Bleb-related Porcine Lymphatic Outflow Is Greater from Subconjunctival compared to Subtenon Blebs

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ABSTRACT

Aim: Understanding the mechanism of fluid outflow by comparing the subconjunctival and subtenon spaces can lead to improved ocular therapeutics. The purpose of the current study is to evaluate subconjunctival vs subtenon lymphatic outflow by creating tracer-filled blebs in each location.

Methods: Porcine ($n = 20$) eyes received subconjunctival or subtenon injection(s) of fixable and fluorescent dextrans. Blebs were angiographically imaged using a Heidelberg Spectralis ([Heidelberg Retina Angiograph] HRA + OCT; Heidelberg Engineering) and bleb-related lymphatic outflow pathways were counted. Optical coherence tomography (OCT) imaging of these pathways was used to assess structural lumens and the presence of valve-like structures. Furthermore, a comparison between tracer injection locations (superior/inferior/temporal/nasal) was made. Histologic analyses for subconjunctival and subtenon outflow pathways were performed, to confirm tracer co-localization with molecular lymphatic markers.

Results: Subconjunctival blebs demonstrated a greater number of lymphatic outflow pathways compared to subtenon blebs in every quadrant [superior: 6.10 ± 1.18 (subconjunctival) vs 0.50 ± 0.27 (subtenon); temporal: 2.30 ± 0.40 vs 0.10 ± 0.10; nasal: 5.30 ± 0.60 vs 0.30 ± 0.21; inferior: 6.00 ± 1.29 vs 0.1 ± 0.1; all comparisons $p < 0.001$]. For subconjunctival blebs, the temporal quadrant showed fewer lymphatic outflow pathways compared to the nasal side ($p = 0.005$).

Discussion: Subconjunctival blebs accessed greater lymphatic outflow compared to subtenon blebs. Furthermore, regional differences existed, with fewer lymphatic vessels temporal than at the other locations.

Clinical significance: Aqueous humor drainage after glaucoma surgery is incompletely understood. The present manuscript adds to our understanding of how lymphatics might influence filtration bleb function.

Keywords: Aqueous outflow, Blebs, Conjunctiva, Glaucoma surgery, Laboratory research, Lymphatics, Subconjunctival, Subtenon.

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INTRODUCTION

Normally, the subconjunctival and subtenon spaces of the eye are potential spaces. They can be expanded in the form of blebs during pathologic states or the treatment of certain eye diseases. Chemosis represents an expansion of these spaces due to total body fluid overload,[1] ocular surgery,[2] or ocular inflammation/infection.[3] In these examples, fluid collects due to alterations in vascular integrity, vascular permeability, or from changes in oncotic pressures. Treating the underlying disorder resolves the chemosis and closes the subconjunctival and subtenon spaces back to their potential states.

Subconjunctival or subtenon blebs can also be created during the treatment of eye diseases. During trabeculectomy glaucoma surgery, direct communication is surgically created from the anterior chamber through a low-resistance sclerostomy into the subconjunctival or subtenon spaces to form a bleb for intraocular pressure (IOP) lowering.[4] These blebs are intended to be permanent. For subconjunctival or subtenon drug delivery, pharmaceuticals are injected into these locations to treat various ocular disorders.[5] However, drug injection blebs are known to disappear quickly,[6] and drug availability maintain only as long as the drug-injection bleb remains. Ocular surgeons frequently experience that subconjunctival antibiotic and steroid blebs given at the end of surgery are gone by the next day. This implies that there must be a bleb-related outflow from subconjunctival and subtenon spaces. Understanding these pathways and the differences between the subconjunctival and

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subtenon spaces can be potentially useful in improving glaucoma surgery or drug delivery.

Bleb-forming minimally invasive glaucoma surgeries (MIGS) have recently become available (XEN gel stent;[7] Allergan) or are under advanced stages of development (Preserflo;[8] Santen Pharmaceuticals). These devices also connect the anterior chamber with either the subconjunctival or subtenon spaces. Specifically for the gel stent, various surgical approaches have been described and can involve an *ab interno* or *ab externo* approach.[9] In both cases, the internal lumen of the gel stent is located in the anterior chamber, but the external lumen of the device can be positioned near the sclera (subtenon: in or under Tenon's layer) or more superficially (subconjunctival: above the Tenon's layer but under the conjunctiva).[10]

For subconjunctival MIGS, considerable debate has arisen as to where the stent should be placed (subconjunctival or subtenon) for best success. Traditional glaucoma drainage tubes (Baerveldts and Moltenos) are most commonly placed subtenon, but successful supratenon (or subconjunctival) placement has been described as well.[11] For subconjunctival MIGS, Yu et al. described that bleb persistence after microfistula stent implantation depended on accessing subconjunctival lymphatic vessels near the external side of the implant.[12] However, Lenzhofer et al. reported that the intra-or subtenon placement of the gel stent outer lumen led to better IOP reduction with lower secondary needling rates at 1-year compared with subconjunctival placement.[10] Therefore, further studies are needed to investigate whether subconjunctival or subtenon bleb-related outflow is better.

Many groups have shown that lymphatics are responsible for draining the subconjunctival and subtenon spaces.[12–19] First, this makes sense as lymphatics are generally considered to drain extracellular spaces. However, blood vessels are an alternative to the historical description of degenerative veins draining glaucoma surgical blebs.[20] Recently, our group studied the drainage pathways of experimentally induced blebs in vertebrate eyes.[21] Lymphatics were implicated based upon structural evidence [(1) OCT presence of semilunar valves in bleb-related outflow pathways pointing in the direction of flow[13,21] and (2) two-photon microscopy[21,22] observation of blind-end tips in bleb-related outflow pathways that were reminiscent of lymphatic capillaries]. Molecular evidence further supported a lymphatic identity as isolated bleb-related outflow pathways expressed lymphatic (PROX1 and podoplanin) but not blood vessel markers.[21]

Therefore, the purpose of this current study is to investigate bleb-related lymphatic outflow from the subconjunctival vs the subtenon spaces by injecting the same tracer in each location. We hypothesize that bleb-related outflow will be greater in one location compared to the other.

METHODS

Study Materials

Postmortem enucleated porcine eyes were obtained (SiouxPreme Packing Company, Iowa; within 48 hours of death). Excess ocular tissue was trimmed to expose bulbar conjunctiva, and the eyes were pinned to Styrofoam.

Injection Protocols

Each porcine eye received a subconjunctival ($n = 10$) or subtenon ($n = 10$) injection of fluorescent dextran tracer in different locations (superior, inferior, temporal, or nasal; 40 total injections).

Fluorescent fluorescein-isothiocyanate-dextran (FITC) (Thermo-Fisher; Waltham, MA; D7136; 500 kD) were diluted to 2.5 mg/mL in a balanced salt solution. In each case, 50 microliters of the tracer were injected into the subconjunctival or subtenon space, near the limbus, and outflow pathways were visualized posterior to that. Figure 1 shows the injection procedure. Videos of the procedure are available as supplementary material online (Clips 1 and 2).

The subconjunctival or subtenon injections were performed by an experienced ophthalmologist surgeon (JYL, GA, or ASH) under microscopic view. To make a subconjunctival bleb, a 30-gauge needle was placed as superficially as possible, directly under the conjunctiva. The conjunctiva was raised with smooth forceps, and the conjunctiva was pierced using the needle (Clip 1 and Fig. 1). After insertion, the needle needed to be clearly visualized with the creation of a bright-colored bleb with sharp margins for this to be considered a subconjunctival injection. For a subtenon bleb, the 30-gauge needle was directed deep, toward the scleral surface (Clip 2 and Fig. 1). The needle tip needed to be hazy and barely visible under the tissue with the creation of a dull-colored bleb with blurred margins to be considered a subtenon injection.

Ocular Surface Lymphangiography and Optical Coherence Tomography

Bleb images were obtained using a Heidelberg Spectralis HRA + OCT (Heidelberg Engineering, Heidelberg, Germany) on the Spectralis confocal scanning laser ophthalmoscope (CSLO) angiographic fluorescein setting as previously described(21). A 55-degree lens was set to a 25-diopter focus. When a suspected outflow pathway originating from the bleb was observed on angiographic imaging, it was counted as lymphatic outflow if it had a lumen with valve-like structures on the anterior segment OCT. To assess this, anterior segment OCT was conducted using the Spectralis HRA + OCT concurrently with ocular surface lymphangiography on the Anterior Segment Module (Scleral Mode). In angiographically positive structures, single-line OCT scans with a 15-degree scan angle (3.9-micron axial and 11-micron lateral resolution; ~4.5 mm) were taken. CSLO angiographic and OCT cross-comparison was made because precise image registration, suitable even for longitudinal patient care, allowed comparisons.[23,24]

Quantitative Analyses of Lymphatic Pathways

To test whether subconjunctival tracer injection in certain locations (superior/inferior/temporal/nasal) was better or worse for accessing
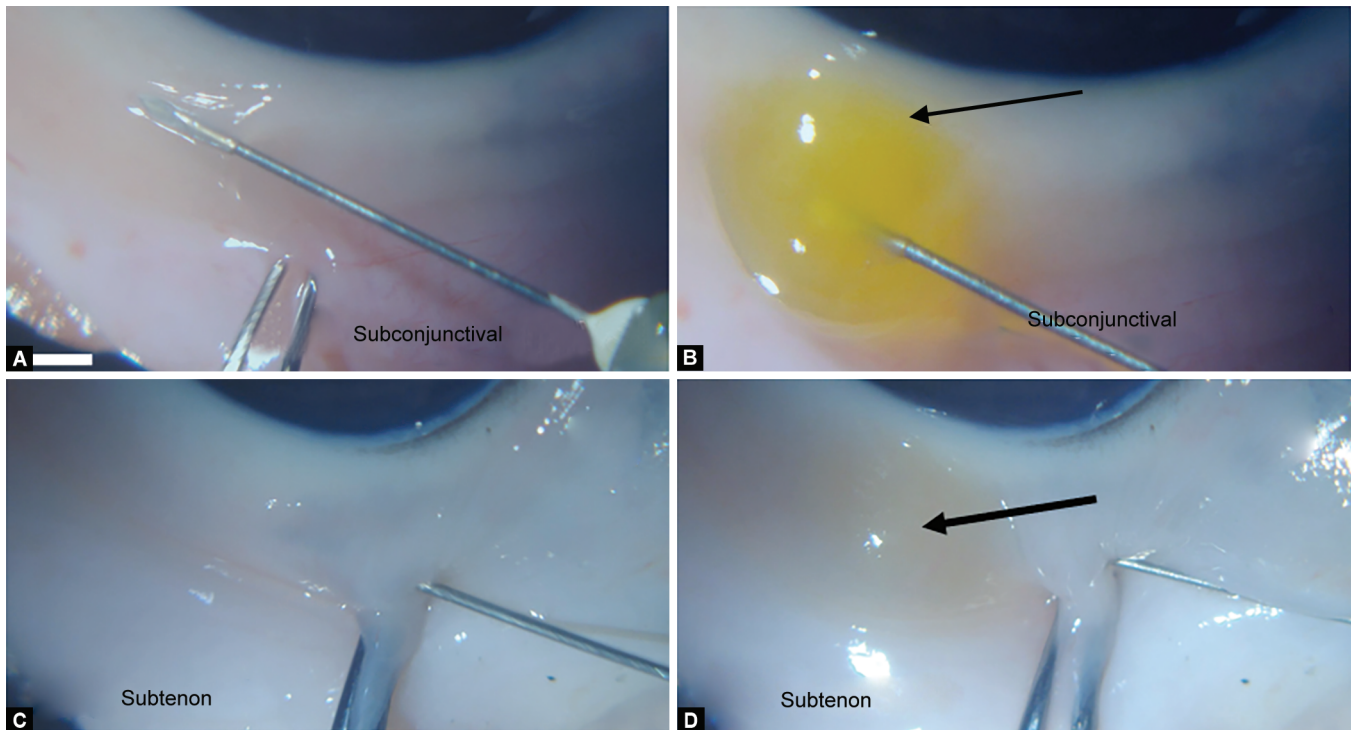
Figs 1A to D: Subconjunctival and subtenon bleb injection technique. (A) Subconjunctival blebs were formed with the needle penetrating the conjunctiva superficially and the needle bevel was always visible; (B) The subconjunctival yellow bleb (black arrow) expanded with clear coloration and borders; (C) Subtenon blebs were formed with the needle aimed deeper (near the scleral surface) with the bevel barely visible; (D) The subtenon bleb (black arrow) was also yellow but dull in color and the edges were less defined owing to deeper positioning. Scale bar: 2 mm

a lymphatic outflow pathway, a Kruskal–Wallis test (PASW, version 9.3) was performed as previously described for segmental outflow determination.[25] Kruskal–Wallis test is a nonparametric statistical test used to detect mean differences across groups. It was chosen in this analysis because of the relatively small sample size. Here, the null hypothesis was that the number of outflow pathways across all quadrants was equal. Rejecting the null hypothesis would mean that there was an overall statistically significant difference in the number of bleb-related outflow pathways across quadrants. *Post hoc*, the different quadrants were compared using six *t*-tests. As each eye generated data points for superior, inferior, nasal, and temporal quadrants, paired *t*-tests were used. Bonferroni's correction was applied to correct for multiple testing (significance only for $p < 0.008$). Independent *t*-tests were used to compare the number of outflow pathways between subconjunctival and subtenon blebs. All quantitative data are shown as mean standard error of the mean (± SEM).

Histology and Immunofluorescence

Given that ocular surface lymphangiography may have missed bleb-related outflow pathways owing to the depth of the pathway (particularly for subtenon injections), immunofluorescence of histological sections was made adjacent to subconjunctival and subtenons blebs. After injection of fluorescent and fixable dextrans and visualization of bleb-related outflow pathways, porcine tissues (including the bleb and outflow pathways) were carefully excised using scissors and placed in paraformaldehyde for 15 minutes at room temperature followed by overnight at 4°C to trap the tracer. The fluorescent FITC-dextrans were fixable due to an attached lysine moiety so that the tracer could

be trapped in the luminal walls of the outflow pathways. This allowed re-identification of the same pathways after histological sectioning as the pathways would be fluorescent. The tissue block was then marked for orientation, placed in Tissue-Tek (Sakura; Torrance, CA), frozen under liquid nitrogen, and 8-micron sections cut (Thermo Scientific CryoStar NX70; Waltham, MA) onto Superfrost slides (Fisher; Pittsburgh, PA). Blebs from two eyes each for subconjunctival and subtenons conditions were assessed with a total of twenty sections cut distal to the bleb for each eye. After blocking sections with 5% bovine serum albumin in PBS and permeabilization with 0.3% Triton X-100 in PBS, sections were incubated with primary and subsequently secondary antibodies at 4°C overnight. Antibodies included: goat polyclonal anti-human PROX1 (1:100; R&D Systems, AF2727; Minneapolis, MN), and anti-goat Texas Red secondary antibodies (1:100; Thermofisher, Waltham, MA). Association for Research in vision and ophthalmology (ARVO) consensus guidelines for identifying ocular lymphatics were adhered to.[26] First, consensus guidelines specifically state that they do not apply to conjunctiva given that lymphatics are so well-established there.[26] In fact, the study of subconjunctival lymphatics is so established that the lymphatics can be identified and studied label-free as solely valve-containing luminal pathways.[27] Despite this, we previously went beyond recommendations and applied consensus guideline criteria to porcine eyes and validated PROX1 as well as other markers.[21] Hence in this work, we proceed with PROX1-only labeling. Slides were mounted with 4′6-diamidino-2-phenylindole (DAPI) containing mounting medium (Vector Lab; Burlingame, CA) and viewed under a Keyence BZ-X700 digital imaging microscope (Keyence; Chicago, IL). Sections were imaged using a 10× or 20× plan-fluor

lens. All images were taken using identical settings for illumination and image capture sensitivity (Keyence imaging software v.f1.51). For FITC-dextrans (EX BP 470/30, DM 495, EM BP 520/35), DAPI (EX BP 360/40, DM 400, EM BP 460/50) and Texas Red (EX BP 560/40, DM 565, BP 630/75), appropriate filters were used.

RESULTS

Subconjunctival and subtenon bleb-related outflow pathways were visualized using fluorescent imaging and OCT (Fig. 2). The pathways were sausage-shaped with invaginations (Figs 2A and D; white arrows). To be considered a true lymphatic pathway, angiographically positive structures were probed using OCT, demonstrating lumens that corresponded with the angiographic signal. Lymphatics normally are collapsed, but they appeared large and dilated in this study due to the pressure head from the injection as well as due to the lack of fluid transport to downstream vessels owing to the postmortem state. Further, these pathways contained semilunar valves (Figs 2C and F, white arrows) in the direction of flow (Figs 2B and E, green dotted arrows point in the same direction as C/F green arrows) that matched invaginations seen on the angiographic image, structurally confirming a lymphatic identity.

Quantitatively, subconjunctival blebs ($n = 10$ eyes) showed a greater number of lymphatic outflow pathways compared to subtenon blebs ($n = 10$ eyes) in every quadrant of porcine eyes [superior: 6.10 ± 1.18 (subconjunctival) vs 0.50 ± 0.27 (subtenon); temporal: 2.30 ± 0.40 vs 0.10 ± 0.10; nasal: 5.30 ± 0.60 vs 0.30 ± 0.21; inferior: 6.00 ± 1.29 vs 0.1 ± 0.1; all locations: 4.93 ± 0.45 vs 0.25 ± 0.12; all comparisons $p < 0.001$; mean $\pm$ SEM] (Fig. 3). Overall difference
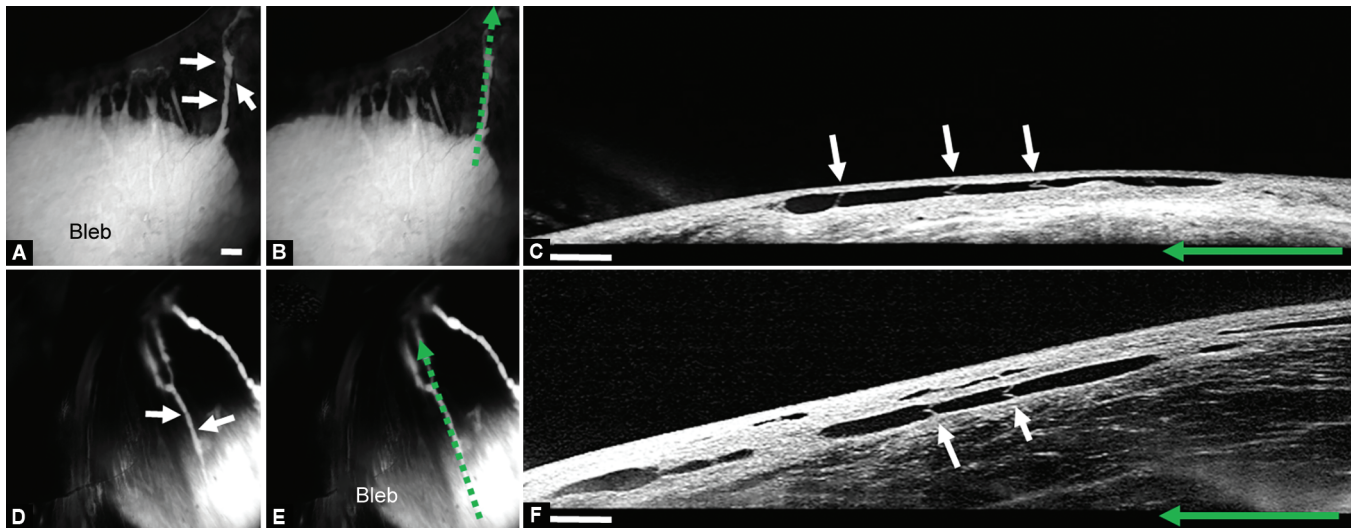
Figs 2A to F: Outflow pathways from subconjunctival and subtenon blebs. (A) Subconjunctival bleb showing an outflow pathway. Invaginations along the pathway (white arrows). The limbus is inferior to the bleb; (B) The same image as A with the OCT B-scan placement is denoted by the dotted green arrow; (C) OCT on the pathway seen in (B) demonstrates a lumen that is very close to the ocular surface with visible semilunar valves (white arrows) that match invaginations in (A). The valves are in the direction of flow off of the bleb [green arrow in (C) matches the direction of the dotted green arrow in (B)]; (D) Subtenon bleb showing an outflow pathway. Invaginations along the pathway (white arrows). The limbus is below and to the right of the bleb; (E) The same image as (D) with the OCT B-scan placement is denoted by the dotted green arrow; (F) OCT on the pathway seen in (E) demonstrates a lumen containing semilunar valves (white arrows) that match invaginations in (D) but is deeper from the ocular surface compared to (B). Again, the valves are in the direction of flow off of the bleb [green arrow in (F) matches the direction of the dotted green arrow in (E)]. Scale bars: 500 microns
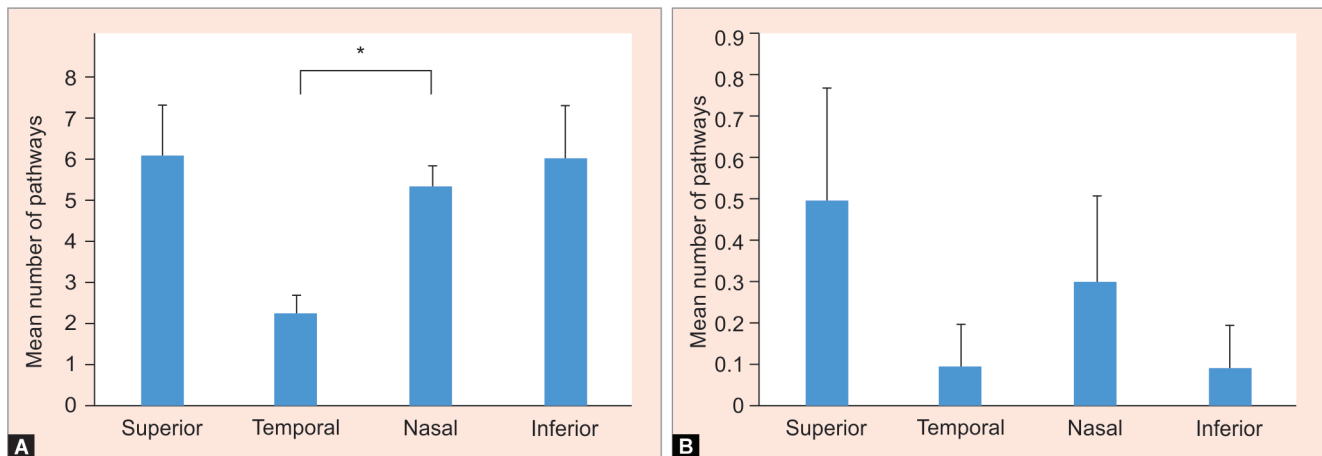
Figs 3A and B: Average Number of outflow pathways per subconjunctival or subtenon bleb. (A) Subconjunctival blebs demonstrated a varying average number of outflow pathways in different quadrants of the eye; (B) Subtenon blebs showed fewer outflow pathways with no differences between the quadrants. Bars show mean $\pm$ SEM. * = $p < 0.008$, accounting for Bonferroni correction

across subconjuctival bleb-related outflow pathway locations was tested using a Kruskal–Wallis test showing that there was an overall difference between the quadrants ($p = 0.004$). Pairwise comparisons were performed showing only a Bonferroni-corrected statistically significant difference comparing temporal and nasal quadrants ($p = 0.005$). The outcomes comparing other subconjunctival quadrants were: superior vs temporal ($p = 0.014$), superior vs nasal ($p = 0.462$), superior vs inferior ($p = 0.960$), temporal vs inferior ($p = 0.014$), and nasal vs inferior ($p = 0.672$). Therefore, non-Bonferroni-corrected results also showed that the temporal quadrant had statistically significantly fewer bleb-related outflow pathways compared to the superior and inferior quadrants. For subtenon blebs, the number of lymphatic outflow pathways did not differ according to bleb location ($p = 0.53$, Kruskal–Wallis test).

Given that subtenon pathways are deeper, it was considered that ocular surface lymphangiography fluorescent imaging may have missed visualization of these pathways because they were too deep. Thus, tissue was fixed (to trap the fluorescent tracer) and histological sections were made immediately adjacent to subconjunctival and subtenon blebs. For subconjunctival blebs, outflow pathways were visualized (FITC-labeled lumens), and these vessels showed co-localized positive immunoreactivity for a lymphatic marker (PROX1) in endothelial cells (Fig. 4 - left panel), replicating prior results.[21] For subtenon blebs, histological sections were cut from the bleb to a point where the fluorescent bleb was no longer apparent. Then, sections cut distal to the bleb showed zero pathways in FITC-labeled

lumens although periodic pathways expressing PROX1 were found (Figs 4 - right panel and 5).

Discussion

The primary result of this study was that subconjunctival blebs accessed a greater number of lymphatic vessels compared to subtenon blebs. Subconjunctival outflow was also segmental, in support of prior research.[14,21] Previous reports suggested in multiple species that more subconjunctival lymphatics were present on the nasal side of the eye.[18,21] This fits a developmental hypothesis where anterior segment lymphatics arise on the nasal side of the eye and then grow temporal.[18] The current report yields a slightly different but similar result with the temporal side of the eye having less lymphatic outflow (as opposed to the nasal side having more) which is also consistent with the above developmental hypothesis.

To further evaluate the segmental lymphatic concept, current results could be combined with prior quantitation of subconjunctival bleb-related lymphatic outflow.[21] Given that tracer-choice was different between the studies [FITC-dextran vs indocyanine green (ICG)], the absolute numbers of outflow pathways could not be compared as opposed to a calculated percentage of bleb outflow pathways per quadrant as a normalized assessment for each eye. Using this normalized method, the results in this work continued to show an overall difference between
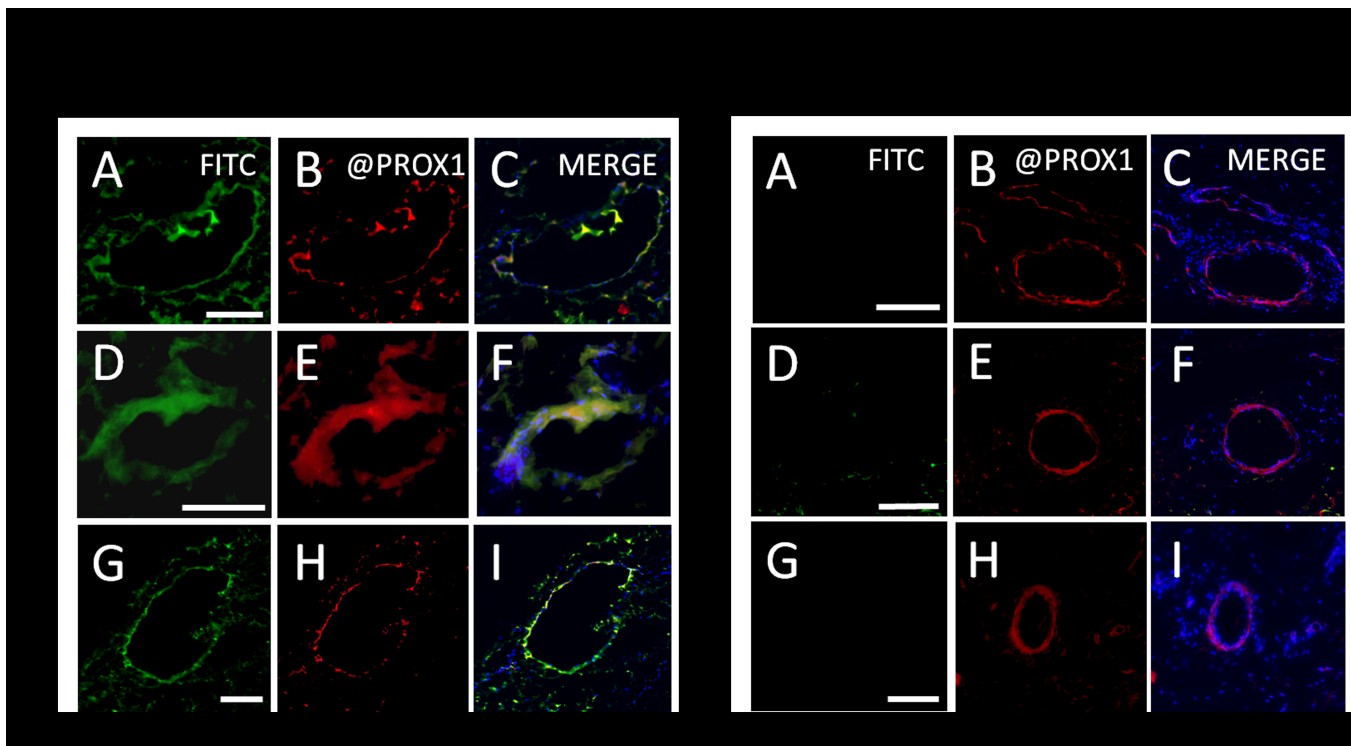
Figs 4A to I: Left panel. PROX1 immunofluorescence of conjunctiva distal to subconjunctival blebs with visible outflow pathways on angiography. Outflow pathways off blebs were fixed with paraformaldehyde to trap fixable and fluorescent dextrans. (A/D/G) Sectioning along those pathways visualized the lumens as fluorescent dextran positive. (B/E/H) Immunofluorescence against PROX1 identified lymphatics. (C/F/I) Fluorescent dextran-positive lumens off blebs overlapped with PROX1 immunofluorescence. **Right panel.** PROX1 immunofluorescence of conjunctiva distal to subtenons blebs without visible outflow pathways on angiography. In the case of the ocular surface, lymphangiography could not visualize deep outflow pathways off subtenon blebs owing to depth, sections were cut distal to subtenon blebs (where no outflow pathways were seen in angiography). (A/D/G) No deep fluorescent dextran-positive lumens were seen. (B/E/H) However, random PROX1 positive structures were still visualized. (C/F/I) Merge with DAPI. Scale bars: all 100 microns
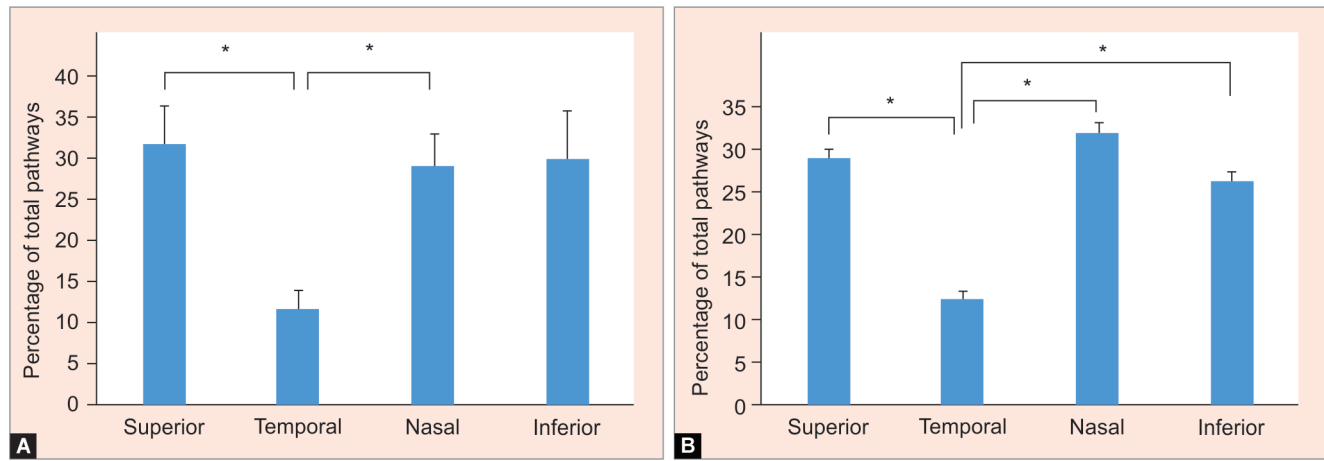
Figs 5A and B: Pooled subconjunctival outflow pathway results. Subconjunctival outflow pathway data was pooled with prior results.[21] Different tracers were used in these two studies which confound counting of the absolute number of pathways. Thus, results were changed to express the percentage of the total number of outflow pathways in each quadrant per eye. (A) Data from this report (*n* = 10 eyes); (B) Pooled data from this report and Akiyama et al.,[21] (*n* = 11 eyes; for total = 21 eyes). Bars show mean ± SEM. * = *p* < 0.008, accounting for Bonferroni correction

the quadrants (Kruskal–Wallis test *p* = 0.003) with the temporal quadrant showing significantly fewer percentages of lymphatic outflow pathways compared to the superior (*p* = 0.007) and nasal quadrants (*p* = 0.007). Using this normalized approach and combined with previous data[21] the percentage of bleb outflow pathways in the temporal quadrant was significantly less than those observed in all other quadrants (superior vs temporal, *p* = 0.003; nasal vs temporal, *p* < 0.001; and inferior vs temporal, *p* = 0.002). So, overall, the regional pattern was consistent between studies, and the overall conclusion is that the temporal side of the eye supports less subconjunctival lymphatic outflow.

Blebs are complex because there are many types of blebs (endogenous chemosis, surgical, and drug-delivery). From a surgical standpoint, ideal bleb formation is likely important for bleb-related glaucoma surgical success. In trabeculectomy, blebs can fail due to sclerostomy closure or scarring of the bleb to the scleral surface. The first problem is addressed by subconjunctival MIGS as the surgical device ensures maintained patency between the anterior chamber and the subconjunctival or subtenon spaces. The second problem is usually addressed by using anti-metabolites (such as 5-fluorouracil[28] or mitomycin-C[29]) to limit scarring. Anti-metabolites are routinely used in both traditional trabeculectomies and subconjunctival MIGS. Nevertheless, both trabeculectomies and subconjunctival MIGS still fail, and this can occur even in the presence of a physical bleb. A third circumstance for bleb failure is where aqueous successfully enters a fully formed bleb but then cannot exit. Loss of lymphatic outflow may be the cause here. Anti-metabolites have been hypothesized to damage lymphatics,[30] and histology of failed trabeculectomy blebs have shown them to be lymphatic.[31]

Location of the bleb or the location of fluid in the bleb may impact how easily fluid exits. Yu et al. suggested that subconjunctival MIGS bleb persistence was associated with a connection to subconjunctival lymphatics.[12] They also described that ocular surface lymphatics were mostly at the subconjunctival level.[14] The results in this report are consistent with these ideas as subconjunctival tracer injection led to easily observable bleb-related outflow pathways while subtenon tracer injection did not. The patterns were also segmental as the temporal side of eyes demonstrated less overall bleb-related outflow (Fig. 3). These

results raise the idea that subconjunctival MIGS should be placed immediate below the conjunctiva on the nasal side of the eye. However, clinical studies have implied that subtenon placement of these surgeries may lead to greater IOP reduction with less secondary needling.[10]

How can better outflow from the subconjunctival space be reconciled with potential greater IOP reduction from subtenon surgical placement? The answer refers back to the overall complexity of bleb biology. While bleb-related outflow is very important, the final surgical result depends on more than just this one factor. For example, while temporal subconjunctival lymphatics are fewer and lymphatics develop from a nasal root, placing glaucoma surgical blebs in either interpalpebral location would be ill-advised because of the risk of infection.[32] One must also consider ease of surgery and post-op scarring. Some surgeons prefer an *ab externo* approach[9] using full dissection of the subtenon space with subtenon stent placement because of greater surgical control. Therefore, it is important to consider that the results of this work do not necessarily conclude that subconjunctival surgical placement is better just as direct nasal placement is ill-advised. Instead, the results state that subconjunctival lymphatic outflow is greater and that multiple factors likely play into the final result of bleb-forming glaucoma surgeries.

One theoretical approach to improving surgical success is then to drive lymphatic formation into blebs. Many MIGS ablate or damage pathways in the eye, such as the trabecular meshwork, without considering the native purposes of these structures.[33] For blebs, antimetabolites necessary to prevent fibrosis may damage subconjunctival lymphatics.[30] Lymphatic growth is endogenously driven by vascular endothelial growth factors (VEGFs). VEGF types-A and B are well-known to proliferate blood vessels[34] in the eye and drive neovascular diseases such as age-related macular degeneration and neovascular glaucoma. Biologics directed against these VEGFs have proven therapeutic benefits. Alternatively, VEGF type-C (VEGFC)[35] is known to specifically promote lymphatic > blood vessel growth with certain mutations (VEGFC-C156S)[36] especially specific for lymphatics. Therefore, driving lymphatic development into a surgical bleb may be one strategy to improve bleb-related outflow in the setting of anti-metabolites by restoring a natural

lymphatic presence. Then decisions regarding surgical placement (subconjunctival vs subtenon) can be based upon other factors.

Several limitations in this study must be discussed. The results in this current research come from *ex vivo* porcine eyes. More *in vivo* research and evaluation of human eyes is needed. In human patients, there are a handful of case reports showing bleb-related outflow pathways after tracer injection.[15–17,19,37] Better work has been performed in post-trabeculectomy patients who received intracameral trypan blue injection during cataract surgery showing that greater IOP reduction is seen when there are a greater number of observed pathways.[15] However, simultaneous structural evaluation in prior reports was absent. Recently, combined tracer-based and structural analysis of subconjunctival outflow were performed in human subjects who had retinal diseases requiring intravitreal drug therapy.[38] ICG was added to lidocaine necessary for anesthesia prior to intravitreal drug injection showing irregular pathways[38] which were clearly different from known venous pathways elicited by aqueous angiographic imaging.[38–40] Further, simultaneous OCT on the bleb-related outflow pathways also showed valve-like structures in human patients, supporting a lymphatic identity. In the future, additional molecular approaches would be beneficial, but this requires surgical removal of human tissue for study. Lastly, the ability to pharmacologically manipulate these pathways eventually needs to be tested in human tissue as well.

In conclusion, this study shows that bleb-related lymphatic outflow is greater in the subconjunctival space compared to the subtenon space and that the temporal side of the eye demonstrates less outflow in *in vitro* porcine eyes.

Clinical Significance

These results have potential implications for glaucoma surgery and ophthalmic drug delivery. Further study is needed to determine if native or induced lymphatic presence/absence can be leveraged to improve patient care.

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Supplement Videos

Clip 1: Subconjunctival Bleb Formation.
Clip 2: Subtenon Bleb Formation.

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