THE IMMUNOPATHOGENESIS OF CANINE OCULAR NODULAR EPISCLERITIS

by

CLARA O. WILLIAMS
(Under the direction of K. Paige Carmichael)

ABSTRACT

Canine ocular nodular episcleritis (CONE) or nodular granulomatous episcleritis develops in the sclera, limboscleral junction and third eyelid and may infiltrate the cornea and its etiology is unknown. This condition is recurrent and inconsistently responds to surgery and immunomodulating medications. We characterized CONE histologically and for the first time immunohistochemically. Forty-two paraffin-embedded specimens obtained from UGA and COPLOW laboratories were stained with H&E and characterized histologically as inflammatory or proliferative. Tissues were stained with Masson's trichrome, Reticulin, CD3, CD79, MAC387, TGFß$_2$, smooth muscle actin (SMA), and desmin. Twenty-three samples (54.5%) were characterized as inflammatory (predominant CD3+ and SMA) and 17(46.5%) as proliferative (predominant TGFß2 and reticulin). CD3+ and TGFß$_2$ were predominant in both groups. The histiocytic-like cells seen in both lesions were non-immunoreactive for MAC387, those cells may be histiocytes in a different stage not expressing MAC387 or may cells from a different lineage.

INDEX WORDS: Canine ocular nodular episcleritis, granulomatous episcleritis,
immunohistochemistry, histochemistry

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DEDICATION

To Frances Goodrich Williams, who gave me Jack.

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To the lit lamp beside the Golden Door:

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Dear Jack, whom by now knows more about immunostains that he would like.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iv
INTRODUCTION 1
BACKGROUND 3
PURPOSE OF THE STUDY 12
EXPERIMENTAL METHODS AND DESIGN 13
Specimens 13
Tissue preparation and processing 13
H&E Staining and Tissue classification 13
Immunohistochemical stains and determination of positive staining 15
Histochemical stains and determination of positive staining 20
Statistical Design 22
RESULTS 23
Signalment 23
Histochemistry 24
Immunohistochemistry 24
STATISTICAL ANALYSIS 43
DISCUSSION 44
CONCLUSIONS 49
REFERENCES 51

LIST OF TABLES

Table 1: Breeds represented in the sample specimens 26

Table 2: Distribution of the lesions according to gender 26

Table 3: Distribution of inflammatory or proliferative lesions according to age, gender and breed 26

Table 4: Anatomic localization of the lesions 27

Table 5: Inflammatory or proliferative characterization compared to lesion location 27

Table 6: Duration of the lesion (weeks, year) 27

Table 7: Duration of the lesion and Inflammatory / Proliferative classification 28

Table 8: Average percentage of the expression levels of the different stains 28

Table 9: Percentage of staining (in a 0 to 100 % scale) of the immunohistochemical and histochemical stains in each one of the tissue samples 29

LIST OF FIGURES

Figure 1: CONE: the section is nfiltrated by histiocytic inflammatory cells, lymphocytes and plasma cells, between collagen bundles. HE. Bar = 200µm. 30

Figure 2: CONE: Collagen bundles within the nodular episcleritis lesion appear glossy and surrounded by lymphocytes and plasma cells. HE. Bar = 200µm. 31

Figure 3: Proliferative lesion with swirls of plump spindle-shaped cells around blood vessels. HE. Bar = 200µm. 32

Figure 4: Prolifetarive lesion with reticulin fibers forming swirls surrounding the inflammatory infiltrates and spindle-shaped cells. Reticulin. Bar = 200µm 33

Figure 5: A proliferative lesion with abundant collagen deposition within inflammatory infiltrate. Masson's Trichrome. Bar = 200µm..... 34

Figure 6: An inflammatory lesion with diffuse infiltration by CD3 positive lymphocytes. CD3. Bar = 200µm. 35

Figure 7: Proliferative lesion with diffuse infiltration of lower number of CD79 positive lymphocytes. CD79. Bar = 200µm. 36

Figure 8: An inflammatory lesion with diffuse MAC 387 positive macrophages infiltration. MAC 387. Bar = 200µm. 37

Figure 9: MAC 387 positive macrophages are found in multifocal clusters through out an inflammatory lesion. Theplum round cells are negative for this immunostain. MAC387. Bar = 200µm. 38

Figure 10: Positive immunoreactivity for TGF β_2 was found through both the inflammatory and proliferative forms of CONE. TGF β_2. Bar = 200µm 39

Figure 11: A proliferative lesion was positive for desmin. The desmin immunoreactive cells are not associated with capillaries or vessels. Desmin. Bar = 200µm. 40

Figure 12: SMA positive staining of an inflammatory lesion revealed cells with elongated cytoplasm, a feature not noticeable on HE stain. Note plump histiocytic cells negative for this immunostain. SMA. Bar = 200µm. 41

Figure 13: Proliferative lesion reveals SMA positive spindle- shaped cells. The plump histiocytic cells were negative for this immunostain. SMA. Bar = 200µm. 42

INTRODUCTION

Canine ocular nodular episcleritis (CONE) is a poorly understood condition that occurs in the ocular adnexa: eyelids, nictitating membrane, bulbar episclera, limboscleral junction and infiltrates the cornea. Some of these nodules are located near or at the limboscleral junction and can easily be excised. Others, while also originating at the limbus, diffusely infiltrate the underlying tissues and can not be completely removed. The etiology of the lesion is unknown. Some consider these lesions to be inflammatory or immune mediated [26] while others consider them to be locally invasive proliferations. Many names have been applied to this condition including nodular granulomatous episclerokeratitis, fibrous histiocytoma, diffuse episcleritis, inflammatory granuloma, proliferative episcleritis and idiopathic granulomatous disease [12, 16, 20].

The plethora of names underscores the lack of understanding of the pathogenesis of these lesions and can make therapy challenging. Surgical and medical approaches have been used independently or combined to treat the condition [26, 32, 33]. Complete excision has proved to be effective in some cases [1, 34] while others require long-term local and/or systemic immuno-modulating therapy [29, 49].

Histologically, the lesion consists of the proliferation of spindle shaped cells and/or round cells [1, 12, 29], macrophages, with focal infiltration inflammatory cells of lymphocytes, plasma cells, and occasionally eosinophils. Extracellular matrix components can be abundant or scarce. These findings, along with the varying responses to therapy, may indicate that there could be different

lesions grouped under the name of CONE. Only histochemical stains have been used to aid in histopathological characterization [1, 6, 11, 12, 20, 29, 35, 47]. Diagnostic techniques such as immuno-histochemical staining (IHC) may allow more accurate characterization of the cell type on this lesion [34]. IHC may also tell us whether the predominant cell type in the lesions is undergoing proliferation. Separating the histological forms of nodular episcleritis into easily recognizable subgroups may lead to a better understanding of the lesion and to a more appropriate therapy.

The cellular in filtrate was characterized using cellular markers **CD3**, **CD79**, for T and B lymphocytes respectively. T and B lymphocytes migrate into inflammatory sites. T lymphocytes are initially activated by macrophages (antigen presenting cell) and produce a variety of mediators to activate monocytes and macrophages. **Mac387** was used for activated macrophages. Macrophages are tissue cells derived from circulating blood monocytes that under the influence of adhesion molecules and chemotactic factors reach extravascular tissue and are capable of phagocytosis. Other macrophage marker (**CD68**) was unsuccessfully attempted.

BACKGROUND

There are few available literature references for canine ocular nodular episcleritis (CONE). Frequently, these references read "Canine ocular nodular episcleritis is a poorly understood condition;"[12, 11] which reflects the inadequacy of previous research and case reports to reach a consensus on the nature of this poorly understood entity. Unfortunately, the lack of consensus still prevails. Although this condition is most often described in dogs [3, 7, 8, 18, 33], there have been similar lesions reported in people and more rarely, in cats [47], a bear [31] and a hedgehog (Dr. Richard Dubielzig, personal communication).

The history of published cases of this lesion begins when subcutaneous pseudosarcomatous fibromatosis (fasciitis) was described as a condition in human medicine for the first time in 1955, in a preliminary review of eight cases of a circumscribed tumor-like lesion localized in the thorax, neck, and arms. Histologically the lesions were reminiscent of fibrosarcoma or angiosarcoma and were non-circumscribed nodules with mucoedematous stroma, into which, extending in all directions, grew large cytoplasmic hyperchromatic spindle cells resembling rapidly proliferating fibroblasts. These cells were considered to be either histiocytes or fibroblasts [17]. There were large numbers of recently developed capillaries. The report had an important impact in the determination of the type and extent of the therapy as formerly the treatment of fast growing fibroblastic masses was extensive surgical excision, while in these cases, surgical excision of the mass was curative [15, 17].

Nodular fasciitis of the eye and adnexa was reported for the first time in ten human cases in 1966. The lesion was considered a benign proliferation of connective tissue, involving the superficial fascia and was found in the eyelids, eyebrow, and scleral insertion of the rectus muscle and corneoscleral limbus. Histologically, there was nodular proliferation of plump, stellate or spindle-shaped cells arranged in parallel bundles or haphazardly with variable amounts of intercellular myxoid ground substance. There were abundant reticulin fibers and moderate amounts of collagen fibers. There were scant infiltrations of lymphocytes and mononuclear cells. The author mentions that "few of the lesions were quite cellular or with areas of more pronounced cellularity" [17]. Also, "one of the most highly cellular lesions had the longest duration, while one of the tumors containing the greatest amount of collagen had the shortest history " [17]. In this article, the lesions were thought to be nodular reactive proliferations of fibroblasts, usually attached to the fascia. Treatment consisted of surgical excision without recurrence [17].

In the veterinary literature, the first reported case of ocular nodular fasciitis appeared in 1967 [1, 2]. A 9-year-old, female, Miniature Schnauzer was examined complaining of a one week duration subconjunctival nodular growth on the temporal limbus of the right eye. Excisional biopsy of the lesion revealed interlacing bundles of connective tissue with numerous capillaries. Fibroblasts with variously shaped nuclei (vesicular to fusiform) compromised most of the mass, and some mitotic figures were evident. Strands of collagen were scattered throughout, and many of the fibroblasts had vacuoles in their cytoplasm. A few giant cells were noticed. Excision of the mass was curative in this case. Seven years later, in 1974, a second report of ocular nodular fasciitis was published, this time in a Collie dog. The mass originated on the limbus and extended into the

superficial cornea. Histologically, the bulk of the mass was composed of a mixture of proliferated capillary-sized blood vessels, histiocytes and fibroblasts arranged in bundles and haphazardly, with mononuclear inflammatory cells at the periphery of the mass [20].

An infiltrative condition that affected mostly Collie or Collie mix dogs was mentioned in a report titled, "Infiltrative corneal lesions resembling fibrous histiocytoma" published in 1976. In this report, four Collies, one Poodle and one cat had unilateral or bilateral masses on the limboscleral junction. These lesions were characterized by continuous growth, a benign appearance and a tendency to recur following excisional keratoplasty. Histological examination revealed connective tissue and histiocytic cells centrally, while there was peripheral lymphoplasmacytic infiltration. Throughout the lesions there were immature fibroblastic cells, with pleomorphic nuclei. The authors considered these features "consistent with either fibrous histiocytoma or ocular nodular fasciitis" [47]. Surgical excision combined with topical antibiotics and topical and repository subconjunctival steroids was necessary to control the recurrence of the condition. In an attempt to reproduce the condition, macerated biopsy tissue from an affected dog was inoculated into the anterior chamber of a guinea pig and into the corneal limbus of an adult Collie dog [47]. Neither recipient developed demonstrable lesions. Also, an explant culture was performed to demonstrate neoplastic stimulus from a minced sample of the biopsy specimen from a dog, but the cultured cells did not undergo transformation nor did they exhibit growth patterns compatible with neoplastic growth [47].

Another report in the same year, a diagnosis of a "proliferative episcleritis" was made on a temporal limbal mass infiltrating the cornea, sclera and bulbar conjunctiva of a two-year-old Boston terrier. Histopathologic examination of the mass revealed numerous fibroblasts with

infiltration of lymphocytes and histiocytes. In the discussion of this paper, the author considers the term proliferative episcleritis more applicable than nodular fasciitis because the involvement of the mass could not be related to Tenon's capsule and the condition was characterized by broad proliferation and infiltration rather than nodular outgrowth. The patient required extensive surgery and local and systemic steroid therapy to address the condition [35].

In a case series published in 1977, canine ocular nodular fasciitis occurred in the limbus and eyelids of young to middle aged dogs, of breeds different than Collie. The condition was non-responsive to topical steroid therapy, but resolved after surgical excision in all the cases. Histopathology revealed solid non encapsulated masses that were cellular and infiltrative in nature. The predominant cells were fibroblasts arranged haphazardly and occasionally forming linear bundles. In H&E stained sections, the loose irregular arrangement of fibroblasts were in a pale myxoid intercellular ground substance. There were focal accumulations of lymphocytes and plasma cells. Special stains with Gridley's reticulin revealed abundance of reticulin fiber formation. Collagen fiber formation was never prominent. The author remarks the importance of the recognition of nodular fasciitis as benign in nature and the fact that excision is curative [20].

In 1982, Fischer published the review of the clinical and histopathological features of sixteen cases of episcleritis and scleritis, and proposed a classification, strictly for the dog, in order to offer a more unifying approach to the study of these disorders. By the time of this publication, terms and such as nodular fasciitis, limbal granuloma, corneoscleral fibrous histiocytoma, sclerouveitis, had arisen to mention episcleritis and scleritis conditions. In Dr. Fischer's proposal, two categories were considered: simple episcleritis and nodular episclerokeratitis.

Histopathologically simple episcleritis consisted of lymphocytes and plasma cell infiltrates. The second category was nodular episclerokeratitis. Clinical findings of episclerokeratitis included a raised fleshy red-gray mass or masses arising at the corneoscleral junction and infiltrating into the adjacent corneal stroma, areas of corneal stromal degeneration, nictitating membrane involvement, bilateralism, breed disposition for the Collie, tendency to recur, a benign nature and responsiveness to local surgical excision, and intralesional injections of corticosteroids. According to the author, the specimens including the infiltrated corneal tissue and adjacent episclera, were characterized by "granulomatous infiltration including lymphocyte, plasma cells, histiocytes and epithelioid cell accumulations, corneal stromal neovascularization with perivascular polymorphonuclear inflammatory cell infiltration and interlacing immature fibroblastic cells and abundant reticulin fiber formation within the corneal stroma" [16]. This review documented that nodular episclerokeratitis lesion had benign behavior [13, 26, 29, 32, 42].

A 1983 paper described the management of fibrous histiocytoma in two Collies, administering Azathioprine (a thiopurine immunosuppressant agent). The authors mentioned that "the terms fibrous histiocytoma, nodular episcleritis, diffuse episcleritis, nodular fasciitis, inflammatory granuloma, conjunctival granuloma, third eyelid granuloma and proliferative keratoconjunctivitis "have been applied to masses composed of fibroblasts, histiocytes, lymphocytes, plasma cells and capillaries, reported on the episclera, sclera, cornea, and nictitans of dogs and cats" [30].

In 1987, Paulsen et al. published a series of 19 cases (1973 to 1985) of nodular granulomatous episclerokeratitis. The disorder was considered idiopathic, bilateral, and responsive to immunosuppressive drugs. The histologic features were those of chronic granulomatous inflammation, with the predominant cell types being histiocytes, lymphocytes and plasma cells.

For the first time, the authors differentiated nodular fasciitis from nodular granulomatous episclerokeratitis describing the former composed primarily of proliferating fibroblasts with abundant reticulin formation. Electron microscopy of one specimen revealed a population of metabolically active histiocytes, with some lymphocytes deemed to be T cells, and fewer plasma cells [35].

Ocular nodular faciitis/fibrous histiocytoma was described affecting an Asiatic bear in 1987. The excised lesion was composed of an edematous haphazard mixture of plump fibroblasts, capillaries, macrophages and lymphocytes. Excision and repository subconjunctival steroids were curative [30].

Different therapeutic approaches for the treatment of CONE were described in 1989, when Wheeler et al published on a series of proliferative keratoconjunctivitis in dogs, treated by a combination of cryosurgery and steroidal therapy. Fibrous histiocytoma, proliferative keratoconjunctivitis, nodular fasciitis, diffuse episcleritis, Collie granuloma, inflammatory granuloma, proliferative-episcleritis, proliferative-keratitis, nodular episclerokeratitis are mentioned as other names give to proliferative keratoconjunctivitis [48].

In 1992, Collins et. al reported idiopathic granulomatous disease with ocular involvement in a dog that exhibited cutaneous, eyelid and bulbar conjunctival masses [6], histopathologic findings for each tissue included granulomatous inflammation with large epithelioid macrophages, plasma cells and lymphocytes [6].

A case series of four Collie or Collie mix dogs was published by Dungan et al in 1993. The authors considered these cases a variant of nodular granulomatous episclerokeratitis due to the fact that there was concurrent intraocular and oral mucocutaneous involvement [12].

The most recent retrospective study was conducted in 1997. Thirty-eight out of 48 cases of primary scleral and episcleral inflammatory disease presented to North Carolina State University between 1980 and 1994 were diagnosed as episcleritis. Histopathology suggested alterations in collagen as the primary stimulus for a cell mediated response. There was also evidence of breed predisposition for Cocker spaniel. Foci of thickened hyalinized collagen fibers indicative of collagenolysis were surrounded by macrophages. The inflammatory response appeared to be directed toward degenerating collagen [11].

Until recently, the histopathological characterization of CONE in veterinary medicine has been based on histochemical stains such as H&E, Alcian blue, PAS, Masson's trichrome, colloidal iron, Toluidine blue, Gomori's methenamine silver, and Wilder's reticulin stain. Similar conditions in human medicine are considered histologically difficult to distinguish from neoplasia; and have been immunohistochemically characterized as nodular fasciitis by having proliferations of fibroblasts and myofibroblasts, that stain positive for smooth muscle actin (SMA)[45], cluster of differentiation 68) CD68 and vimentin [34, 44].

SMA is the most important marker of intermediate filaments. It can be positive in pericytes, myofibroblasts and smooth muscle cells [14]. **Desmin** is the major protein of intermediate filaments in smooth muscle, not found in vascular tissue [25]. **Vimentin** and intermediate filament

protein present in cells of mesenchimal origin [25]. The immuno stain **vimentin** is of value in the differential diagnosis of undifferentiated neoplasms [34, 44]. Although it can differentiate between most epithelial and mesenchymal cells, is not a good differentiator between mesenchymal stains.

CD68 is a monocyte/macrophage associated antigen which is mainly located in lysosomes. CD68 antibody recognizes macrophages in a variety of human tissues (histiocyte marker) 34, and immunolabels large macrophages and plasma cells in dogs 5. It reacts with plasmacytoid T-cells that are believed to be of moncyte/macrophage origin. It does not react with granulocytes and its precursors. The antigen is very formalin-sensitive and requires heat induced epitope retrieval.

CD3 and CD79 are also mononuclear markers. CD3 antigen is highly specific for T cells. The CD3 antibody recognizes early thymocytes and is assumed to be an early sign to a T cell lineage. CD79 is an antibody useful to demonstrate B cells 28.

p53 is a nuclear protein that acts as a transcription factor that is involved in inhibiting cell proliferation when DNA damage occurs, and triggering apoptosis. Normal p53 protein has a very short half-life. The mutant p53 leads to increased half-life of the protein and its accumulation in tumor cells, detectable by immunohistochemistry. It is considered a neoplastic proliferation marker 28.

Transforming growth factor-beta **($TGF\beta_2$)** is a multifunctional peptide with pleiotropic effects that controls proliferation, differentiation and other functions in many cell types. **$TGF\beta_2$** was used to demonstrate fibroblastic proliferation [9, 10]. In low concentrations, **$TGF\beta_2$** induces the synthesis of **PDGF**, and stimulates fibroblasts chemotaxis and production of collagen and

fibronectin, being implicated in the fibrosis elicited in chronic inflammatory states. **TGFß$_2$** is also known for inhibiting the degradation of extracellular matrix by metalloproteinases, is a potent immunosuppressant and can triggers apoptosis. **TGFß$_2$** is produced by almost all cells, and all cell membranes have receptors for it [28].

PURPOSE OF THE STUDY

We hypothesize that CONE represents two distinctive histological lesions and that this may explain the variability of the clinical course. The purpose of this retrospective study is to characterize CONE histologically (IH) and immunohistochemically (IHC) in order to probe this hypothesis. A distinctive IH and IHC profile may aid the practitioners with the correct identification of the lesions may help in the selection of the appropriate therapeutic approach.

EXPERIMENTAL METHODS AND DESIGN

Specimens

Forty two paraffin embedded archived specimens with a primary histopathologic diagnosis of ocular nodular episcleritis obtained from the biopsy submissions from 1988 - 2005 to the Department of Pathology and Athens Diagnostic Laboratory at The University of Georgia and the Comparative Ophthalmology Pathology Laboratory of Wisconsin, University of Wisconsin-Madison (COPLOW). Initially, hematoxylin and eosin (**HE**) stained slides were reviewed for the presence of adequate tissue and accurate diagnosis. Sections from cases of interest were stained for imunohistochemistry **CD3**, **CD79**, **Mac387**, **smooth muscle actin**, **desmin**, **Gomori's reticulin**, **Masson's trichrome** and **TGFß$_2$** .

Tissue preparation and processing

Forty two formalin fixed, paraffin embedded tissue blocks were sectioned at 3 μm. Replicate sections were placed on Super Frost/Plus Slides (Fischer scientific, Pittsburg, PA) deparaffinized in two successive xylene baths for 8 minutes each. After deparaffinization, they were rehydrated in a series of graded alcohols.

H&E Staining and Tissue classification

Once deparaffinized and rehydrated, the section were stained with (H&E) and examined under the microscope. The samples were divided into two categories, inflammatory or proliferative.

Inflammatory tissues were those in which there were high numbers of epithelioid macrophages, granulomas (focal infiltration of neutrophils surrounded by macrophages), focal or diffuse infiltration of the epithelium by neutrophils, plasma cells, occasional mast cells and collagen degeneration with adjacent inflammatory (neutrophilic or granulomatous) reaction. Proliferative lesions were those in which spindled shaped cells were predominant. These cells, interpreted as fibroblasts were found in sheets, or whorls and around capillaries and well formed vessels. Fewer plasma cells and no eosinophils or collagen degeneration was noted.

The tissues were immunohistochemically stained with **CD3**, **CD79**, and **Mac387**. **CD3** is a marker for T lymphocytes, which are ready available effector cells present in sites of acute inflammation, and develop a reciprocal relationship with macrophages in chronic inflammation, resulting in a focus where these two kinds of cells (macrophages and T cells) persistently stimulate each other until the triggering event is removed. The anti **CD79** alpha antibody has been demonstrated to react against B lymphocytes [5]. Mouse antihuman calprotectin (**Mac387)** was applied to demonstrate macrophages [19]. **Smooth muscle actin** and **desmin** [25] were applied to characterize intermediate filaments (major protein of intermediate filaments found in smooth muscle and in the Z disks of squeletal and cardiac muscle). **SMA** (intermediate filament) is the most important marker of myofibroblasts. Desmin is the major protein of intermediate filaments in smooth muscle. It is also found in the Z disks of skeletal and cardiac muscle, but not in vascular tissue [25].

TGFß$_2$ was performed to demonstrate proliferation [9, 10]. **TGFß$_2$** induces the synthesis of platelet derived growth factor **(PDGF)**, and stimulates fibroblasts chemotaxis and production of collagen

and fibronectin, being implicated in the fibrosis elicited in chronic inflammatory states. It also inhibits degradation of extracellular matrix by metalloproteinases, is a potent immunosuppressant and can triggers apoptosis. These special stains were performed in the DAKO automated stainer. **TGFß$_2$** was performed manually.

Other histochemical stains applied besides **HE** were **Gomori's reticulin** and **Masson's trichrome** to demonstrate deposition of reticulin and collagen fibers, and characterize the extracellular matrix.

Immunohistochemical stains and determination of positive staining

The protocols followed for each one of the stains are described as follows.

CD79: After deparafinization and rehydration of the tissues, the antigen was retrieved with buffer citrate (0.01M pH 6) under pressure cooker (Cell Mark) for fifteen minutes. Then the sections were allowed to cool for fifteen minutes. In the automated stainer (DAKO, Carpinteria, CA) the sections were rinsed with Tris base pH 7.6. Endogenous peroxidase activity was blocked by incubating for five minutes in H_2O_2 (CVS Pharmacy, Athens, GA). The primary antibody (CD79) M7051 antibody (DAKO, Carpinteria, CA) was applied to the sections in a 1:100 dilution with antibody Plain Diluent (DAKO, Carpinteria,CA). This monoclonal antibody reacts to an intracytoplasmic epitope and is used for demonstration of B cells in many mammalian species [24]. The primary antibody was added to all slides except for the negative control which received only antibody diluent. The tissues were allowed to incubate for one hour at room temperature and then rinsed with Tris buffer saline (TBS). The secondary biotinylated antibody (Zymed superpictures HRP Polymer conjugate broad spectrum, San Francisco, CA)

was placed on all tissue sections, which were incubated for ten minutes, and rinsed with TBS. The substrate chromogen (3, 3'-Diaminobenzidine {DAB}) was applied for twelve minutes. The sections were counterstained with hematoxylin. The sections were placed in a series of graded ethanol (95% and 100%) followed by two xylene rinses. The tissue sections were then cover-slipped with Permount media.

CD3: After deparafinization and rehydration of the tissues, the antigen was retrieved with buffered citrate (0.01M pH 6) in a pressure cooker (Cell Mark) for fifteen minutes. Then the sections were allowed to cool for fifteen minutes. In the automated stainer (DAKO, Carpinteria, CA) the sections were rinsed with Tris base pH 7.6. Then the peroxidase was blocked and incubated for five minutes (H_2O_2, CVS Pharmacy, Athens,GA). The primary antibody (CD3) Rabbit A0452 antibody (DAKO, Carpinteria, CA) was applied to the sections in a 1:100 dilution with Antibody Plain Diluent (DAKO, Carpinteria,CA). This monoclonal antibody is a pan T cell marker. The primary antibody was added to all the slides except for the negative control which received only antibody diluent. The tissues were allowed to incubate for one hour at room temperature and were rinsed with tris buffered saline (TBS). The secondary biotinylated antibody (Zymed superpictures HRP Polymer conjugate broad spectrum, San Franscisco,CA) was placed on all tissue sections and incubated for ten minutes, and then rinsed with TBS. The substrate chromogen (DAB) was then applied for twelve minutes. The sections were then counterstained with hematoxylin with five dips in the solution. The tissue slides were then placed in a series of graded ethanol (95% and 100%) and then in two xylene rinses. The tissue sections were then cover-slipped with Permount media.

Mac387: After deparafinization and rehydration of the tissues, the antigen was retrieved with buffer citrate (0.01M pH 6) under pressure cooker (Cell Mark) for fifteen minutes. Then the sections were allowed to cool for fifteen minutes. In the automated stainer (DAKO, Carpinteria, CA) the sections were rinsed with Tris base pH 7.6. The peroxidase was blocked and incubated for five minutes (H_2O_2 CVS Pharmacy, Athens, GA). Then endogenous avidin and biotin were blocked applying avidin for ten minutes, rinsing, and then biotin for ten minutes (Biotin blocking system, DAKO cytomation, Carpinteria, CA), then rinsing. The primary antibody (M0747) antibody (DAKO, Carpinteria, CA) was applied to the sections in a 1:100 dilution with Antibody Plain Diluent (DAKO, Carpinteria,CA). The primary antibody was added to all the slides except for the negative control which received only antibody diluent. The tissues were allowed to incubate for forty five minutes at room temperature and then rinsed with TBS. The secondary biotinylated antibody (DAKO LSAB2 Link HRP) was placed on all tissue sections and incubated for twenty five minutes, and then rinsed with TBS. Then LSAB2 StrepAvidin HRP was applied for another twenty five-minutes. The substrate chromogen (DAB) was then applied for twelve minutes. The sections were then counterstained with hematoxylin. The tissue slides were then placed in a series of graded ethanol (95% and 100%) and then in two xylene rinses. The tissue sections were then cover-slipped with Permount media.

SMA: After deparaffinization and rehydration of the tissues, the antigen was retrieved with buffer citrate (0.01M pH 6) under pressure cooker (Cell Mark) for fifteen minutes. Then the sections were allowed to cool for fifteen minutes. In the automated stainer (DAKO, Carpinteria, CA) the sections were rinsed with Tris base pH 7.6. The peroxidase was blocked and incubated for five minutes (H_2O_2 CVS Pharmacy). Then endogenous avidin and biotin were blocked

applying avidin for ten minutes, then rinsed, and biotin for ten minutes Biotin blocking system, DAKO cytomation, Carpinteria, CA), then rinsed. The primary antibody (M0851) (DAKO, Carpinteria, CA) was applied to the sections in a 1:100 dilution with Antibody Plain Diluent (DAKO, Carpinteria, CA). The primary antibody was added to all the slides except for the negative control which received only antibody diluent. The tissues were allowed to incubate for forty five minutes at room temperature and then rinsed with TBS. The secondary biotinylated antibody (DAKO LSAB2 Link HRP) was placed on all tissue sections and incubated for twenty five minutes, and then rinsed with TBS. Then the LSAB2 StrepAvidin HRP was applied for another twenty-five minutes. The substrate chromogen (DAB) was then applied for twelve minutes. The sections were then counterstained with hematoxylin. The tissue slides were then placed in a series of graded ethanol (95% and 100%) and then in two xylene rinses. The tissue sections were then cover-slipped with Permount media.

Desmin: After deparaffinization and rehydration of the tissues, the antigen was retrieved with buffer citrate (0.01M pH 6) under pressure cooker (Cell Mark) for fifteen minutes. Then the sections were allowed to cool for fifteen minutes. In the automated stainer (DAKO, Carpinteria, CA) the sections were rinsed with Tris base pH 7.6. The peroxidase was blocked and incubated for five minutes (H_2O_2, CVS Pharmacy). Then endogenous avidin and biotin were blocked applying avidin for ten minutes, then rinsed, and biotin for ten minutes (Biotin blocking system, DAKO cytomation, Carpinteria, CA), then rinsed. The primary antibody (MU072-UC) (Biogenex, San Ramon, CA) was applied to the sections in a 1:100 dilution with Antibody Plain Diluent (DAKO, Carpinteria,CA). The primary antibody was added to all the slides except for the negative control which received only antibody diluent. The tissues were allowed to incubate

for forty five minutes at room temperature and then rinsed with TBS. The secondary biotinylated antibody (DAKO LSAB2 Link HRP) was placed on all tissue sections and incubated for twenty five minutes, and then rinsed with TBS. Then the LSAB2 StrepAvidin HRP was applied for another twenty five minutes. The substrate chromogen (DAB) was then applied for twelve minutes. The sections were then counterstained with hematoxylin. The tissue slides were then placed in a series of graded ethanol (95% and 100%) and then in two xylene rinses. The tissue sections were then cover-slipped with Permount media.

TGFß$_2$: The sections were deparaffinized by successive xylene baths. Initially, the slides were placed for five minutes in three different containers with a xylene bath (one time in each). Then, they were rehydrated in a series of graded alcohols (100%, 95% and 70%). Then they were rinsed in distilled water for three minutes. The process of antigen retrieval started by placing the slides in a 0.01 M pH 6 citrate solution for five cycles of five minutes each in the microwave (700 W power). The sections were then rinsed and allowed to cool for three minutes in distilled water. The excess water was removed using a kimwipe around the specimen. Using the DAKO LSAB + Kit, Peroxidase protocol the specimens in the slides were covered with 3% hydrogen peroxide and placed in an incubation chamber for 5 minutes. The slides were rinsed with Tris base pH 7.6 and placed in a Tris bath. Then enough primary antibody TGFß$_2$ (V rabbit polyclonal Santa Cruz Biotechnology) was applied in a 1:200 dilution to cover the specimens and allow to incubate in the chamber for thirty minutes. Then the slides were rinsed and biotinylated secondary antibody was applied and incubated for 15 minutes, at room temperature. Then the slides were rinsed again, and excess buffer removed. Streptavidin peroxidase was applied to cover the specimen and incubated for 15 minutes. Then the slides were rinsed and

DAB was applied to the slides and allow to incubate five minutes. The slides were then rinsed and the chromogen properly disposed.

Sections were counterstained by being immersed in a bath of aqueous hematoxylin for five minutes and dipped in blueing agent. Dehydration of the sections was performed placing them in a series of graded ethanols (70%, 95%, and 100%) for three minutes each time, and finally in three xylene rinses for three minutes each time. The sections were removed from the last xylene container and were allowed to dry. The tissue sections were then cover-slipped with Permount media.

The set of immunohistochemically stained sections was thoroughly evaluated under a light microscope. A cell was considered positive by the presence of well pigmented intracytoplasmic brown granules. A total of 100 cells in 10 high power fields were counted. The percentage of positive stained cells was obtained by counting the number of positively stained cells among the total cells.

Histochemical stains and determination of positive staining

Gomori's reticulin: Once the sections had been deparaffinized and rehydrated, they were oxidized in potassium permanganate for one minute, washed in tap water for two minutes, then covered with potassium metabisulfite solution for one minute, and washed again in tap water for two minutes. Sections were then covered with ferric ammonium sulfate solution for one minute, then washed in tap water for two minutes, followed with two changes of distilled water 30 seconds each. Slides were then impregnated in silver solution for two minutes, rinsed in distilled

water for 20 seconds, covered with formalin solution (for reduction) for three minutes and washed in tap water for three minutes, they were then toned in gold chloride for 10 seconds, and rinsed in distilled water, reduced in potassium metabisulfite solution for one minute, fixed in sodium thiosulfite solution for one minute, and washed in tap water for two minutes. Slides were dehydrated in 95% alcohol, absolute alcohol and cleared, cover-slipped with Permount media.

Masson's trichrome: After the tissue sections had been deparaffinized and rehydrated to distilled water they were left in Bouin's Mordant overnight at room temperature. Then they were washed in tap water until the yellow color disappears, and stained in working Weigert's iron hematoxylin solution for ten minutes, washed in running water for 10 minutes and covered with Biebrich scarlet-acid fuchsin solution for six minutes. Following which then rinsed in distilled water, covered the slides with phosphomolybdic-phosphotungstic acid solution for three minutes and rinsed again. The slides were covered with aniline blue solution for five minutes, rinsed in distilled water, covered with glacial acetic acid solution for three to five minutes and then dehydrated and cover- slipped with Permount media.

The tissue sections stained with **Gomori's reticulin** and **Masson' trichrome** were evaluated under a light microscope. Positive staining was considered when coal black tint for reticulin and blue tint for Masson's were noticed in the tissue fibers. Ten high power fields were evaluated and the positive staining was also expressed in percentage, obtained by comparing the area of positive fiber staining, to the non stained areas.

Statistical Design

The following statistical analyses were performed using SAS V 8.2 (Cary, NC). An exact binomial test was utilized to test if the proportion of samples that were proliferative was significantly different than 50% which would be expected by chance. The quantitative assessment of expression of primary antibody staining was compared for differences in expression between types of staining using an analysis of variance (ANOVA (2-sided, α=0.05)). Multiple comparisons were made using Tukey's test (2-sided, α=0.05). Staining expression levels were represented on a 0-100% scale. PROC GLM was utilized in SAS for the analysis.

RESULTS

The initial examination of hematoxylin and eosin (**HE**) stained slides under a light microscope revealed two histologically distinctive conditions: one in which the predominant inflammatory type were epithelioid macrophages, sometimes forming granulomas, although there was frequent infiltration of neutrophils and fewer plasma cells, eosinophils and mast cells (Fig. 1). There was frequent collagen degeneration, creating a focal inflammatory reaction infiltrated by neutrophils and surrounding lymphocytes and macrophages (Fig 2). Twenty three (54.8 %) samples were characterized as inflammatory. In the second condition, the predominant type of cell was variable sized spindled-shaped, organized in sheets and whorls (Fig 3). Sometimes there were nodular aggregates of lymphoplasmacytic inflammatory cells and clusters of histiocytic cells. There were few neutrophils. Eosinophils or mast cells were not observed, nor was there evidence of collagen degeneration. Nineteen of the samples (42.2%) were considered proliferative.

Signalment

Twenty different breeds were represented in the sample set. Of these, Labrador retriever and Cocker spaniel breeds were over-represented (Table 1). The lesions were evenly distributed between the two genders, showing no predilection for male or female (Table 2). The condition affected mature dogs (average age of presentation of inflammatory and proliferative lesions is 7.9 years and 7.8 years respectively) and there was no difference in the age of onset of inflammatory vs. proliferative forms of canine ocular nodular episcleritis (CONE). The Cocker spaniels developed the inflammatory type of lesion more frequently than any other breed.

Most lesions were unilateral (92.85%) and the most frequent locations of the lesion were corneoscleral and scleral (Table 4). Both inflammatory and proliferative lesions were seen more often at the corneoscleral location. Interestingly, the three bilateral lesions were inflammatory (Table5). Fourteen (33%) of the lesions were of unknown duration. Eleven (33%) of the lesions were less than 4 weeks old (Table 6). Four (9.5%) of the lesions classified as proliferative were present less than four weeks. At least ten (23.8%) of the inflammatory lesions were present after four weeks (Table7).

Histochemistry

Histochemical stain **Gomori's reticulin** was considered positive if there were dark black staining fibers. Histochemical stain **Masson's trichrome** was considered positive for collagen if there was evidence of blue staining fibers. Fibers positive for **Masson's trichrome** and **Gomori's reticulin** were found in both types of tissues (inflammatory and proliferative) in different proportions. **Gomori's reticulin** staining (Fig 4) was present in about even proportions in proliferative (12.9 %) and inflammatory (12 %)lesions. In the inflammatory lesions, there was greater (8.9%) **Masson's trichrome** staining (Fig 5) compared to the proliferative (3.2%) lesions (Tables 8 and 9).

Immunohistochemistry

Immunohistochemical stains **CD3**, **CD79**, **Mac387**, **TGFß$_2$**, **desmin** and **smooth muscle actin (SMA)** were considered positive if there was brown granular intracytoplasmic staining (DAB) (Tables 8 and 9).

All the cases (32.6% in the inflammatory and 28.23% in the proliferative) but two stained positive for **CD3** (Table 8). The **CD3** infiltration was mostly diffuse in both types of lesions (Fig 6). On occasions the **CD3** positive cells were organized in multiple clusters in the proliferative lesion. Positive staining for **CD79** (Fig 7) was present in low percentages in both inflammatory (7.6%) and proliferative (4.41%) lesions (Table8). **Mac387** macrophage marker (antibody mouse antihuman calprotectin) positive cells were diffusely scattered (Fig 8) on the tissue or arranged in focal clusters (Fig 9) in either inflammatory (9.34%) or proliferative (7.6%) samples. The cells interpreted as epitheliod macrophages (histocytic-like) in **HE** did not stain with this antibody.

All but one case had diffuse positive staining for **TGFß$_2$** (26.73% inflammatory and 35.8% proliferative). The positive cells were evenly scattered throughout the tissue (Fig 10). **Desmin** antibody stain was positive in only one case (Fig 11). **SMA** was seen in spindle shaped cells present in both inflammatory (12.39%) and proliferative (6.71%) lesions. The percentage of **SMA** positive staining in inflammatory tissues was greater than in proliferative tissues. **SMA** staining revealed plump spindle-shaped cells not previously visible in the inflammatory lesions (Fig 12). The positive cells were found in whorls around areas of extracellular matrix (Fig 13).

TABLES

Table 1: Breeds represented in the sample specimens

Breeds in the Sample			
Breed	**Number of cases**	**Breed**	**Number of cases**
Bichon	1	Golden retriever	2
Labrador ret	6	Sheltland sheep d	1
Dachshund	1	Springer spaniel	1
Pekapoo	1	Airdale	1
Cocker spaniel	7	Rottweiler	1
Schnauzer	1	Chihuahua	1
Greyhound	2	French poodle	1
Pug	1	Terrier mix	2
Beagle	1	Chow Chow	1
Shi tzu	1	German shepherd	1
Mixed	3	Unknown	4

Table 2: Distribution of the lesions according to gender

Gender		
Male	**Female**	**Unknown**
19	19	2

Table 3: Distribution of inflammatory or proliferative lesions according to age, gender and breed

Distribution of lesions			
	Age(years)	**Male**	**Female**
Inflammatory	**7.9**	**10**	**11**
Proliferative	**7.8**	**9**	**8**

Table 4: Anatomic localization of the lesions

Anatomic localization	
Localization of the lesion	**Number of cases**
Retrobulbar	1
Corneoscleral	15
Scleral	14
Third eyelid	3
Eyelid	1
Intraocular	1
Unilateral	39
Bilateral	3

Table 5: Anatomic distribution of inflammatory and proliferative to lesions

Location of inflammatory and proliferative lesions		
Lesion location	**Inflammatory**	**Proliferative**
Retrobulbar	1	-
Corneoscleral	10	6
Scleral	8	6
Third eyelid	1	2
Eyelid	1	-
Intraocular	-	1
Unilateral	20	19
Bilateral	3	-

Table 6: Duration of the lesion (weeks, year)

Lesion Duration		
Duration of the lesion	**Number of cases**	**Percentage %**
< 4 weeks	11	26
< 1 year	10	24
1 year	3	7
> 1 year	4	10
Unknown	14	33

Table 7: Duration of inflammatory and proliferative lesions

Duration of the Lesion					
	< 4 weeks	**< 1 year**	**1 year**	**>1 year**	**Unknown**
Inflammatory	7	7	2	1	6
Proliferative	4	3	1	2	7

Table 8: Average percentage of the different stains

Average percentage of the different stains								
	CD3	**CD79**	**Mac387**	**TGFß$_2$**	**Desmin**	**SMA**	**Masson's**	**Reticulin**
Inflammatory	**32.6**	**7.6**	**9.34**	**26.73**	**0**	**12.39**	**8.9**	**12**
Proliferative	**28.23**	**4.41**	**7.6**	**35.8**	**0.78**	**6.71**	**3.2**	**12.9**

Table 9: Percentage of staining (in a 0 to 100 % scale) of the immunohistochemical and histochemical stains in each one of the tissue samples

Biopsy number, inflammatory or proliferative characterization and percentage of positive stains										
#	I/P	Biopsy #	Mac387	CD3	CD79	TGFB	Desmin	SMA	Masson	Reticulin
1	I	00RD1106	0	25	5	0	0	15	0	0
2	I	01RD071	5	50	30	30	0	5	0	30
3	I	01RD344	5	10	0	5	0	0	20	20
4	I	03RD1090	25	40	10	40	0	0	5	15
5	I	95A51609	5	60	5	40	0	20	0	10
6	I	A977024901	0	30	10	10	0	10	0	0
7	I	A977024902	5	40	5	10	0	5	0	10
8	I	00RD1165	5	15	5	15	0	0	60	5
9	I	01RD557	0	10	0	15	0	0	30	10
10	I	01RD1156	10	50	30	40	0	30	0	15
11	I	01RD0059	5	15	5	15	0	15	5	20
12	I	02RD1029	5	60	15	10	0	25	0	20
13	I	03RD0025	10	25	0	30	0	50	40	5
14	I	94A45925	5	50	0	40	0	5	0	10
15	I	00RD0780	20	40	5	10	0	5	5	20
16	I	02RD184	5	60	15	80	0	50	0	30
17	I	03RD0074	40	40	10	40	0	5	0	10
18	I	01RD889	10	70	5	15	0	5	5	10
19	I	00RD0448	40	10	5	50	0	40	30	40
20	I	00RD1065	10	20	0	40	0	0	0	10
21	I	94A49146	0	5	10	10	0	0	5	10
22	I	94A491462	0	5	5	40	Unknown	Unknown	Unknown	Unknown
23	I	A9819631	5	20	0	30	0	0	0	15
24	P	04RD0553	5	60	5	40	10	10	5	20
25	P	03RD1533	5	30	0	40	0	0	5	5
26	P	01RD1119	10	40	5	40	0	0	5	10
27	P	01RD0233	10	50	10	10	0	15	0	50
28	P	00RD0301	5	10	5	50	0	0	10	20
29	P	01RD0473	10	40	5	30	0	5	0	40
30	P	01RD1398	5	40	5	40	0	0	5	0
31	P	01RD1144	10	50	10	40	0	15	5	15
32	P	01RD558	10	10	5	10	0	15	10	0
33	P	02RD1052	5	15	0	60	5	0	0	5
34	P	02RD1602	5	20	5	40	0	5	0	10
35	P	B02 717	5	5	0	10	0	5	Unknown	0
36	P	B90 26242	0	5	0	Unknown	Unknown	Unknown	Unknown	Unknown
37	P	03RD1133	30	10	0	50	0	10	0	5
38	P	03RD1106	5	0	0	40	0	0	5	50
39	P	03RD1475	5	40	15	10	0	45	0	5
40	P	03RD0169	5	25	5	40	0	0	5	5
41	P	A93341211	0	0	0	50	0	0	0	5
42	P	A93341212	0	30	0	10	0	0	5	5

I: Inflammatory P: Proliferative

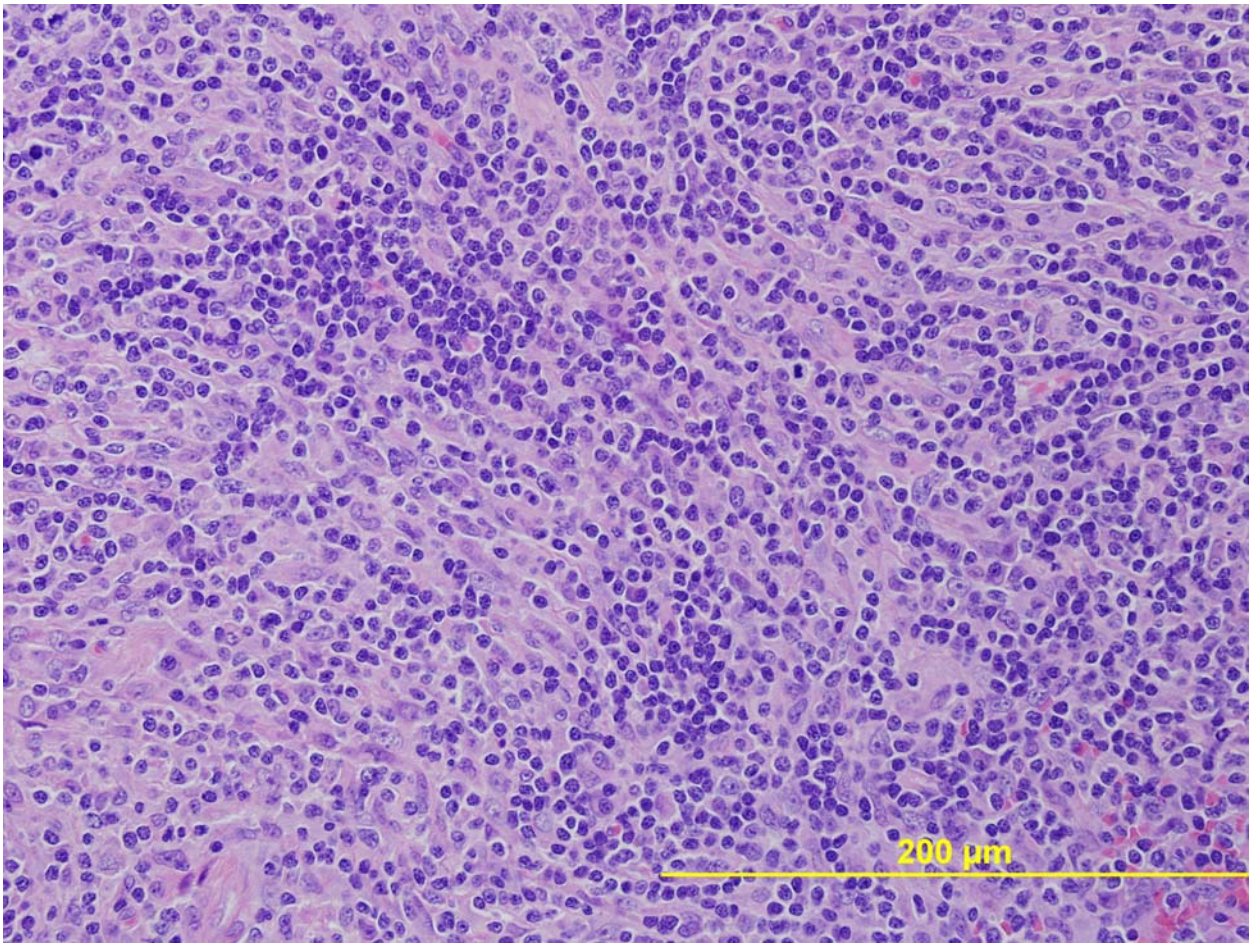

Figure 1: CONE: the section is nfiltrated by histiocytic inflammatory cells, lymphocytes and plasma cells, between collagen bundles. HE. Bar = 200µm.

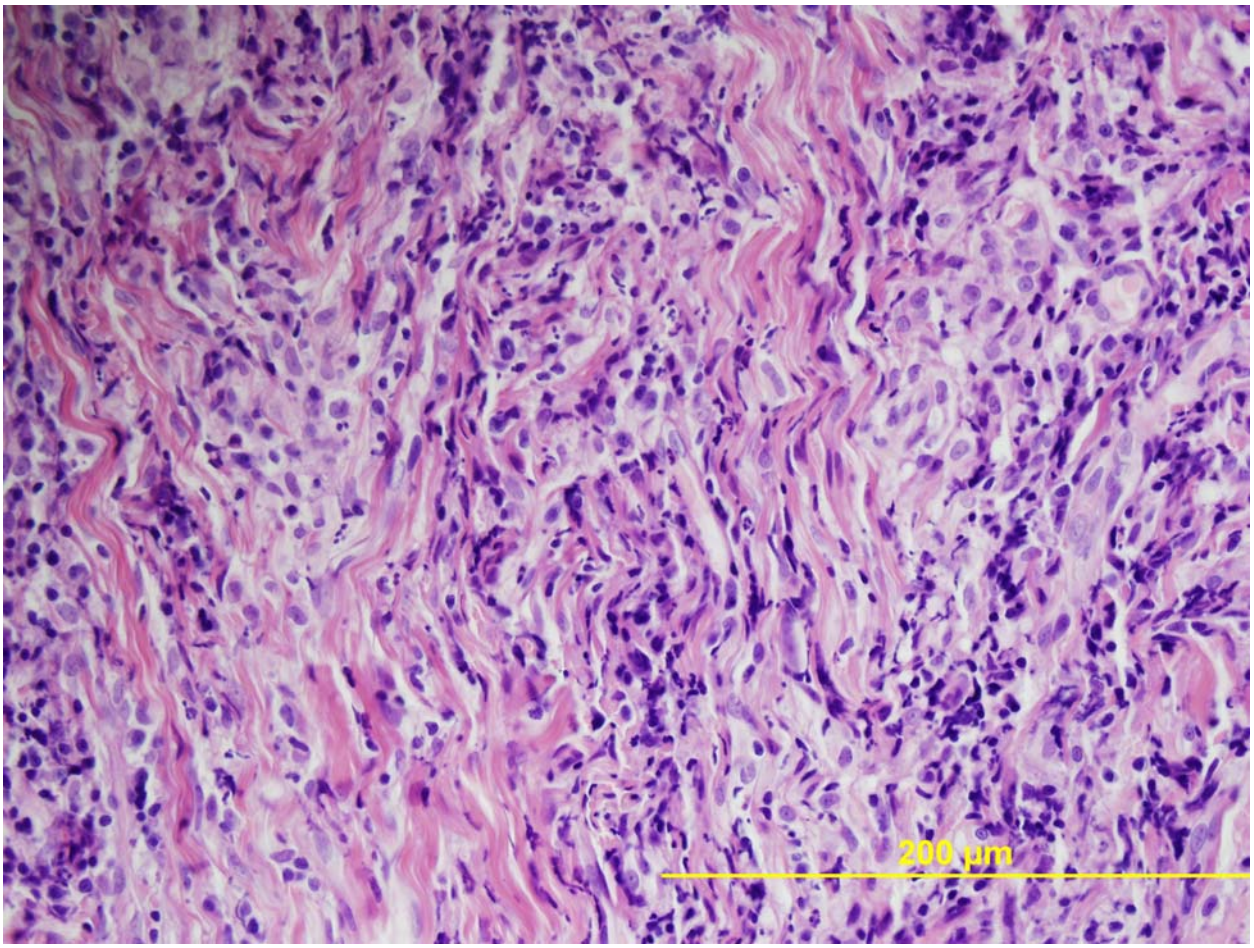

Figure 2: CONE: Collagen bundles within the nodular episcleritis lesion appear glossy and surrounded by lymphocytes and plasma cells. HE. Bar = 200µm.

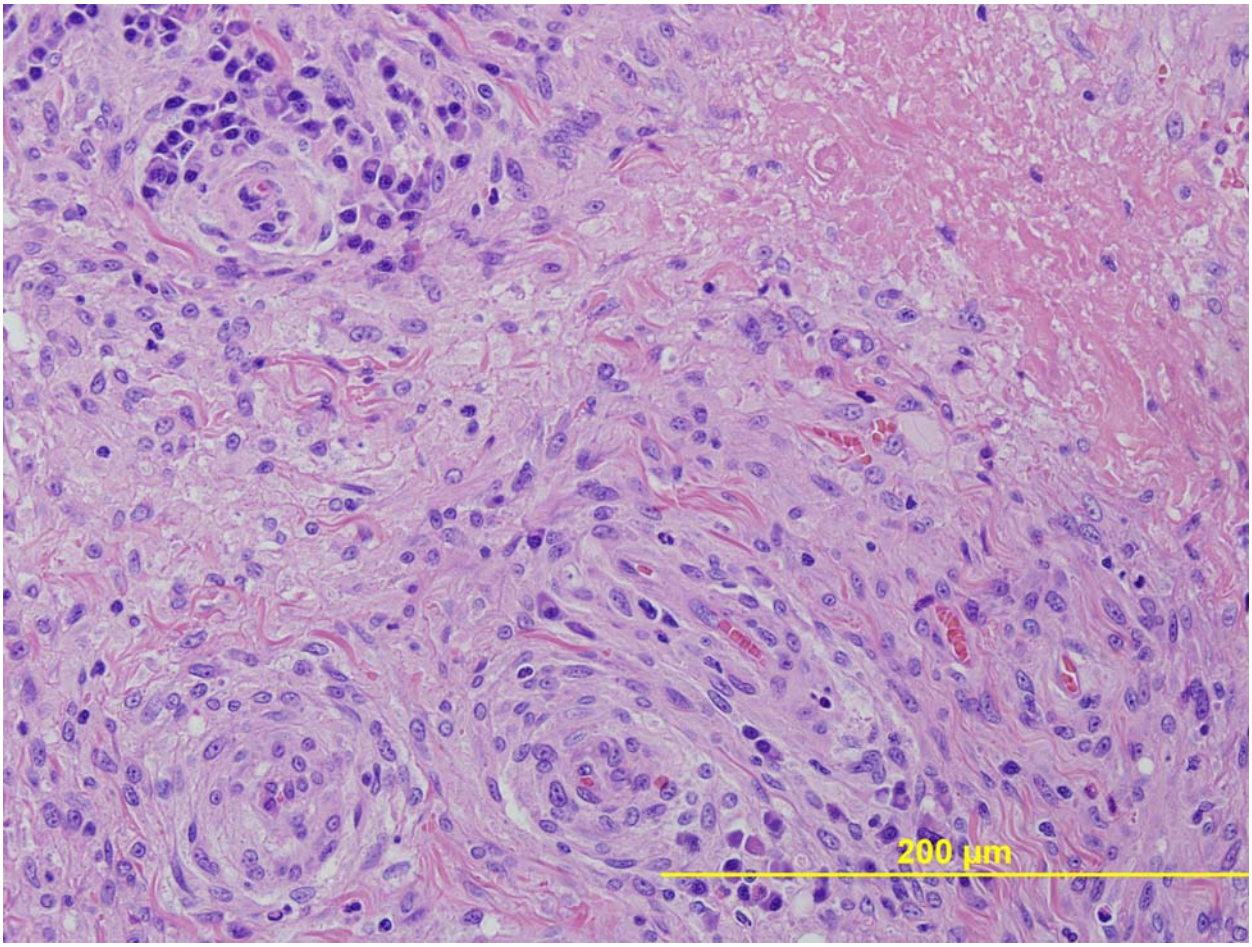

Figure 3: Proliferative lesion with swirls of plump spindle-shaped cells around blood vessels. HE. Bar = 200µm.

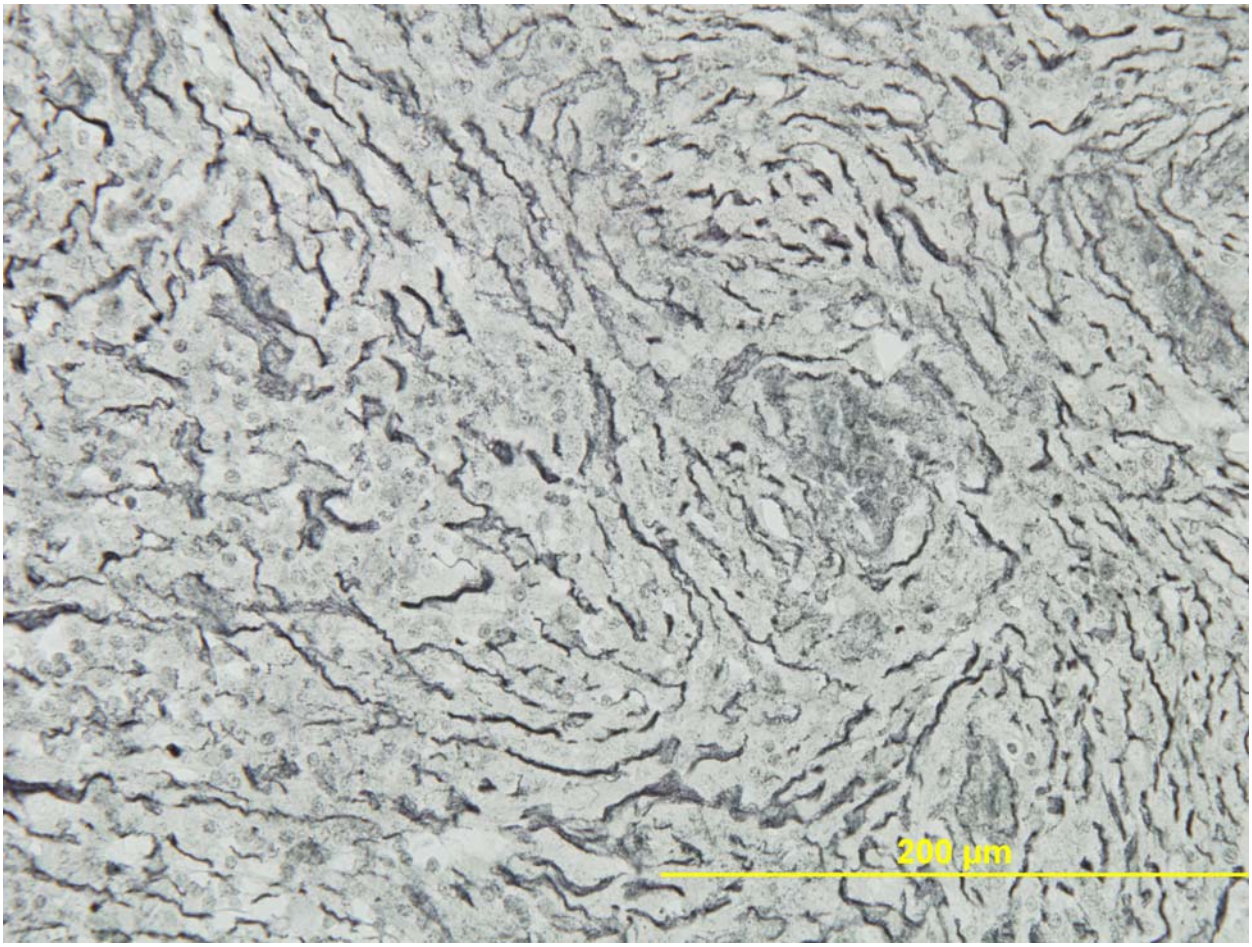

Figure 4: Prolifetarive lesion with reticulin fibers forming swirls surrounding the inflammatory infiltrates and spindle-shaped cells. Reticulin. Bar = 200µm

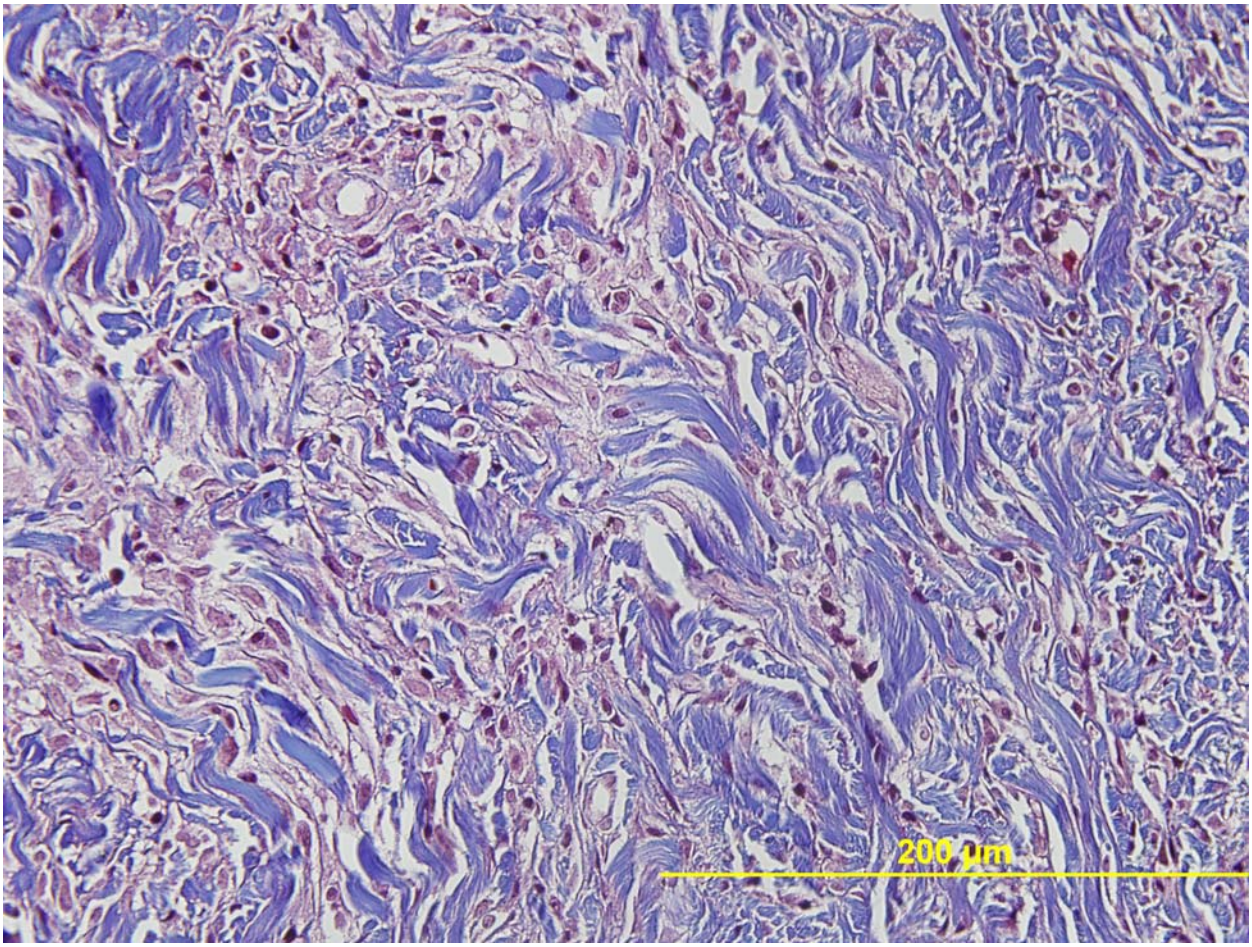

Figure 5: A proliferative lesion with abundant collagen deposition within inflammatory infiltrate. Masson's Trichrome. Bar = 200µm

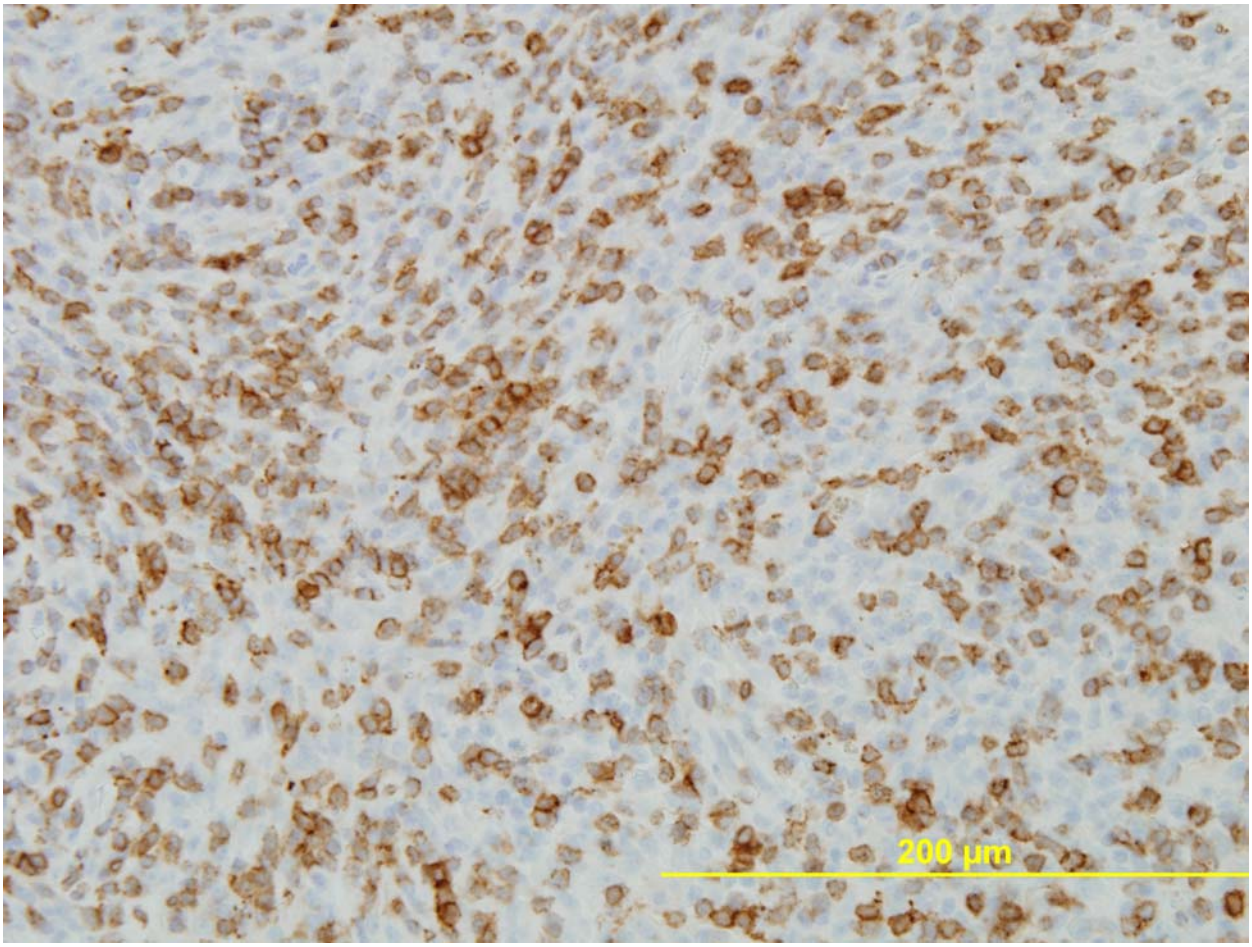

Figure 6: An inflammatory lesion with diffuse infiltration by CD3 positive lymphocytes. CD3. Bar = 200µm.

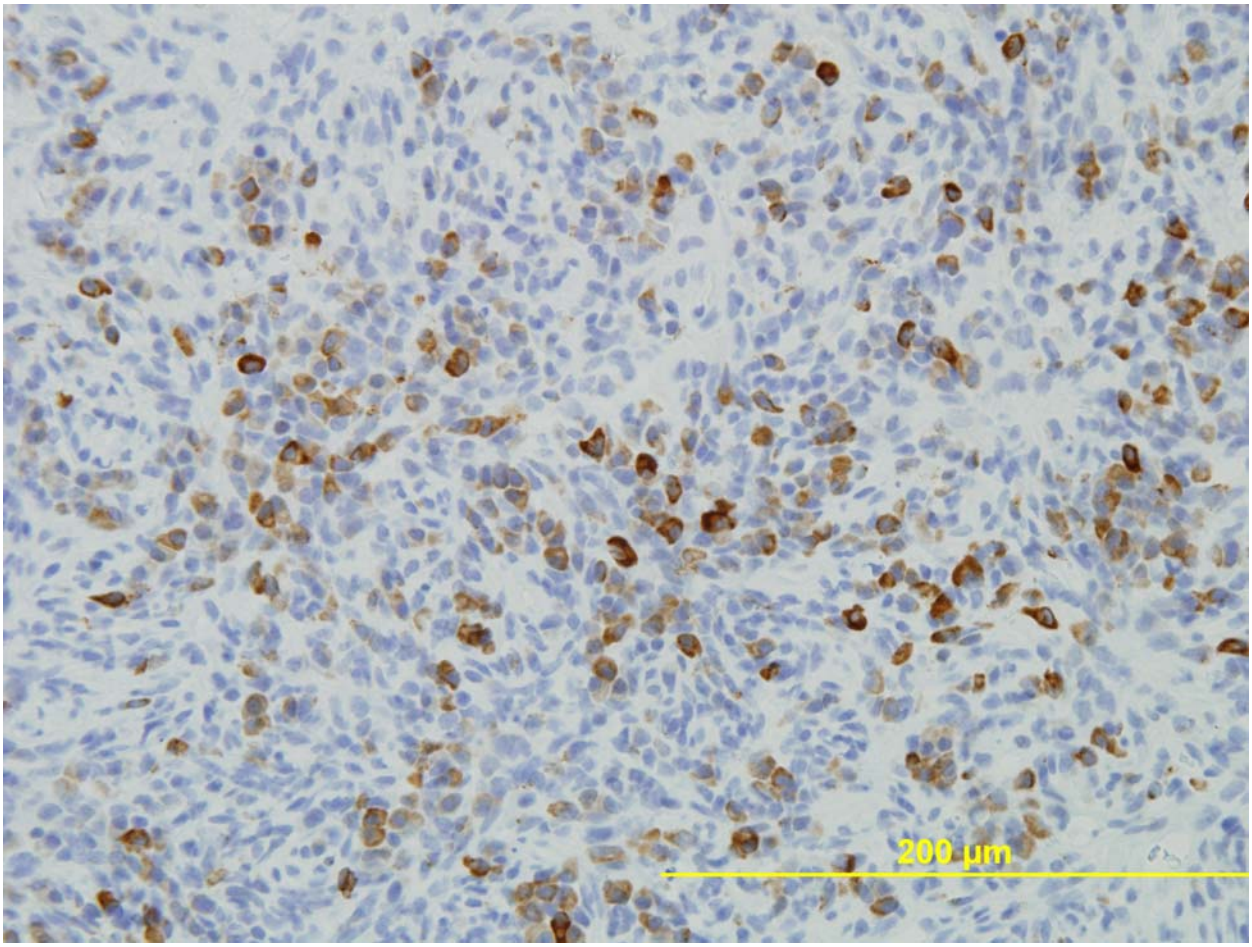

Figure 7: Proliferative lesion with diffuse infiltration of lower number of CD79 positive lymphocytes. CD79. Bar = 200µm.

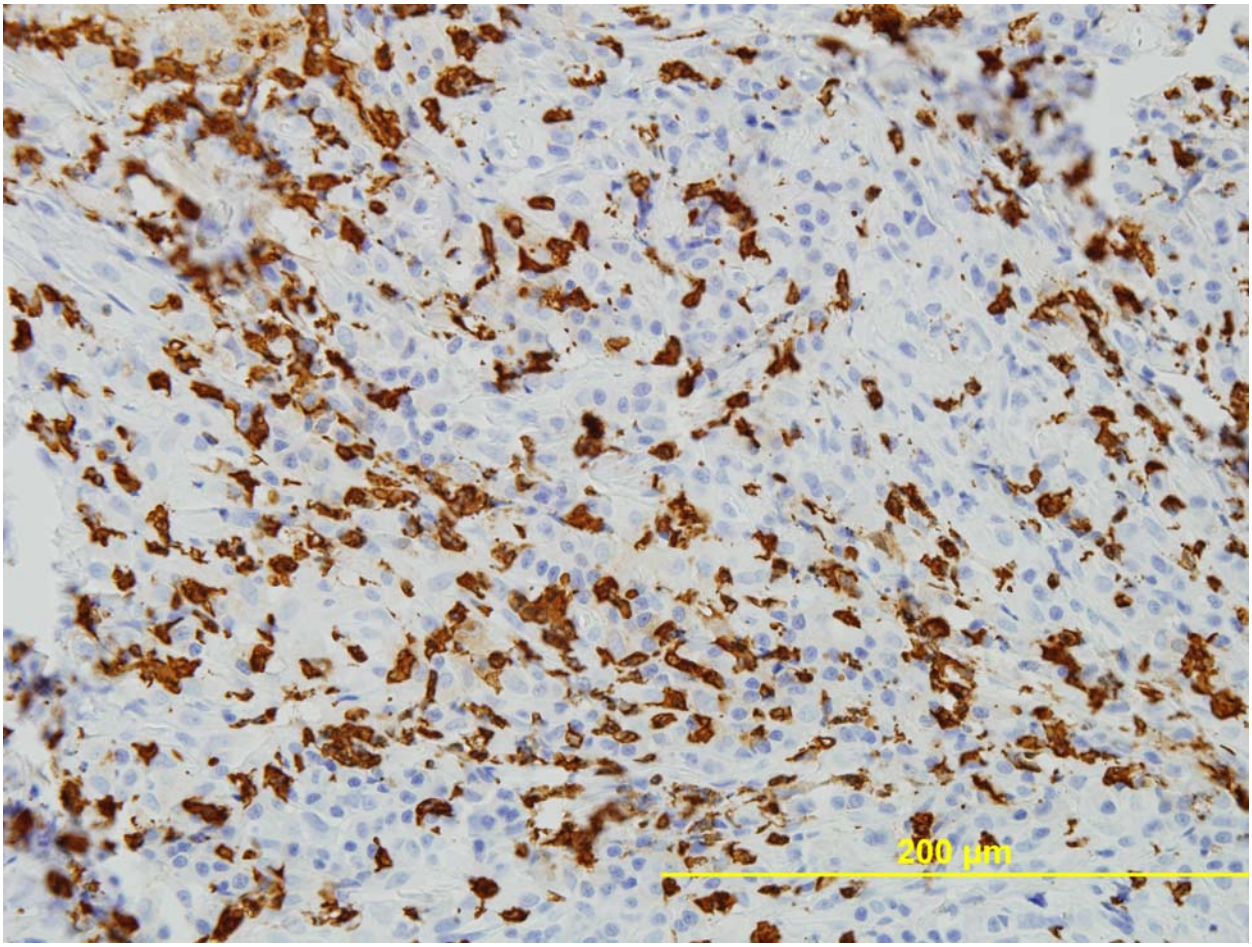

Figure 8: An inflammatory lesion with diffuse MAC 387 positive macrophages infiltration. MAC 387. Bar = 200µm.

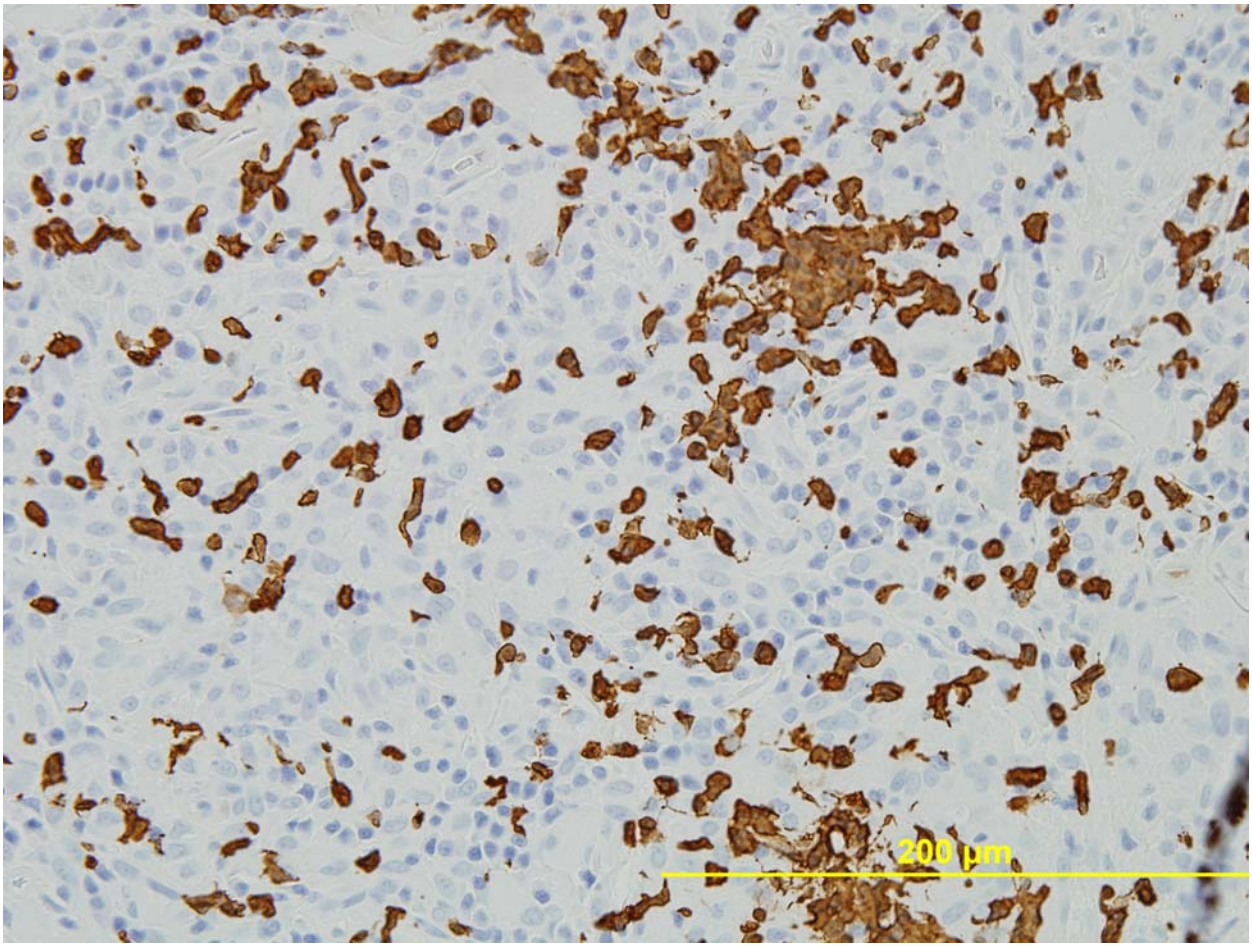

Figure 9: MAC 387 positive macrophages are found in multifocal clusters through out an inflammatory lesion. Theplum round cells are negative for this immunostain. MAC387. Bar = 200µm.

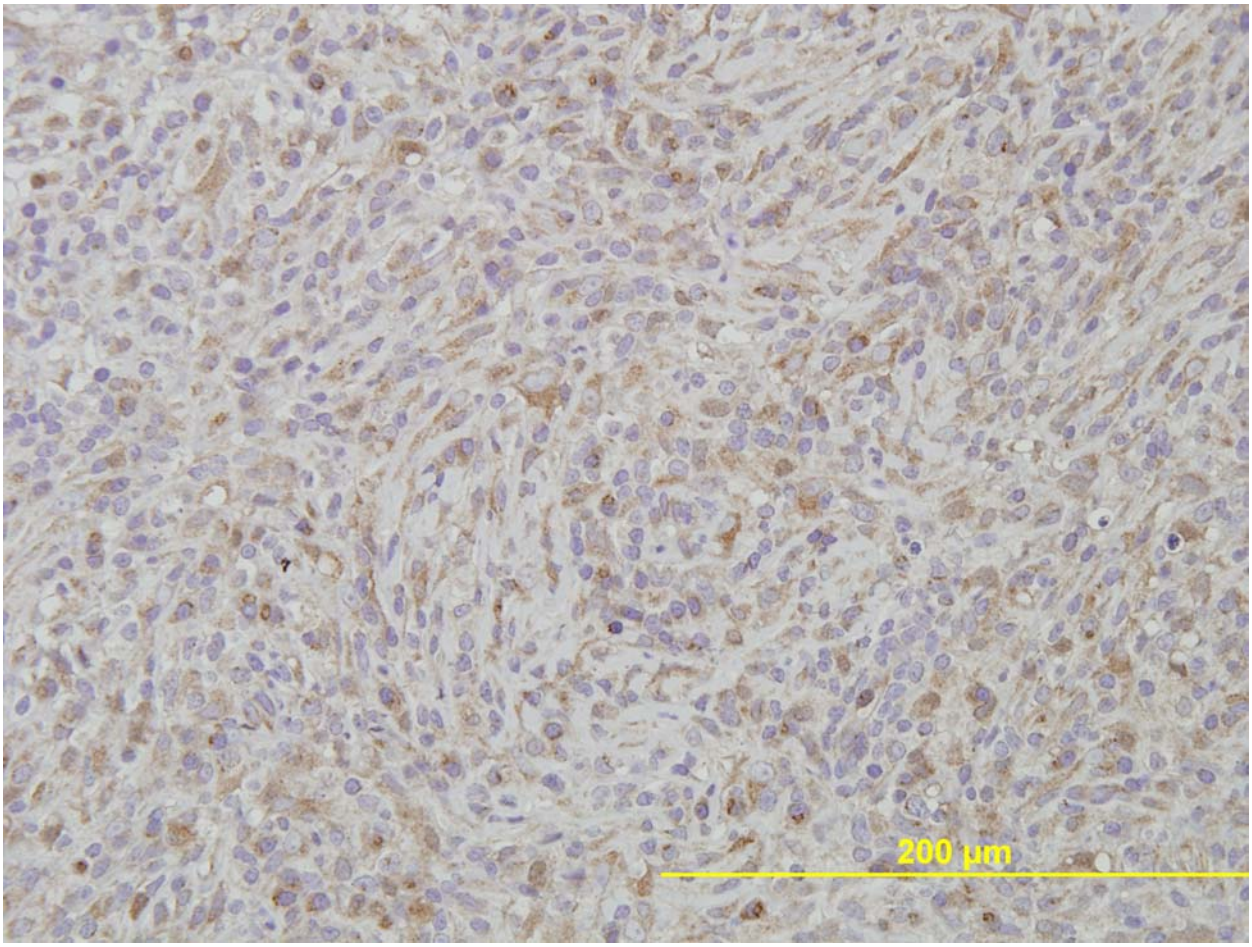

Figure 10: Positive immunoreactivity for TGF β_2 was found through both the inflammatory and proliferative forms of CONE. TGF β_2. Bar = 200µm

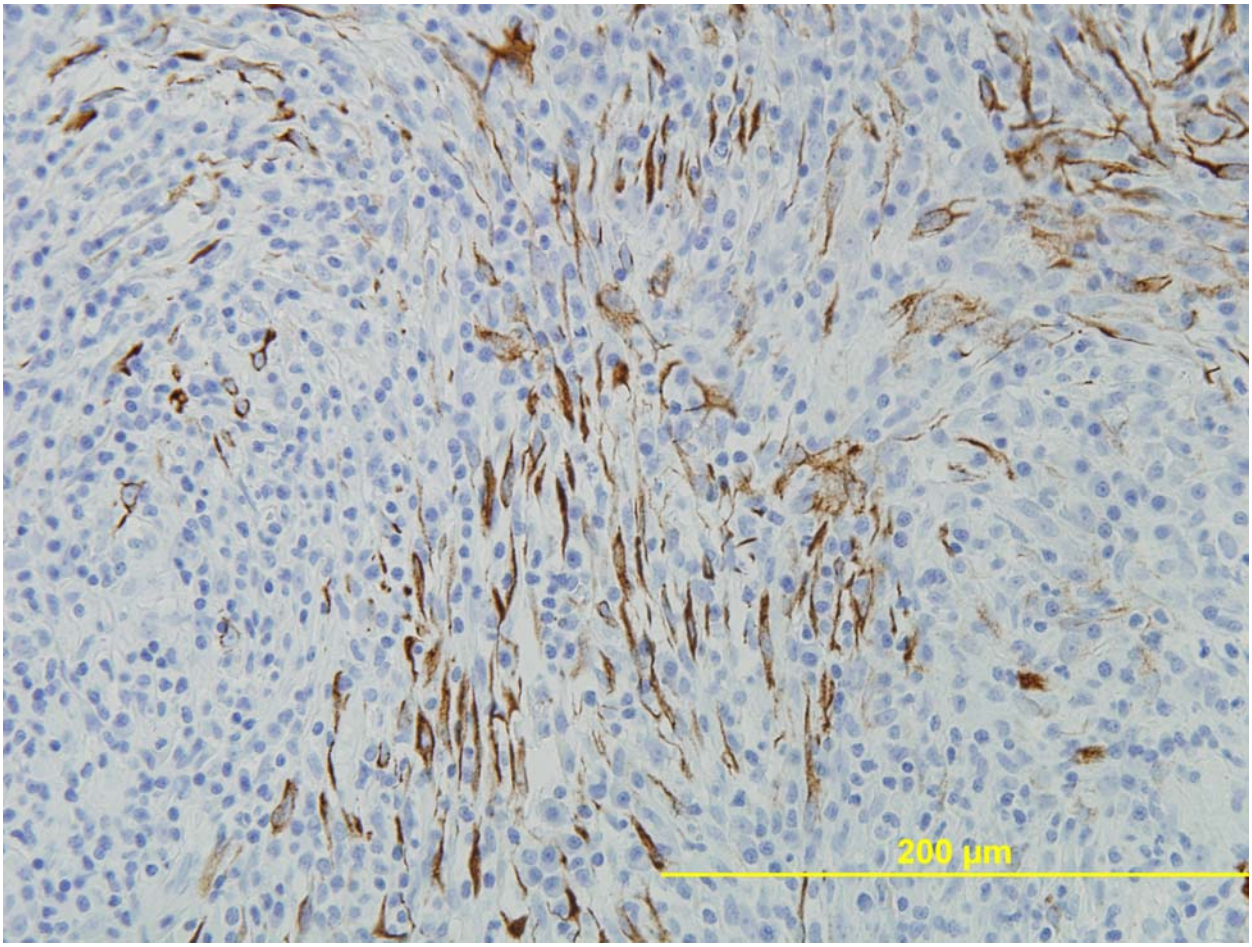

Figure 11: A proliferative lesion was positive for desmin. The desmin immunoreactive cells are not associated with capillaries or vessels. Desmin. Bar = 200µm.

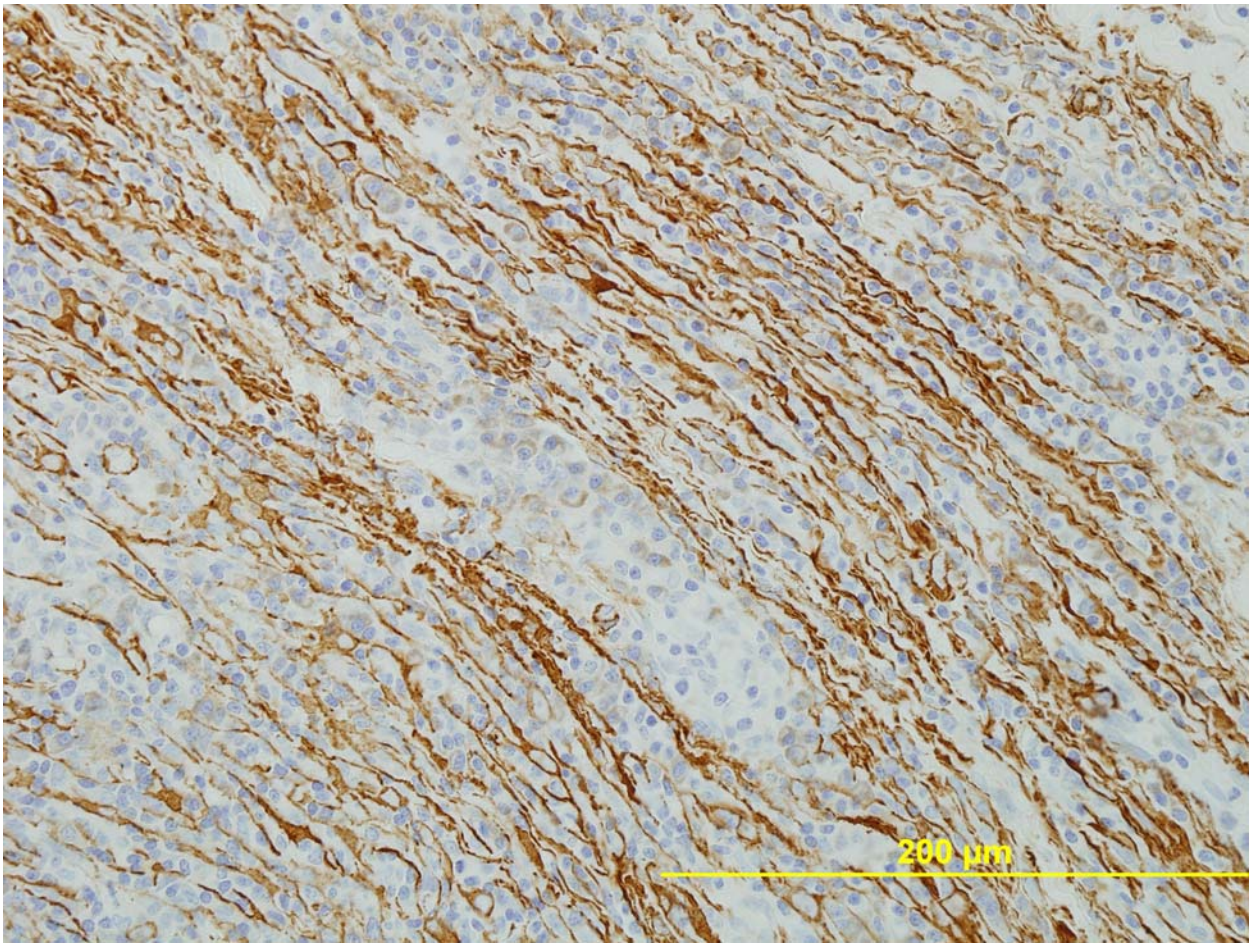

Figure 12: SMA positive staining of an inflammatory lesion revealed cells with elongated cytoplasm, a feature not noticeable on HE stain. Note plump histiocytic cells negative for this immunostain. SMA. Bar = 200µm.

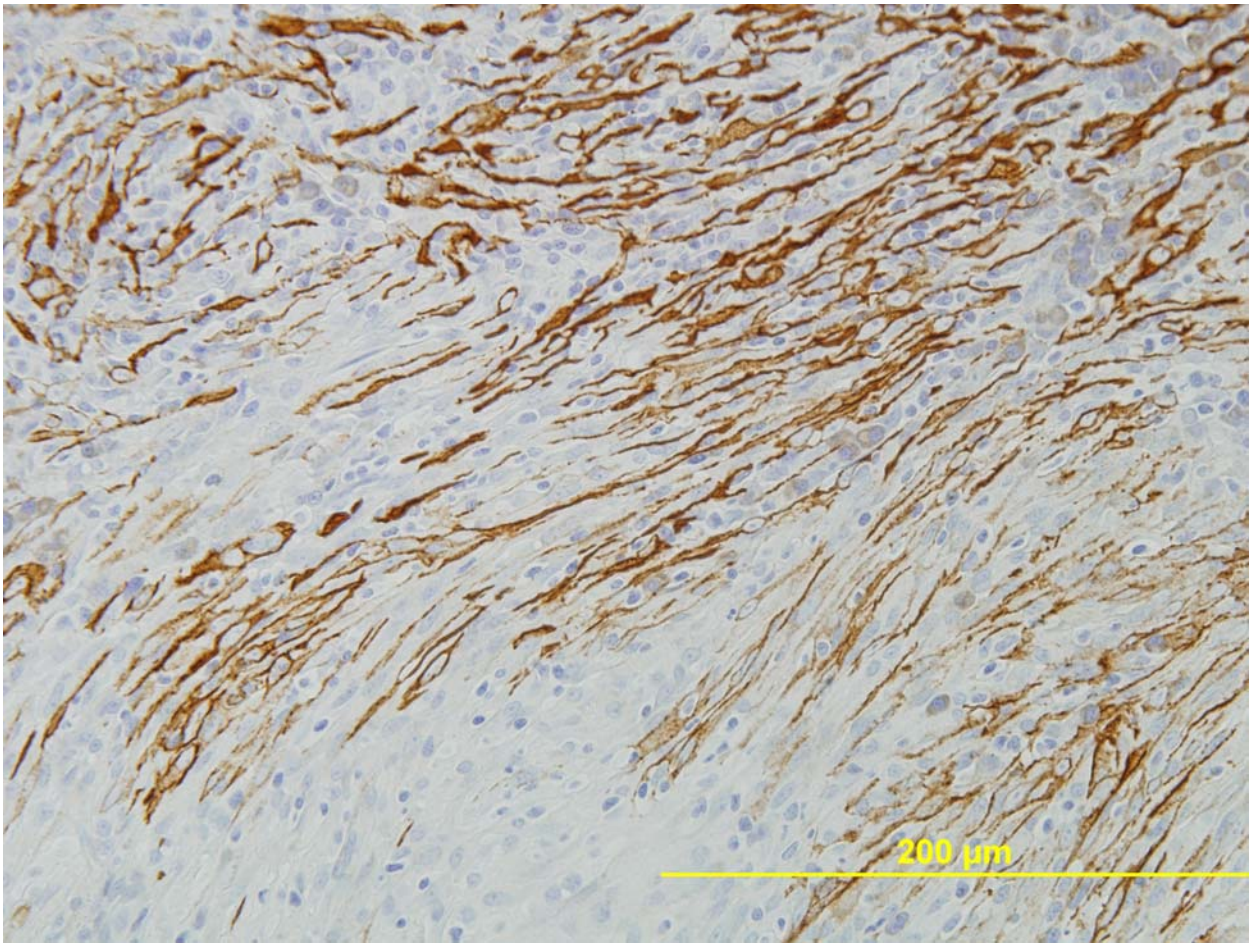

Figure 13: Proliferative lesion reveals SMA positive spindle- shaped cells. The plump histiocytic cells were negative for this immunostain. SMA. Bar = 200µm.

STATISTICAL ANALYSIS

The different stains were analyzed by Tukey grouping ($p < 0.0001$). There were statistically significant differences in expression levels between stain types **TGFβ 2** and **CD3** compared to the rest of the stains and formed a group apart. **Reticulin, SMA, Mac387**, **Masson's trichrome**, **CD79** and **desmin** were statistically similar compared to each other.

When the different stains were analyzed for correlation to duration of the lesion and age of the patient, there were no significant correlations.

The different stains were also analyzed by Tukey grouping for differences of expressions levels according to localization. There were no statistical differences in expression levels between localizations with counts ≥ 3 (limbus, scleral and third eyelid) for any stain.

Comparing the expression levels of stain and the inflammatory or proliferative tissue samples, there was no significative difference between inflammatory and proliferative lesions occurrence.

In inflammatory samples, there was a higher percentage of **CD3** and **SMA** compared to proliferative samples. **TGFβ 2** and **reticulin** were expressed in higher percentage in proliferative samples

DISCUSSION

Canine ocular nodular episcleritis (CONE) is a poorly understood condition in dogs that responds variably to immunomodulating therapy. There is still no consensus regarding to its etiology and pathogenesis. In our study, we hypothesized that the clinical condition represents two distinctive histological lesions and this may explain the variability of the clinical course. Our study included 42 dogs with CONE lesions in the episclera, limboscleral junction, corneoscleral junction, sclera, third eyelid and eyelids. Of these, 23 were classified as inflammatory and 19 as proliferative. The average age of dog was affected by both lesions was 7.8 years, with dogs as young as 10 months and as old as 15 years. There was no statistical correlation between age, gender, duration of the lesion with the histological characterization.

Cocker spaniels and Labrador retrievers were more frequently affected overall, but there was no breed of dog preferentially affected with one or the other histological lesion. The predilection of this condition in Cocker spaniels in our study has also been reported in the literature [11]. Labrador retrievers could be overrepresented in the sample, as it has become a more popular breed in the last two decades and therefore maybe more frequently presented for consultation than other breeds. There is no previous reported affinity of this breed for episcleral conditions. The absence of Collie and Collie mix dogs in the sample constitutes the main difference between ours and previous studies [16, 47], and may be explained by the change in popularity of this breed and therefore, the lesser frequency for consultation. The lesion described in Collies (Collie granuloma) is very similar to the inflammatory lesion in our study [20, 30, 47].

In our study, the lesion was significantly more frequently localized at the corneoscleral junction. Even though the episclera involves other anatomic structures as the third eyelid and base of the extraocular muscles insertion, the susceptibility of the limboscleral junction to develop inflammatory or neoplastic conditions could be explained by its exposure to the environmental elements (air, UV light, allergens) [9]. In addition, in our study, among the CONE lesions characterized as proliferative, there were one intraocular and one retrobulbar masses. Intraocular progression of the lesion has previously been reported in veterinary medicine [36, 37, 38, 39, 40, 41]. Retrobulbar location of CONE lesion could also be primary.

The majority of the lesions (93%) were unilateral; however, there were three bilateral lesions. These three bilateral lesions were characterized as inflammatory and were located at the corneoscleral junction. The reason these lesions were bilateral is uncertain, but systemic conditions such as ehrlichiosis or toxoplasmosis have been linked to episcleritis [50]. One possibility is that foci of collagen degeneration are triggered by an immune mediated event. It is also possible an actinic response may incite CONE at these highly susceptible locations.

Duration of the lesion was derived from the data obtained from the submission forms, and was divided arbitrarily into lesions present less than four weeks, less than one year, one year and more than one year. There was no data available for duration of the lesion in thirty percent of the samples. Out of the known data, 26 % of the lesions were present less than four weeks, and 25 % were present less than one year. Four samples characterized as proliferative, were present for less than four weeks. It can be argued that the lesions may have gone unnoticed for a longer period, as in the retrobulbar case, but the other three proliferative lesions were present for less than four

weeks on the corneoscleral (2) and scleral (1) locations that should have been easily noticed. Conversely, there were three cases consistently inflammatory after one year of initial presentation. These later results agree with what has been found in human literature [17].

CD3 marker and **TGFß$_2$** immunostains were statistically present higher levels than the other stains, in both inflammatory and proliferative lesions.

TGFß$_2$ is a growth factor with pleiotropic effects. **TGFß$_2$** is produced by almost all cells, and all cell membranes have receptors for it. In low concentrations, **TGFß$_2$** induces the synthesis of **PDGF**, and stimulates fibroblasts chemotaxis and production of collagen and fibronectin, being implicated in the fibrosis elicited in chronic inflammatory states. **TGFß$_2$** is also known for inhibiting the degradation of extracellular matrix by metalloproteinases, is a potent immunosuppressant and can triggers apoptosis [28]. In this study, was highly expressed in both inflammatory and proliferative lesions. Both types of lesions in the study are active sites of cell growth and proliferation. Additional and more selective proliferation markers are necessary to characterize cellular proliferation present at these lesions.

Mac387 staining was almost evenly present in both types of lesions. Surprisingly, the **Mac387** positive cells were not the cells interpreted on HE as histiocytes, but smaller cells surrounding histiocytes, and occasionally scattered throughout the tissue or forming small clusters of cells. The cell thought to be histiocytes could be macrophages that do not express in its epitope the calcium binding protein (MRP14) that **Mac387** binds to [19].

There was no significant expression of **CD79** in either condition, inflammatory or proliferative. **HE** staining revealed few plasma cells in both types of lesions. Plasma cells are terminally differentiated end product of B cell activation and may produce antibodies against the antigen in the inflammatory site or degraded tissue components.

SMA stained positive in 57% of the samples, and the majority were inflammatory lesions. **SMA** (intermediate filament) is the most important marker of myofibroblasts [25]. Myofibroblasts are present in traumatized tissue and are thought to arise from fibroblasts, smooth-muscle cells, endothelium, pericytes, and myoepithelium [14]. The positive **SMA** distribution in both types of lesions could be explained by the fact that there was active proliferation even in the inflammatory type of lesion. Myofibroblasts and fibroblasts are not likely to respond to immunomodulatory therapy. These findings may explain the poor response to immunomodulatory therapy noted in some cases of CONE.

Desmin is the major protein of intermediate filaments in smooth muscle. In our study, only one specimen had 10% positively staining for desmin. We believe that in this single positive sample, desmin may have stained intermediate microfilaments in smooth muscle [25]. The reason for the negative desmin staining in the other samples is uncertain. This particular sample was fixed for approximately the same amount of time as the others, so fixative-associated epitope washing was not thought to be a factor.

The extracellular markers **Gomori's reticulin** and **Masson's trichrome** were positive in both types of lesions, inflammatory and proliferative, indicating synthesis of extracellular matrix.

Degenerated collagen fibers (collagenolysis) were found in the **HE** stain, surrounded by inflammatory infiltrate. As expected, there were more fibers (reticulin positive) in the proliferative samples.

The results in our study lead us to speculate that CONE may represent a single disease process that has both an inflammatory and a proliferative component. The initial lesion of collagenolysis and a subsequent inflammatory reaction can either remain inflammatory or progress to a proliferative form. Wounding and the subsequent proliferative repair process, can lead to neoplasia [20]. For example ocular sarcomas in cats are preceded by trauma to the eye or are associated with the persistent presence of ocular foreign material in the eye. We speculate that in CONE persistent inflammation/repair leads to the proliferation of fibroblasts and myofibroblasts through unknown mechanisms. Similarly, in vaccine reactions, the initial inflammatory lesion with fibroplasia is thought to transform into proliferative lesion that may lead to neoplasia. Possible mechanisms include oncogenes, and expression or increased levels of growth factors [21]. Based upon our results, we speculate that if the CONE lesion remains inflammatory, it is more likely to be amenable to immunomodulatory therapy. If it progresses to a proliferative lesion, immunomodulation is not effective and the lesion must be excised. Additional studies are necessary to prove this theory.

CONCLUSIONS

CONE (canine ocular nodular episcleritis) is a mainly unilateral lesion that equally affects middle-aged male or female dogs. The condition is characterized by a fleshy elevated lesion, that occur more frequently at the limbus or sclera, but can also occur on the third eyelid, eyelids and sporadically appear in the retrobulbar aspect of the globe or invade it intraocullarly.

Based on the hematoxylin and eosin staining, CONE represents two distinctive histological lesions: One in which the predominant inflammatory type of cells were interpreted as epithelioid macrophages, sometimes forming granulomas, although there was frequent infiltration of neutrophils and fewer plasma cells, eosinophils and mast cells. There was evident collagen degeneration, creating a focal inflammatory reaction infiltrated by neutrophils and surrounding macrophages. This condition was considered inflammatory.

In the other condition, the predominant type of cell was spindle-shaped, organized in sheets and whorls. Even though inflammation was not a prominent feature in these tissues, there was multifocal inflammatory infiltration of macrophages, small lymphocytes and occasional neutrophils, especially around and/or close to small caliber vessels. Fewer neutrophils and no eosinophils nor mast cells were observed. There was no evidence of collagen degeneration. This condition was considered proliferative.

These two characterizations of CONE may represent different outcomes of a condition triggered initially by the same event (chronic irritation and wound healing process). The initial lesion of collagenolysis and a subsequent inflammatory reaction can either remain inflammatory or progress to a proliferative form, maybe due to alteration in growth factors regulation and oncogenes expression. The lesions that remain inflammatory are responsive to immunomodulatory therapy. If the lesion becomes proliferative, excision is required.

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