Chapter

The Extracellular Matrix of the Human and Whale Cornea and Sclera: Implications in Glaucoma and Other Pathologies

Elena Vecino, Noelia Ruzafa, Xandra Pereiro, Ane Zulueta, Alfredo Sarmiento and Alejandro Díez

Abstract

The cornea is the transparent part of the eye that allows light to enter into the eye and reach the retina, thereby activating the neurons that will send messages to the brain. The sclera is the hard-white part of the eye, and its main function is to provide structure and form to the eye, and to support the retina. Indeed, while the cornea best performs its main functions when transparent and it is capable of adapting its curvature to allow the eye to focus, the sclera must be opaque and hard to function correctly. Both structures are mainly composed of collagen, some elastic fibres and ground substance, all components of the Extracellular Matrix. The disposition of the collagen fibres and the amount of ground substance around the fibres is responsible for the differences in the aspect of both these structures. In this chapter, for the first time we have compared the structure and ultrastructure of the cornea and sclera in humans and the whale adult (18mts) *Balaenoptera physalus*, the second largest animal on the planet. We will discuss how the differences in their structure may be related to the maintenance of intraocular pressure in their distinct environments, which is of particular clinical interest as increased intraocular pressure is one of the main causes underlying the development of open angle glaucoma.

Keywords: cornea, sclera, extracellular matrix, structure, ultrastructure, collagen, whale, eyes

1. Introduction

The cornea and sclera are the two most external structures of the eye and the extracellular matrix (ECM) plays a crucial role in their activity. While the cornea is transparent and located in the front the eye, the sclera is the white part that forms the rest of the eye, giving it its spherical form, and providing hardness and structural protection to the internal part of the eye. Different types of collagen constitute the core of both structures, which is surrounded by the so-called "ground substance" that lies between the collagen fibers and around the few cells that are present in these elements. The viscoelastic properties of the cornea and sclera define the distensibility of the eye, which is related to the control these structures exert over intraocular pressure. Moreover, the cornea fulfils its main functions at the

interface of the eye with air or water (depending on the habitat). Indeed, the cornea is the principal refracting surface of the dioptric system of the eye, which is why it is transparent, avascular, viscoelastic and quite resistant to deformation.

The structural and chemical composition of the ECM of very large eyes like the whale's eye has been little studied. The human eye measures approximately 2.3 cm in diameter and the two whale's eyes that we have analyzed were 12 and 13 cm in diameter (**Figure 1**). In this chapter we compare the morphological and structural aspects of the human cornea and sclera with those structures in one of the largest eyes ever studied. We consider that at least some of the differences in the structure of these eyes is likely to help adapt the whale to its very extreme conditions of life. These animals live between two very different habitats, capable of rapidly shifting between the water surface and the very deep sea, experiencing huge changes in pressure that their eye can only support without deforming thanks to the strong structure of both the cornea and sclera.

Here we will consider the two structures separately, the cornea and sclera, although there is a continuation between both in the eye. The composition of both structures is very similar, mainly comprised of an ECM that contains collagen, as mentioned above, although the organization of the collagen fibers in each differs underlies their distinct viscoelastic characteristics. Quick-freezing and the deep-etching methods have been used in ultrastructural studies of the collagen fibers in the cornea and sclera, demonstrating that corneal collagen fibers were separated by moderate interfibrillar spaces. By contrast, scleral collagen fibers were organized compactly, with fewer interconnecting filaments. In the sclera, the collagen fibers have a wider diameter (around 200 nm) than those in the cornea (around 40 nm), and the periodicity of the collagen striations was variable in each structure, although in the sclera these striations were difficult to detect because of the surrounding ground substance [1]. Here we used several techniques to study the whale's cornea and sclera, from classical histochemical trichromic staining (**Figure 2**), fluorescent light microscopy (**Figure 3**) to scanning electron microscopy (SEM) (**Figures 4** and **5**), in addition to Raman spectroscopy (**Figure 6**). While microscopy will enable us to determine the structure and ultrastructure of the tissue, Raman spectroscopy is a technique that can be used to optically probe the molecular changes in the tissue. The result of this technique is a spectrum

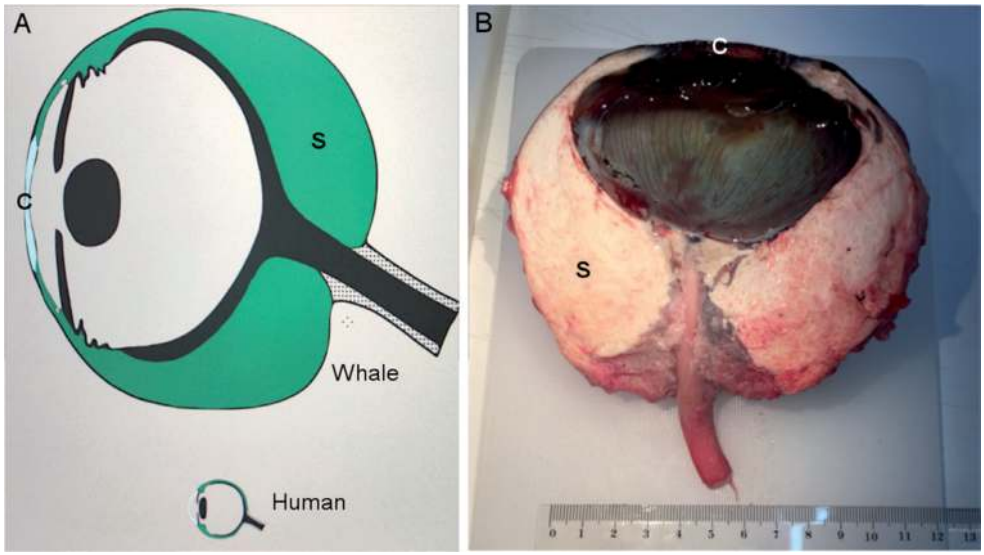

Figure 1.
(A) Scheme of the whale and human eye in proportional scale. In green the sclera and in blue the cornea (B) Picture of half of the whale's eye. (C) cornea, (S) Sclera.

characterized by shifts in wave numbers, which in many cases can be associated with the vibration of particular chemical bond (or single functional group) in the molecule [2]. We will describe the two structures cornea and sclera, comparing human and whale main differences.

2. Methods

Four methods were used to study the extracellular matrix. Two light microscopy methods, for that purpose the cornea and sclera were fixed for 12 h with paraformaldehyde (PAF) 4% and for the other two techniques of electron microscopy a post fixation with 2,5% glutaraldehyde for 2 h was performed after the previous fixation with PAF. The first histological technique used was Masson's trichrome staining, performed in 5 micrometers paraffin sections to visualize the collagen fibers in blue/green from the extracellular matrix (**Figure 2**). The second technique used was fluorescence microscopic technique, to determine the organization of the keratocytes. For that purpose the nuclei of the keratocytes were stained with DAPI in cryostat sections (14 micrometers) (**Figure 3**). The third technique was the scanning electron microscopy (SEM) to visualize the ultrastructure of the matrix components, for that purpose, small portions (few mm^2) of cornea and sclera were dehydrated in increasing gradation of alcohol followed by complete dehydration with hexamethyldisilazane (HDMS), then the pieces were oriented in the platform

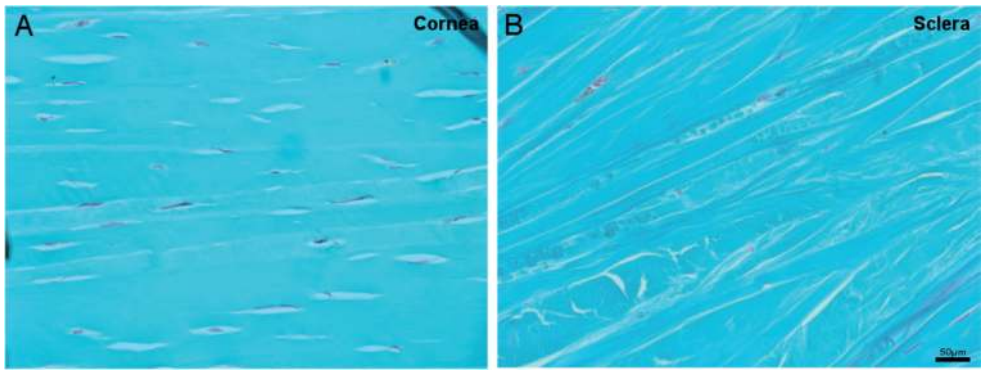

Figure 2.
Trichrome staining of paraffin sections from cornea (A) and sclera (B) of whale's eye. Note the linear and parallel organization of the fibres in cornea and the different orientation of the fibres in the sclera. Scale bar 50 µm.

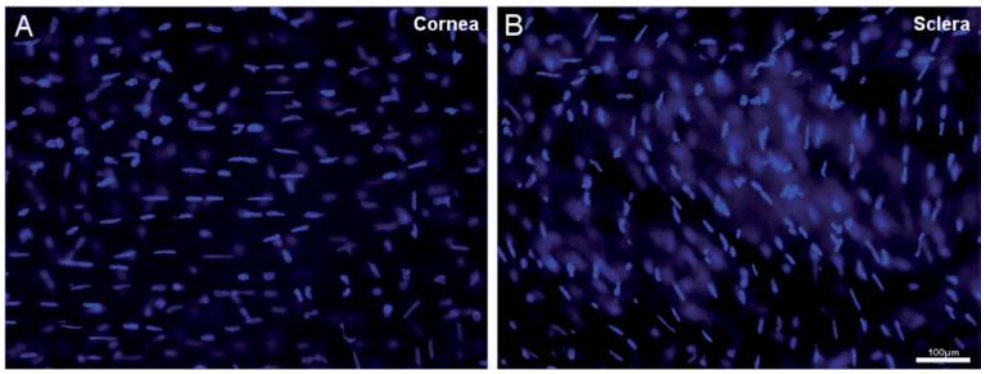

Figure 3.
Fluorescence microscopic picture of the nuclei of the keratocytes. Note their distribution and orientation in cornea (A) and sclera (B) stained with DAPI. In the cornea there are lower number of keratocytes and they are more organized than in the sclera. Scale bar 100 µm.

of the microscope and coated with gold (**Figures 4** and **5**). The last technique was RAMAN microscopy, for that purpose small portions of cornea and sclera were dehydrated as for the SEM, but the HDMS step was not carried out. Thus, the samples were analyzed with a confocal InVia Raman (Renishaw) connected to a spectrophotometer and an excitation laser of 785 nm was connected to a Leica microscope to register the spectro of the different tissues (**Figure 6**).

3. The cornea

As indicated above, the cornea is a transparent organ that allows the light to enter into the eye. The features that contribute to its transparency are a thin epithelium, the absence of blood vessels and its chemical composition, mainly comprised of collagen and some important ground substance, with very few cells. The cornea has five main parts: (1) the epithelium; (2) Bowman's layer; (3) the stroma; (4) Descement's layer; and (5) the endothelium. In this chapter we will concentrate on the stroma, of which the ECM is the main component.

In humans the cornea is approximately 0.5 mm thick, while in the whale's eye it measures 3 to 4 millimetres. In both cases it is composed almost entirely of collagenous lamellae. The collagen fibres are organized in lamellae approximately 6 mm in diameter but with certain variability in their width and thickness. The lamellae are arranged parallel to the corneal surface and sometimes they form loose fibrillar networks. The collagen fibres within the bundles lie parallel to each other, and they are uniform in size and spacing, a feature produced by the cementing ground substance that is distributed regularly between the fibres (**Figure 2**). In the most peripheral cornea, the lamellae gradually adopt a less regular orientation and little-by-little their structure approximates to the organization in the sclera [3]. The collagen fibres in the central cornea vary in diameter between 21–65 nm in humans [4], data that is consistent with that found in our human SEM preparations.

A few specialized fibroblasts called keratocytes can be found between the collagen fibres, and they are responsible for the synthesis of the collagen and ground substance. Only a small proportion of the cornea is occupied by these cells, around 2–3% in humans, and as such, it is generally considered an almost acellular structure [5]. The small number of cells present in the corneal stroma, the avascular nature of this structure and the very well-organized collagen lamellae, all contributes to the characteristic transparency of the cornea (**Figures 2** and **3**).

The ground substance in the cornea consists of mucoproteins, glycoproteins and other substances exclusive to collagen, and it forms a cement like filling in the space between the corneal fibres. In 1969, using alkaline lead, citrate and uranyl acetate staining, 2 nm diameter filaments were seen to exist at right angles to the collagen fibres that they connected, postulating that these were the proteoglycans that bind to the corneal collagen D-period [6]. Using cationic dyes (alcian blue, cuprolinic blue, cupromeronic blue) in a critical electrolyte mode, the presence of proteoglycans was confirmed. Later studies described these proteoglycans to be keratan sulphate (lumincan) and dermatan sulphate (decorin) in the cornea [7], while only dermatan sulphate proteoglycan was found in the sclera, bound to the same sites as in cornea [8]. It was subsequently proposed that these molecules play a role in maintaining the relative positions of the fibrils, which is important for corneal transparency [9, 10]. So far, the most sophisticated and less invasive technique to study the ultrastructure of the cornea, without affecting the physiological state of hydration is the X-ray [11] and this will help in the future for a better understand the pathophysiology of the cornea.

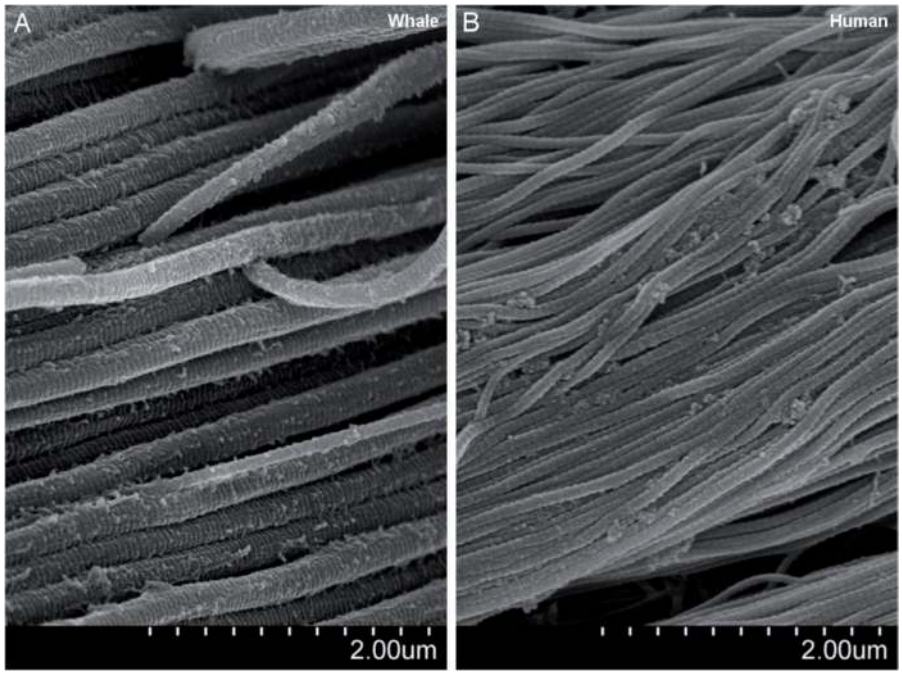

Figure 4.
Scanning electron microscopy pictures of the whale's (A) and human (B) corneas. Note the distribution of the fibers in laminar bundles. Scale in μm.

The corneas of the whale studied are oval in shape, with axes of 5 x 3 cm, and they have a convex outer surface. The corneal thickness varies between the centre, where it is 2.5 mm thick, and the periphery where it is thicker, measuring 4 mm at the corneal-scleral boundary. The diameter of the corneal collagen fibres also differed significantly between the human and whale. Thus, while in humans the corneal fibres are around 60 nm in diameter, in whale they measure around 200 nm (**Figure 4**). The composition of the collagen is probably very similar in both species, not least because their histochemical staining is very similar, also resembling that of the pig, rat and mouse cornea. Moreover, and in addition to SEM and TEM, when the whale cornea and sclera was studied by Raman spectroscopy, the characteristics of the peaks for the collagen components were similar to those in humans [12].

4. The sclera

The sclera is the white part of the eye and it is relative thin, ranging from 0.6 mm in the anterior part to 1 mm in the posterior part of human eyes. However, the sclera is very thick in large whales like the fin whales that we have studied, and it measures 3 to 4 cm at the back of the eye, although it is thinner (0,5 cm) in the anterior part (**Figure 1**). This thick and quite hard structure serves as a coffer in which the sensitive parts of the eye like the retina can be protected from the intense pressures these animals are exposed to when swimming in the deep seas.

The ECM of the human sclera is mainly composed of type I, III, V and VI collagen. The principal function of type I collagen is to resist tension, while type III collagen is considered essential in maintain the structure of expandable organs and type V collagen has been implicated in controlling fibril diameter. Type V collagen also fulfils a role in anchoring to the basement membrane and adjacent stromal matrix, a function it shares with type VI collagen [13]. In the sclera, the collagen

fibrils have various diameters, ranging from 25 to 230 nm. Although these collagen fibrils form bundles, their arrangement is more heterogeneous in the sclera than in the cornea. These collagen bundles vary in width and thickness, often sprouting branches and intertwining with each other, at least in humans [14]. Moreover, in the sclera there is a narrower interfibrillar distance than in the cornea and the ground substance is more abundant, impairing the discrimination of the band periodicity of the collagen fibres. Indeed, it has been necessary to use special treatments and atomic force microscopy to describe the differences in the periodicity of the collagen bands between the cornea and sclera [15].

In transverse section of the eye the human sclera is thinner towards the corneo-scleral junction, while it thickens in the medial direction, posterior to the vitreous chamber, where it joins the bundle of the optic nerve. The dorsal part of the sclera is larger than the ventral domain, which means that the optic nerve can exit the eye with a ventral disposition. The collagen fibres that make up the sclera are mainly embedded in the ground substance and the characterization of the different types of collagen fibres has been achieved in humans by immunogold EM staining [16]. The fibres are tightly packed and arranged in different directions, which provides the eyeball with strength and shape (**Figures 2B** and **5**). Close to the corneo-scleral limbus, large blood vessels circulate not far from the angle, forming a ring. In the sclero-corneal stroma of the limbus there is a large number of pigmented cells and numerous channels are present in this area that form the well-developed trabecular meshwork responsible for draining aqueous humour toward the veins.

The analysis of the whale's sclera using Raman spectrometry showed us that even when the thick sclera is quite hard (with a texture like a spongiosum bone), hydroxyapatite does not appear to be present and thus, we concluded that the hard sclera is not ossified Indeed, when comparing the spectrometry fingerprint of human bone with that from the whale's sclera, both structures share collagen peaks (**Figure 6**). The sclera is likely to be important in preserving the shape of the eyeball, shielding it from the effects of the deforming forces. Indeed, this large eye can be retracted or protruded thanks to a large muscle that surrounds the optic nerve

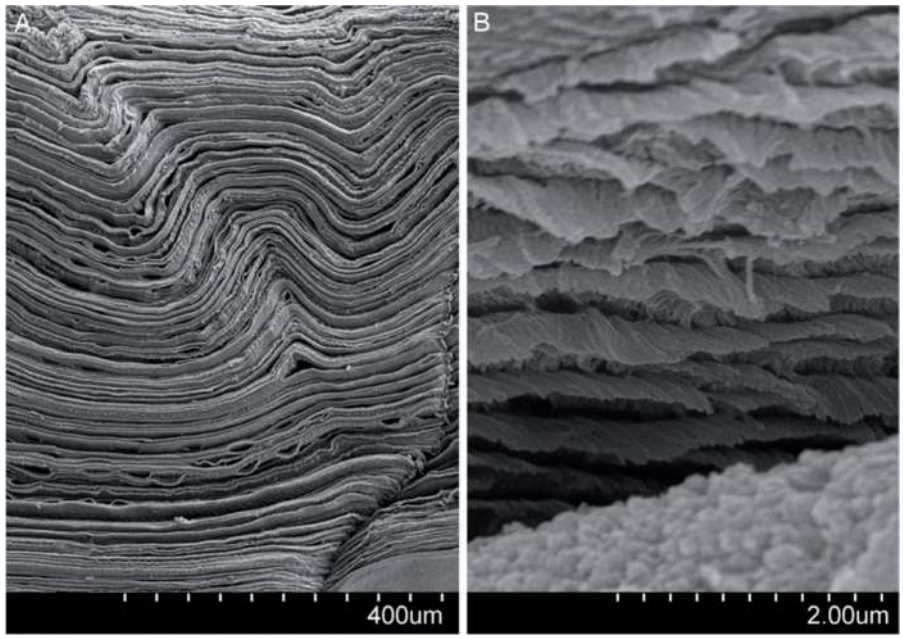

Figure 5.
Scanning electron microscopic pictures of (A) and (B) are both sclera collagen fibers from whale's sclera. (A) lower and (B) higher magnification. Scale in μm.

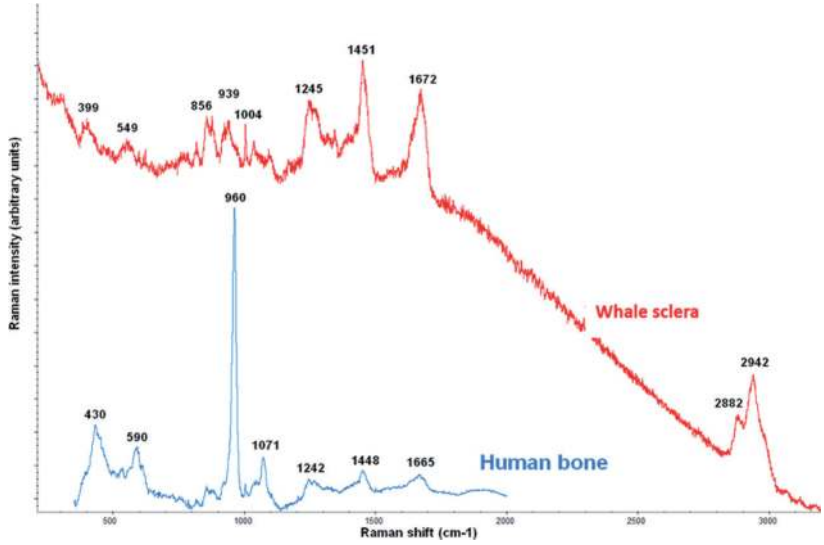

Figure 6.
Raman spectrometry (A) overlapping the spectra from human bone (blue) and whale's sclera.

and that is full of blood vessels, the ophthalmic rete [17]. It is possible that this large muscle also helps the eye and a thick sclera resist the pressure of the deep seas and avoid eye deformation.

5. Implications of the cornea and sclera extracellular matrix in glaucoma and other eye pathologies

Glaucoma is the main cause of blindness in the world. Although there are several types of glaucoma, the most common is characterized by an increase in the intraocular pressure (IOP) that induces neurodegeneration in the retina. Indeed, glaucoma leads to the death of the retinal ganglion cells that are the responsible for sending visual information from the eye to the brain, thus causing blindness [18]. The increasing of the intraocular pressure is due to the elevated secretion of aqueous humour or to a reduction in the evacuation of it, mainly through the trabecular meshwork. So far, in the human eye it has not been detected any sensor to detect and control the intraocular pressure. Interestingly, encapsulated sensory corpuscles are specialized nerve endings located in the corneo-scleral area that do not have a very clear function. These have been found in different cetaceans and in the whale *Balaenoptera acutorostrata*, they were also found in the buccal cavity. It has been speculated that these corpuscles might play a role in detecting and controlling the pressure in different areas of the eye including the sclera [19].

The thickness of the cornea is very important and has to be taken into consideration in order to measure IOP correctly. Since the way to measure the IOP is through the cornea, the instruments used must be adapted to the mean cornea thickness. However, in order to correct the defects, a refraction technique has been developed that involves correcting the curvature of the cornea by reshaping the stroma of the cornea with a laser, LASIK surgery. The thickness of the cornea is critical to be able to perform this surgery, particularly since the mean cornea thickness in humans is 500 um and it reaches a maximum of 600 um, and LASIK surgery should not be performed on thinner corneas. After LASIK surgery, the patient should retain a minimum of 250 μm corneal thickness. In this sense, IOP measurements can vary depending on the thickness of the cornea, being underestimated in patients with

thinner corneas and overestimated in patients with thicker corneas. Another side effect of re-shaping a thin cornea is the deformation in the central part, which can alter corneal curvature, so-called keratoconus. This is a phenomenon that leads to a gradually bulging of the cornea outwards into a cone shape, which causes blurry, distorted vision. In order to correct this keratoconus crosslinking of the collagen fibers should be performed by applying UV light to the collagen fibers, thereby reinforcing the structure of the cornea. The UV light together with the application of riboflavin (vitamin B2) will enhance the bonds between collagen fibers in the stroma of the cornea [20]. It is also hypothesized that stiffening of ocular structures, including cornea and sclera may be related to the pathogenesis of glaucoma [21].

Another alteration to the cornea that can influence IOP measurement is the prolonged use of contact lenses. Initially, contact lenses can induce a flattening of the cornea during the first months of use, but prolonged use can cause a thinning of the cornea with some deformation. Thus, we can conclude that prolonged use of contact lenses negatively influences corneal physiology. Aging can also change central and peripheral corneal thickness. By using ultrasonic pachymetry in 250 patients aged 9 to 97 years, it was concluded that central corneal thickness increases significantly with age, whereas the degree of symmetry decreases [22]. Accordingly, there are different factors that can affect corneal thickness and thus, IOP measurements, which could influence the detection and treatment of glaucoma.

The sclera provides a tough fibrous support structure for the retina and optic nerve, fulfilling a biomechanical function that may be crucial in glaucoma. Several studies have assessed collagen fiber architecture in order to identify if uniaxial (one preferred direction) or biaxial (two directions) collagen organization of the sclera is related to glaucoma. So far, changes in fiber orientation have been detected between glaucomatous and non-glaucomatous eyes, although it could be an adaptation to the elevated pressure and it is not clear if there is a predisposition to glaucomatous axon damage [23]. However, the very hard, strong and thick sclera present in the whale's eye means there is no capacity for distension or structural modification. As such, any elevation in IOP in whales would be sensed by the retina. The other structure in the eye that is sensitive to IOP is the lamina cribosa (LC) or cribiform plate that forms a scaffold for the passage of the optic nerve's axon bundles, anchoring the bundles to each other and to the sides forming the optic nerve. It reinforces the posterior eye, protecting it from injury at the site of optic nerve exit. The LC is subject to mechanical strain as it lies at the border between two different compartments subject to pressure: the anterior compartment to IOP and the retrobulbar compartment to that of the cerebrospinal fluid [24]. Hence, the LC has been proposed as the main site controlling the pressure that represents the insult to retinal ganglion cell axons in glaucoma [25]. Moreover, the LC thickness and the posterior displacement of its components have been associated with the rate of progressive retinal fiber layer thinning and the severity of glaucoma. Changes in the structure of the LC have been found in patients with glaucoma, indicating that these structural changes could provide information regarding the evolution of glaucoma [26]. However, in our large exanimated animals, the LC of the whales is as hard as the sclera, which means it will be very difficult for it to deform. Thus, in these animals there is a very limited possibility for the eye to deform in response changes in the IOP.

6. Conclusions

In conclusion, we have evaluated the structure of the eye in the second largest mammalian on the planet, the long fin whale, considering the possible functional consequences of its features. These eyes are around 150 times larger than the human

eye, although their structure is very similar and their ECM components are also comparable, albeit in different proportions. Thus, the cornea and sclera are thicker, adapting to the whale's ecosystem and to the physiology of their body size. The very large structures and the rigid ECM lead us to consider the implication of the ECM in eye diseases like glaucoma and keratoconus, which in these animals will be very difficult to explain in the context of their very distinct dimensions and structure.

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Conflict of interest

The authors declare no conflict of interest.

Notes/thanks/other declarations

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