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AUTHORS: Rick Sanchez and Julie T. Daniels

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Limbal Stem Cells Deficiency in Companion Animals; Time to give something back?

R F Sanchez[1], J T Daniels[2]

[1] Royal Veterinary College, Queen Mother Hospital for Animals, Hawkshead Lane, North Mymms, Hatfield, England, AL9 7TA
[2] Department of Ocular Biology and Therapeutics, UCL Institute of Ophthalmology, London, England, EC1V 9EL

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Abstract
Experimental animals have been used extensively in the goal of developing sight saving therapies for humans. One example is the development of transplantation of cultured limbal epithelial stem cells (LESC) to restore vision following ocular surface injury or disease. With clinical trials of cultured LESC therapy underway in humans and a potential companion animal population suffering from similar diseases, it is perhaps time to give something back. This review will explore the current challenges and future research directions that will be required to develop and deliver cultured stem cell therapy to veterinary ocular surface patients.

Introduction

The human animal bond has existed for as long as recorded history permits us to look back in time. Animals are our companions, some serve as guides to humans in need, others assist us in rescue, search and policing missions, for better or for worse some are part of the food chain that sustains our life, they are carriers and reservoirs of diseases that can affect the human population and they form part of the small and large animal models that help us understand and treat diseases that affect us. For example, in the development and safety testing of numerous therapeutic products including pharmaceuticals and more recently cell therapies. We have learned much from their contributions to medical research and it may now be possible to reverse-translate this knowledge for the benefit of veterinary patients. One area of particular interest is the cornea, where significant advances have been made in the treatment of blinding human ocular surface diseases.

The cornea and tear film constitute the major refractive area of the companion animal eye with up to +45D of refractive power in dogs and cats. The range of accommodation varies widely between species (e.g. +1D to +7D in dogs and +2D to +8D in cats Vs. +15D in humans, +19D in raccoons and up to +34D in rhesus monkeys (Ofri R 2007)). Companion animals have a cone-rich area (e.g. area centralis) but no fovea and dichromatic day vision (e.g. deuteronopia). Some raptor bird species have two foveas with a super-rich cone photoreceptors density, tetrachromatic vision that spans into the UV light spectrum and an

equally impressive accommodative capability. In contrast to the human eye, the companion animal eye is designed to see exceptionally well at night. It has a cornea with a large diameter, a pupil that dilates widely in low levels of light, and a highly reflective tapetum lucidum in the central-to-superior choroid that increases photoreceptor stimulation due to its structural properties (e.g. cellular tapetum with zinc cysteine or rivoflavin reflective crystals in most carnivores and reflective collagen in the tapetum fibrosum of most hervibores). Despite interspecies differences, corneal transparency is crucial to vision in every eye, and vision is as pivotal to our survival and wellbeing as it is to the survival and wellbeing of most animal species we share our lives with.

The companion animal eye is affected by a variety of corneal diseases and many of these are directly related to corneal epithelial cell health. It is therefore fair to say that in animals, as in humans, limbal epithelial stem cells also play a key role in maintaining corneal health and, hence, vision.

Corneal epithelial stem cell research is centered on human diseases. Some of this research has used corneal tissues of rodents and rabbits (Kinoshita S *et al* 1981, Nagasaki T *et al* 2003, Kadar T *et al* 2011, Das P *et al* 2013, Luesma MJ *et al* 2013), bovines (Sun *et al* 2006), pigs (Notara M *et al* 2011), dogs (Wood JA *et al* 2012) and even goats (Yin JQ *et al* 2013). Yet, as the focus of these studies are humans, our understanding of the anatomy and physiology of healthy corneal epithelial stem cells in most animal species is rather poor, and it is practically inexistent in disease. Yet animal models are routinely used for ocular surface (and other) cell therapy development and safety testing.

LSC biology

Davanger and Evensen first postulated the localization of corneal epithelial stem cells was the limbus (Davanger M, Evesen A, 1971). Since, a great deal of work has followed. The development of the X, Y, Z hypothesis for corneal epithelial cell maintenance concluded that vertical growth from the basal cell layer to the surface (X) and centripetal migration of cells (Y) equaled cell loss from the corneal surface (Y) (Thoft and Friend, 1983, Sharma and Coles, 1989, Beebe and Masters, 1996). This complemented the original idea that stem cells were localized in the limbus and supported further studies that explained how corneal epithelial stem cells in the limbus self-renewed and were the source of transient amplifying cells that replenished the basal cell layer (Kinoshita *et al* 1981; Tseng 1989).

The vectors of force that naturally develop in the cornea during epithelial cell movement create a striped, swirled or vortex pattern as described in mouse models of epithelial cell movement (Nagasaki T *et al* 2003). It is of interest to note that small dogs with corneal disease may develop superficial corneal scarring and pigment in strikingly similar patterns (figures 1 and 2).

Currently, it is widely accepted that limbal epithelial stem cells (LESC) are found in the region of the limbal palisades of Vogt (Goldberg MF *et al* 1982). There, the

basal epithelial layer of humans undulates and creates a series of stem cell niches that contain fibroblasts, melanocytes and blood vessels (Secker GA and Daniels JT, 2009, Dziasko *et al* 2014). The niches are also reported to contain Langerhan's cells (Baum JL 1970) and T-lymphocytes (Vantrappen L *et al* 1985). In addition, recent studies in mice described the presence of a novel type of interstitial cell called telocytes in association with stem cell niches that also contain nerve endings in addition to blood vessels (Luesma MJ *et al* 2013). The niches in humans were shown to contain putative LESC in areas described as limbal crypts and focal stromal projections, which showed a regional variation around the limbus with a predominant localization in the superior and inferior corneal quadrants and absence nasally and temporally (Shortt AJ *et al* 2007; Dziasko *et al* 2014). Similar findings have been described in pigs, which were recommended by the authors as a candidate model for the study of cultured human limbal epithelial cell transplantation (Notara M *et al* 2011). Limbal epithelial crypts described as 'distinct anatomical extensions from the peripheral aspect of the limbal palisades' have also been suggested as a human niche for LESC (Dua SH *et al* 2005). Recently there was a suggestion that oligopotent stem cells might exist over the entire ocular surface, including the conjunctiva, although there is an accumulation of stem cells at the limbus (Majo F *et al* 2008).

Until the studies described above demonstrated that the basal epithelial layer of the human limbus (and its unique microarchitecture) was proven to host LESC, much of this information had to be extrapolated from studies performed in rabbits and mice. A very similar situation applies today to many of the domesticated and wild animal species.

Limbal palisades may be directly observed in up to 80% of human eyes with a table mounted slit lamp biomicroscope (Townsend WM 1991). It is possible similar structures might be occasionally visible with the use of a hand-held slit lamp biomicroscope in some veterinary patients although there are no reports presenting solid evidence for or against this finding. One study in equines suggests the palisades are not visible in horses (Moriyama H *et al* 2014). Palisades and niches have been described in mice and pigs but the authors did not indicate if they could be directly observed with slit lamp biomicroscopy (Notara *et al* 2011, Luesma MJ *et al* 2013). Until the microstructure of the limbus of domestic, and in particular, companion animals is studied and described in the same detail as the limbus of humans, the significance of interspecies similarities and differences remains unknown.

Since the study by Shortt et al that utilized in vitro and in in vivo confocal microscopy to describe the limbal stem cell niches in humans (Shortt AJ *et al* 2007) other studies have used similar methods (Kobayashi A *et al* 2005, Takahashi N *et al* 2009, Barbaro V *et al* 2013). An interesting report of a canine veterinary patient with metaherpetic disease of the corneas used in vivo corneal confocal microscopic to demonstrate the conjunctival phenotype of the diseased corneal epithelium (Ledbetter EC *et al* 2013). However, the authors did not include healthy controls to allow for comparisons of limbal microanatomy. A recent study in equines reported the localization of LESC in the equine limbus through immunohistochemistry although microstructural studies of the horse

limbus were not included (Moriyama H *et al* 2014).

Lastly, it is also important to mention some of the putative roles of LESCs and their potential interaction with other corneal cells, such as mesenchymal stromal cells of the limbus (Dziasko *et al* 2014). Mesenchymal stromal cells derived from the limbus are through to have an important immunosuppressive role (Bray LJ 2013) and limbal stem cell deficiency (LSCD) is associated to inflammation of the corneal surface (Holland EJ and Schwartz GS 1996, Schlötzer-Schrehardt U 2005). The barrier effect created by LESC against conjunctivalization and vascularization of the cornea has also been studied in rabbits (Kruse *et al* 1990), dogs (Brunelli *et al* 2007) and mice (Luesma MJ *et al* 2013). However, much still is to be elucidated about these potential roles of LESC in health and disease.

Identification of stem cells

A great deal is known about the characterization of LESC, which has been reviewed elsewhere (Chee KY *et al* 2006, Shortt AJ *et al* 2007, Qi H *el al* 2008, Secker GA and Daniels JT 2009). However, a definitive LESC marker has not been proven to date. This identification challenge is due in part by the fact that LESC cohabitate with transient amplifying cells and terminally differentiated cells, which complicates the quest for a single cell type (Schlötzer-Schrehardt U and Kruse FE 2005, Qi H *et al* 2008). LESC might be less than 10% of the limbal basal cells and the transient amplifying cells around them are likely to share some of the same characteristics (Cotsarelis G *et al* 1989, Wolosin *et al* 2000).

Putative markers that deserve a mention include the positive markers ΔNp63α, K19, integrin-alpha-9, vimentin, ABCG2 and NGF and its receptor, as well as negative or differentiation markers CK12, CK13, connexin and involucrin (Schlötzer-Schrehardt U and Kruse FE 2005, Chee KYH *et al* 2006, Shortt AJ *et al* 2007, Qi H *et al* 2008, Barbaro V *et al* 2013). The marker p63 is an essential transcription factor for epithelial differentiation and it is expressed in its alpha-isoform in wounded/activated corneas but not in cultures started from a resting/unpertubated cornea (Barbaro V *et al* 2013). Although the marker ABCG2 has been suggested as possibly being the single most useful cell surface marker for LESCs, a combination of several markers has been suggested as being the most useful approach to LESC identification (Schlötzer-Schrehardt U and Kruse FE 2005). Interestingly, a recent demonstration of the clustered location of LESC in the human limbus supports the findings that ABCG2 and p63α are distributed in a similar fashion along the human limbus (Shortt AJ *et al* 2007).

The localization of p63 in central basal epithelial cells of the cornea of humans (Chen *et al* 2004; Dua *et al* 2003) and rats (Chee KYH *et al* 2006) put the usefulness of p63 into question. In addition, a study of the acute phase of epithelial healing in the central cornea that used fresh cadaveric humans corneas, demonstrated it was possible to find a p63 pattern with a gradient that extended all the way to the central cornea in the absence of a limbus (Chang CY *et al* 2008). It was also suggested that the presence of p63+ cells in the central cornea is indicative of the migration of cells from the limbus in the

damaged/activated cornea and that the α isoform of ΔNp63 is only present in the basal layer of the limbus of the resting cornea (Barbaro V *et al* 2013).

A recent study in equines attempted the first localization of LESCs in the horse limbus through immunohistochemical cell identification using p63, cytokeratin 14 and cytokeratin 3, and it reported the successful culture of these putative cells (Moriyama H *et al* 2014). The study did not report the exact limbal localization of the LESCs other than they were located in areas where the limbus was pigmented. Despite the morphological study of the cells grown in culture, it is possible that in the absence of a definitive marker, some of the cells grown might have been transient amplifying epithelial cells and not LESCs.

Perhaps the most compelling marker of human LESC to date is ABCB5, a gene recently shown to be required for corneal development and repair in mice and humans (Ksander BR *et al* 2014). A role for ABCB5 in companion animal ocular surface maintenance is as yet unknown.

Diseases of the LESC

Limbal epithelial stem cell deficiency (LSCD) leads to corneal opacification through conjunctivalization and vascularization of the transparent cornea (Dua HS 1998, Dua HS *et al* 2000, Ahmad S 2012). The incidence of conjunctivalization in corneal disease has not been reported or studied in equal measure in domesticated animals as they have been in humans. One possible explanation is that simple methods that demonstrate the presence of conjunctivalization such as impression cytology are not commonly employed in veterinary ophthalmology. One study encourages the use of impression cytology and immunohistochemistry to search for the expression of K12 and MUC-1 (Barbaro V *et al* 2013). This study found that K12 expression was seen in corneal epithelium but never in the conjunctiva and that MUC-1 was seen in the conjunctival epithelium and not in the healthy corneal surface (Barbaro V *et al* 2013). A more ubiquitous use of impression cytology would increase the collective knowledge of the incidence of corneal conjunctivalization and would help develop our understanding of the effect of various corneal diseases in the animal cornea.

Independent of the use of simple diagnostic tools, the fact is that our understanding of the complex intercellular chemical messenger system and genetics that regulate the corneal epithelial cell cycle in the mammalian eye is still relatively limited. Injury, genetic diseases and alterations of the limbal stem cell niche microenvironment can lead to LSCD (Kadar T *et al* 2011). Among these, chemical, heat or radiation burns and chronic inflammatory conditions are widely recognized sources of damage to LESCs in humans with LSCD (Dua HS 1998). Impaired repair mechanism of the corneal epithelial layer through Herpes simplex, neuroparalytic keratitis, drug toxicity, contact lens keratopathy and diseases such as pterygium, aniridia and Stevens Johnson syndrome are also associated to varying degrees of LSCD in humans (Dua HS *et al* 2000).

Several corneal diseases in small animals also lead to vascularization, conjunctivalization and/or pigmentation of the corneal surface. Yet, despite a wealth of knowledge of conditions potentially associated with LSCD in domesticated animal species, and despite decades of scientific papers and textbooks dedicated to veterinary ophthalmology, LSCD is not commonly reported or discussed in the existing literature.

Corneal changes typically seen in LSCD include corneal conjunctivalization with vascularization, fibrosis and inflammatory infiltrate, and they give the cornea a dull, opaque and irregular appearance (Dua HS and Joseph A 2003, O'Callaghan AR and Daniels JT 2011). Similarly to humans with LSCD, there are corneal conditions in animals in which corneal disease is strongly suspected to result in LSCD, such as with canine herpes virus -1 keratitis (Ledbetter EC *et al* 2013). Chemical damage to the cornea in dogs can lead to corneal changes that also share many similarities with LSCD in humans (Christmas R 1991). It is possible that chronic irritation and/or immune mediated conditions such as canine keratoconjunctivitis sicca, lymphocytic-plasmacytic keratitis (aka chronic superficial keratitis or corneal pannus), and pigmentary keratitis might develop into, or in part be caused by LSCD. The same might be said of some of the corneal changes seen in eosinophilic keratitis that develops in cats, horses or rabbits. Last but not least, naturally occurring aniridia has been described in a breed of dog, although it is considered a very rare disease, and it is not described whether or not the corneas of these dogs might be affected by LSDC as they are in humans with aniridia (Villagrasa M 1996, Hunter LS *et al* 2007).

Recently, a report of a canine with possible LSCD and conjunctivalization of the cornea caused by Canine Herpes Virus -1 was also diagnosed with a neurotrophic keratitis (Ledbetter EC *et al* 2013). Interestingly, the authors postulated based on the studies of others (Cavanah HD and Colley AM 1989, Lambiase A *et al* 2000, Touhami A *et al* 2002, Ueno H *et al* 2012), a potential negative effect of corneal sensory nerve depletion on the LESC leading to or aggravating the LSCD. This resonated with the findings in the recent study describing the presence of telocytes in stem cell niches of mice that also contained nerve endings (Luesma MJ *et al* 2013) and the well-known relationship between corneal epithelial health and a healthy corneal nerve supply (Naoyuki Y *et al* 2005). The dog cornea has approximately 10 corneal nerve trunks and the cat cornea approximately 13 (Chan-Ling T 1989 and Barnett PM *et al* 1991). Although generally speaking this makes the cat cornea more sensitive to that of dogs, sensitivity also depends on the shape of the skull. There are three basic skull shapes, dolicocephalics (long skulled dogs such as rough collies), mesaticephalic (medium sized skulled dogs and cats, such as Labradors and domestic short haired cats) and brachycephalic (short sized skull dogs and cats, such as Bulldogs and Persian cats). Brachycephalic dogs and cats have the lowest number of corneal nerve trunks and thus lower corneal sensitivity than the rest of the skull shapes (Blocker T *et al* 2001). Spontaneous severe pigment changes are seen in the corneal surface of some brachycephalic dog breeds (Figure 3) and it is possible this may be in part related to a comparatively poor corneal sensation and the effect of corneal nerve amount and/or health on the LESC microenvironment. As mentioned earlier, indirect

pathologic events that produce an abnormal microenvironment for LESCs are known to play a role in LSCDs. Pathological events in the limbal stroma through exposure to sulfur mustard were postulated to cause delayed LESC death in rabbits through such a pathway (Kadar T *et al* 2011).

There is evidence to support the role of UVB induced damage to LESC niches in mice resulting in LSCD (Das P *et al* 2013). Once resolved, Lymphocytic-plasmacytic infiltrate of the cornea in dogs (a.k.a. chronic superficial keratitis) can lead to chronic corneal scarring with long lasting pigmentation in breeds with heavily pigmented limbus, like German shepherds (Figures 4 and 5). Interestingly, this condition is worsened by the effect of solar radiation although the use of UV-blocking contact lenses did not appear to have an effect on the progression of the disease in one study (Denk N *et al* 2011). This study did not mention if the lenses covered the limbus. Although dogs wore the lenses for approximately six months, the geographic location where the study took place (Munich, Germany) has median solar UV radiation that is predictably lower than in warmer latitudes or higher altitudes. A similar study in a location with a higher solar UV radiation might have led to different results.

There are genetic factors that regulate LESC proliferation in the bovine cornea (Sun *et al* 2006) and many cytokines that affect corneal wound healing and originate in the fluids bathing the cornea (Welge-Lussen U *et al* 2001), as well as corneal keratocytes (West-Mays JA and Dwivedi DJ 2006) and corneal epithelial cells (Rolando M and Zierhut M 2001). More recently, nerve growth factor was described as an essential support for stem cell renewal and, importantly, as a probable growth factor with critical regulatory functions in the LESC niche of the human limbus (Qi H *et al* 2008). These findings have started to shed some light on the potential auto-regulatory roles of LESCs.

Finally, much remains to be elucidated on the role of telocytes, the anti-inflammatory role of mesenchymal stromal cells of the limbus and their relationship to LESCs, and the role played the Langerhan's cells and T-lymphocytes of the LESC niche during inflammation.

Treatment of LSCD - A window into the future

Before the effects of LESC treatments can be fully understood or even predicted for veterinary patients, gaining a comprehensive understanding of the microanatomy of the corneal limbus, as well as the biology and identification of LESC of different species, is paramount. A first step must be the localization of LESCs in the limbus of a variety of companion animals, and studying if LESC niches and palisades of Vogt also exist in other species besides humans, pigs and mice (Shortt AJ *et al* 2007, Notara M *et al* 2011, Luesma MJ *et al* 2013). The study of methods to isolate, grow and transport LESC onto the animal cornea for transplantation would naturally follow this.

A review by Holland and Schwartz of the historical development of epithelial transplantation for corneal LSCDs in humans describes the methods that have developed over the years and that range from conjunctival to limbal transplantation (Holland and Schwartz 1996). In addition, there are a wide variety of tissues stem cells may originate from, and all may be used for culture with therapeutic purposes (Secker GA and Daniels JT, 2009).

It is clear that the use of transplants has achieved a great deal of success in the treatment of LSCDs in humans and in the few experimental trials in dogs reported to date (Holland and Schwartz 1996, Brunelli *et al* 2007). If any of the diseases of the companion animal cornea that might be LSCDs are confirmed as such, it seems probable that LESC transplants would be successful in their treatment too.

In addition to the dog described by Ledbetter *et al* (2013), the only other corneal disease described as LSCD in a canine patient was related to an accidental alkali burn (da Cunha *et al* 2010). However, the diagnosis was supported by the extrapolation of results from similar problems in humans, and not through investigation of the disease process in the dog cornea. In this single case report, the eye was treated with an autologous limbal transplant only a few hours after it had sustained an acute chemical injury. It is possible that in this case corneal re-epithelialization would have been possible without surgical intervention. The only other study to describe epithelial transplantation treatments in the veterinary ophthalmic literature written in English is an experimental study performed in dogs (Brunelli *et al* 2007). The authors demonstrated that limbal transplantation was associated with repithelialization and that experimental destruction of the limbus was associated with conjunctivalization of the corneal surface (Brunelli *et al* 2007).

The body of evidence to diagnose and treat LSCD in animals is small though promising. However, until more data is gathered and studies similar to those carried in human corneal epithelial cell cultures and cadaveric eyes are repeated for a variety of veterinary species, blinding corneal conditions that affect the wellbeing of animal species and that are potentially linked to a LSCD will go untreated.

The difficulties and clinical relevance of LESC localization should not be underestimated. Sampling the most appropriate part of the limbus to collect and grow LESCs is challenging in the absence of a definitive LESC marker and needs to be tightly targeted so as to not lead to LSCD of the donor eye (O'Callaghan AR and Daniels JT 2011).

One of the aims of a future investigative effort should focus on understanding the differentiation of LESC during corneal disease and repair. This knowledge might help focus the selection of specific areas of the limbus or other parts of the cornea for use in transplants or for the creation of a cell line culture with therapeutic purposes. The results of one study in an in vitro human corneal model using fresh cadaveric corneas demonstrated the freshly wounded central cornea is capable of regeneration in the absence of LESC over the first 12 hours after injury (Chang CY *et al* 2008). This highlighted the role of transient amplifying cells of the central cornea in the acute response after injury. It also demonstrated with one of its models that the limbus does not respond to injury for at least 12 hours (Chang CY *et al* 2008). Another study in mice highlighted the

role of central corneal cells for the maintenance of an intact epithelium in corneal homeostasis, while limbal stem cells were shown to be instrumental during major corneal repair (Majo F et al 2008).

Conclusions

Limbal epithelial stem cells play a pivotal role in maintaining corneal epithelial health and thus corneal clarity. Extrapolation of findings from one species to another is not always possible or prudent. Studies to date have largely been carried out in animal corneal tissues but have focused on the LSCDs that affect humans. LSCDs also occur in animals. Developing a deep understanding of the microanatomy and physiology of the limbus and corneal cell turnover in all species is of paramount importance. The successful treatment of LSCDs in humans and animals, and validation of comparative studies between species depend on this knowledge.

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Figure 1. Shih-Tzu with trichiasis and a corneal swirl due to mild superficial scarring.

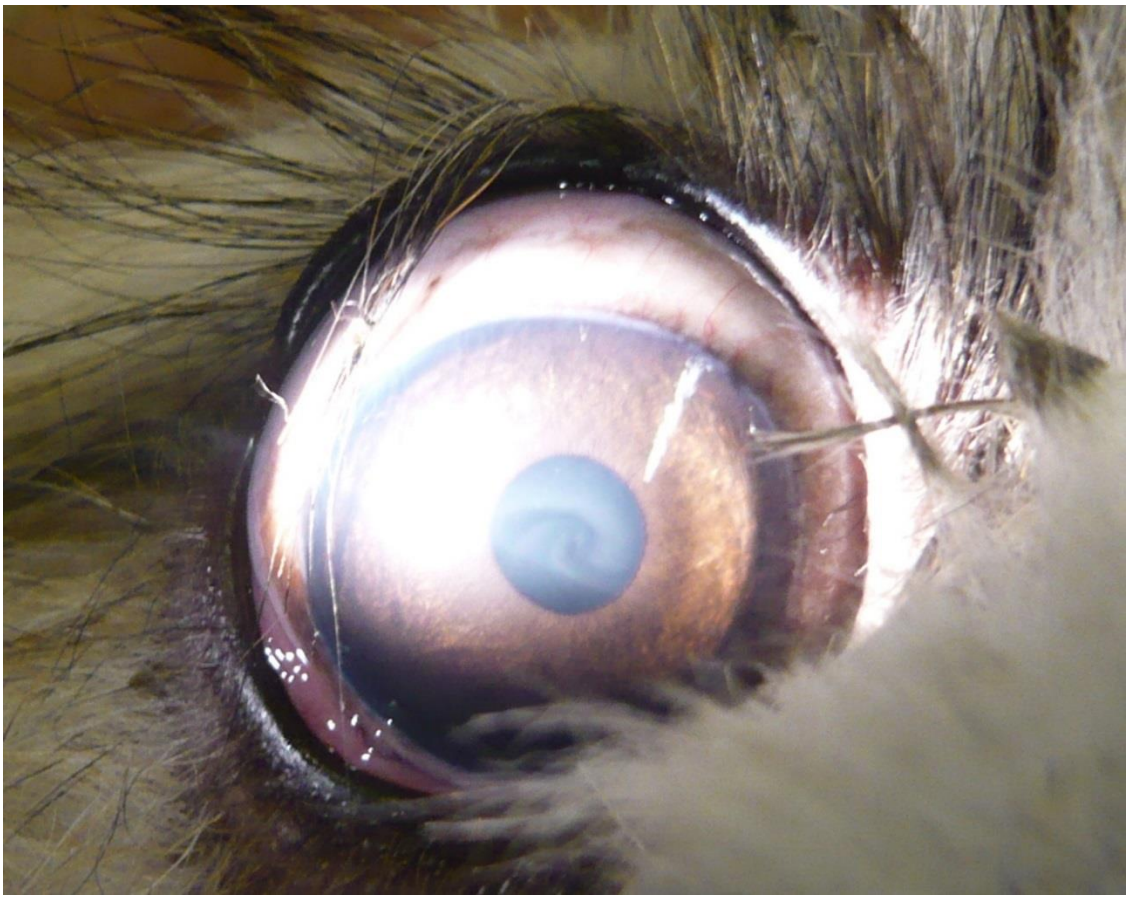


Figure 2. Young Pug with early Pigmentary keratitis-keratopathy and a pigmented swirl.

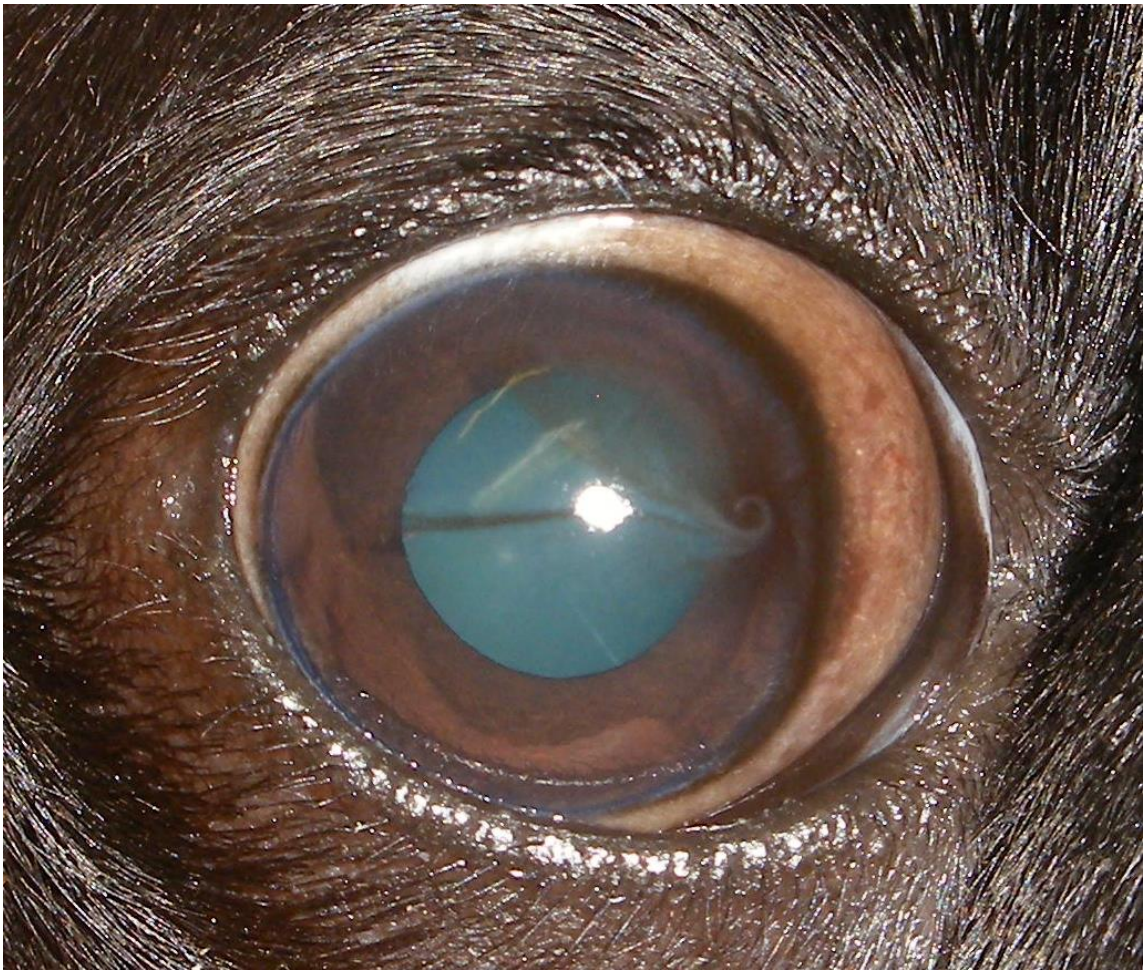


Figure 3. Pigmentary Keratitis-keratopahty in a Pug obscuring the medial to central cornea.

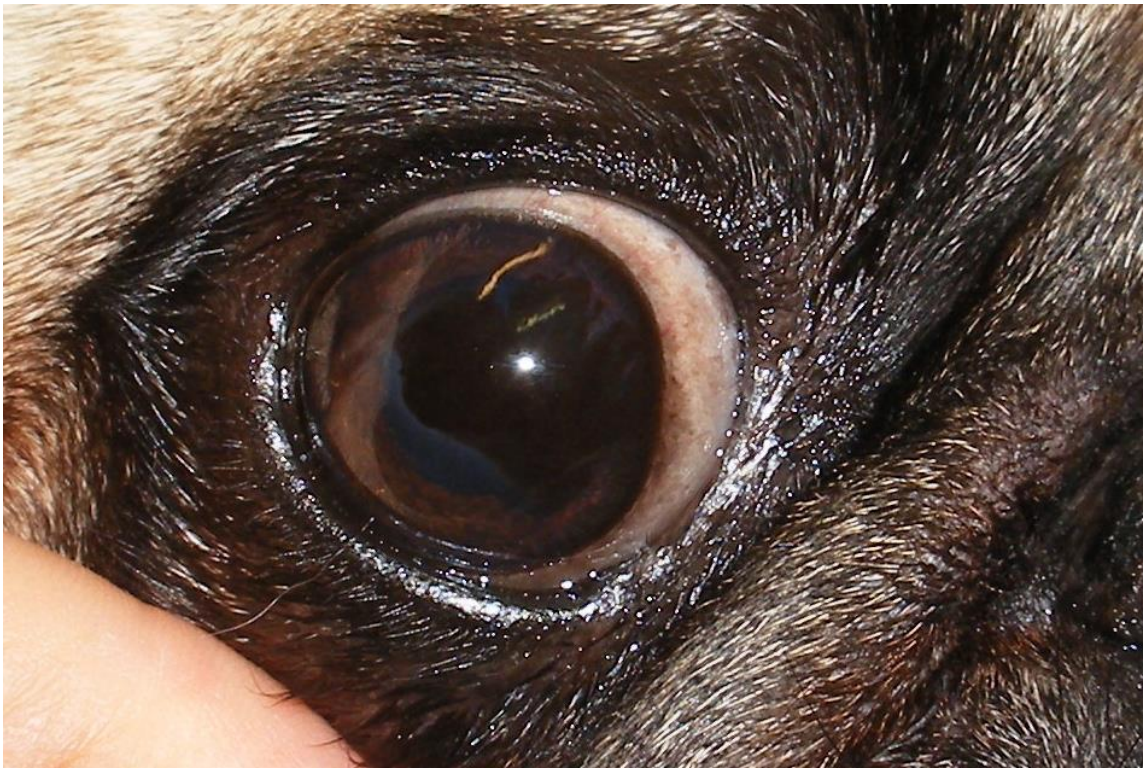
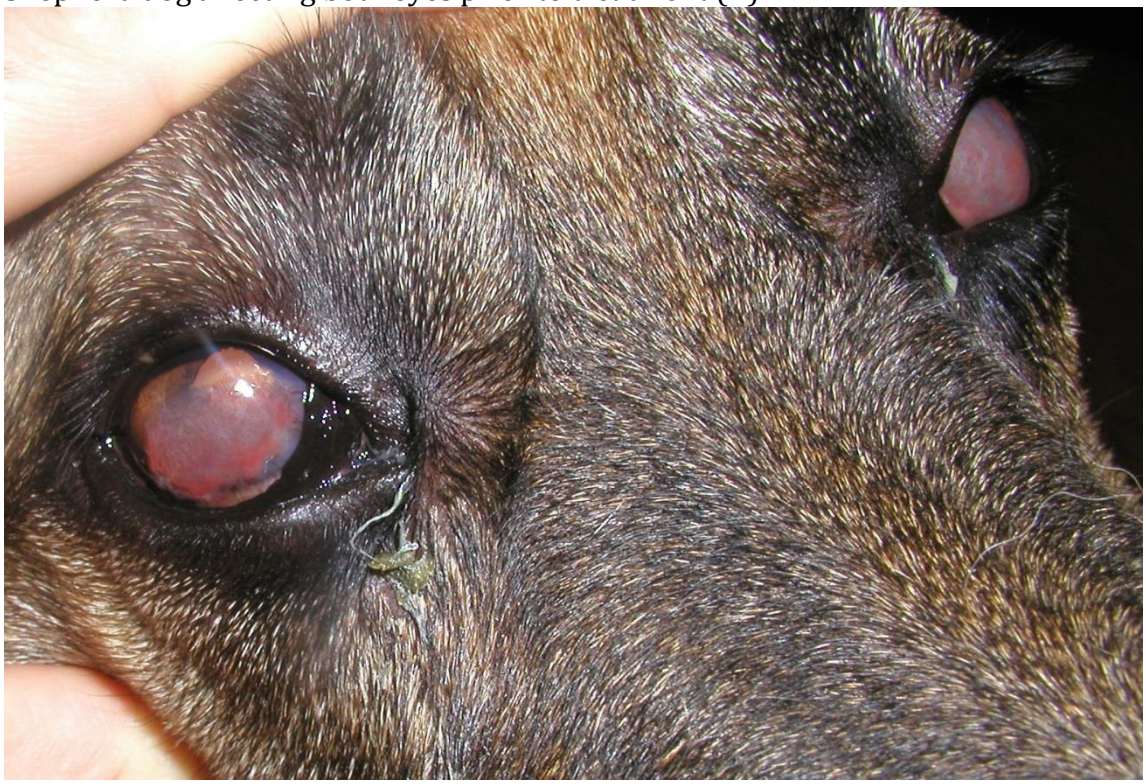


Figure 4. Lymphocytic-plasmacytic keratitis in an 8 year old, male German Shepherd dog affecting both eyes prior to treatment (A).



Same dog one month after treatment with a topical steroid. The inferior cornea is pigmented and scarred (B).