial-subepithelial junction. They are rare except in children. These types are considered more of phases of migration of the nevus cells from the basal epithelium to the conjunctival stroma.

Malignant transformation was estimated to be <1% [21, 35]. However, new onset in middle age or later in life, unusual location (i.e., fornix, tarsus, caruncle, plica), large lesions more than 10 mm in diameter, prominent feeder vessel or intrinsic vascularity with hemorrhage, non-cystic lesions, and non-mobile lesions (i.e., fixed to the underlying episclera) are clinical indications to excise the lesion [36].

3.2.2. Complexion-associated melanosis (CAM)

CAM, also known as racial melanosis, is a benign bilateral conjunctival lesion found among darkly pigmented individuals [8, 12]. It is typically observed in the peri-limbal area and uncommonly in the fornix or palpebral conjunctiva. On examination, variably pigmented non-cystic flat lesions are observed.

3.2.3. Primary acquired melanosis (PAM)

PAM is defined as melanocytic proliferation in the conjunctival epithelium. It is more frequent in light-skinned individuals and is usually unilateral. It typically begins insidiously in the middle age. Sunlight exposure may be a risk factor in the development of PAM. It may originate from an abnormality in neural crest as it has also been seen in patients with neurofibromatosis [37].
It presents as a flat brown, superficial, non-cystic, solitary, patchy, diffuse, or multifocal pigmentation involving bulbar, fornical, and palpebral conjunctiva or cornea. Amelanotic PAM can be occasionally seen [8, 21, 38]. Cellular atypia, determined by biopsy and careful histopathological examination, aided by immunohistochemical staining (using Melan-A stain to highlight the melanocytes), is the principal risk factor for progression to melanoma (Figures 8 and 9). In one study of 311 eyes with PAM, lesions without atypia or with mild atypia demonstrated 0% progression into melanoma. On the other hand, 13% of patients having PAM with severe atypia progressed into melanoma [21].

Clinical indications to perform a biopsy include ≥5 mm lesion diameter, progression, thickening, the appearance of a nodule, vascularity, involvement of the palpebral conjunctiva or cornea, patients with personal or family history of dysplastic nevus syndrome, and history of ocular or extraocular melanoma [12].
Conjunctival melanoma, although rare, represents the second most frequent malignant conjunctival lesion after squamous cell carcinoma [39]. In the past, its evolution almost invariably resulted in an unfavorable prognosis, resulting in orbital exenteration in an attempt to eradicate the highly invasive disease. It represents a challenge for the clinician and pathologist because it can present in several pictures and originates from apparently benign lesions such as conjunctival nevi [40]. Conjunctival melanoma most frequently affects white individuals with an incidence varying from 0.24 to 0.80/1,000,000 population [41, 42]. It is more frequent in elderly individuals with a mean age ranging from 55 to 70 years [41–47]. Although rare in
young people, there are reports of conjunctival melanoma cases in patients less than 20 years of age [48, 49]. There is no significant difference between men and women [41–47].

Conjunctival melanoma arises from PAM in 75% of cases, preexisting nevus in 20%, and de novo in 5% [8, 12]. Systemic risk factors include dysplastic nevus syndrome, neurofibromatosis, and xeroderma pigmentosum [38]. Sunlight exposure is also suggested as a cause in the development of bulbar conjunctival melanoma. The most frequent location of conjunctival melanoma is the bulbar conjunctiva in the peri-limbal area, but it can occur in any location, such as the palpebral or fornical conjunctiva, the plica, or the region of the caruncle [45, 47, 50, 51].

Clinically, conjunctival melanomas may have variable presentations. Classically, it presents as a mass or an elevated pigmented conjunctival lesion. In some cases, it may appear more diffuse or multiple, with poorly defined borders, particularly when associated with PAM [22]. Less commonly, conjunctival melanomas can present as a pink or reddish pigmented lesion or can be even amelanotic, making it difficult to recognize and, thus, delaying its diagnosis and treatment [47]. Moreover, the recurrence of conjunctival melanoma after excision is typically amelanotic [12]. Repeated and continuous contact of the conjunctiva from an adjacent eyelid margin melanoma may cause a secondary conjunctival melanoma (implantation melanoma) [52]. Like SCC, conjunctival melanoma is classified according to the AJCC-TNM classification. Conjunctival melanomas can metastasize regionally to pre-auricular and submandibular lymph nodes. Distant metastasis involves the brain, liver, skin, and bone [53].

Conjunctival melanocytic intra-epithelial neoplasia (C-MIN) is a term used to describe lesions exhibiting proliferation of melanocytes. Scoring of C-MIN is based on several factors including the pattern of horizontal and vertical epithelial involvement, the degree of cellular atypia, nuclear and cellular diameter, and the presence of nucleoli and mitotic figures. Then, C-MIN is graded from 0 to 10 with 0 corresponding to an absence of any melanocytic proliferation or atypia (i.e., melanosis only), 1–4 corresponding to the severity of PAM (i.e., mild, moderate, and severe atypia), and 5–10 corresponding to conjunctival melanoma in situ (Figure 10) [54].

![Figure 10. Histopathological appearance of conjunctival melanoma in situ with atypical melanocytic proliferation involving the full thickness of the conjunctival epithelium (original magnification ×200 periodic acid-Schiff).](image-url)
The lesions usually demonstrate atypical melanocytic proliferation characterized by abundant cytoplasm, prominent nucleoli, and atypical mitotic figures with invasion of the underlying conjunctival stroma as well as the adjacent epithelium. Atypical melanocytic proliferation can be limited to the epithelium in the early stages in cases arising from PAM (melanoma in situ) with radial spread in a similar way to cutaneous melanomas. The entire lesion should be removed in one piece without touching it by excising it along the limbus to prevent seeding of the tumor cells in the surgical area.

Pathological examination of the excised lesion should include observation of important features such as ulceration, thickness of the tumor and its predominant histologic cell type (i.e., epithelioid or spindle), and the vertical growth phase. Other important characteristics include lymphocytic infiltration, vascular or perineural invasion, and the mitotic activity detected using Ki-67 index. In addition, microscopic satellitosis—defined as separate nests of tumor disconnected from the main malignant mass—should be also observed [55].

Evident histopathological features that are associated with worse survival include tumor thickness more than 2 mm, the presence of ulceration, epithelioid morphology, higher count of mitotic figures (>1/mm²), lymphovascular invasion, and microsatellitosis [55, 56]. Extra-bulbar conjunctival melanoma is found in a multivariate analysis study to be associated with poor outcome [57].

Immunohistochemical (IHC) stains for melanocytes such as Melan-A red, MART-1, S-100 protein, HMB-45, and the cell proliferative marker Ki-67 may help to identify melanocytic problematic cases. In conjunctival melanomas, the immunohistochemical expression of HMB-45 and Ki-67 is higher than what is observed in PAM or conjunctival nevi [58]. Beta-catenin is an IHC marker that was more strongly expressed in conjunctival melanomas compared to nevi and PAM. Thus, its role in conjunctival melanomas is different than cutaneous melanoma, in which loss of beta-catenin expression has been associated with a more aggressive course [58]. Programmed cell death protein 1 (PD-1) and its interaction with its ligand PD-L1 studied in patients with conjunctival melanoma has shown increased risk of distant metastases and worse survival when expressed by the tumor [59].

BRAF is a human gene that encodes a protein called B-Raf, a proto-oncogene. Conjunctival melanoma is one of the BRAF mutation-associated malignancies. A higher chance of distant metastasis might be seen in conjunctival melanomas expressing BRAF mutations. Conjunctival melanoma and cutaneous melanoma show resemblance in the significance of this type of mutation and its relevance to the clinical presentation [57, 60].

Advanced therapy of conjunctival melanomas using cryotherapy, radiotherapy, and chemotherapy has lowered the frequency of surgical exenteration. It is essential to perform periodic systemic screening in high-risk patients [54]. Local recurrence after therapy ranges from 50 to 70% at 10 years with an overall mortality rate of 25% at 10 years and more than 30% at 15 years [21]. Multifocal melanomas, extra-limbal location, incomplete surgical excision, and the lack of additional treatment are considered to be risk factors for recurrence [54, 57].
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Conflict of interest

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