Elevated blood/lymphatic vessel ratio in pterygium and its relationship with vascular endothelial growth factor (VEGF) distribution

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Summary. Introduction. Pterygium is a conjunctival fibrovascular tissue growth on the cornea. The pathogenesis of pterygium involves several factors such as the presence of active angiogenic factors. Expansion of the lymphatic microvasculature has also been hypothesized. This study examines the activity of the angiogenic/lymphangiogenic factor VEGF and the expression of vascular and lymphatic endothelial proteins in pterygia and normal conjunctival tissues. Materials and methods. Primary grade 2 pterygium (n=20) and normal conjunctiva (n=20) biopsies were obtained during surgery after written informed consent. mRNA expression for CD31, podoplanin, and VEGF (isoforms VEGF-A and VEGF-165) were determined by qRT-PCR. Tissue samples were also processed for immunohistochemical techniques to examine the lymphatic and vascular endothelium (anti-D2-40, anti-CD31 respectively) and VEGF-A and VEGF-C levels and distribution. Results. VEGF-A gene expression levels failed to differ between the healthy and pterygium tissues. However, expression of its more angiogenic isoform, VEGF-165, was significantly higher in the pterygia. Immunohistochemistry revealed the greater presence of VEGF-A, compared to VEGF-C, in pterygium than conjunctiva, both in blood vessels and extracellular matrix. In addition, pterygia showed higher expression levels of the endothelial junction protein CD31. Lymphatic marker D2-40 expression was slightly augmented in this pathological tissue. The ratio between blood and lymphatic vessel counts was 1.05 in the normal conjunctiva and 3-fold this value in pterygium. Conclusion. In pterygium, while both lymphangiogenesis and angiogenesis take place, the formation of new blood vessels is the most relevant event, correlating with the increased expression of vascular endothelial CD31 and an elevated blood/lymphatic vessel ratio. The presence of high levels of VEGF-A in both vessel networks and extracellular matrix in human pterygium tissue may have a major impact on angiogenesis in this pathological tissue.

Key words: Pterygium, Lymphangiogenesis, VEGF, Podoplanin, Angiogenesis

Introduction

Pterygium is a common invasive ocular disease in which a fleshy fibrovascular tissue from the bulbar conjunctiva encroaches onto the cornea (Jin et al., 2003; Kaufman et al., 2013). The growth arises from activated and proliferating limbal epithelial stem cells (Dushku and Reid, 1994; Chen et al., 1994; Chui et al., 2011). Histologically, pterygium consists of a squamous metaplastic and globlet cell hyperplastic epithelium and a subjacent activated highly-vascularized connective

tissue showing inflammation and matrix remodeling (Chui et al., 2008, 2011). Several authors have described tumor-like features of this tissue such as its extraordinary invasiveness and fast recurrence after excision, along with p53 overexpression. This has determined that pterygium, traditionally considered a chronic degenerative growth disorder, is now interpreted as a neoplastic-like growth disorder (Schneider et al., 2006). Although several risk factors like chronic irritation, inflammation or viral infection have been proposed (Hill and Maske, 1989; Coroneo, 1993; Wang et al., 2000; Nolan et al., 2003; Chalkia et al., 2013), extensive epidemiological studies have indicated it is an ultraviolet radiation-related disease (Di Girolamo et al., 2006; Chiang et al., 2007; Chui et al., 2008).

This main etiological factor therefore seems to be responsible for the up-regulation of multiple pro-inflammatory cytokines, growth factors and matrix metalloproteases. These factors are important for the fibrosis, angiogenesis, inflammation and extracellular matrix remodeling that characterize pterygium (Zhou et al., 2016).

Several fibroangiogenic growth factors such as basic fibroblast growth factor (b-FGF), heparin-binding epidermal growth factor (HB-EGF), connective tissue growth factor (CTGF), insulin-like growth factor-binding protein (IGFBP)-2, epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) have been detected in pterygium, suggesting their direct or indirect implication in the pathogenesis of pterygium (Lee et al., 2001; Marcovich et al., 2002; Jin et al., 2003; van Setten et al., 2003; Gebhardt et al., 2005).

The VEGF family of growth factors is a group of ligands for endothelial cell-specific VEGF tyrosine kinase receptors with a key role in regulating the formation of the circulatory system and the development of vessels from pre-existing ones, angiogenesis and lymphangiogenesis. The VEGF family includes, among others, the homodimeric protein VEGF-A, the most important member of the VEGF family involved in promoting angiogenesis in inflammation, wound healing, tumors, and endothelial cell proliferation, survival and migration, and VEGF-C and VEGF-D related to lymphangiogenesis (Keck et al., 1989; Leung et al., 1989; Byrne et al., 2005). These factors perform their functions through three receptors located on endothelial cells and other cell types. VEGF-A binds to VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). VEGFR-2 is an important receptor in angiogenesis and seems to mediate almost all known cell responses to VEGF (Robinson and Stringer, 2001; Byrne et al., 2005). The function of VEGFR-1 is less well known, but it is thought to modulate the function of VEGFR-2. VEGF-C and VEGF-D, but not VEGF-A, are ligands of the third receptor (VEGFR-3), which mediates lymphangio-genesis.

Regarding ocular diseases, VEGF is a main growth factor and has been attributed roles in increasing vascular permeability, angiogenesis and lymphangio-genesis (Witmer et al., 2003). VEGF has also been found to contribute to the pathogenesis of a series of ocular neovascular diseases in which its expression is dependent on the transcription factor HIF-1 (Hu et al., 2014; Meng et al., 2017). Several studies have highlighted the role of this potent angiogenic factor in the development of pterygium (Gebhardt et al., 2005; Aspiotis et al., 2007; Meng et al., 2017), suggesting antiangiogenic strategies as primary or adjuvant treatment for pterygium (Mauro and Foster, 2009; Bahar et al., 2012; Mak et al., 2017).

In this context and considering the scarce data available on lymphangiogenesis in pterygium, this study examines the gene and protein expression of vascular and lymphatic endothelial markers and the blood/lymphatic vessel ratio in relation to VEGF-A and VEGF-C activity as the main regulators of angiogenesis and lympahngiogenesis in pterygium.

Materials and methods

Patients

The specimens examined were primary nasal pterygia in grade 2 and conjunctival tissues obtained during surgery. The study protocol was approved by the Ethics Committee of the University Hospital Principe de Asturias and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants. Inclusion criteria were: a primary fleshy and active pterygium that invaded the cornea by more than 3 mm, no previous trauma or surgery in the affected eye and no ocular or systemic disease such as diabetes, vascular disease or reactive connective tissue disease.

For the different techniques, 20 specimens of normal conjunctiva were obtained during cataract surgery and 20 specimens of pterygia were collected during surgery for pterygium. Mean age ± standard deviation (SD) was 50.24±15.82 years for the control subjects and 47.62±10.18 years for the patients with pterygium.

Immediately after harvesting the tissue samples, the normal conjunctiva and pterygia specimens were placed in sterile minimal essential culture medium and transferred at 4°C to the laboratory. Each tissue sample was divided into two parts and one fixed in F13 solution and embedded in paraffin for immunohistochemical studies and the other frozen at -80°C until use for RNA extraction. So, all the samples were used in all the different studies.

Surgical techniques

Pterygium surgery was performed under local anesthesia induced by the instillation of tetracaine chlorhydrate eye drops (Colirio Anestésico Doble®, Alcon Cusí, Barcelona, Spain) followed by a subconjunctival injection of 0.5 ml of 2% lidocaine diluted 1:200,000 in epinephrine into the body of the pterygium using a 25-gauge needle. The pterygium was dissected from the cornea with a crescent surgical blade.

The body of the pterygium, including the adjacent Tenon's capsule, was then dissected and excised with Wescott scissors, exposing the bare sclera. Mitomycin C (MMC) 0.02% (0.2 mg/ml) was applied directly to the scleral bed for 1 min and the ocular surface was washed copiously with a balanced salt solution. There was no need for cauterization. The area of the conjunctival defect was measured with a caliper and a conjunctival limbal autograft of the same size as the defect was obtained from the supertemporal quadrant of the bulbar conjunctiva. The graft was placed with the epithelium side down on the cornea and brought close to the area of bare sclera with the limbal edge facing the wound. The conjunctival autograft was secured using fibrin glue (Tisseel VH; Baxter, Vienna, Austria). The two components of fibrin sealant were loaded separately in two syringes. One drop from each was placed over the recipient bed and the graft was quickly flipped over onto the pterygium defect and smoothed out. Postoperatively, topical tobramycin and dexamethasone eye drops (Tobradex®, Alcon Cusí, Barcelona, Spain) were instilled four times daily for 1 month. Specimens of conjunctiva were obtained from the normal superior bulbar conjunctiva at a distance of least 5 mm from the sclera-corneal limbus in subjects undergoing cataract surgery. The width of the control bulbar conjunctiva extracted in the surgical procedure was similar to the pterygium area.

Real-time reverse transcription-polymerase chain reaction (qRT-PCR)

The samples were collected and stored at -80°C until RNA isolation. RNA was extracted using the guanidine-phenol-chloroform isothiocyanate method with TRIzol® (Invitrogen, USA). After breaking and homogenizing every tissue sample, these were centrifuged, and RNA recovered from the aqueous phase, precipitated with isopropanol and centrifuged several times with 70% alcohol to clean the RNA pellet. RNA concentrations were measured using a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific Inc., USA) by measuring absorbance at 260 nm. RNA quality was verified with 260/280 nm and 260/230 nm ratios.

Reverse transcription (RT) was conducted using oligo dT primers (Amersham, USA) and the M-MLV reverse transcriptase enzyme (Invitrogen, USA) to synthesize complementary DNA (cDNA) from 50 ng/μl of total RNA. To verify the absence of genomic DNA, a negative RT without M-MLV enzyme was run.

cDNAs were diluted 1:20, and 5 μl of this dilution plus 10 μl of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, USA) and 1 μl (6 μM) of each primer (forward and reverse) were used for the qPCR in a final volume of 20 μl. This qPCR was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, USA) using the following specific human primers: CD31: 5'-ACT GCA CAG CCT TCA ACA GA-3' (sense) and 5'-TTT CTT CCA TGG GGC AAG-

3' (antisense); PODOPLANIN: 5'-TGA CTC CAG GAA CCA GCG AAG-3' (sense) and 5'-GCG AAT GCC TGT TAC ACT GTT GA-3' (antisense); VEGF-165: 5'-GAC AAG AAA ATC CCT GTG GGC-3' (sense) and 5'-AAC GCG AGT CTG TGT TTT TGC-3' (antisense); VEGF-A: 5'-ATG ACG AGG GCC TGG AGT GTG-3' (sense) and 5'-CCT ATG TGC TGG CCT TGG TGA G-3' (antisense)and GAPDH: 5'-GGA AGG TGA AGG TCG GAG TCA-3' (sense) and 5'-GTC ATT GAT GGC AAC AAT ATC CAC T-3' (antisense).

The cycling conditions were: an initial stage at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C (each primer) for 30 s, and 72°C for 1 min. Products were subjected to 2% agarose gel electrophoresis and visualized with UV light. Gene expression was normalized against the expression of the constitutive gene glyceraldehyde 3-phosphate-dehydrogenase (GAPDH).

All data are expressed as the mean $\pm$ SD. All statistical tests were performed using the Graph Pad Prism 5 package (GraphPad Software, Inc. La Jolla, CA, USA). Mean data were compared among groups using the Mann-Whitney U-test. Significance was set at $P<0.05$.

Immunohistochemistry

Samples for light microscopy were embedded in paraffin after fixing in F-13 solution and 5-μm-thick sections obtained. The sections were deparaffinized in xylol and graded alcohols and rehydrated and equilibrated in phosphate-buffered saline (pH 7.4). As primary antibodies, we used mouse monoclonal anti-human PECAM-1 (CD31) (Clone WM-59, P-8590, 1:100 dilution) (Sigma-Aldrich, Merck, Darmstadt, Germany), mouse monoclonal anti-VEGF-A (Clone JH121, ab28775, 1:50 dilution) (Abcam, Cambridge, UK), mouse monoclonal anti-VEGF-C (Clone 197CT7.3.4, ab191274, 1:50 dilution) (Abcam, Cambridge, UK) and mouse monoclonal anti-human podoplanin (Clone D2-40, M3619, 1:100 dilution) (Agilent, Dako, CA, USA) antibodies. As a secondary antibody, a goat anti-mouse IgG-Biotin antibody (B0529, 1:300 dilution) (Sigma-Aldrich, Merck, Darmstadt, Germany) was used. The antigen-antibody reaction was detected by the alkaline phosphatase-labeled avidin-biotin procedure. A negative control of the technique was run without the primary antibody. After immunostaining, nuclei were counterstained with acid hematoxylin and the tissue sections examined by light microscopy. CD31 protein was positively expressed in the endothelium of blood and lymphatic vessels, while only the lymphatic vessels expressed podoplanin.

For labeling detection and semiquantification at least 10 microscopy fields (400x magnification) per sample were examined. 400x magnification has been used in order to increase the number of fields to observe. Digitalized histological images were captured using a digital camera (Axiocam HR, Zeiss) fitted to the light microscope (Carl Zeiss, Oberkochen, Germany). The

scale used for semiquantification was based on previously described (Pérez-Rico et al., 2014), as follows: 1, undetectable staining (<10%); 2, minimum staining (10-25%); 3, moderate staining (25-50%); 4, strong staining (50-75%); 5, maximum staining (75-90%); and 6, almost complete staining (>90%). Semiquantification was performed by two independent observers in a blinded fashion.

Despite the score on each of the samples using this six-group scale, the different protein expression and the intensity of staining were joined in three categories: (1-2) < 25%, (3-4) 25-75% y (5-6) >75%.

Blood/lymphatic vessel ratio

Vessels positive for CD31 and podoplanin immunostaining were quantified in the different groups by performing counts in 10 microscopy fields (200x magnification) per sample using a Zeiss light microscope (Carl Zeiss, Oberkochen, Germany). Blood and lymphatic vessels are positive for CD31, while only the lymphatic vessels express podoplanin. All positive vessels for the CD31 and podoplanin were counted in consecutive tissue sections and the difference between them gave us the number of blood vessels. Results were expressed as mean total number of positive vessels for each subject group. Quantification was performed by two independent observers in a blinded fashion. The ratio of blood/lymphatic vessels was calculated using the values of the counts.

Results

VEGF gene and protein expression

In the qRT-PCR analysis, we examined the expression of the VEGF-A messenger and its isoform VEGF-165 (more frequent and angiogenic isoform) in the pathological and healthy tissue samples. VEGF-A expression was similar in both groups showing no variations in mRNA quantity. However, significant differences in VEGF-165 were observed between both groups. Despite the high deviation of the mean in the pathological group due to the large expression difference in each patient, expression was significantly higher in this group (p<0.05) compared to the conjunctival samples (Fig. 1).

Overall, our immunohistochemical study of VEGF-A revealed greater VEGF-A labeling in the pterygium versus conjunctiva samples (Fig. 2). VEGF-A expression in the healthy samples was undetectable to minimum (1-2 or 0-25%) in most patients, and only two patients showed moderate to strong levels (3-4 or 25-75%) (Table 1). The protein was located in the epithelium, in the plasma membrane inside small blood vessels, among blood cell components and in the endothelial cells lining the blood vessels (Fig. 2).

Expression of VEGF-A in the pterygium samples was quite heterogeneous. The majority of patients showed high expression levels (3-4: 25-75%) mainly in the subepithelial connective tissue, associated with extravasation of the growth factor from active blood vessels inside the connective tissue. However, in a quarter of these patients, expression levels were similar to those observed in the control group (1-2: 0-25%) (Table 1, Fig. 2).

The immunohistochemical study of VEGF-C revealed greater labeling in the pterygium versus conjunctiva samples (Fig. 3). VEGF-C expression in the healthy samples was undetectable to minimum (0-25%) in 13 patients, and seven patients showed moderate to strong levels (3-4 or 25-75%) (Table 1). The protein
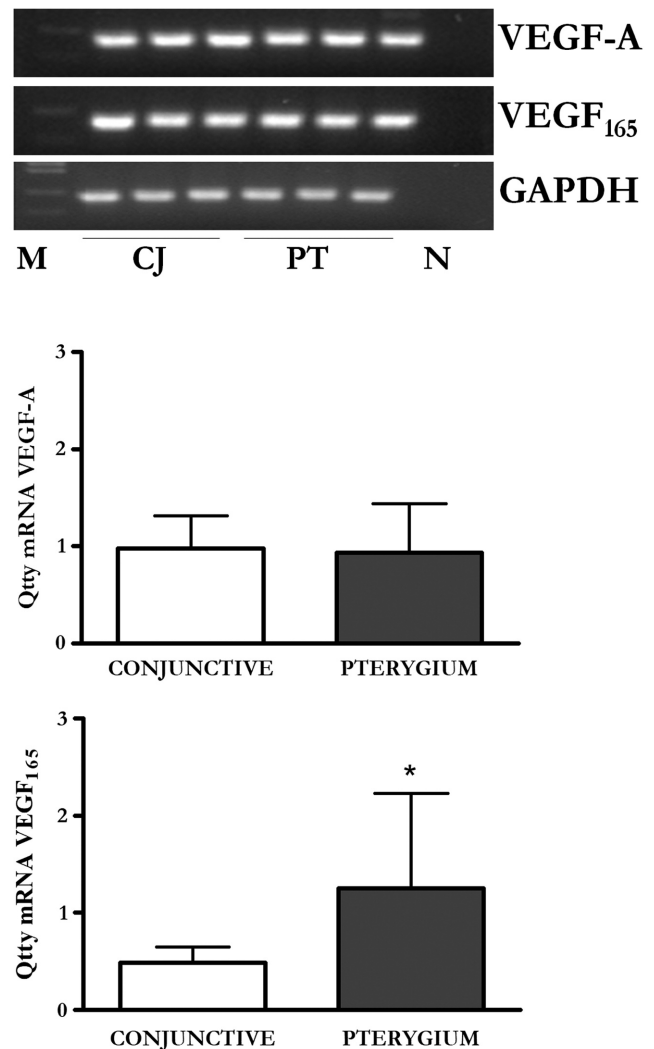

Fig. 1. Relative quantification of VEGF-A and VEGF-165 mRNA in conjunctival and pterygium tissue by qRT-PCR. Results are the mean ± SD. Agarose gel product (top panel). Gene expression was normalized to the expression recorded for the reference gene GAPDH. (M: molecular weight markers, N: negative control, CJ: conjunctival tissue, PT: pterygium tissue). *, P<0.05.

expression was located mainly in the epithelium and in the wall of some of the vessels and some isolated cells of the subepithelial connective tissue. The plasma inside the small vessels was not marked (Fig. 3).

Expression of VEGF-C in the pterygium samples was much higher showing 10 patients with 3-4 expression levels (3-4: 25-75%), 9 with 1-2 levels and 1 patient with maximum staining. The increase in
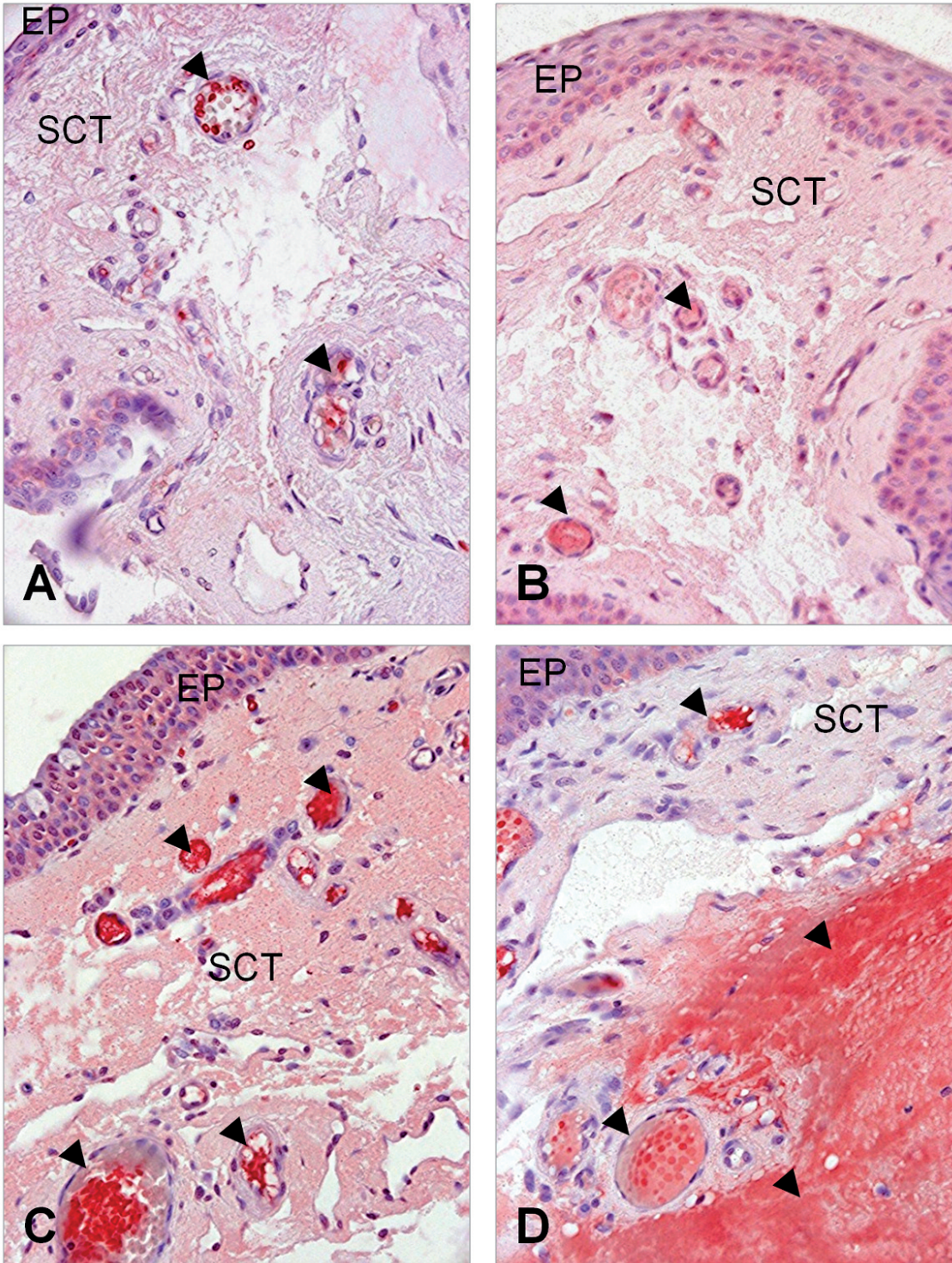

Fig. 2. Immunohistochemical expression of VEGF-A in conjunctival **(A,B)** and pterygium tissue **(C,D)**. Greater VEGF labeling was observed in the pterygium versus normal conjunctival samples. Protein expression was located mainly in the plasma membrane and endothelial cells of the blood vessels in the subepithelial connective tissue, and associated with extravasation of the growth factor from active blood vessels. (EP: epithelium; SCT: subepithelial connective tissue; ▶: areas of labeling). x 400.

expression was observed mainly in the epithelium and subepithelial tissue where the wall of most vessels appeared intensely marked, as well as some areas of the extracellular matrix and some positive immunolabeled cells in this connective tissue (Table 1, Fig. 3).

CD31 gene and protein expression

Our qRT-PCR analysis revealed significant differences between the two tissues in the expression of platelet endothelial cell adhesion molecule (PECAM-1),
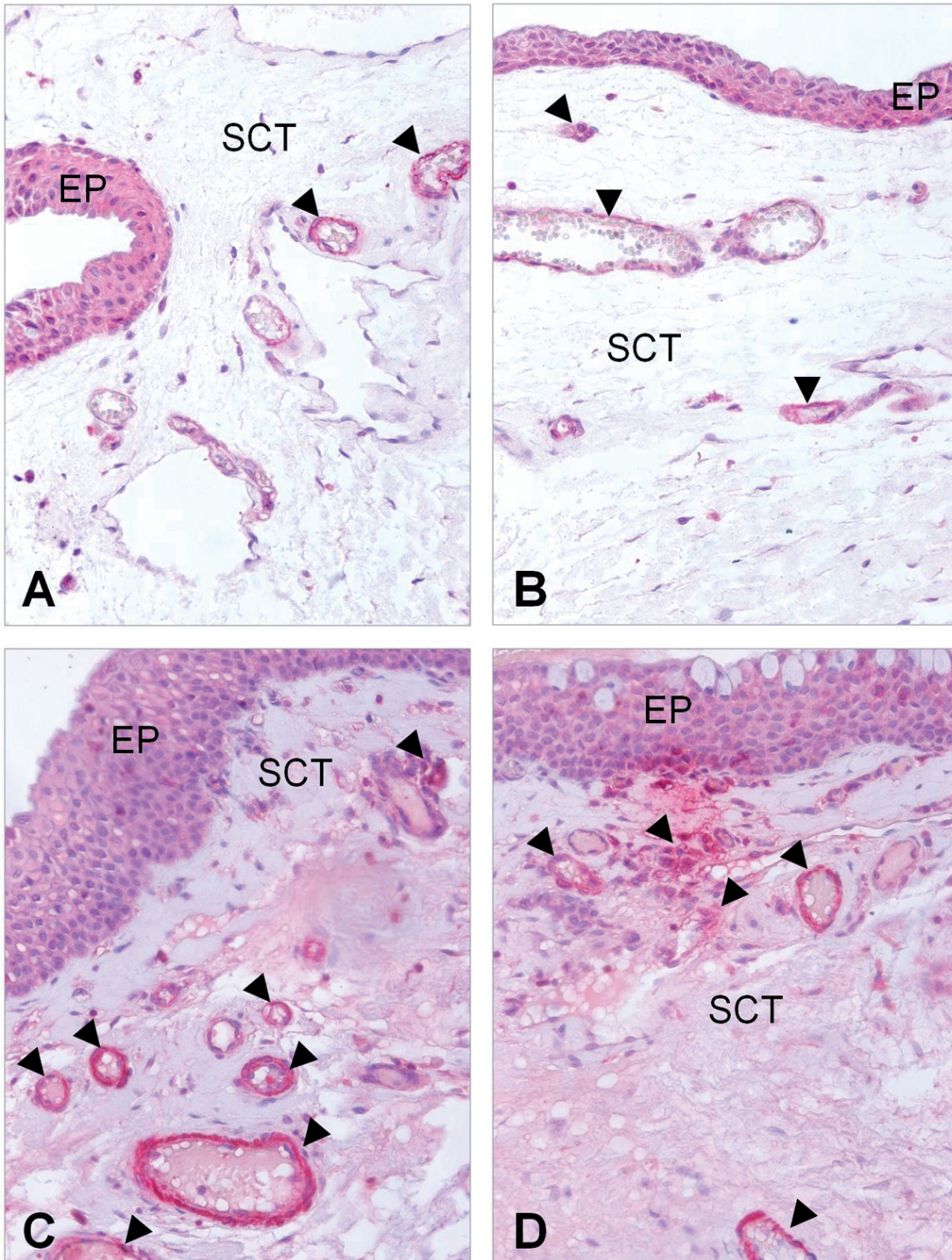

Fig. 3. Immunohistochemical expression of VEGF-C in conjunctival **(A,B)** and pterygium tissue **(C-D)**. Greater VEGF labeling was observed in the pterygium versus normal conjunctival samples. Protein expression was mainly expressed in the subepithelial tissue where the wall of most blood vessels appeared intensely marked, as well as some areas of the extracellular matrix and some positive immunolabeled cells in this connective tissue. (EP: epithelium; SCT: subepithelial connective tissue; ▶: areas of labeling). x 400.

also known as cluster of differentiation 31 (CD31). Thus, greater gene expression was detected in the pathological group (p<0.05) than in the healthy conjunctival tissue (Fig. 4).

In general terms, immunohistochemical labeling for CD31 was higher in the pterygium versus conjunctival samples (Fig. 5). Almost all subjects in the control group showed minimal expression (1-2: 0-25%) and only three showed moderate to strong levels (3-4: 25-75%) (Table 1). Protein expression was located mainly in the endothelial cells coating the inside of the blood vessels (Fig. 5).

In the pterygium group, CD31 expression was very different and almost all patients showed moderate to strong labeling (3-4: 25-75%) (Table 1) accounted for by the significant increase produced in subepithelial connective tissue vessels (Fig. 4).

PODOPLANIN/D2-40 gene and protein expression

In the qRT-PCR analysis, the expression of podoplanin, the transmembrane mucoprotein recognized by the D2-40 monoclonal antibody selectively expressed in the lymphatic endothelium, showed no significant differences between the control conjunctiva and pterygium groups (Fig. 6).

Almost all individuals in the control group showed minimal expression (1-2: 0-25%) in the immunohisto-

chemical labeling, while only four of them showed moderate to strong levels (3-4: 25-75%) (Table 1). The protein was located in the endothelial cells lining the lymphatic vessels such that these vessels could be identified among the blood vessels (Fig. 7).

However, compared to the control group, more than double the number of patients in the pterygium group showed moderate to strong labeling levels (3-4: 25-75%).

Blood/lymphatic vessel ratio

In the two groups, we also quantified vessels immunopositive for CD31 and podoplanin. Significantly higher (p<0.001) numbers of blood vessels were observed in the pterygium than control (Fig. 8). Mean ± SD were 16.09±3.33 in the control conjunctiva group versus 107.37±11.64 in the pterygium group.

The number of vessels positive for D2-40 immunohistochemistry in the pterygium group was also significantly higher (p<0.05) than in the healthy conjunctiva group. The number of lymphatic vessels observed in the pterygium tissue was 2-fold (31.62±5.15) with respect to the healthy conjunctiva (15.27±4.58).

When blood and lymphatic vessels were compared within the same group of patients, similar numbers of both types of vessel were observed in the control group. However, a significantly higher number of blood vessels relative to lymphatic vessels was detected in the pterygium tissue (p<0.001). This led us to calculate the blood/lymphatic vessel ratio within each of the study populations confirming the significantly higher
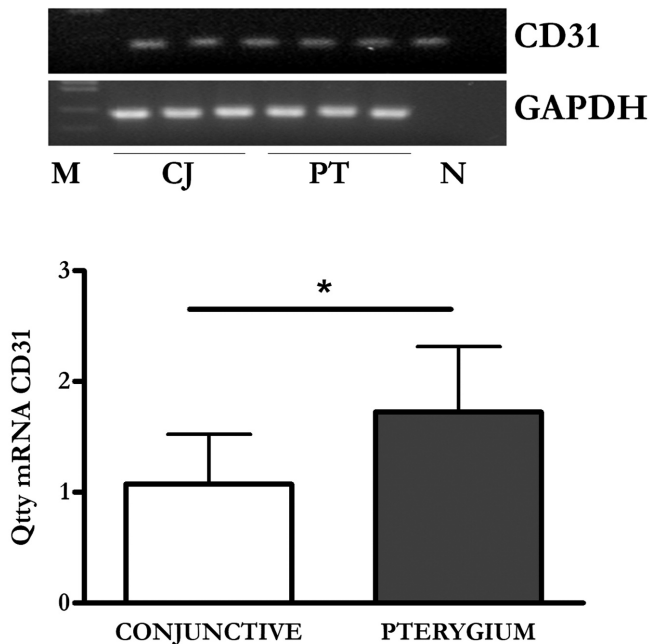

Fig. 4. Relative quantification of CD31 mRNA in conjunctival and pterygium tissue by qRT-PCR. Results are the mean ± SD. Agarose gel product (top panel). Gene expression was normalized to the expression recorded for the reference gene GAPDH. (M: molecular weight markers, N: negative control, CJ: conjunctival tissue, PT: pterygium tissue). *, P<0.05.

Table 1. Contingency table showing the number of patients with different scores within each study group.

	SCORE	NORMAL CONJUNCTIVA	PTERYGIUM
VEGF-A	1-2	18	5
	3-4	2	13
	5-6	0	2
VEGF-C	1-2	13	9
	3-4	7	10
	5-6	0	1
CD31	1-2	17	2
	3-4	3	16
	5-6	0	2
D 2-40	1-2	16	11
	3-4	4	9
	5-6	0	0

Semiquantification of protein expression levels of VEGF-A, VEGF-C, CD31 and D 2-40 in the immunohistochemical labeling. Labeling intensity scores were combined into three categories. The scale used for semiquantification was: 1: undetectable staining (<10%); 2: minimum staining (10-25%); 3: moderate staining (25-50%); 4: strong staining (50-75%); 5: maximum staining (75-90%); and 6: almost complete staining (>90%).

blood/lymphatic vessel ratio (p<0.001) in the pterygium patients than the control subjects (Fig. 8).

Discussion

Pterygium is a human eye disease produced by chronic exposure to ultraviolet light. The disease presents as an invasive centripetal fibrovascular growth on the eye surface causing corneal tissue destruction, matrix alteration, chronic inflammation and neovascularization. Several fibroangiogenic growth factors like VEGF have been identified in pterygium, suggesting their direct or indirect role in the pathogenesis of pterygium (Lee et al., 2001; Marcovich
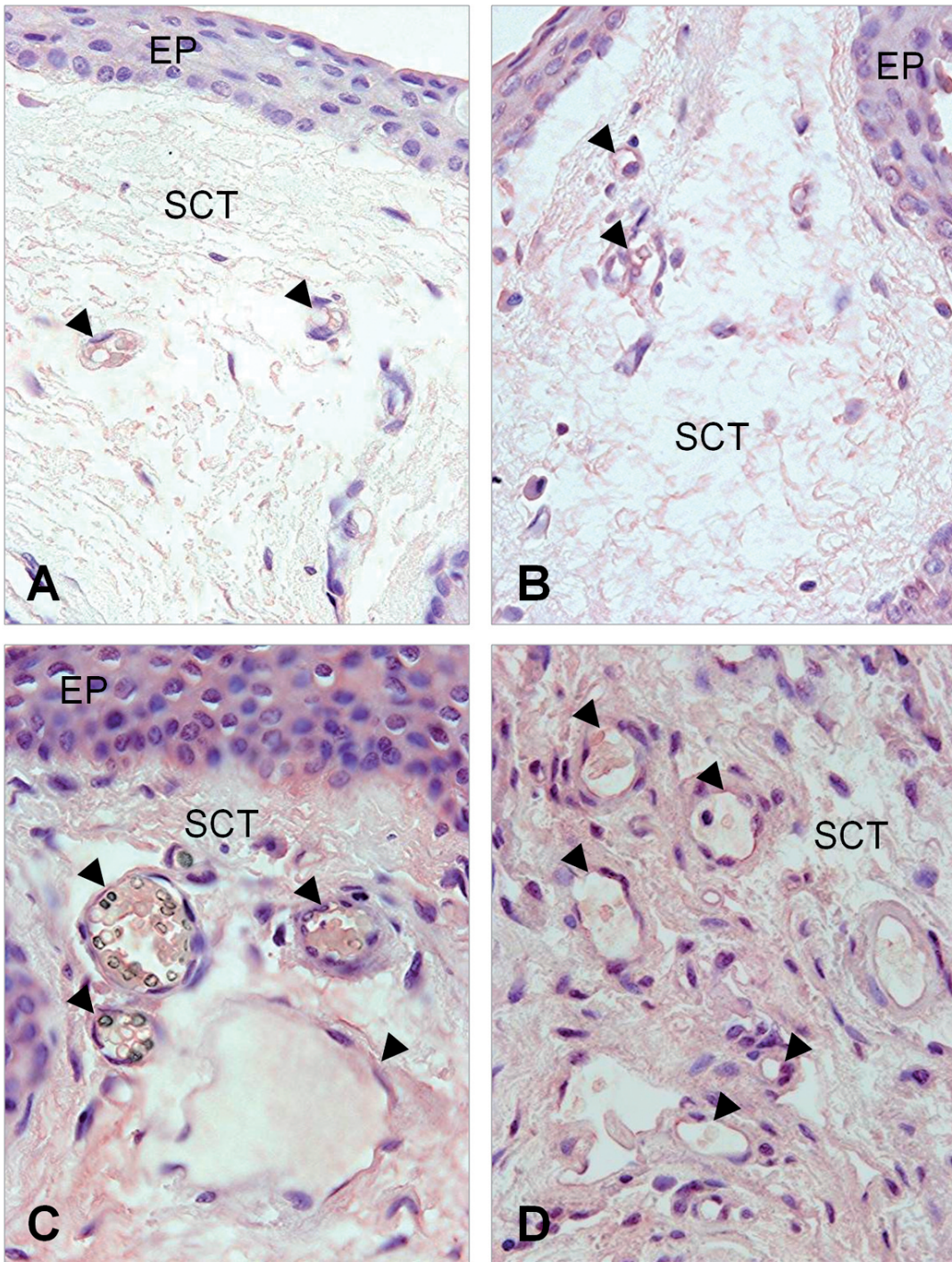

Fig. 5. Immunohistochemical expression of CD31 in conjunctival **(A,B)** and pterygium tissue **(C,D)**. Greater labeling was observed in pterygium versus normal conjunctiva owing to the significant increase produced in vessels in the subepithelial connective tissue of the pterygium. (EP: epithelium; SCT: subepithelial connective tissue; ▶: areas of labeling). x 640.

et al., 2002; Jin et al., 2003; van Setten et al., 2003; Gebhardt et al., 2005). In this study, we compared in pterygium and in healthy conjunctival tissue the gene and protein expression of several markers of vascular and lymphatic vessels and the ratio between blood and lymphatic vessel numbers in relation to the distribution of VEGF-A and VEGF-C, as the main regulator of angiogenesis and lymphangiogenesis.

Vascular endothelial growth factors (VEGFs) are a family of secreted signaling polypeptides that act through a family of receptor kinases in endothelial cells involved in the formation of the circulatory system, vasculogenesis, and the development of vessels from pre-existing ones, angiogenesis/lymphangiogenesis. The VEGF family includes VEGF-A (also called VEGF), the most important member of the VEGF family involved in promoting angiogenesis and VEGF-C and VEGF-D in lymphangiogenesis (Keck et al., 1989; Leung et al., 1989; Byrne et al., 2005). Humans express the alternately spliced VEGF-A isoforms of 121, 145, 165, 183, 189 and 206 amino acids in length (Robinson and Stringer, 2001). VEGF-165 appears to be the most abundant and potent isoform, for that reason it has been examined in this work.

In our study, gene expression of VEGF-A messenger RNA was similar in both groups showing no variations in mRNA quantity. However, the expression of the VEGF-165 isoform was significantly higher in the pterygium tissue (p<0.05) compared to the conjunctival samples. On the contrary, other authors (Detorakis et al.,

2010) have reported higher VEGF-A mRNA levels in pterygium than conjunctiva. This discrepancy could be explained by the fact that the authors compared more advanced pterygia with less advanced ones, and VEGF-A mRNA was also significantly correlated with postoperative recurrence. Other studies (Ling et al., 2012a) have also shown that compared with primary pterygia, VEGF-A and VEGF-C mRNA levels are significantly up-regulated in recurrent pterygia.

Our immunohistochemistry/protein analysis revealed greater VEGF-A labeling in pterygium than healthy conjunctiva, not only in blood vessels but also in the extracellular matrix. VEGF-A expression in the pterygium tissue was very heterogeneous, and most patients showed high expression. However, a quarter of the patients in this group showed expression levels that were similar to those recorded in most of the control group subjects. In addition, the pterygium samples showed significantly higher gene and protein expression levels of the endothelial junction protein CD31, which serves to identify all the vasculature including blood and lymphatic vessels.

Although many studies have shown the occurrence of angiogenesis during the formation of pterygia, the unbalanced expression of angiogenic stimulators and angiogenic inhibitors controls the formation and progression of pterygia. Jin et al. (2003) reported that pterygium tissues contain drastically reduced levels of pigment epithelium-derived factor, an angiogenic inhibitor, and increased VEGF levels, in accordance with our results. Immunohistochemical studies have also revealed VEGF overexpression in pterygium along with the presence of abundant CD31 (Aspiotis et al., 2007), as noted here, but also a characteristic absence of thrombospondin -1 (TSP-1), an inhibitor of angiogenesis (Taraboletti et al., 1990; Roberts, 1996; Aspiotis et al., 2007). Other authors (Marcovich et al., 2002) also observed the overexpression of VEGF and von-Willebrand factor (vWF) in pterygium tissue. Other authors (Lee et al., 2001) have described increased levels of nitric oxide and VEGF in pterygium tissue but, unlike our observations, expression was strongly visible mainly in the epithelium of the head portions of pterygia specimens.

Although angiogenesis and the blood vascular component has been well characterized in pterygium, the literature lacks much information regarding lymphangiogenesis and the outgrowth of lymphatic vessels, and specifically the blood/lymphatic ratio in pterygium. The development of lymphatic vessels that occurs both in normal settings such as inflammation or wound healing, or as part of many pathological processes, is triggered by the vascular endothelial growth factors VEGF-C and D, which cause the proliferation and migration of endothelial cells (Ferrara et al., 2003). We have shown in this study that the levels of VEGF-C are increased in the pterygium with respect to the normal conjunctiva, although this increase is less important than that of VEGF-A. The most frequently
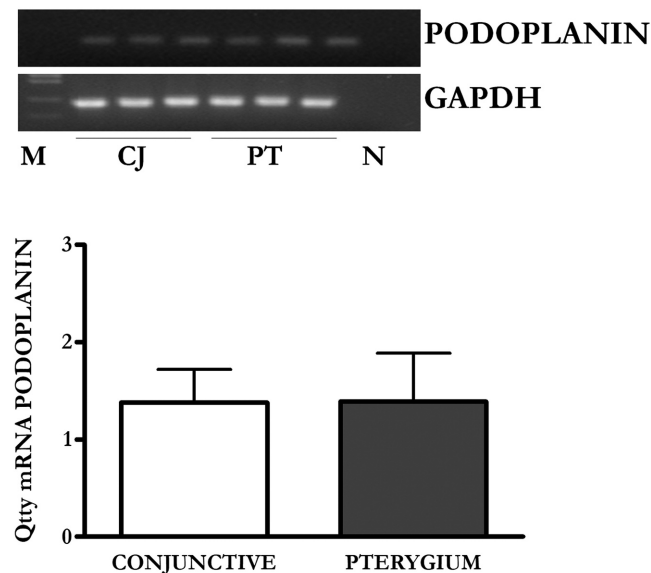

Fig. 6. Relative quantification of PODOPLANIN mRNA in conjunctival and pterygium tissue by qRT-PCR. Results are the mean ± SD. Agarose gel product (top panel). Gene expression was normalized to the expression recorded for the reference gene GAPDH. (M: molecular weight markers, N: negative control, CJ: conjunctival tissue, PT: pterygium tissue).

used lymphatic vessel marker in morphological diagnostics is LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1). However, there is so far little evidence of the presence of the lymphatic component in human pterygium identified through the immunoreactive podoplanin (PDPN), a 36-43-kDa mucin-type transmembrane mucoprotein recognized by the D2-40 monoclonal antibody. This molecule was given the name podoplanin due to its expression on kidney podocytes and it has been shown that podoplanin is selectively
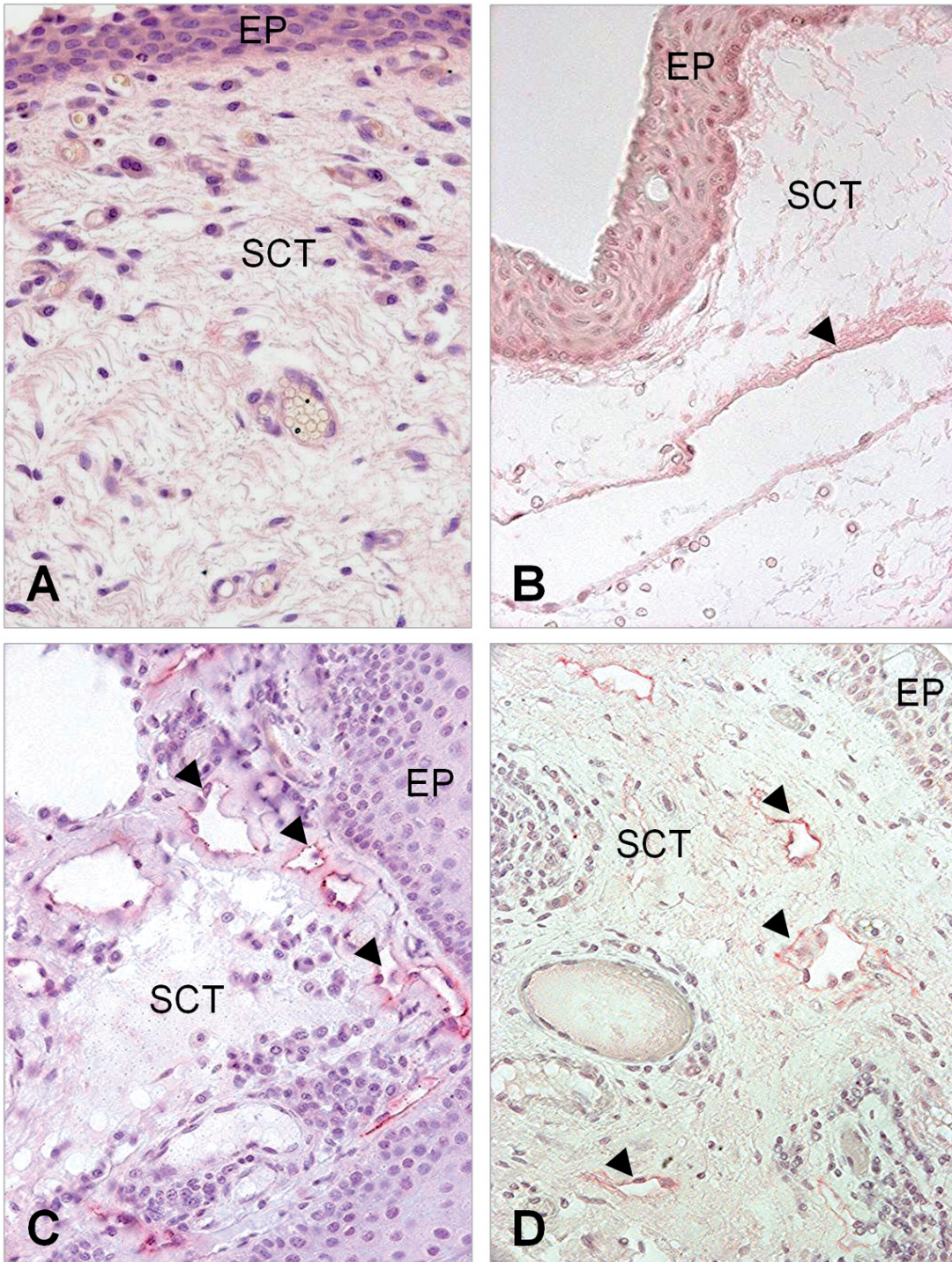

Fig. 7. Immunohistochemical expression of PODOPLANIN using the D2-40 antibody in conjunctival (**A,B9** and pterygium (**C,D**) tissue. Protein expression was located in the endothelial cells lining the lymphatic vessels, highlighting these vessels against the unstained blood vessels. (EP: epithelium; SCT: subepithelial connective tissue; ▶: areas of labeling). x 400.

expressed in the endothelium of lymphatic capillaries but not in the blood vasculature. Subsequent investigations have described podoplanin expression in lymphangiomas, Kaposi sarcomas, and in a subgroup of angiosarcomas and emphasized the utility of this protein as an immunohistochemical marker in the diagnosis of lymphatic endothelial-derived tumors (Kahn et al., 2002).

In the present study, we detected similar podoplanin mRNA expression in the conjunctival and pterygium tissue. However, moderately to strongly labeled protein levels of podoplanin were recorded in more than 2-fold of the pterygium patients than control subjects. The number of vessels testing positive for D2-40 immunohistochemistry in the pathological group was also significantly higher than in the control group, and lymphatic vessel numbers were double in the pterygium with respect to the conjunctiva. However, the largest differences in vessel counts emerged between blood vessels, which were around ten times higher for the pterygium than healthy conjunctiva. In summary, both lymph and blood vessels were found to be increased in the pathological tissue. However, the 3-fold increased blood/lymphatic vessel ratio observed in pterygium confirms the predominance of the blood component over the lymphatic component in this tissue.

Other authors (Lin et al., 2013) have also found greater blood vessel densities (BVD) than lymphatic densities (LVD) in Grade 2 pterygia. However, they have reported a much faster lymphangiogenesis than angiogenesis increase describing significantly greater numbers of LYVE-1 positive lymphatic vessels and upregulation of VEGF-C in cases of more severe disease (Ling et al., 2012b). The same authors showed similar patterns in recurrent pterygium reporting a significant though non-parallel relationship between angiogenesis and lymphangiogenesis (Qi et al., 2012) and a significant increase in the latter in recurrent versus primary pterygium (Ling et al., 2012b). These authors suggested investigation of antilymphangiogenesis therapy strategies to improve the efficacy of pterygium interventions.

In agreement with our findings, one of the few studies based on the use of D2-40 antibody for lymphatic vessel identification (Fukuhara et al., 2013) detected a significantly higher lymphatic vessel density and also VEGF-C/VEGFR-3 in pterygium compared with normal conjunctiva. These authors proposed a blockade of this pathway by chemical agents to inhibit pterygium progression. However, these authors noted no differences in blood vessel density nor in its proportion relative to lymphatic vessel density in pterygium versus normal conjunctive, while we observed a ratio that was higher by 3-fold in pterygium. Other studies also based on podoplanin have also detected a significantly higher lymph microvascular density from 1 to 3 vessels/field versus 7 to 10 in pterygium (Cimpean et al., 2011).

Besides the number of patients or sample size limitations of our study, we should mention several potential sources of variability such as genetic and inter-individual patient differences and the reproducibility of the methods. Continued research in this area is strongly
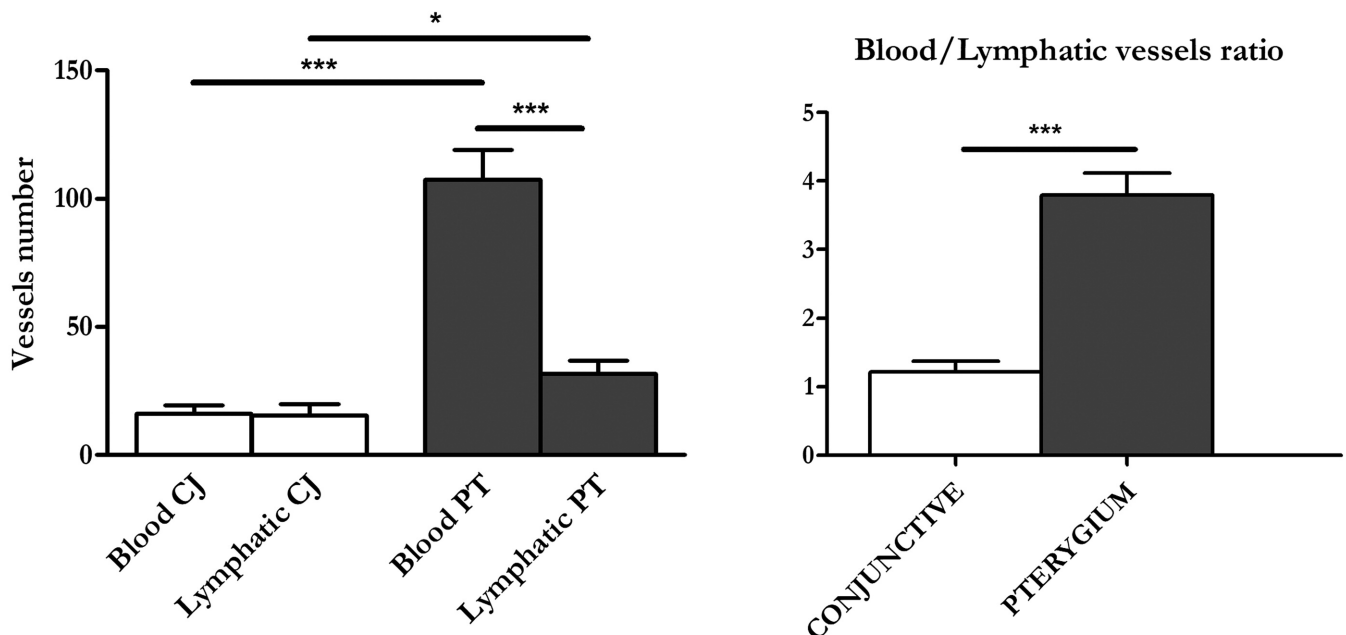

Fig. 8. Quantification of blood and lymphatic vessel numbers and blood to lymphatic vessels ratio in conjunctival and pterygium tissue. Results are the mean ± SD. (CJ: conjunctival tissue, PT: pterygium tissue). *, P<0.05, ***, P<0.001.

recommended as the VEGF family is quickly emerging as an important target for the development of future strategies to treat diseases involving angiogenesis and lymphangiogenesis.

In conclusion, our findings indicate that both lymphangiogenesis and angiogenesis are stimulated in pterygium. The formation of new blood vasculature is the most relevant event, and this was correlated here with the increased expression of vascular endothelium CD31 and a higher blood/lymphatic vessel ratio. The presence of high levels of VEGF-A in both the vessels and extracellular matrix of human pterygium tissue may point to an active role of this factor in driving angiogenesis.

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