**Histology and Histopathology**
*From Cell Biology to Tissue Engineering*

# Absence of lymphatic vessels in non-functioning bleb capsules of glaucoma drainage devices

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**Summary.** Purpose. To evaluate the presence and appearance of blood and lymphatic vessels in non-functioning bleb capsules of glaucoma drainage devices (GDD).

Materials and methods. Non-functioning (n=14) GDD-bleb capsules of 12 patients were analyzed by immunohistochemistry for blood vessels (CD31, vascular endothelium), lymphatic vessels (lymphatic vessel endothelial hyaluronan receptor-1 [LYVE-1] and podoplanin) and macrophages (CD68).

Results. CD31+++ blood vessels and CD68+ macrophages were detected in the outer layer of all specimens. LYVE-1 immunoreactivity was registered in single non-endothelial cells in 8 out of 14 (57%) bleb capsule specimens. Podoplanin-immunoreactivity was detected in all cases, located in cells and profiles of the collagen tissue network of the outer and/or the inner capsule layer. However, a colocalization of LYVE-1 and podoplanin as evidence for lymphatic vessels was not detected.

Conclusions. We demonstrate the presence of blood-vessels but absence of lymphatic vessels in non-functioning bleb capsules after GDD-implantation. While the absence of lymphatic vessels might indicate a possible reason for drainage device failure, this needs to be confirmed in upcoming studies, including animal experiments.

**Key words:** Glaucoma drainage devices, Bleb capsule, Lymphatic tissue, Baerveldt, Lyve-1, Podoplanin

## Introduction

The implantation of episcleral glaucoma drainage devices (GDD) has been established as one of the preferred surgeries in a wide range of glaucoma indications (Dietlein et al., 2008; Minckler et al., 2008; Rosentreter and Dietlein, 2010). The efficacy of GDD has been demonstrated in various studies (Gedde et al., 2007, 2012; Goulet et al., 2008). Success rates with IOP≤14 mmHg are described in 63.9% of all implants 5 years after surgery in the Tube Versus Trabeculectomy Study and are similar to the trabeculectomy (Gedde et al., 2012). The main reason for failure following poor filtration and an increase in IOP is an excessive fibrotic encapsulation of the bleb (Molteno et al., 2003).

Up to now, only a few immunohistology studies exist evaluating the bleb capsules after GDD surgery in eyes with non-functioning and/or functioning implants: in functioning blebs, with the exception of very early capsules (younger than 2 months), Molteno et al. described a typical histological organization of the capsules. They detected two typical layers: a moderate cellular outer layer of regularly arranged collagen fibers and an inner avascular hypocellular layer of regularly arranged swollen and fragmented collagen fibers; both layers contained fibroblasts and macrophages with different morphology (Molteno et al., 2006, 2009). McCluskey et al. investigated the role of metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMP) in functioning blebs of Molteno implants. They

detected TIMP-2 expression in most bleb capsules and hypothesized that bleb capsules undergo a cycle of collagen breakdown and renewal throughout life (McCluskey et al., 2009). Välimäki et al. compared functioning and non-functioning blebs in different types of GDD and were able to demonstrate different patterns of MMPs and TIMPs depending on the filtration function (Välimäki and Uusitalo 2015). In functioning GDDs, they found a marked but not evident increase of MMP-9 compared to non-functioning bleb capsules using immunohistochemistry. Moreover, TIMP-3 was more prominent in the outer layer of functioning GDDs, whereas non-functioning capsules showed a more diffuse arrangement in all layers. Additionally, they detected a different pattern of structural proteins with an increase of fibronectin, tenascin, collagen IV and smooth muscle actin (SMA) expression in a small sample size taken from non-functioning capsules. They concluded that these findings indicate the presence of an active wound healing process, suggesting a possible reduction in filtration through the bleb wall (Välimäki and Uusitalo, 2014).

The lymphatic system transports immune cells and proteins (Bock et al., 2013; Hos et al., 2015). In addition, it plays an important role in fluid homeostasis by draining interstitial fluid from tissues and returning it to the vascular system (Bock et al., 2013; Hos et al., 2015). It is well known that lymphatic vessels proliferate in inflammatory processes mediated by lymphangiogenic factors of macrophage and granulocyte origin (Cursiefen et al., 2004; Alitalo et al., 2005). Regarding the presence of lymphatic tissue, the human eye is exceptional: while some outer parts of the eye (including extraocular muscles, lacrimal gland, limbus, and conjunctiva) contain lymphatic vessels under physiological conditions (Gausas et al., 1999; Cursiefen et al., 2004; 2006; Heindl et al., 2011; Hos et al., 2015), there is an absence of lymphatic tissue in the inner eye (choroid, retina and sclera) as well as the central cornea, a situation that has been referred to as the "immune privilege of the eye" (Medawar 1948; Streilein 2003; Schroedl et al., 2008, 2015; Herwig et al., 2014; Heindl et al., 2015). However, in ocular diseases like infection or tumor, or following open globe injury, several studies revealed pathologically induced growth of lymphatic tissue (Cursiefen et al., 2002, 2011; Heindl et al., 2011, 2013; Wessel et al., 2012). On the other hand, recent investigations demonstrated that the Schlemm's canal as part of the aqueous humor drainage system is a lymphatic-like vessel and responsive to prolymphangiogenic factors (Aspelund et al., 2014).

Recently, we have shown that the aqueous humor, which is drained via Schlemm's canal or GDD, contains several antilymphangiogenic factors such as vasoactive intestinal peptide (VIP) and α-melanocyte stimulating hormone (α-MSH) (Bock et al., 2016). These inhibited lymphangiogenesis *in vivo* and *in vitro* and could potentially in larger quantities also affect lymphangiogenesis around GDD (Bock et al., 2016).

Since GDD surgery is performed by implantation of the device in lymphatic vessel-containing conjunctiva, and the lymphatics-free sclera, we were interested in whether lymphatic tissue is involved in the encapsulation of bleb filtration in GDD. Therefore, the purpose of the present study was to evaluate the presence and appearance of lymphatic vessels in fibrotic bleb capsules of GDD.

## Materials and methods

We retrospectively analyzed tissue derived from 15 eyes of 14 patients who underwent bleb capsule excision after previously performed Baerveldt surgery between January 1, 2013, and December 31, 2015, in the Department of Ophthalmology, University Eye Hospital, Cologne, Germany (surgeon: TD). We included all patients who underwent bleb capsule excision because of poor function. We had to exclude one patient because of poor quality of probes and one patient because of incomplete data. Furthermore, one patient received an excision two times within the study interval. Another patient underwent a bleb capsule excision on both eyes (patient characteristics are given in Table 1).

The excision was performed as described previously and the resected tissues were fixed in 0.075 M phosphate buffered saline (PBS) containing 4% formaldehyde (Rosentreter and Dietlein, 2010). Specimens were then dehydrated in increasing concentrations of ethanol (70-99%) and embedded in paraffin (Micro-Cut Paraffin, catalog #24201, Polysciences, Warrington, USA) at 52-54°C. Step sections with a thickness of 5 $\mu$m were cut and floated on deionized water at 50°C and mounted as single sections on Starfrost glass slides (Knittel-Glaeser, Braunschweig, Germany). Slides were subsequently dried at 37°C for 24 h. Step sections were routinely stained with H&E (hematoxylin and eosin; Merck, Darmstadt, Germany), PAS (periodic acid-Schiff; Merck) and van Gieson (van Gieson iron hematoxylin and van Gieson solution; Merck).

*Immunocytochemistry*

Immunohistochemical stains for podoplanin (monoclonal mouse anti-human, clone D2-40; ready-to-use; catalog #N1607; Dako, Glostrup, DK) and LYVE-1 (rabbit anti-human, dilution 1:50; Zytomed, Berlin, Germany) were performed. In addition, immunohistochemical stains for CD31 (monoclonal antibody mouse anti-human, clone JC70A; dilution 1: 20; catalog #M0823; Dako, Glostrup, DK) as control for endothelial immunoreactivity and CD68 (monoclonal antibody mouse anti-human, clone KP1; dilution 1: 100; catalog #M0814; Dako, Glostrup, DK) for macrophage immunoreactivity were performed. 5 $\mu$m thick sections were cut using a sledge microtome (Leitz 1400, Wetzlar, Germany). After deparaffinization and rehydration, heat-mediated antigen retrieval was performed for podoplanin, LYVE-1 and CD31 with TRS buffer (Target Retrieval

Solution, pH: 9.0, Dako, Glostrup, DK) for 30 min (for podoplanin, CD31) and 20 min (for LYVE-1) in 96°C water bath. For CD68, antigen retrieval was performed by fast enzyme (Fast Enzyme, catalog #ZUC059-015, Zytomed Systems, Berlin, Germany). Washing steps were performed with a wash buffer (DCS Labline, catalog #WL583C2500, DCS, Hamburg, Germany). To detect tissue immunoreactivity the labeled streptavidin-biotin immunoenzymatic antigen detection method with horseradish peroxidase (HRP) and chromogen 3-amino-9-ethylcarbazole (AEC) was used. The specimens were incubated with the primary antibody (diluted with DCS Labline antibody dilution buffer, catalog #ALI20R500, DCS, Hamburg, Germany) incubated in a humidity chamber for 30 min (for podoplanin, CD31) and 45 min (for LYVE-1, CD68) at room temperature (RT). Endogenous peroxidase was blocked with 3% hydrogen peroxide (Emsure ISO, Merck, Darmstadt, Germany) diluted in wash buffer for 10 min followed by incubation with the biotinylated secondary antibody for 10 min (Polylink, DCS Detection Line, DCS, Hamburg, Germany). After this step, the specimens were rinsed with wash buffer (DCS Labline, catalog #WL583C2500, DCS, Hamburg, Germany). The specimens were incubated for another 10 min with the streptavidin-HRP-complex (DCS Detectionline Peroxidase Label (HRP), DCS, Hamburg, Germany) followed by incubation for 3 min with AEC (AEC+ High Sensitivity, Dako, Glostrup, DK). Counterstaining was performed with Hemalaun (Merck, Darmstadt, Germany).

Negative controls were employed for each antibody and resulted in absence of immunoreactivity. Due to the presence of lymphatic vessels, conjunctiva served as positive control for podoplanin and LYVE-1. Blood vessels from the placenta were used as positive control for CD31 and macrophages from the tonsil as positive control for CD68.

LYVE-1 (lymphatic vessel hyaluronan receptor-1) is one of the most common markers for lymphatic endothelial cells and is expressed on lymphatic endothelium, but also detected on cells other than lymphatic endothelium (Banerji et al., 1999; Jackson, 2004). Another well-established marker for endothelial cells in lymphatic capillaries is podoplanin, a mucin-type transmembrane protein (Breiteneder-Geleff et al., 1999). However, both markers, LYVE-1 and podoplanin, are also expressed in cells other than lymphatic endothelium (Breiteneder-Geleff et al., 1997; Mouta Carreira et al., 2001; Sleeman et al., 2001). CD31 is physiologically mainly expressed on blood vascular endothelium (Albelda et al., 1991; DeLisser et al., 1997). CD 68-antibody, a transmembrane glycoprotein, was used to define macrophages (Holness and Simmons 1993). Immunohistochemical staining was analyzed by 3 independent investigators (R.S., K.R.K., and L.M.H.) in a masked manner using digital photographs of the histological specimen. The photographs were taken by the same microscope and camera and were presented standardized on the same display. Grading was categorized in a semiquantitative way and four groups were created that matched the amount of immunoreactive profiles: 0 absent (or artifact), +sporadic, ++few and +++many. For each category a group of four reference pictures were used to compare and categorize the specimen (Fig. 1). The results of the three investigators were averaged. Lymphatic vessels were defined by co-localization of LYVE-1 and podoplanin immunopositive staining according to the international consensus guidelines (Schroedl et al., 2014).

*Immunohistochemistry*

For double immunohistochemistry of LYVE-1 and podoplanin, the following protocol was applied: slides were de-paraffinized and antigen-retrieval applied (20

**Table 1.** Bleb capsule excisions after glaucoma drainage implant: summary of patient data and results of immunohistochemistry.

| Specimen | Patient | Eye | Age (y) | Sex | Glaucoma diagnosis | Mean IOP (mmHg) | Time to Excision | Re-Excision | CD68 | CD31 | LYVE-1 + cells | Podoplanin + clefts/cells |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | OD | 13 | M | congenital | 35 | 1.6 y | no | + | +++ | + | +++ |
| 2 | 2 | OD | 20 | M | aphakic | 21 | 1.5 y | no | ++ | ++ | + | ++ |
| 3 | 3 | OD | 26 | F | ICE | 26 | 1.9 y | no | ++ | +++ | ++ | +++ |
| 4 | 4 | OS | 50 | M | Steroid induced | 28 | 5.1 y | yes | + | +++ | ++ | ++ |
| 5 | 5 | OS | 5 | M | aphakic | 35 | 10.5 mo | no | + | +++ | - | + |
| 6 | 6† | OD | 5 | F | congenital | 31 | 4.2 y | yes | + | ++ | - | + |
| 7 | 7 | OD | 9 | F | inflammatory | 36 | 1.1 mo | no | ++ | ++ | ++ | +++ |
| 8 | 8 | OS | 58 | F | Silicone Oil induced | 26 | 2.5 y | yes | + | ++ | - | ++ |
| 9 | 9‡ | OS | 6 | F | congenital | 23 | 6.3 y | yes | ++ | ++ | - | ++ |
| 10 | 6† | OD | 5 | F | congenital | 27 | 4.5 y | yes | ++ | ++ | - | +++ |
| 11 | 10 | OS | 70 | M | POAG | 27 | 3.2 y | no | + | +++ | ++ | +++ |
| 12 | 11 | OS | 60 | F | POAG | 26 | 7.8 mo | yes | + | ++ | - | +++ |
| 13 | 9‡ | OD | 7 | F | congenital | 36 | 11.2 mo | no | ++ | +++ | ++ | +++ |
| 14 | 12 | OD | 63 | F | POAG | 24 | 3.7 y | no | + | +++ | ++ | +++ |

ICE: Iridocorneal Endothelial Syndrome, IOP: intraocular pressure, POAG: Primary Open-Angle Glaucoma. Immunhistochemical staining, -: absent (or artefact), +: sporadic, ++: few, +++: many. †: specimen from the same patient and eye, ‡: specimen from the same patient but fellow eye.

min in 10 mM sodium citrate buffer, pH 6.0, at 85°C). Slides were rinsed in 0.05M tris buffered saline (TBS, 5 min at RT; Roth, Karlsruhe, Germany), and sections were incubated for 1h at RT in TBS containing 5% donkey serum (Sigma-Aldrich, Vienna, Austria), 1% bovine serum albumin (BSA, Sigma-Aldrich), and 0.5% Triton X-100 (Merck, Darmstadt, Germany). After three 5 min rinses, sections were incubated with an anti-human LYVE-1 (raised in rabbit, 1:50, Acris, Herford, Germany) and anti-human podoplanin (raised in mouse, 1:50, Serotec, Düsseldorf, Germany) both diluted in TBS/BSA/TritonX-100 (over night at RT). Additionally, various PROX1 antibodies were applied in human skin samples as a positive control (raised in mouse: Acris, Herford, Germany, AM08261PU-N; Sigma-Aldrich, Vienna, Austria, P0089; raised in rabbit: Novus Biologicals, Centennial, USA, NBP1-18605) but revealed absence of immunoreactivity, despite several antigen retrieval protocols, and were therefore not applied in this study. After rinsing in TBS (three times, 5 min), binding sites of primary antibodies were visualized with corresponding Alexa488-, and Alexa555-tagged antisera (1:1000; Invitrogen, Karlsruhe, Germany, in TBS/BSA /Triton X-100;) for 1h (RT), followed by additional rinsing in TBS (three times, 5 min). Slides were incubated with 4',6-Diamidino-2 phenylindole dihydrochloride (DAPI; 1:4000, stock 1 mg/ml, VWR, Vienna, Austria, 10 min), rinsed three times 5 min in TBS and were embedded in TBS-glycerol (1:1 at pH 8.6). Negative controls were performed by omission of the primary antibodies during incubation and resulted in absence of immunoreactivity. All antibodies tested were raised against human epitopes and were successfully applied in earlier studies in human tissue (CD68 (Holness and Simmons 1993; Auw-Haedrich et al., 2007), /CD 31 (Muller 1995; Välimäki and Uusitalo 2015), /LYVE-1 (Cursiefen et al., 2002; Jackson, 2004; Schlereth et al., 2014), /podoplanin (Kaiserling et al., 2003; Herwig et al., 2014)).

### Documentation

In order to document single and double immunohistochemistry, a confocal laser-scanning unit (Axio ObserverZ1 attached to LSM710, Zeiss, Göttingen, Germany; ×20 dry or ×40 and ×60 oil immersion objective lenses, with numeric apertures 0.8, 1.30, and 1.4, respectively; Zeiss) was used. Sections were imaged using the appropriate filter settings for Alexa555 (568 nm excitation, channel 1, coded red), Alexa488 (488 nm excitation, channel 2, coded green), and DAPI (345 nm excitation, channel 3, coded blue). Colocalization of same structures in channel 1 and channel 2 resulted in yellow mixed color.

The present clinicopathologic study was performed in conformance with the tenets of the Declaration of Helsinki. The approval of the institutional review board or the ethics committee was not required in this instance.

### Results

The bleb capsule excision was performed at mean 965 days (range; 35 days to 6.3 years) after surgery (by a single experienced surgeon avoiding crush artifacts; TD). The mean intraocular pressure (IOP) was 29 mmHg (range; 21-36 mmHg). Our patient cohort consisted of a variety of glaucoma diagnoses; primary open-angle glaucoma (n=3), congenital glaucoma (n=3), aphakic glaucoma (n=2), steroid-induced glaucoma (n=1), inflammatory glaucoma (n=1), iridocorneal endothelial syndrome (n=1) and silicone oil induced glaucoma (n=1). In 9 of 14 cases the patient underwent capsule excision for the first time. Five of 14 specimens were taken from repeated excision; where two of them have an additional collagen matrix implantation (Ologen®) before. A summary of patient's data is given in Table 1.

The histological structure of all cross sections showed similar arrangements: the inner layer, which surrounded the GDD, showed an avascular hypocellular structure with a mostly high density of collagen fibres (Fig. 2). The outer layer presented a higher cell concentration and varying grade of vascularization as well as a looser collagen network (Fig. 2). Table 1 shows the immunohistochemical staining of the specimens. Addition grading was performed in a semiquantitative way per specimen into four groups: absent (-), sporadic (+), few (++) and many (+++). There was no correlation of the mean intraocular pressure (IOP) at the time of bleb capsule excision and our immunohistochemical findings.

### CD31+ blood vessels in the bleb capsules

In the cross sections, a clear CD31 positive signal surrounding a vessel lumen was detected in all specimens exclusively in the outer layer in a various range from sporadic to many staining (Table 1, example case: Fig. 3A). We could not find any relationship between the number of CD31+ blood vessels and the time interval from implantation or repeated excision.

### LYVE-1+ cells but no classical lymphatic vessels are detectable in the bleb capsules

The bleb capsules were analyzed using the lymphatic markers LYVE-1 and podoplanin. In our cohort 8 out of 14 (57%) specimens presented LYVE-1 immunoreactivity in the cross sections (example case: Fig. 3C). Two out of 8 (25%) specimens showed sporadic, and 6 out of 8 (75%) specimens presented few LYVE-1 positive cellular immunoreactivity, respectively. It is notable that all but one probe (n=5), after re-excision, displayed absence of LYVE-1 immuno-reactivity.

The LYVE -1+ cells were single, non-endothelial cells. Almost all of them were located in the outer layer (except one specimen in the interface from the inner to

the outer layer) and mostly assembled relatively close to each other. A correlation between the appearance of blood vessels and LYVE1+ cells was not obvious.

*Podoplanin+ cells*

Podoplanin+ structures were detected in all cases to various extents from sporadic to many staining. All were located in clefts of the collagen tissue network of the outer and/or the inner layer of the bleb capsules (example case: Fig. 3D. In 5 out of 14 (36%) specimens, the positive stainings were registered only in the inner layer. Three out of 14 (21%) cross sections showed podoplanin positive staining just in the outer layer. The
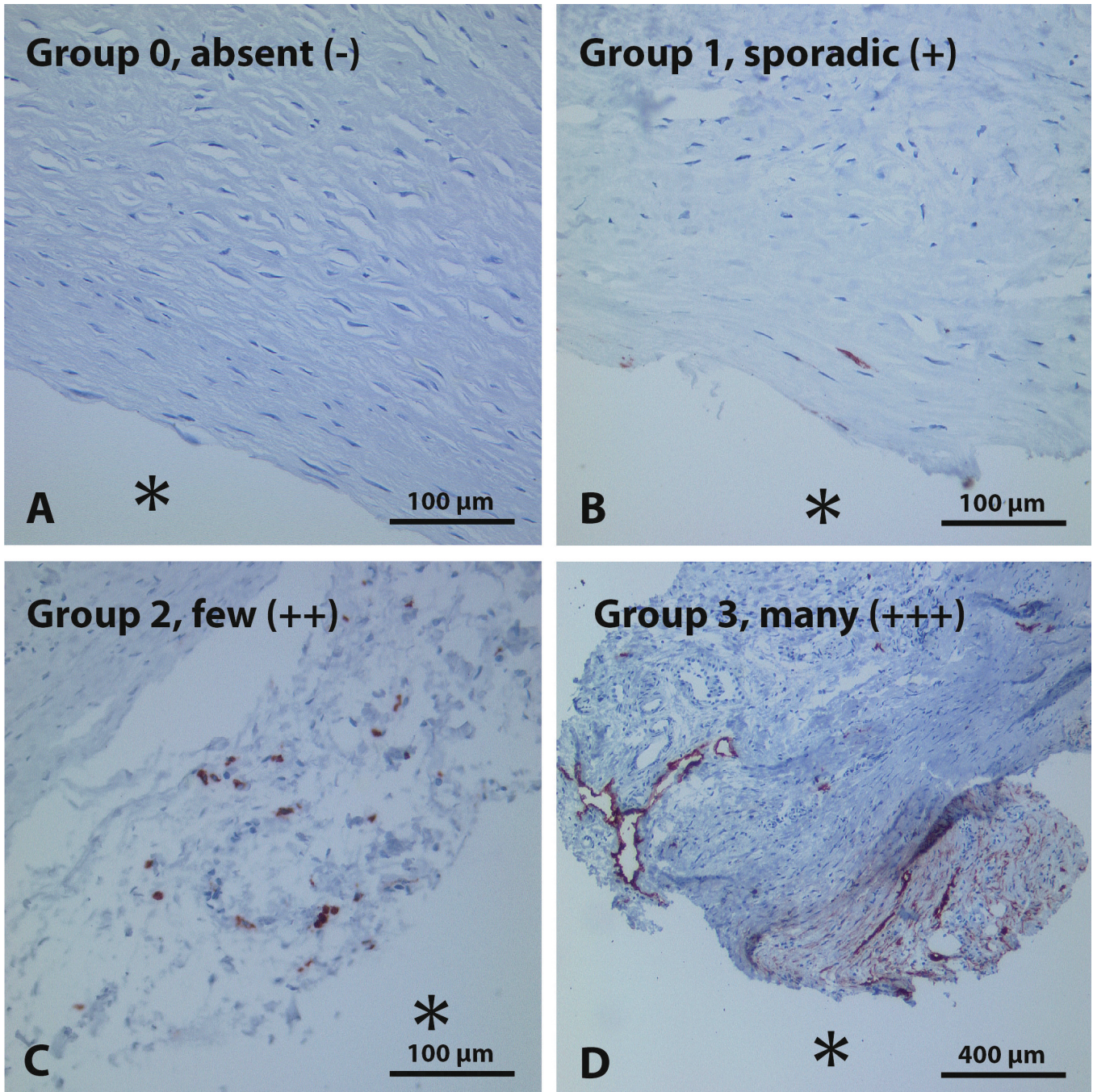


**Fig. 1.** Grading samples immunohistochemistry (asterisk denotes bleb cavity).

remaining 6 specimens (43%) presented a staining in the inner and outer layer of the bleb capsules. Variations of the time interval after excision or repeated capsule excisions did not show marked differences in staining. No typical lymphatic vessel staining pattern was observed.

### CD68+ macrophages in the bleb capsule

In our case series we were able to detect CD68+ positive cells in all specimens with sporadic to few immunoreactivity (example case: Fig. 3B). They were located in the outer layer of the bleb capsules and mostly presented in the neighborhood of blood vessels. There was no marked relationship between immunostaining and the time interval from implantation or repeated excision.

### Confocal laser scanning microscopy

In order to better identify podoplanin positive clefts/cells (see Table 1) that could be possibly associated with lymphatic vessels, all specimens received an additional investigation applying double immunohistochemistry against LYVE-1 and podoplanin, followed by confocal microscopy (Fig. 4C,F,I). In all of the specimens investigated, single immunoreactivity of the markers could be confirmed, while a colocalization of LYVE-1 and podoplanin was not detected. Fig. 4 presents exemplary cases.

### Discussion

Our study demonstrates for the first time that there are blood vessels, but no classical lymphatic vessels in the outer bleb capsule after GDD-implantation. The histological arrangement of our cases after Baerveldt surgery are in line with the findings after Molteno implants, which are characterized by a moderately cellular vascularized outer layer of regularly arranged collagen fibres and an inner avascular hypocellular layer of altered collagen fibres (although we did not specify the collagen content in our study) (Molteno et al., 2009). We were able to show that there is no indication for
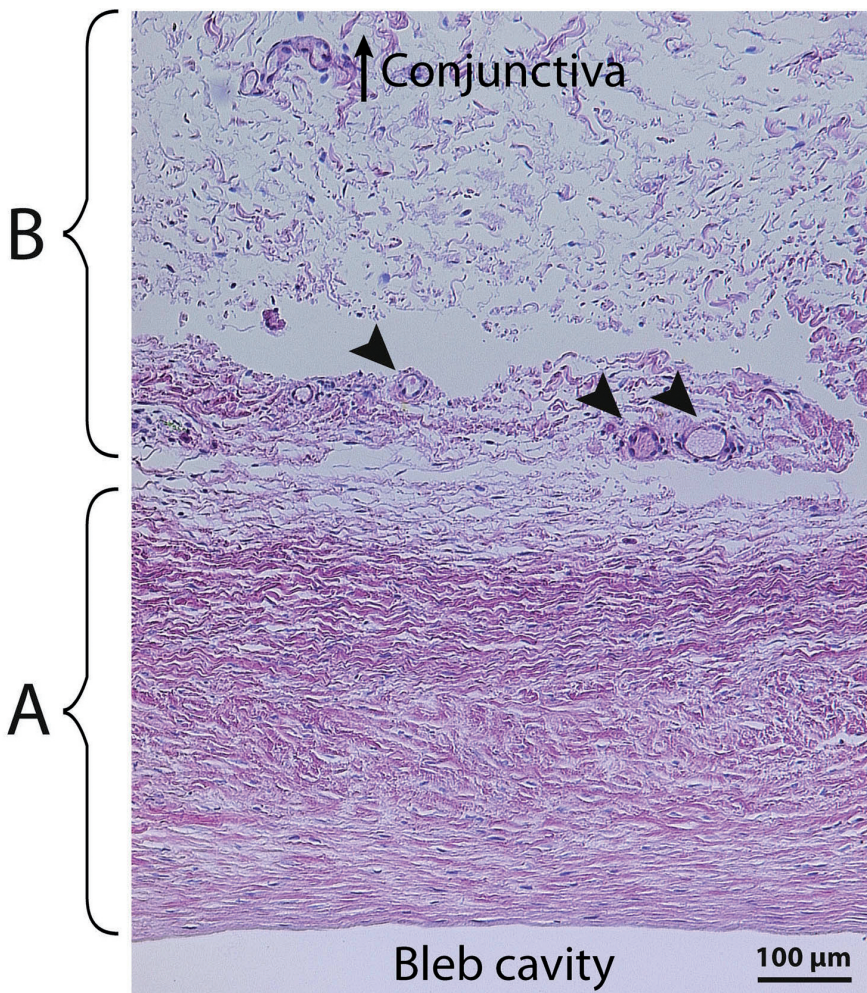


**Fig. 2.** Typical histological structure of Glaucoma drainage device (GDD) capsules. The figure shows a cross section of a 10.5 month old capsule (hematoxylin and eosin staining). **A.** Inner layer: High density of collagen fibres, avascular, hypocellular. **B.** Outer layer: Lower density of collagen fibres, vascularized (arrowheads), higher cell concentration.

classical lymphatic vessels in the analyzed bleb capsules.

Nevertheless, our investigations did show LYVE-1+ cells in multiple specimens of non-functioning bleb capsules. LYVE-1+ cells were already found in the uveal tract (Schroedl et al., 2008, 2015) and in the human sclera (Schlereth et al., 2014). They may act as precursor
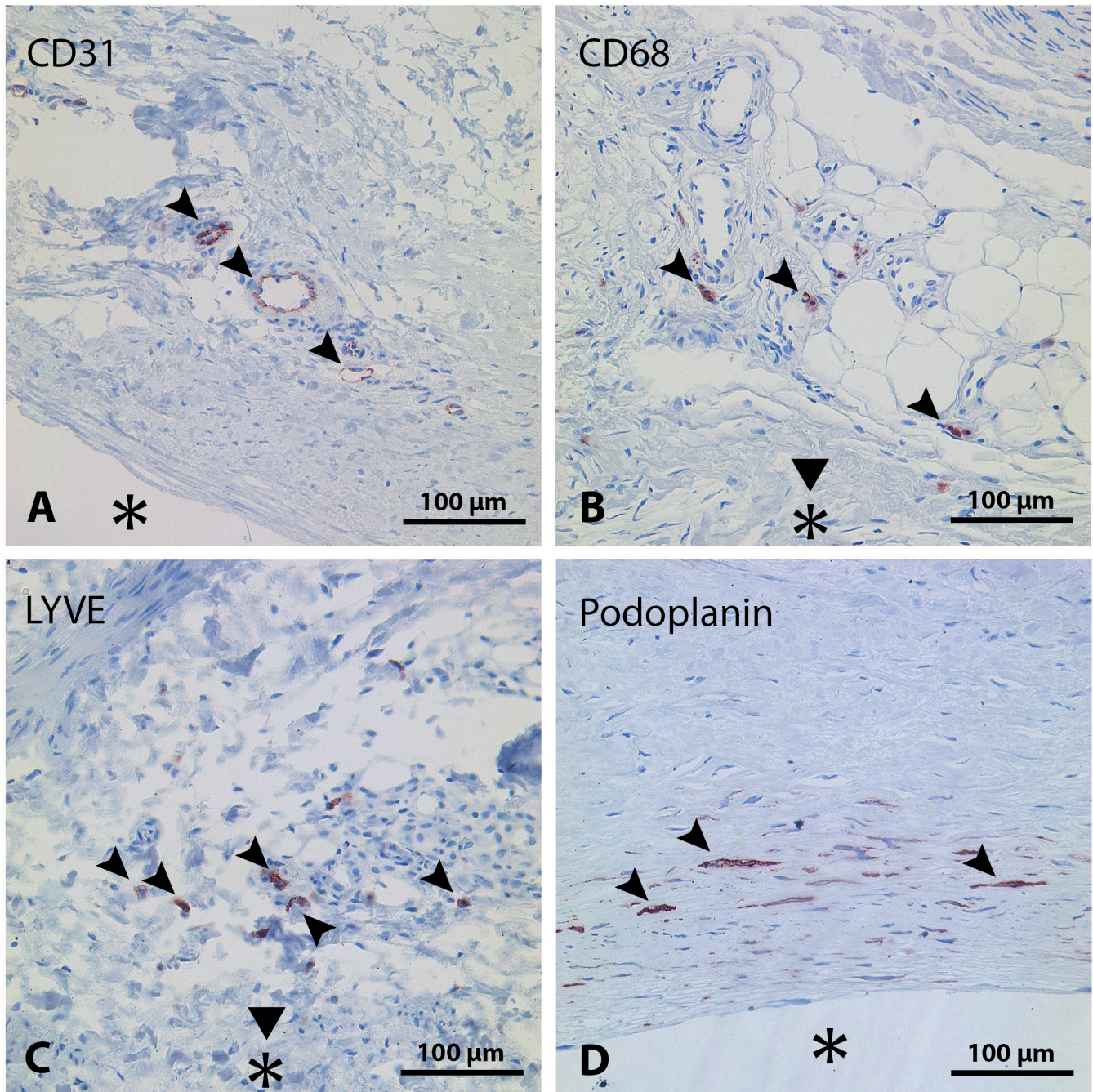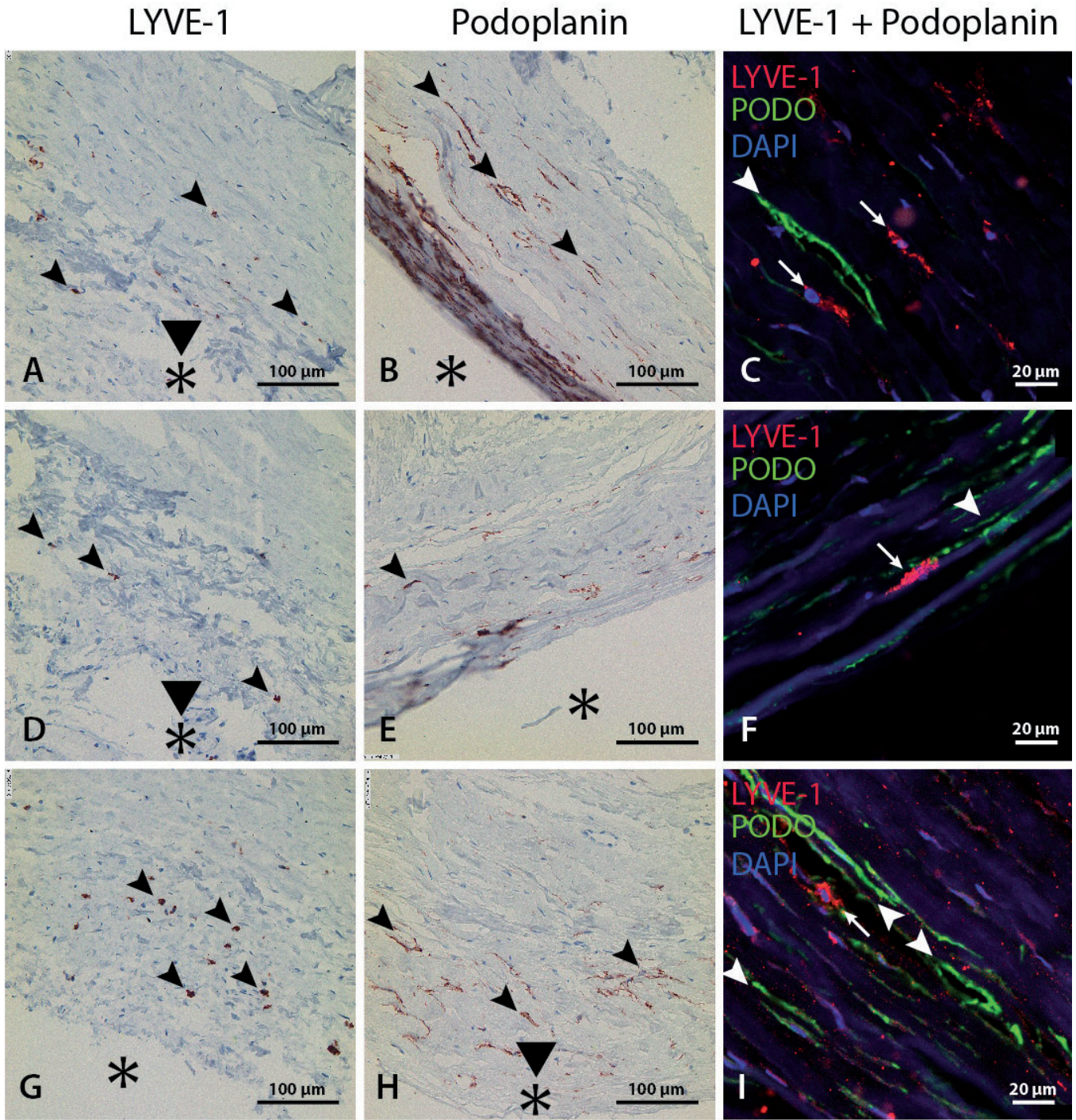


**Fig. 3.** Exemplary presentation of immunohistochemical staining of different GDD bleb capsules for CD31, CD68, LYVE-1 and podoplanin. **A.** CD31. Blood vessels (arrowheads) were clearly detected by CD31 exclusively in the outer layer of the capsule (asterisk shows bleb cavity) (example case of patient no. 1). **B.** CD68+ macrophages (arrowheads) were detected in the neighborhood of blood vessels of the outer layer (example case of patient no. 8; fat tissue is related to a subconjunctival fat prolapse; white arrowhead/asterisk points to the direction of the bleb). **C.** Single non-endothelial LYVE-1 positive cells (arrowheads) were assembled in groups and located in the outer layer (example case of patient no. 7; white arrowhead/asterisk points to the direction of the bleb). **D.** Podoplanin positivity was registered in cells and clefts (arrowheads) of the outer and/or inner layer of the capsules, but did not show a typical vessel lumen (asterisk shows bleb cavity) (example case of patient no. 12).

cells for the de novo formation of ocular lymphatic vessels in pathological disorders (Xu et al., 2007).

According to the current understanding, new lymphatic vessels grow from pre-existing lymphatics and bone marrow-derived lymphatic endothelial precursor cells (Maruyama et al., 2005). On the other



**Fig. 4.** Exemplary presentation of immunohistochemical staining of the same specimen (patient no.11) in light microscopy and confocal laser scanning microscopy. **A, D, G.** Shows LYVE-1 positive cells (arrowheads) in light microscopy. **B, E, H.** Presents podoplanin positive clefts (arrowheads) in light microscopy. **C, F, I.** LYVE-1 (red; arrows) and podoplanin (green; arrowheads) were not co-localized in confocal microscopy (single optical sections). Blue represents DAPI. The dense podoplanin-positivity at bottom of B is interpreted as fibroblast reaction in a chronic inflammatory environment (Quintanilla et al., 2019).

hand, a lymphatic vasculogenesis is also under debate. Martinez-Corral et al. found dermal lymphatic vessels formed independently of sprouting from veins (Martinez-Corral et al., 2015).

Lymphangiogenesis has an important role in tissue repair and tumor growth (Bock et al., 2013; Hos et al., 2015, 2016, 2017). During inflammation, lymphatic vessels play a key role by regulating the transport of lymphocytes to the lymph nodes (Hos and Cursiefen 2014; Ulvmar and Makinen 2016). It is well known that they proliferate during inflammatory processes (Alitalo 2011). Proinflammatory cytokines induce VEGF-C expression in a variety of cells and regulate lymphatic growth and survival (Bock et al., 2013). Moreover, in inflammatory tissue, granulocytes and macrophages intensively produce lymphangiogenic factors (Cursiefen et al., 2004; Maruyama et al., 2005). In chronic inflammation, lymphatic hyperplasia and/or enhanced lymphangiogenesis was described in a number of chronic human diseases e.g. ulcerative colitis as well as in inflammatory arthritis, psoriasis and chronic airway inflammation in mice (Kaiserling et al., 2003; Kunstfeld et al., 2004; Baluk et al., 2005; Zhang et al., 2007). A reason for the lack of lymphatic vessels in our specimens could be the low grade of inflammation. Nevertheless, the detected LYVE-1+ cells might be macrophages with participation in lymphangiogenesis under certain circumstances (e.g. under acute postoperative conditions) (Schroedl et al., 2008). Whether the observed LYVE1+ cells do also participate in the regulation of vascular tone, as known from other tissues, is currently not clear (Lim et al., 2018). Furthermore, also a temporaly and therefore undetectable existence of lymphatic vessels seems possible (prio to or after the time point of bleb excision. Interestingly, however, we found no LYVE-1+ cells in most of the repeated capsule excisions. One possible explanation could be infiltration of the tissue due to the acute stimulus of the GDD after implantation and a re-infiltration is missing after an excision of the infiltrated tissue.

Furthermore, recent evidence suggests aqueous humor exhibits anti-lymphangiogenic effects via VIP and alpha-MSH (Bock et al., 2016). The high amount of drained aqueous humor in GDD could indeed be partly responsible for its alymphatic nature (Bock et al., 2016).

While there seems to be a lack of permanent lymphatic vessels in non-functioning bleb capsules, conclusions regarding bleb failure cannot be drawn at this point. E.g., mast cells are also relevant for scarring and might also be involved in bleb failure, especially in glaucoma patients (Chang et al., 2009); however, other authors were unable to detect a change in numbers of mast cells following filtration surgery (Gwynn et al., 1993). Also, while there might be a correlation between postoperative outcome (e.g. IOP) and (macroscopic) bleb vascularization (Klink et al., 2011), we were unable to find any correlation between our immunohisto-chemical findings and the wide range of mean IOP at the time of bleb capsule excision in our small sample size.

Desirable would be an investigation which compares non-functioning and a larger number of functioning blebs regarding the appearance of lymphatics. However, such an investigation is not feasible, because the excision of functioning blebs is not possible for ethical reasons. In principle, it is conceivable that lymphatics play a role in the filtration of functioning GDD like that described for an aqueous outflow pathway after trabeculectomy but has to be proven in an animal model for obvious reasons (Benedikt, 1976).

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