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SPECIAL ISSUE REVIEW

Lymphatic vessels of the eye – old questions – new insights☆

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Dedicated to Prof. Prof. h. c. Dr. med. Michael Földi with deep gratitude, who has not only supported our research project, but has often been of ground-breaking importance to us.

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ABSTRACT

Due to its accessible position and tissue heterogeneity, the eye is ideally suited for studying the lymphatic system. As early as the 19th century, questions about the origin and function of this system were discussed. For example, whether *Schlemm*'s canal, which is of particular importance in the pathogenesis of glaucoma, is a lymphatic vessel, or does this vascular system begin with finger-shaped protuberances? Despite the discovery of lymphatic endothelial molecules and the use of molecular imaging technologies, these questions are still discussed controversially today.

Leber demonstrated in 1873 with a solution consisting of two dyes of different particle size that only the smaller particles from the anterior chamber of the eye filled the episcleral and conjunctival veins around the corneal margin. He believed to have proven – to be read in the historical review of our article – that the *Canalis Schlemmii* in humans is a venous circular vessel and not a lymphatic vessel. In our own investigations, we reduced the rather contradictory and complex question of whether there are lymphatic vessels in the eye to the question of whether there are drainage connections between the different sections of the eye and the lymphatic system or not. With different radioactive tracers and combined with unilateral ligation of cervical lymph vessels, we observed outflow from the subconjunctival and retrobulbar space, from the anterior chamber and the vitreous body. The rate of discharge of the radioactive tracer was determined by the radiopharmaceutical and injection site. In analogy to the lymphatic drainage of the head we found a segmental drainage of lymphatic substances on the eye. Vitreous humour and retrobulbar space were drained by lymphatic vessels, predominantly to the deep cervical lymph nodes, while anterior chamber and subconjunctival space drains predominated over the superficial cervical lymph nodes. Eyeball tattoos – as loved by some fan communities – should therefore cause a coloured staining of the superficial cervical lymph nodes. The boundary of the drained segments would be in the area of the eyeball's equator.

According to the textbooks, the lymph is actively removed from finger-shaped initial segments via precollectors and collectors with properly functioning intraluminal valves and smooth muscle cells in the vessels' media. In patients with spontaneous conjunctival bleeding, however, we observed phenomena in the conjunctival lymph vessels, which ca not be explained with old familiar ideas. At nozzle-shaped vessel constrictions separation of blood components occurred. The erythrocytes formed partially a so-called fluidic "resting bulk layer". Parallel vessel parts caused a retrograde filling of already emptied segments. These observations led our experimental investigations.

In the literature, there are different scanning electron microscopy (SEM) images of lymphatic endothelial surfaces; nevertheless they are unassigned to a particular vessel segment. In the conjunctiva, we studied the question whether there is a dependence between vessel diameter and the surface characteristics of endothelial cells (after unfolding by lymphography). A constantly applied photo-mathematical procedure for all specimens allowed determining the size of the cross sections. The specimens were randomized into seven groups with diameters of 0.1–1.0 mm and above and examined by SEM. In the smallest vessels (diameter = 0.11 mm), the impressions of the occasionally occurring nuclei in the lumen were clearly impressive. With increasing diameter, these impressions were lost and the individual endothelial nuclei could no longer be identified. Rather, one recognized only wall-like structures. In vessels of intermediate diameter (0.3–0.4 mm), structures could be seen on the surface similar to reticular fibres. With increasing diameters, their prominent character weakened. In the group with diameters above 0.5 mm, wavy surface structures were shown. Finally, in vessels of diameters over 1.0 mm, a uniform, flat surface was observed. Regardless of the collection site of the specimens, we found certain surface characteristics related to the vessels' calibre.

☆ This paper belongs to the special issue Human lymph vessels.

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In further investigations by means of interstitial dye lymphography, we were able to demonstrate in the conjunctiva that under increasing injection pressure, additional vessels stained from finger-shaped processes. At least in the conjunctiva, the existence of so-called "blind-ending initial segments" seems doubtful (despite the fact that initial segments or "initial lymphatics" would begin in periphery, not end). Rather, these are likely to be temporary filling states. SEM investigations were carried out on the internal structure of these dome-shaped vessel parts by means of a specially developed preparation technique. Despite numerous variants in the lymphographic design of the blind bags – in the form of finger, balloon, dome, piston, pyramidal, double-humped and spearhead-like endings – slot-shaped, lip-shaped and saw blade-like structures were repeatedly found, similar to a zipper. These findings suggest preformed connections to the next segment and may control lymphatic flow.

To clarify the retrograde fluid movements, we examined the lymph vessels' valves or those structures that were previously interpreted as valves. The different structures found could be subdivided into three groups. The lack of common bicuspid structures provides an explanation for retrograde fluid movement. That nevertheless a directional flow is possible, is explained by the flow model developed by Gerhart Liebau.

Conjunctival lymphatics show intraluminal structures by double contrast injection, which we divided into four groups due to anatomical differences:

a. Gusseted or fan-shaped lamellae that insert on the vessel wall.
b. Filamentous or columnar intraluminal structures.
c. Larger dividing wall-like structures that extended far into the lumen and imitated valve-like structures.
d. Complex structures with valve-like and filamentous parts, which appeared stretched in the lumen. These structures were predominantly found in vessels with a diameter of about 1.0 mm.

An accurate statement about the occurrence of certain intraluminal vascular structures in certain vascular calibres was possible only conditionally. However, complex and extended structures (group d) were found almost exclusively in larger vessel calibres (diameter > 0.9 mm). The structures are reminiscent of published findings in the "collector channel orifices of *Schlemm*'s canal". They should play an important role in the regulation of the intraocular pressure, or the balance between production and outflow of the aqueous humour. The influence of such structures on the function of the lymphatic vessels is not yet known. As an approach models could be used, which for instance are applied in the water industry for the drainage, the degradation of introduced substances, or the detention pond. The latter serves for the retention and purification of drainage water (storage, treatment and reuse of drainage water). Dead zones, barriers, short-circuit currents and swirling are further hydraulic terms. Can intraluminal vascular structures, for example, affect the lymphatic flow and thus the mechano-sensitivity of lymphatic endothelial cells?

Whatever interpretation model we use, the warning of the Swiss anatomist His from 1862 is still true today that all theories about the formation and movement of lymph should be based on precise anatomical basics. This review article therefore tries to make a contribution therefore. Despite knowing of lymphatic endothelial molecules, despite the discovery of the role of lymphangiogenic growth factors in diseases and the use of molecular imaging technologies, we still know too little about the anatomy and function of the lymphatic system.

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1. Introduction

Recent contributions on the origin and function of the lymphatic vessels of the eye create the impression that we are not only adequately informed about this subject, but also that old controversies from the last century and before have been solved, such as the questions "*Are there lymphatics in the eye?*", "*Do [initial] lymphatics start as finger-shaped evaginations?*", or "*Is Schlemm's canal a lymphatic vessel?*" Are these questions really solved, or are old debates fought out with new arguments?

The endothelial cells of *Schlemm's* canal express VEGFR-3 and are therefore responsive for VEGF-C; quite similar to lymphatic endothelial cells (Aspelund et al., 2014), but are not positive for D2-40 and LYVE-1 (Herwig et al., 2014).

Koina et al. (2015) conclude in their paper on the 'Evidence for lymphatics in the developing and adult human choroid' that "*the system of blind-ended initial lymphatic segments seen just external to the fenestrated vessels of the choriocapillaris is ideally placed for recirculating extracellular fluid and strategically placed for immune surveillance. The presence of a system of lymphatic-like channels in the human choroid provides an anatomical basis for antigen presentation in the posterior eye, with a possible route from the eye to the sentinel lymph nodes, similar to that already described for anterior eye lymphatics.*"

Shortly afterwards this paper was criticized by Heindl et al. (2015) entitled "*Sufficient evidence for lymphatics in the developing and adult human choroid?*": "*This study does not meet the recently published consensus criteria on the immunohistochemical detection of ocular lymphatic vessels, and therefore, in our opinion, requires critical revision.*"

On March 21, 2017, Michael Jeltsch (Wihuri Research Institute, Helsinki) expresses himself in a similar way in his personal communication to the authors: "*There may be lymphatic vessels in the choroid, as postulated in 1996* (Junghans et al., 1996)*, but the Koina et al. Paper has so many weird results that I want to have better data before I believe that. It contains many statements that are incompatible with the observations of others (for example, Prox-1 not in the nucleus, but in the cytoplasm). Also, the paper contains some contradictions in itself: e.g. figure 10-Q is similar to figure 1M, the 'macrophages' do not cluster around the capillary in figure 10-Q, and they are well distributed evenly throughout the image field. Either 10-Q or 1L-M is misinterpreted; etc. Ultimately, everything depends on the subjective classification of the vessels in the TEM images as 'lymphatic' or 'blood vessel'. The authors write themselves: 'presumably' lymphatics (but the many mistakes in the paper do not mean that there are 100% no lymphatics in the choroidea).*

That Koina and co-workers could not accept the criticism was to be expected. From their reply, which appeared a few weeks later, two sentences are should be quoted here (Chan-Ling et al., 2015):

"*The ultrastructural characteristics of lymphatics presented in our study are well defined and compelling. Recent studies have demonstrated, for the first time, the existence of lymphatics; in the central nervous system (CNS), citing our work and our criteria for ultrastructural characterization of lymphatics and similarly using the presence of anchoring filaments associated with lymphatic capillaries as the defining ultrastructural criteria for the identification of lymphatics.*"

Schrödl et al. (2015) come to a similar assessment as Heindl and co-workers. They used the lymphoid markers LYVE-1, PDPN, PROX1, FOXC2, VEGFR3, CCL21, which were combined with α-smooth muscle actin and 4′,6-diamidino-2-phenylenediols (DAPI) for their immunohistochemical studies. Single, double and triple marker combinations were documented with confocal microscopy. Messenger RNA analysis for CCL21, FOXC2, LYVE-1, PDPN, PROX and VEGFR3 was performed in choroid and skin. Finally, the authors conclude: "*By combining several lymphatic markers, single cells expressed these markers, but classical lymphatic vessels were not detected in the human choroid. Therefore, the healthy adult human choroid must be considered a-lymphatic, at least with the markers applied here.*"

What are the difficulties of histological identification of lymphatic vessels? Even with modern methods, the question remains as to how completely the endothelial cell layer in the vessels is stained by antibodies. This cannot be guaranteed with some antibodies and methods of presentation. Therefore, "*the use of more than one lymphatic endothelial marker or a marker panel is recommended [. . .]*" (Schroedl et al., 2014). For example, in the letter cited above,

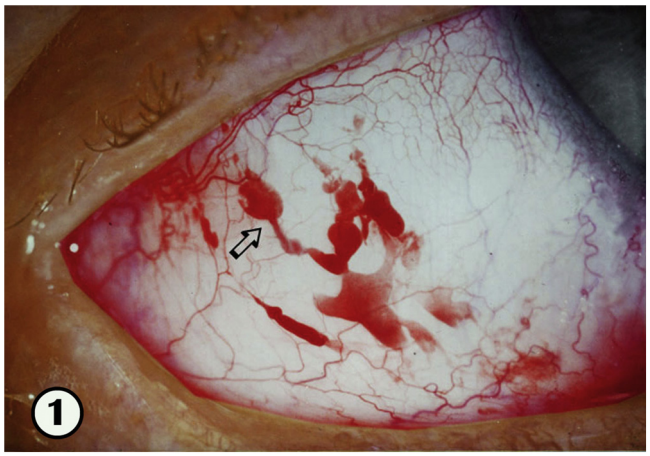


Fig. 1. Spontaneous blood filling of lymph vessels of the conjunctiva (right eye). Filling direction from the bottom to temporal top. At the nozzle-shaped segment boundary (arrow), a reverse flow of serum was observed.

Jeltsch commented on the question of finger-shaped protuberances in the area of the initial lymphatic vessels as follows: "*In the case of an immunohistochemical depiction with LYVE-1, a complete (but not necessarily uniform) staining is usually given in lymph capillaries. Mainly because the cutting thicknesses that are used are quite thin and the incubation times are quite long. Of course, it is possible that some parts of the lymphatic capillaries are not shown, but that all blind-ending protuberances are caused by insufficient staining, I consider excluded.*"

Exceptions in the location of these usually invisible channels are the lymphatic vessels in the conjunctiva of the eye. Here under abnormal circumstances, but also with spontaneous bleeding, these vessels can be observed with unarmed eyes. The following figure (Fig. 1) shows temporally deployed lymphatics in a 72-year-old patient following a conjunctival cyst surgery. The cherry-stone-sized cyst was nasally and had no connection to the lymphatic vessels. In addition to the characteristic pumpkin-seed form, complex structures, partially parallel and filled with blood, could be visualized. Under the slit lamp microscope at one point a discrete retrograde flow of fluid through the "nozzle-shaped" segment boundary (arrow) was observed, a fluid-dynamic situation that is incompatible with previous ideas of lymph transport over valve-filled vascular sections. The further transport of the blood occurred in the seated patient in batches with the blink of an eye temporal-cranially, with balloon-shaped parts alternated with very thin and not unfolded sections. Twenty-four hours later no more blood could be detected. These structures were again "invisible". In another 47-year-old patient, bleeding and filling of sausage-shaped conjunctival lymphatic vessels also occurred following conjunctival injury. In this case, a mirroring or layering was observed with the slit lamp microscope in a balloon-shaped section. In the lower half, more erythrocytes had sedimented. Above this, the balloon was transparently filled with serum-like fluid (Grüntzig, 1986, Fig. 8). The erythrocytes formed fluidically a so-called "resting bulk layer". Already Elschnig (1915) reported the same with a "blood effusion into the lymph vessels of the eyeball's conjunctiva": "*In several cases I could detect a layering of the blood, a lowering of the red blood corpuscles in the plasma to the deepest place of the hoses.*" Furthermore, the human eye can be invaded secondarily by lymphatic vessels if the eye wall is opened by trauma (Wessel et al., 2012).

In patients, the conjunctival lymphatic vessels may be stained for diagnostic purposes (e.g. differential diagnosis of conjunctival cyst or lymphangiectasis) with 2.5% Patent Blue V solution. In the following example, we injected 0.01 ml into the initially anesthetized conjunctiva nasal-superiorly, obscuring the dye depot by

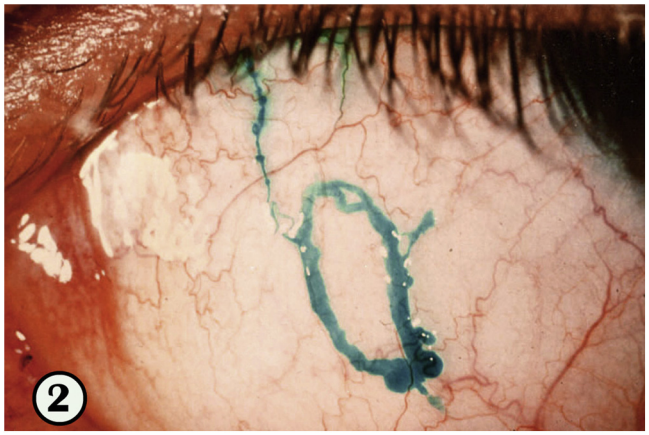


Fig. 2. Ring-shaped conjunctival lymphangiectasis, patent blue injection nasally above (left eye, male, 25 years).

the upper lid. The depot disappeared completely within 24 h, irritation symptoms were not observed. The conjunctival swelling turned out to be an annular lymphangiectasia (Fig. 2). In most cases only a small part of the conjunctival lymphatic network is stained in lymphography. Nevertheless, in congestions it can lead to the depiction of a dense vascular network with partly cylindrical vessel strands (Grüntzig, 1982, Fig. 45b).

In the subcultural movement "Modern Primitive", which deals with body modifications, so-called "eyeball tattoos" and "scleral tattooing" have achieved a high priority. "Tattoo artists" try to inject ink or other dyes under the conjunctiva to discolour the white of the eye. Owners proudly show their "eyeball tattoos" on YouTube. In the meantime, the public has become accustomed to tattoos on the skin, they are no longer shocking (Rauers, 2008; Ferreira Santos da Cruz et al., 2017). Information about risks and costs can be accessed via the Internet. Yet nothing is known about the long-term drainage of these dyes from the eye area. From other parts of the body, it is known that nearby lymph nodes stain as brightly as the skin (Laux et al., 2016).

2. Historical contributions to the lymphology of the eye

We probably owe the first representations of lymphatic vessels in the area of the eye to Mascagni (1787). These were injection specimens of the lids of cadavers. It was not until a century later that his portrayals were supplemented by Most (1905) who was able to detect two lymphatic drainage networks with different drainage routes in the eyelids. In 1793, Schreger published a work entitled "*Theoretische und praktische Beiträge zur Kultur der Saugaderlehre*" (Theoretical and practical contributions to the culture of lymphology) (Schreger, 1793). Therein he reports (with an illustration) on a patient with a generalized exanthema and concomitant swelling of the lymphatics in the conjunctiva – probably the first account of such vessels in the conjunctiva. Teichmann succeeded by means of interstitial injections of cadavers to produce an unsurpassed representation of conjunctival lymphatic vessels (Teichmann, 1861, pp. 66f). In one of his two illustrations (Plate VIII Fig. 2) the arcade-shaped lymphatic vessel network beginning at the edge of the cornea can be seen enlarged at the upper edge. Besides, he found single smaller vessels (finger-shaped protuberances?), "*which seemed to run towards the centre of the cornea. Whether they really go on in that direction, or whether they describe, what seems to me to be the most probable, only a larger arc and then return to the net, I could not decide. [. . .] In the places where the branches of the net unite with each other, one finds extensions, the branches themselves, however, are very thin.*" After a more or less radial course "*they gather in a common vessel*" parallel to the limbus and reach their largest diameter. Teichmann called these collecting vessels "border vessels". He commented critically on the technique of injection in the conjunctiva (Teichmann, 1861, p. 115): "*In many organs, for instance the conjunctiva, injections of the lymphatics are among the most difficult works I know; here, under 100 eyes, I have hardly received so many that they would have been suitable for the investigation. The most difficult to achieve are complete injections.*"

Whereas the existence of conjunctival lymphatics was no longer in doubt since Teichmann's work, in the second half of the 19th century there arose a scientific dispute over the occurrence of lymphatics in the eye itself, their origin and lymph drainage.

The contribution of the Swiss anatomist Wilhelm His (1831–1904) "*On the roots of the lymphatic vessels in the skins of the body and on the theories of lymphogenesis*", published in "Zeitschrift für wissenschaftliche Zoologie 1862–1863", begins with the sentence (His, 1863): "*Of the open questions that have been raised and discussed for more than 200 years [sic!] in the anatomical literature on the lymphatic system, there is probably no physiological interest that immediate as the question of the origin of the lymphatic vessels in the organs of the body.*"

Shortly afterwards, His published an essay entitled "*Lymphatic vessels of the retina and the optic nerve*" (His, 1866), in which he demonstrated after intravascular dye injections "sheath tubes" around the blood vessels, which he regarded as "withdrawal routes of the retinal lymph". The results of this work were based on the work published in 1865 "*On a perivascular canal system in the nervous central organs and on its relations to the lymphatic system.*" (His, 1865)

Brenner (2015) mentions this presentation in relation to the work of Iliff et al. (2012) on a paravascular "pathway" within the CNS and that this channel system, "*also known as Virchow-Robin's spaces, was already described by His in 1865.*"

The current literature (e.g. Aspelund et al., 2015; Jessen et al., 2015) describes this perivascular canal system as a 'glymphatic system' because of its 'dependence on glial water channels.' Pathological extensions of this channel system can be visualized by MRI and can provide evidence of an increased risk of stroke and the development of neurodegenerative diseases (Gaberel et al., 2014; Ramirez et al., 2016).

His (1866, p. 262) found sheath tubes in the retina particularly abundant in the "*inner optic sheath, in which they can easily be injected. For now I think I have to look at these optic nerve lymph channels as the retinal pathways of the retinal lymph.*"

In the paper published in 1863, His mentions the history of lymphology and describes the prevailing dogmas of 1800 about the open beginnings of the lymphatic vessels (His, 1863). The art of filling the lymph vessel beginnings with mercury or other masses reached its peak and a certain termination at the beginning of the 19th century. With the expansion of microscopic anatomy, so His (pp. 228f), "*the art of injecting lymphatic vessels was lost along with a good portion of the other more delicate dissecting technique, and gradually, under the authority of the histological textbooks, more and more the conviction took place in which we grew up younger, that everything that was formerly said about the beginning of lymphatics belonged to a myth. It is an essential merit of Teichmann to have finally once again taken up the matter where they had left our predecessors almost 30 years ago, and to have shown by the pictures of his magnificent specimens, what can be achieved by the so often disregarded methods of injection.*"

He summarized the results of his own research as follows (His, 1863, p. 229): "*From all the parts that I have examined regarding their lymphatic vessels, I have found that the first roots of the system are entirely devoid of their own, isolable wall. They are channels buried in the connective tissue of the cutis, mucous membranes, etc., which,*

to illustrate it with coarser images, behave no differently towards their environment, such a not brick-lined tunnel towards the surrounding rock, or a smooth borehole to the board that it is led through."

When the famous contribution "Studies on the change of fluid in the eye" appeared in 1873, the author, Theodor Leber, had just become a new full professor of ophthalmology at the Georg-August-University in Göttingen. With his own experiments, Leber had refuted Schwalbe's (Schwalbe, 1870) "most recent" view that the anterior chamber of the eye should be regarded as a large lymphatic space and the meshes of *Schlemm*'s canal (SC) as smaller lymph spaces communicating with it (Leber, 1873, p. 92). Leber found that physiological saline drained off easily into the anterior chamber, similar to the carmine solution, which, as in the manner described by Schwalbe, "*after a very short time fills the networks of episcleral and conjunctival veins around the edge of the cornea*" (Leber, 1873, pp. 98f). However, this does not succeed with Berlin Blue (which is known not to be diffusible). To confirm his assumption that there is no open connection between the anterior chamber of the eye and the lymphatic system, a simple *experimentum crucis* offered him the injection of a mixture of both dyes. The mixture now had a violet colour. Thus he received constantly – also in experiments on living animals – (p. 102) "*a red injection of the circum-corneal vessels, just as if carmine solution alone had been applied, never the vessels were injected blue or violet.*" And his résumé read (p. 106): "*The Canalis Schlemmii or Circulus venosus in humans is a venous vascular corona of plexus-like character.*"

Was it therefore clarified that the SC could not be a lymphatic channel? Or did Leber, like other researchers on other parts of the eye, only produce an artefact? Thus, those "corneal tubes" or "Saftkanälchen" in the cornea described as the beginning of the lymphatic system by Bowman (1849) and von Recklinghausen (1862) have also been rejected as artefacts.

By means of air injection into the cornea, Bowman's tubes can be displayed relatively easily. At injection, we first observed a cloud of smaller and larger columns at the tip of the cannula. These columns or tubes appeared partially bundled and layered in patterns. They appeared suddenly, lightning like cracks tearing the ice of a lake. With continued injection, they will spread on the entire cornea and end at the edge of the cornea. Rarely, i.e. in about 10% of cases, lymph vessels in the conjunctiva also filled with air. In addition to the lamellar bundled "tubes" there were longer and larger vessel-like structures, probably perinerval "fabric coatings" (Grüntzig and Hollmann, 1998, Fig. 3). Enzyme-histochemically, precipitations appeared in the cornea, which impress like lymphatic vessels. However, transmission electron microscopic examination revealed that these were not endothelium-lined lymphatic vessels (Pfankuchen, 1991).

In 1909, Bartels published a detailed report on the sources of error in the methods of injection used in the study of the lymphatic system (Bartels, 1909). Eisler (1930) went one step further and disqualified all previous injection results on the eye as artefacts, claiming that effluent from the clefts in endothelium-clad lymphatics was never detected. This contribution had a disastrous effect on younger ophthalmology in that the perivascular space as a possible transport route for lymphatic substances was banned from discussion for many years. For example, Fowlks and Havener (1964) described the outflow of dye from the anterior chamber of living rabbits in perivascular channels of the ciliary body, the communication of these spaces with the suprachorioidea, but interpreted the further transport as "re-secretion" into the posterior chamber.

We owe Michael Földi and his colleagues decisive impulses to the revision of the then prevailing opinion about the drainage connections between the eye and the lymphatic system. In animal experiments, an operative blockade of the cervical lymphatic vessels (Földi et al., 1963) led to an "autoinjection of the lymphatic system with lymph" in the head, as well as the histological demonstration of oedematous, enlarged, pre-lymphatic transport pathways. These findings are supplemented by the electron-microscopic work of Casley-Smith on alterations in the area of the pre-lymphatic transport pathways after cervical lymph blockade (Casley-Smith, 1976). Casley-Smith also described dilatations of perivascular spaces in the retina, iris, and optic nerve after cervical lymph blockage, as well as filling them with protein-rich oedema fluid (Casley-Smith et al., 1978).

Rejections of corneal transplants and glaucoma, coupled with the discovery of lymphatic endothelial cell markers, have recently strengthened research efforts to find new solutions to old questions. Markers include the vascular endothelial growth factor receptor-3 (VEGFR-3), podoplanin, a membrane-bound glycoprotein that is highly expressed on lymphatic endothelial cells, LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1), and Prox 1 (Prospero homeobox protein 1). For more details on selected key molecules in lymphatic research and their properties see also a current review in this Journal (Jha et al., 2018).

Even with the new markers, no endothelium-lined lymphatics could be detected in the cornea. In the case of the pterygium, lymphatic vessels only reach the cornea within the conjunctival connective tissue (Grüntzig, 1982, Fig. 27). In the case of vascularized corneas, the detection of intracorneal lymphatics has been successful (Faure et al., 1970; Collin, 1974). Based on a immunohistochemical investigation of lymphatic vessels in vascularized human corneas (Cursiefen et al., 2002), Cursiefen et al. (2003) reported: "*We herein summarize the current evidence for lymphangiogenesis in the cornea and describe its molecular markers and mediators. [. . .] Whereas corneal angiogenesis in vascularized high-risk beds provides a route of entry for immune effector cells to the graft, lymphangiogenesis enables the exit of antigen-presenting cells and antigenic material from the graft to regional lymph nodes, thus inducing alloimmunization and subsequent graft rejection.*"

The complexity of the answer to the question of ocular lymph vessels can be demonstrated at *Schlemm*'s canal. Lymphatic vessel or not? If the molecular markers are more important than anatomical, functional and ontogenetic aspects, then this endothelium-lined canal may be interpreted as a lymphatic vessel. A clinician without additional physiological training can hardly classify recent findings about the endothelia in this channel system in their meaning; for instance the complexity of the explanatory model for the aqueous drainage by means of "expression regulation by wall shear stress". In a review entitled "*Aqueous Humor Dynamics: A Review*", Goel et al. (2010) (References reduced): "*Trabecular and vascular endothelial cells are mechanosensors that direct vessel wall self-organization in order to optimize wall and shear stress. Pressure and shear stress-mediated signals in endothelia initiate a series of responses at the cellular, molecular, and genetic levels, and induce both rapid responses and slow adaptive changes that regulate pressure and flow* (Davies et al., 1997; Ingber, 2002). *These processes are not linear but are part of a highly complex interactive network in which an alteration in any component requires a contemporaneous adjustment of numerous other components in an interactive fashion resulting in long-term homeostasis* (de Jong, 2002; Ingber, 2003)."

3. Own investigations

When we began to study the ocular lymphatic system 40 years ago, we did not try to answer the complex question, "Are there any lymphatic vessels in the eye?" We reduced it to the question of the existence of drainage connections between the eye and lymph nodes of the cervical lymphatic system.

3.1. Detection of drainage connections between eye and lymphatic system

In animal experiments (rabbits), we injected unilaterally minute amounts (each 50 μCi in a volume of 0.06 ml) of radiolabeled colloids liable for lymphatic transport into orbit, anterior chamber, vitreous and subconjunctival space (Grüntzig et al., 1977b,c; Schicha et al., 1977). The following tracers were injected: ^{99m}Tc sulphur colloid (particle diameter approx. 30 nm, corresponding to the diameter of **i**ntermediate **d**ensity **l**ipoprotein), ^{99m}Tc-microcolloid (particle size approx. 3 nm), ^{99m}Tc-human albumin (2–5 nm), ^{198}Au-colloid (approx. 5 nm). The activity distribution was registered up to 16 hours after injection in vivo with a gamma camera and connected computer to kinetic processes such as outflow from the injection site, activity excretion and liver, kidney and lymph node accumulation capture. In addition, the trial was combined with a bilateral cervical lymph blockage surgically created by Mrs. Etelka Földi (Casley-Smith et al., 1978). If there were no transport connections between the eye and the lymphatic system, this procedure would not influence the results. At the end of the experiment, the animals were dissected and the eye, retrobulbar space, optic nerve, brain, cerebrospinal fluid, superficial and deep cervical lymph nodes, inguinal lymph nodes, skin, musculature, blood, liver, spleen, kidneys, thyroid and bone were removed. Tissue samples were measured in the borehole sample changer and the activity concentrations per gram of tissue and organ activity in percent of total applied activity were determined.

After injection in the **retrobulbar space** a significant concentration of the activity could be observed for the most part in the equilateral *Lymphonodulus cervicalis profundus* (Grüntzig et al., 1977a). By the cervical lymph blockade the removal of lymphotropic substances from the retrobulbar space was largely inhibited. After injection in the **anterior chamber** a significant concentration could be observed for the most part in the equilateral *Lymphonodulus cervicalis superficialis* (Grüntzig et al., 1977b). After **intravitreal** injection drainage to the bilateral deep cervical lymph nodes could be observed (Grüntzig et al., 1978a). After injection into the **subconjunctival space** a significant accumulation of activity could be registered in the equilateral *Lymphonoduli mandibulares* and *cervicales* (Grüntzig et al., 1978b).

The data substantiate segmental lymph drainage from the eye: vitreous body and retrobulbar space for the most part into the deep cervical lymph nodes (*Lymphonoduli cervicales profundi*), anterior chamber and subconjunctival space for the most part into the superficial cervical lymph nodes (*Lymphonoduli cervicales superficiales*).

The rate of discharge of the radioactive tracer was determined by the radiopharmaceutical and injection site. ^{99m}Tc albumin was the fastest with small particle size and ^{99m}Tc sulphur colloid with a relatively large particle diameter was the slowest. For ^{99m}Tc microcolloid, the anterior chamber showed the highest drainage rate after 6 h, followed by the subconjunctival space and the vitreous humour. The retrobulbar room had the lowest runoff. The removal took place in various ways: First, via the systemic circulation with subsequent radiopharmaceutical-dependent distribution predominantly in the liver and kidneys and partial excretion; second, via lymphatic drainage to the regional cervical lymph nodes. The activities enriched in the cervical lymph nodes were relatively low after anterior chamber injection and after vitreous injection. On average above the factor 100, the corresponding values were higher after retrobulbar and subconjunctival injection.

Details including results and discussions have been published (Grüntzig et al., 1977a,b,c; Schicha et al., 1977, 1978; Grüntzig et al., 1978a,b, 1979). A detailed record of the transport path of the radioactive tracers was not possible due to the experimental setup used. Possible outflow channels were so-called "pre-lymphatic channels" ("prelymphatics"), for which the terminus technicus "tissue channels" has recently been preferred (Brenner, 2014).

3.2. Prelymphatic pathway between vitreous body, optic nerve and cervical lymph nodes?

A possible outflow via such structures was investigated by animal experiments in the optic nerve. Ulrich had already described such an outflow in 1889 with the help of ink injections into the vitreous body in rabbits (Ulrich, 1889). More recent work was published by Hayreh (1966, 1978) and Gärtner (1968).

In our investigations, we used rabbits for intravitreal ink injection, similar to Ulrich (1889).

In seven rabbits Indian ink was injected into the vitreous body and afterwards its drainage was controlled histologically (frozen sections). In five of these seven rabbits the investigation was combined with a bilateral dissection of the cervical lymph nodes. The lymph node dissection caused a massive deceleration of the outflow of Indian ink; the perivascular connective tissue showed a clear oedematous relaxation. The histological evidence suggests that vitreous body, the interstitium between glia cells of papilla, the pial tissue between nerve bundles in the optic nerve, the perivascular space around the central vessels of the retina and the subarachnoid sheath of the optic nerve may be interpreted as prelymphatic pathway (Grüntzig and Huth, 1977).

Hayreh wrote in 2016 that rabbit findings should not be transposed to primates (Hayreh, 2016). Rabbits have no *lamina cribosa* (LC) and thus the outflow of fluids and particles from the vitreous into the optic nerve is possible, and in addition: "*the loose perivascular tissue surrounding the central artery and its branches in the optic nerve septa seen in the rabbit does not exist in man and primates*". In fact, rabbits today are considered critically as experimental animals for glaucoma research because of the sparse LC.

A multi-layered LC with close three-dimensional similarities to the primate LC is described in the cat eye (also pig and dog eye). The size of the LC diameter and the variability of the single pores within the LC are comparable in cat, pig and dog species and, again, match the situation in the primate (May, 2008).

In addition to our ink-based rabbit experiments (Grüntzig and Huth, 1977), we have studied the effects of chronic cervical lymphostasis on cats. The effects in regions directly drained by lymphatics (facial skin and retrobulbar tissue), and in regions primarily drained by prelymphatics (cerebral cortex, retina, papilla, iris, choroid and Circle of Willis) were studied ophthalmoscopically and electron microscopically. It was found that in all regions there was considerable oedema, increases in protein concentration and excess collagen, together with increased numbers of macrophages. The lymphatics, where these existed, had many open junctions: both these vessels and the prelymphatics were very dilated, with considerable increases in protein concentration (Casley-Smith et al., 1978).

In the *dura mater* of the human optic nerve, lymph capillaries were found in post-mortem examinations (with light microscopy, transmission and scanning electron microscopy). The highest concentration of lymphatic capillaries was found in the bulbar part of the dura behind the ocular globe (Killer et al., 1999).

3.3. In vivo animal experiments with a water-soluble X-ray contrast medium

In own in vivo animal experiments (rabbit, dog, baboon, rhesus monkey) the efferent lymph vessels of the conjunctivae and eyelids could be shown as far as the jugular vein by direct and indirect application of Iotasul® (Schering AG, Berlin), a water-soluble X-ray contrast medium (Grüntzig et al., 1981, 1982).

3.4. Segmental lymphatic drainage of the eye

Our previous studies on rabbits and cats revealed that there are drainage connections between the eye and the cervical lymphatic system. By a cervical lymph blockade in which the cervical lymph nodes are resected and their afferent lymphatics ligated, the discharge of lymphatic substances is significantly reduced, the perivascular space around the central vessels of the optic nerve ("prelymphatic pathway") appears loosened and dilated oedematous histologically. In analogy to the drainage of the head area, as for instance the gingiva (Rohen, 1975), it can be assumed that there is also a segmental outflow of substances liable for lymphatic transport from the eye.

According to our experimental results, vitreous humour and retrobulbar space were drained mainly by the lymphatic vessels running to the deep cervical lymph nodes, while anterior chamber and subconjunctival space drain predominantly to the superficial cervical lymph nodes (in eyeball tattoos one would expect a coloured staining). Following Magari-Nishimura (1962), the boundary of the drained segments could be sought in the area of the eyeball's equator.

3.5. Fine structure of conjunctival lymphatics

When we started our investigations, we assumed that the anatomy of the conjunctival lymphatic system was known. The works by Teichmann (1861), Knüsel (1924), Orts Llorca (1930), Nataf and Delon (1953) or Bussaca (1955) seemed to prove this assumption. These publications included the ideas of finger-shaped initial segments and pointed-arched valves, which Knüsel described and depicted in 1924 (Knüsel, 1924, Figs. 7, 8). Similar pointed-arch-shaped patterns were registered on mesenteric lymphatics and interpreted as invaginations, single valves and double-leaflets (Vajda, 1966). Castenholz (1984a, Fig. 14) described a corresponding pattern in corrosion casts of the subepithelial lymphatic plexus (rat tongue) as "*a typical V-shaped impression caused by a valve*". We also initially misinterpreted such entities as valves protruding into the vessel lumen (Grüntzig, 1986).

Animal experiments in the conjunctiva (cattle) in post-mortem lymphography (Fig. 3) showed that finger-like protuberances were able to re-join the remainder of the lymphatic network as the injection proceeded. The filling of the vessels took place discontinuously, which was documented in a film (Grüntzig, 1981). Slit lamp microscopy was not possible to differentiate into lymph capillaries, pre-collectors or collectors. In several publications there are photographs of different endothelial surfaces (Castenholz, 1984b, Fig. 5b), but to which vessel sections are they assigned? We avoided this topographical uncertainty by choosing the vessels' diameter as the reference for the endothelial surface of different vessel-types.

3.6. Is the endothelial structure dependent on the lymphatic vessel's diameter?

To clarify this question, post-mortem conjunctival interstitial lymphography was performed on 150 bovine eyes using a combined Berlin blue dye, air and fixative injection (Grüntzig et al., 1987).

The injection required careful fixation of the bulb to be examined. For this purpose, the bovine eye was mounted on a semicircular recessed polystyrene carrier and the conjunctiva stretched with pins in a circular manner. For injection we used a 2 ml holding syringe with a disposable cannula whose diameter was 0.1 mm. The puncture site was parallel to the limbus located about 1 mm away from the limbus district. This area was particularly well suited for injection, since the conjunctiva is immovably fused with the sclera and an injection of the underlying sclera is avoided. Investigation was done stereo-microscopically (slit-lamp).

Fig. 3. Conjunctival lymphatic network (cattle) after Berlin blue lymphography with finger-shaped protuberances. Injection point limbus rim above with cannula entry channel (arrow). Cannula diameter 0.42 mm.

Ninety of these specimens were processed for advanced scanning electron micrographs (SEM). To avoid drying, the conjunctiva was drizzled with saline during the injection, and then with 2% glutaraldehyde during the preparation. Due to the gapless preparation of the specimen, a precise identification of the preparation site was ensured. A constantly applied photo-mathematical algorithm (Hollmann, 1995, pp. 148f) was used to determine the diameters.

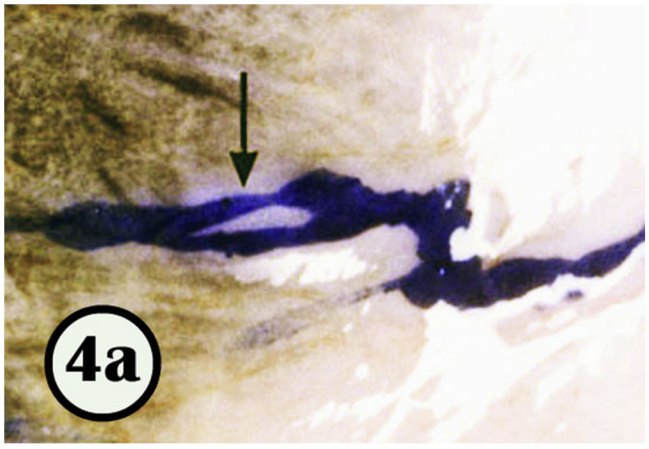
The specimens were randomly categorized in seven groups defined in terms of their "transmitted light cross sections" with diameters of 0.1–1.0 mm and above and examined by SEM.

In the smallest vessel calibres (D = 0.11 mm) (Fig. 4a), the impressions into the lumen of the occasionally occurring cell nuclei clearly imposed. The cytoplasm was displaced by filling pressure of the injection mass, and the appearance of large clearances between the cell nuclei aroused (Fig. 4b). With increasing diameter, these free spaces were lost and the individual cell nuclei could no longer be identified. Rather, only wall-like structures were recognized by the probable combination of many smaller structures. The cells moved closer to each other, or the intraluminal pressure was no longer able to change their dimension so much.

For medium diameters (0.3–0.4 mm) structures similar to reticular fibres were visible on the endothelial surface as structural features (Fig. 5). In smaller cross-sections, they visually stood out clearly from the intimal surface without, however, losing their integrity to it.

With increasing diameters, their prominent character vanished and they were recognizable only as a faint drawing. In the group with diameters of 0.5 mm, the SEM showed a rampart-like, almost wave-like surface structure due to the uniform arrangement. The width of the "ramparts" seemed to be hardly below 10 μm. Width and height decreased with the vessel's diameter as well as the prominence of fibrous structures.

The intima at diameters above 1.0 mm (Fig. 6a) showed a uniform, flat surface texture. It was characterized by a regular, reticulate and weak furrowing. The endothelium seemed to be traversed at depth at these sites by a variety of reticulated fibres. They gave



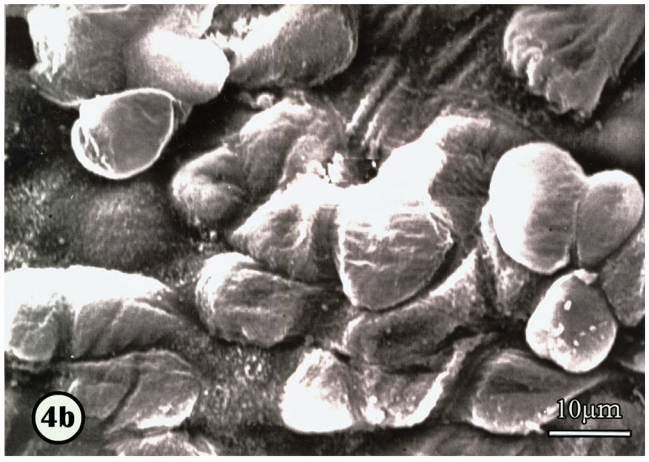


Fig. 4. (a) Conjunctival lymph vessel (bovine), Berlin blue filling. The corneal limbus is oriented to the left edge of the image. Arrow marks interface (cross section 0.1 mm) for SEM examination. (b) Scanning electron micrograph (SEM) of the lymphatic vessel endothelium with a vessel cross-section of 0.1 mm at the interface of Fig. 4a. Sharply delimited and contrasting core impressions of the endothelial cells. These occur frequently in small groups.

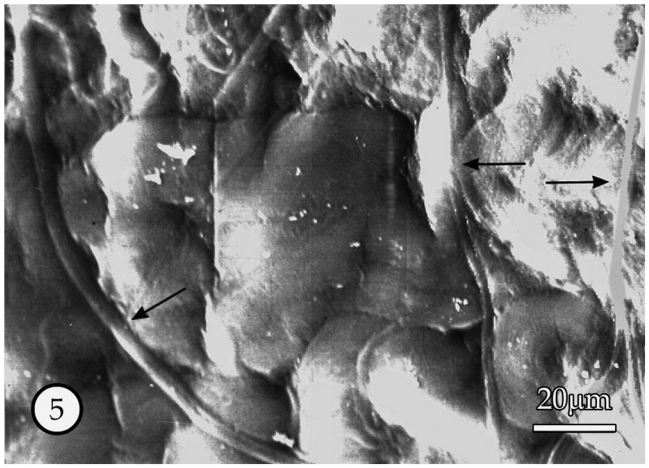
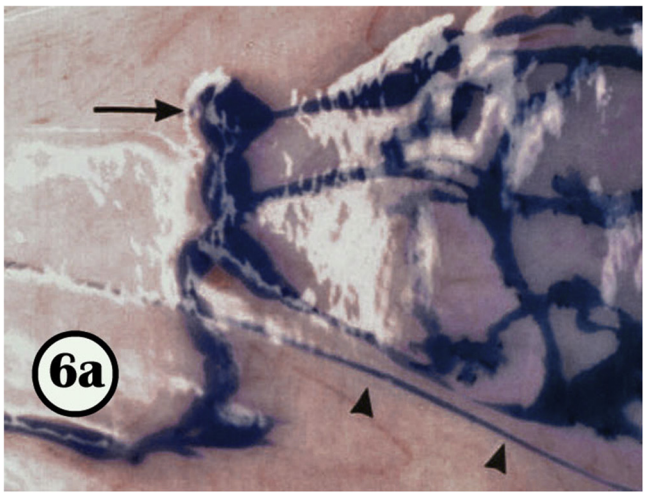


Fig. 5. SEM image of the lymph vessel endothelium with a vessel cross-section of 0.31 mm. The endothelial cells appear wall-like, arranged close together. In addition, structures resembling reticular fibres are found (arrows).



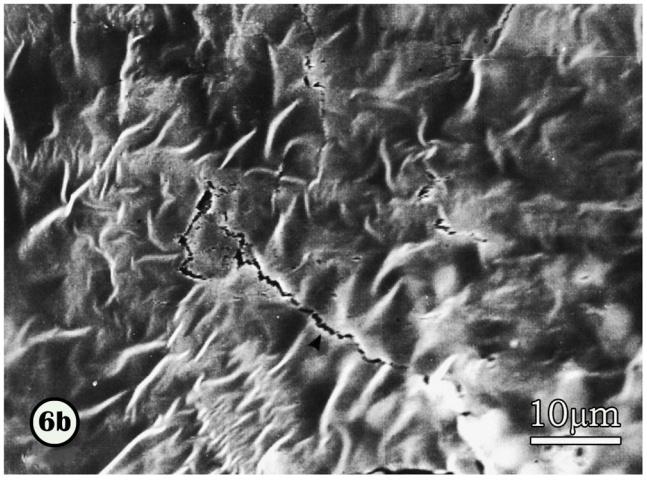


Fig. 6. (a) Conjunctival lymph vessel (bovine), Berlin blue filling. The corneal limbus is oriented to the right edge of the image. Arrow marks interface (cross section 1.45 mm) for SEM examination. Light reflexes caused by dripping glutaraldehyde. At the bottom of the image a randomly filled blood vessel (arrowheads). (b) SEM view of the inner lymph vessel surface at a vessel cross-section of 1.45 mm. The surface structure is characterized by reticular and weak furrowing. There are no core impressions. Fixation-related tear artefacts (arrowhead).

the impression of superficial cracks that did not continue into the depths (Fig. 6b).

After an arbitrary categorization and size classification of the lymphographically unfolded vessels, independent of the place of collection of the preparations, certain surface characteristics of the intima, which were oriented on the vessel calibres, were to be determined by SEM examination. With certain restrictions, previously published intima depictions could also be classified according to size. Individual cell nuclei emerging into the lumen (Grüntzig, 1982; Castenholz, 1984b) are found predominantly in the smallest and smaller vessel calibres (0.1–0.3 mm), whereas only large vessel calibres (1.0 mm and above) display an endothelial surface that is generally smooth and in its depth appears to be crossed by fine fibres (Nolte, 1992, Fig. 132).

Further investigations were carried out on the so-called "initially finger-shaped lymphatics" in the conjunctiva, which were observed in lymph vessel depictions.

3.7. *Finger-shaped initial segments: only temporary filling states?*

Brenner focused in a review on this topic under the excellent title "*Initial lymphatics – myths and truths*" and wrote (Brenner, 2014): "*First, initial lymphatics do not 'end', but can begin at best*

blindly. The designation of the direction, that is, the determination of where the beginning and where the end is, should nevertheless be based on the direction of the flow or transport of the lymph. Thus, it is clear that initial lymphatics can only begin. The question remains whether, and if where, they start as blindly beginning finger-shaped structures."

Already Bartels (1909) tried to clarify the structure of these finger-like vessel sections histologically. On his specimens it can be seen that these structures do not represent the beginning or end of a vessel, but are part of an uninterrupted network of lymphatic vessels, although in parts of this network no injection compound has penetrated, so that the vessel segments remain invisible when viewed macroscopically. These observations led Bartels to suggest that the unoccupied sections were newly created, but not yet unfolded lymphatic channels within the framework of the development of the lymphatic system, and thus were impervious for the injection mass.

We examined these finger-, balloon-, dome- and piston-shaped segments post-mortem at the conjunctiva of bovine eyes by means of indirect lymphatic vessel injection. At the same time, the injection pressure was measured and the progressive filling of the lymphatic vessel network documented photographically (Grüntzig et al., 1990, Fig. 3). We achieved the following results:

During lymphography numerous coarser and finer, tube-like projections (sacculations) filled, from which further progression of the coloured solution could only be observed when higher injections pressures were applied. We were able to prove that such segments had connections to neighbouring vessels as well as forming plexus of their own. Based on our findings we have reason to doubt the existence of tube-like initial segments in the lymphatic system. Rather in the conjunctiva of the bovine eye this system has the structure of a fine network where tube-like segments can be demonstrated as temporary filling stages.

Why did former investigators repeatedly confirm these finger-shaped segments as the beginning of the lymphatic system and did not question this notion? The answer seems easy. These investigators assumed that their chosen depiction was complete and thus allowed to draw an accurate conclusion about the organization of this channel system. This applies both to our own injection specimens and to specimens by means of hydrogen peroxide drops (Kraus, 1957) and to images obtained by means of corrosion technology (Wenzel-Hora et al., 1987). Also, the histological images of Vajda and Tomcsik (1971) in one plane cannot detect vessel continuations or connections in another plane. Another SEM image of such a piston-shaped initial segment exists made by Castenholz (1984b, Fig. 8b). It is a corrosion cast (rat tongue, Mercox®). In addition to core impressions, the initial segment also shows "*a narrow connection between the lumen's cast and a cast of the interstitium (arrow)*". This might not be an interstitial cast, but the beginning filling of a following segment. Ignored by the author, a lip-shaped retraction remained at the tip of the piston-shaped initial segment, indicating a potential vascular attachment. What kind of internal structure could leave such a delicate furrow in the filling? What does the "blind end" (or the "blind beginning") look like?

3.8. The internal structure of the dome-shaped "blind end"

To clarify this question, we attempted to depict the internal vault structure of finger-shaped segments by scanning electron microscopy. Was there an evidence for a possible opening at the "blind end", i.e. a connection to a following segment?

In the conjunctiva of bovine eyes, double-contrast lymphography (Berlin blue solution/air) under slit-lamp microscope was used to search specifically for finger shaped segments (Grüntzig et al., 1994). The conjunctiva had previously been prepared by fixing in polymeric resins. A proportion of the specimens were amenable to examination under scanning electron microscope.

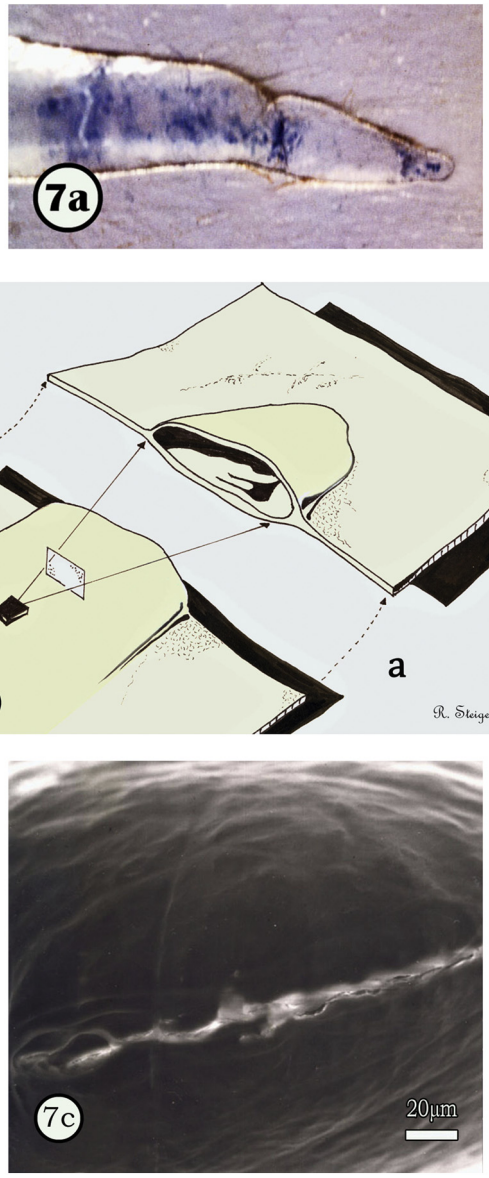


Fig. 7. (a) Finger-shaped conjunctival lymph vessel (bovine), Berlin blue air filling. Vessel cross section at the top 0.2 mm. (b) drawing of Fig. 7a. Cutting guide (a), SEM beam path (b). (c) SEM image of the segment tip of (a). Aperture-shaped structure of zippered or saw blade-like shape in the dome.

The results (Grüntzig et al., 1997): Notwithstanding the observed great variation in the shape of the lymphographically obtained blind-ending structures – in the form of terminations shaped variously like fingers, balloons, domes, pistons or pyramids, terminations with two humps, and terminations shaped like spear heads – scanning electron microscopy revealed within the fornices many relatively uniformly shaped structures in the form of fissures, configured with lips and saw-tooth edges, rather like zip fasteners.

In our publication, lymphatic vessel processes are depicted with corresponding SEM images (Grüntzig et al., 1997). Some of the zipper or saw blade-like boundaries were already partially opened slightly. By way of example, a finger-shaped segment according to the Berlin blue air Injection is reproduced here (Fig. 7a). By way of drawing, the incision and the ray path of the SEM were recorded (Fig. 7b). The SEM image showed a slightly open saw blade-like structure in the dome (Fig. 7c). Further photographs can be found in the doctoral thesis of Steiger (1995).

These findings are suggestive of preformed connections to neighbouring segments. This appears to be another element, in addition to the familiar valve-like structures, for controlling the circulation of lymph.

3.9. Fine structure of conjunctival lymphatic valves

Despite new insights, our ideas about the transport of lymph from the initial canalicular system to the lymph nodes are still debatable. Is this because even younger authors continue to assume the "properly functioning intraluminal valves" of textbooks, e.g. Scallan et al. (2016)? Engineering models and the derived interpretation of lymph transport are also based on this myth (Nipper and Dixon, 2011). And at Bazigou and Makinen (2013) we even find these ideas in the title: "*Flow control in our vessels: vascular valves make sure there is no way back*". The most recent publication of this group also uses this "no-way-back-legend" again (Ulvmar and Mäkinen, 2016).

We noticed retrograde fluid movements under physiological conditions, as well as the sedimentation and formation of a "resting bulk layer" (erythrocytes at haemorrhage into the lymphatic system) (Grüntzig, 1986). In the depiction of the conjunctival lymphatic system at the border between cornea and conjunctiva by means of Berlin blue and subsequent air injection, the dye was expelled by the following air. Here, too, we were able to observe reflux movements of the dye solution. In short vessels, after expulsion of the dye, retrograde filling in the following segment with dye resulted from a parallel small-lumen vessel in the sense of a small roundabout. But this filling could also be done in the opposite direction (Hollmann, 1995, Fig. 83). In contrast to the cascading filling of lymphatic vessels with numerous anastomoses, blood vessels filled rapidly, progressing radially to the periphery. With careful preparation, fine-meshed lymphatic vessel networks could be visualized in a superficial conjunctival layer, which seemed to communicate with lower-lying lymphatic vessel areas via "blind-sac-like" processes. Visualized blood vessels coursed in a layer in-between, i.e. between superficial and deeper lymphatic networks.

Investigations with contrasting by Berlin blue and air as well as Berlin blue and Mercox® showed arch-shaped structures within the lymph vessels' lumina (Grüntzig et al., 1987, Fig. 5). Stereoscopically, however, we recognized that these were not valves, but hump-like vessel bulges (bulbi) whose dye accumulations projected over one another in an arc. This may also apply to the structures depicted by Knüsel and other authors, which were interpreted as valves (Knüsel, 1924, Figs. 7b and 8). This misinterpretation has persisted until today also in investigations of the lymphatic vessels with endothelial markers. Here we rely on the works of Norrmén and collaborators (Norrmén et al., 2009; Norrmén et al., 2011), where – in the latter publications – they marked these arcuate colour enhancements with arrows in Fig. 3A (image P7) and referred to them as flaps, as they also interpreted the confluence of two vascular fractions as collectors: "*Postnatally, the network has remodeled into mature collecting vessels, with high VEGFR-3 expression in luminal valves (arrows) [. . .]*"

According to prevailing doctrine, lymph vessel valves in the sense of a pressure-suction pump cause a centripetal outflow of the lymph. The lymph should be emptied after its formation in the initial area in the direction of so-called pre-collector and collector vessels (Castenholz and Zöltzer, 1985). The pre-collectors should already have valves that are of particular importance for the pumping function or the further transport of the lymph (Kubik, 1952). Backflow is not possible due to the morphology of the valves (Mazzoni et al., 1987). The majority of authors see in the valves bicuspid structures, with the downstream free cusp edges each have a common starting point (Vajda and Tomcsik, 1971; Gnepp and Green, 1980). According to recent studies, endothelial cells also participate in the regulation of lymphatic transport as a function of the luminal or transmural flow (Breslin and Kurtz, 2009; Miteva et al., 2010). Such currents at the endothelia should be responsible for the upregulation of PROX1 and FOXC2 to form valves in the pre-collectors and collectors (Sweet et al., 2015).

In order to clarify our observations of retrograde fluid movements, we investigated the fine structure of the lymphatic vessel valves or those structures that were previously interpreted as valves (Grüntzig et al., 1994).

In the conjunctiva of bovine post-mortem eyes (n = 100) segmental connections of lymphatic vessels with a diameter of less than 1 mm were opened specifically under control of slit lamp microscope after having undertaken an interstitial double contrast lymphography (solution of Berliner blue/Air). Some of these specimens (n = 20) could be investigated further on with the scanning electron microscope. That procedure allowed for matching the patterns of flow and vessels structures in the beginnings of the lymphatic system observed with the slit lamp microscope congruent with those structures shown by the scanning electron microscope.

The following segmental connections could be demonstrated:

a. Lymphatic vessels, which flow like a pipe into the wall of another segment (Grüntzig et al., 1994, Fig. 2). In the SEM image (Grüntzig et al., 1994, Fig. 5b) it can be seen that the opening is formed in equal parts by the vessel wall and the tubular leaflet. The portion forming the free edge of the leaflet appears somewhat thickened.
b. Laterally arranged oval connections (Grüntzig et al., 1994, Fig. 3) with valve resembling an aperture. The iris-shaped leaflet between the two segments is thickened thread-like at the free edge. Over these shunt-like compounds sections of identical caliber and partially parallel extending larger vessels were filled, whereat these singular leaflets seemed to open only at pressure increase in the "switching segment".
c. Segments of lymphatic vessels arranged in a line, which flow into one another between two hump-shaped protrusions with bicuspid valvular structures. These structures may correspond to the lymphatic bulbs described in the literature (Pfuhl and Wiegand, 1940) and invaginations (Vajda and Tomcsik, 1971).

In the latter connection structure, we observed details that might explain a retrograde fluid motion. For example, in the region of the segment transition (Fig. 8a), two leaflets projecting from opposite sides into the lumen with a central opening (arrowhead) can be seen by means of a slit-lamp microscope. The injection site at the limbus is aligned with the upper edge of the picture. The filling took place against the direction of the arrow. The starting points of the leaflets, visible as narrow colour-intensive lines, appear to be placed at a certain distance from each other on the vessel wall. The SEM image (Fig. 8b) confirms this observation. The longitudinal oval vessel cross section is at this point 0.7×0.29 mm. At the opposite angles one recognizes two depressions (red dots) which correspond to the cuspids- or bulbus-like protuberances observed by slit-lamp microscopy. From there, two membranous leaflets project diagonally into the vessel lumen. They frame a round hole-like opening with a diameter of approx. 180 μm. The attachments of the two leaflets on the vessel wall (arrows) are relatively far apart, about 170 μm. One of the leaflets appears curled, an indication that the vessel was not under maximum filling pressure. Therefore we can assume that there was no pressure-related artefact of the leaflets' attachment. The filling took place against the direction of the arrows.

The lack of a common but clearly separated attachment of the leaflets that we have proved in some cases contradicts the theory of a competent valvular system throughout all lymphatic vessels discussed hitherto in the literature. The most important prerequisite for the function of a valve is a complete closure of all its

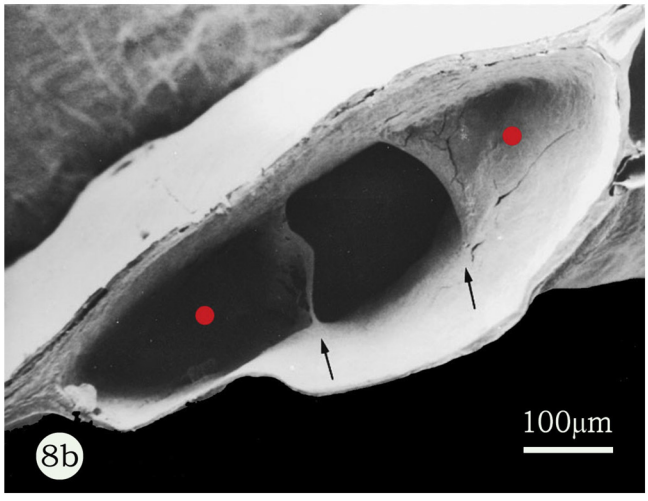


Fig. 8. (a) Conjunctival lymph vessel (bovine) Berlin blue air injection. Vessel segments with two-conical connection opening (arrowhead, vessel cross-section 0.3 mm). Filling direction against the arrowhead. (b) SEM image of the segment connection marked in (a). Double-leaf segment connection with round hole-like central opening. The leaflets are separated (arrows). Lymphatic bulbs (red dots). Filling direction against arrowheads. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

components, since each gap allows a return flow of the liquid to be conductive. Regardless of the size of the leaflet, they become ineffective at the latest when back-flowing liquid builds up in front of the leaflets and the expanding vessel wall leads to the diverging of the two leaflets. The result is a return flow through the resulting gap in distally located vessel sections. A separation of liquid components, as we observed in haemorrhage, would also be possible.

3.10. Directed flow without flap valves

Summing up our observations, the result is such a complex picture that a fluid-dynamic interpretation hardly seems possible. Nevertheless, under physiological conditions lymphedema does not occur. How could one explain a directed lymph flow with and without valves, without musculature of the vessels and with ring-shaped canal structures?

Liebau described models of valveless pumps for similar phenomena in the bloodstream (Liebau, 1954; Mahrenholtz, 1963; Jung, 2011). Conditions for a directed flow under such conditions are:

- Impulsive and periodically generated pressure fluctuations.
- Differences in diameter of the piping system on both sides of the pulsation point.
- Elasticity differences in the pipe segments.
- Mass differences.

One of the features of the conjunctival lymphatic vessels are cross-sectional differences, which might occur also in other organs but were not proven there. The vessels are partially parallel and are connected anatomically and functionally by lateral branches. In part, this creates annular structures allowing for a circular flow. The different expansion behaviour of individual vessel sections in lymphography suggests differences in elasticity. Pressure fluctuations could for instance be triggered by blinking or eye movements. Thus, there are possibilities of a directed liquid movement according to the flow principle of Liebau (Grüntzig and Hollmann, 1998, Fig. 10).

3.11. Discovery of further intraluminal structures

Further investigation has led to the observation of dye accumulations during lymphography, which appeared localized on the lymphatic vessel wall in a pan-shaped well (Grüntzig et al., 1987, Fig. 8). The surface seemed a bit retracted at these points. But since no dye could have come on the vessel wall from the outside, an explanation had to be found inside the lymphatic vessel. To solve the puzzle, we cut – after appropriate fixation (Grüntzig et al., 1994) – these segments open. To our astonishment, we saw thread-like or trabecular-like internal structures, at the base of which dye particles had remained. The results reminded us of previous observations in conjunctival biopsies. For example, in a patient in an ectatic conjunctival lymphatic vessel, an S-shaped curved connection between two prominent endothelial nuclei was shown by scanning electron microscopy (Grüntzig, 1982, Fig. 7). In 1983, in a conjunctival lymphatic vessel of another patient, we registered a filamentous structure between the endothelium at the vessel wall and a valve-like subdivision of the lymphatic vessel (Grüntzig et al., 1983, Fig. 8), which we later interpreted as resistance to expansion (Grüntzig, 1986). Wenzel-Hora and Behrens von Rautenfeld later published similar findings (Wenzel-Hora et al., 1987, Fig. 11).

If one wants to locate such structures in the conjunctiva during slit-lamp microscopy during lymphography, lymph capillaries, pre-collectors and collectors, as we know from the textbooks (Kubik, 1993, pp. 2ff), cannot be distinguished. Both in animal experiments and in vivo in humans, we often saw thin and thick vessel canals running parallel. Were the thinner vessels lymph capillaries (or "initial lymphatics", see Brenner, 2014), the thicker channels perhaps pre-collectors or collectors? Out of wide vessels thin vessels retrograde filled up again, which again ran parallel above the larger vessel. According to the subdivision cited above, this terminology assumes a progressive outflow, which we were unable to confirm at the conjunctiva either in vivo or in animal experiments. For us in lymphography it was never predictable when, where and how

more vessels or vascular provinces filled. Was the lymphatic flow regulated by valves, or were there other structures involved?

We performed indirect lymphography on the conjunctiva of 550 post-mortem enucleated bovine eyes and selectively analysed intraluminal dye accumulations (as described above), both by slit-lamp and scanning electron microscopy (n = 90) (Grüntzig and Hollmann, 1998). Instead of a subdivision in pre-collectors and collectors, we used the vessel diameter as a comparable size. The vessel diameters were determined using a specially developed photomechanical method (Hollmann, 1995). The diameter of the vessels unfolded and examined by double contrast injection varied between 0.4 and 1.4 mm. In each of the cases we suspected that intraluminal structures could be detected, which we categorized as follows (for illustrations, see Hollmann (1995) and Grüntzig and Hollmann (1998)):

a. Gusseted or fan-shaped lamellae that insert on the vessel wall.
b. Filamentous or columnar intraluminal structures. The vascular lumina appeared at the insertion sites by the expansion pan-like drawn inwards.
c. Larger dividing wall-like structures that extended far into the lumen and imitated flap-like structures. Flaps, however, could be clearly differentiated.
d. Complex structures with sail-shaped and filamentous parts, which appeared stretched in the lumen. These structures were predominantly found in vessels with a diameter of about 1.0 mm.

How complex these intraluminal lymphatic vessel structures can be is demonstrated here by an example. First, the expanded lymph vessel segment after double contrast injection (Fig. 9a). In the middle of the picture you can see a slightly constricted segment transition with two tinted humps. The arrow outside the following segment indicates the flow direction. In the vicinity of the following segment, two complex spot-shaped dye accumulations dominate (white arrows). In between, delicate bluish dye shadows can be seen. The vessel cross-section at the interface (line) was 0.9 mm. At the interface in the direction of the effluent, a triangular structure was found in the reflected-light microscope. The tips seemed to advertise on the vessel wall and open a leaflet-shaped structure. The SEM image showed a cross-section at first glance of many fibres (Fig. 9b). In the centre of the vessel, one could see a smaller, sail-shaped formation, which was stretched to the "back" (more precisely, in the direction of flow). The leaflet seemed to be fastened with numerous reticular fibres in all directions. Complex structures were found on the vessel wall that could correspond to the speckle-shaped dye enrichments in Fig. 9a. With vessel cross sections over 1.4 mm, the density of the intraluminal structures seems to decrease again (Fig. 10). Systematic investigations are missing.

These intraluminal lymph vessel structures are reminiscent of findings in the "*collector channel orifices of Schlemm's canal*" (Bentley et al., 2016), which presumably play an important role in the regulation of intraocular pressure, or the balance between production and outflow of aqueous humour. The authors write: "*The anatomical structures identified in this study indicate a possible regulatory role in fluid dynamics by Schlemm's canal by virtue of collector channels and their associated structural entities such as flap-like structures, septa, and tubule-like structures.*"

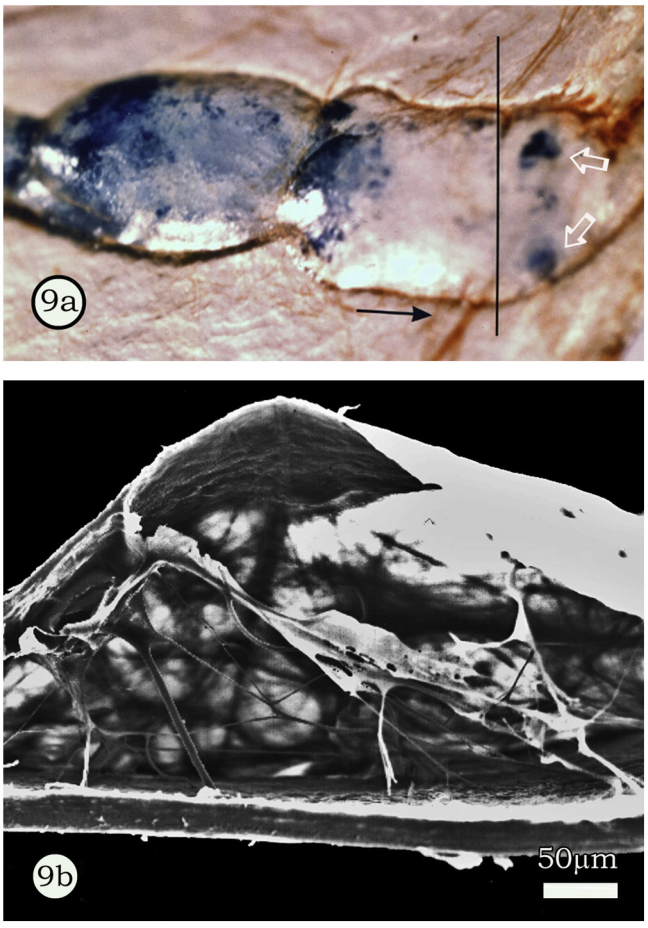


Fig. 9. (a) Conjunctival lymph vessel (bovine). Berlin blue air filling. Vessel cross-section at the interface (black line) 0.9 mm, filling direction black arrow. Borderline two dot-shaped dye enrichments (white arrows), between them delicate dye shadows. (b) SEM image of the segment marked in Fig. 9a. View into the lumen in filling direction. Vessel cross section 0.9 mm.

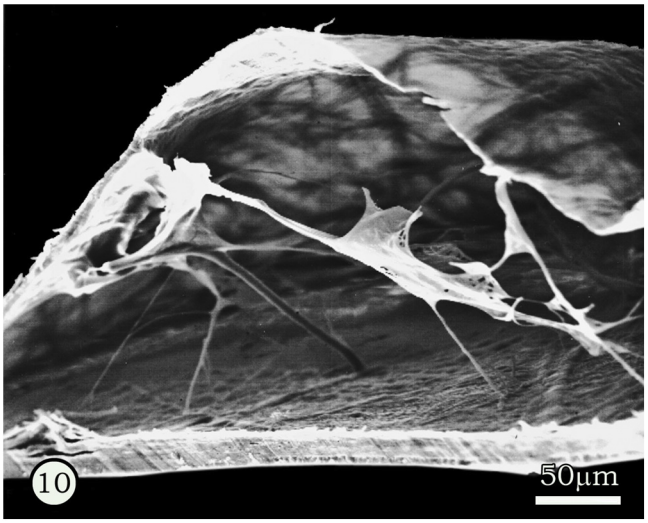


Fig. 10. SEM image of intraluminal lymphatic vessel structures with a lumen cross-section of 1.40 mm.

4. New theories also require precise anatomical foundations

The above-mentioned fluid flow in the absence of valves could indeed be explained by Liebau, but not the influence of the internal structures thereon. For this we would have to use other ideas, such as they are used for example in the water industry for drainage, degradation of introduced substances and backwater-shelters (buffer storage, infiltration ditch). There one knows the term of the detention pond. It serves for the retention and purification of drainage water (storage, treatment and reuse of drainage

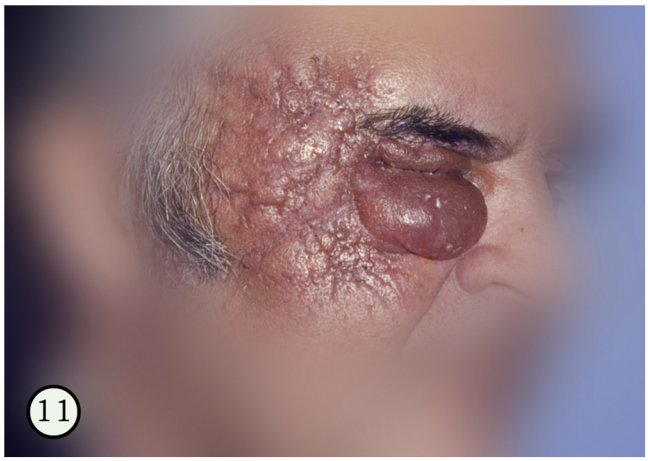


Fig. 11. Eyelid oedema caused by scarring in the area of the draining pre-auricular lymph vessels following a traffic accident (right eye).

water). Dead zones, short circuit currents and swirling (short-circuiting and swirling) are other hydraulic terms. "Dead zones" refer to areas of low mixing of the ingredients, which reduces the effective reaction volume available. A "short-circuit flow" occurs when, due to an unfavourable arrangement of inflow and outflow, the non-cleaned content gets directly into the outlet. By placing barriers, the effectiveness of the detention pond can be improved (Steidl and Kalettka, 2016). Encouraged by examples in water protection, one easily succumbs to the temptation to transfer these findings – albeit with appropriate reservations – to our observations, with new questions or impetus for further investigation.

The flapless lymphatic spaces of amphibians are known to store large quantities of fluid (Bartels, 1909). The lymph nodes in mammals also serve as lymphatic depots. In addition, in other parts of the mammalian lymphatic system, ampoule and sac-like extensions were found, which could possibly serve to store fluid (Kubik, 1952). They would temporarily hold back the drainage of the lymph, serving as a kind of restraint system. What roles do the intraluminal structures play we observed? Do they serve as "barriers" that reduce the flow rate? Do they increase the "filtering effect" of these vascular sections by increasing the contact time between lymph and endothelial cells? So they are more than just a "strain protection"? Does manual lymphatic drainage affect such internal structures? Are such structures and their functions changed in diseases such as lymphedema or inflammation?

After accidents in the facial area there can be scars and oedema. In the depicted patient (Fig. 11), scarring occurred in the draining lymphatic vessels following injury from a car accident. These caused secondary lymphedema (Földi et al., 1998) on the right upper and lower eyelids. After skin flap plastic surgery with resection of the scar area, the lymphedema remitted. What transformations did the internal structures of the lymphatic vessels undergo in these scar areas?

Xerophthalmia, caused by vitamin A deficiency, causes the conjunctiva and cornea to dry out. Xerophthalmia is a widespread cause of blindness in the so-called "developing countries". In our latitudes, we rather encounter the "dry eye syndrome". It is estimated that 15 million people in Germany have problems with dry eyes (Bundesverband der Augenärzte Deutschlands e.V., 2013). In the US, the cost of the disease was estimated at $ 55.4 billion per year (Yu et al., 2011). Are the conjunctival lymphatic vessels involved in this disease, or is their retention and clearance function involved? The rodent-propagated Hantaviruses in America are known to cause dramatic changes in barrier function on the lymphatic endothelial cells without destroying the endothelium (Mackow et al., 2013). They block the receptor and signal transmission, similar to what we know about the intestinal epithelial cells of cholera toxin. One consequence of Hantavirus infection is fatal acute pulmonary oedema, referred to as Hantavirus pulmonary syndrome (HPS; MacNeil et al., 2011). In Germany, Hantavirus infections have also been observed in recent years. (According to the Robert Koch Institute at Berlin, 1.713 infections were reported in the year 2017 (Hofmann et al., 2018)). In the case of the "dry eye syndrome" (Javadi and Feizi, 2011), is there also a change in intraluminal structures or interference in the signal network? At least, Goyal et al. (2010) could demonstrate lymphatic vessels unaccompanied by blood vessels growing toward the centre of corneas with dry eye syndrome, accompanied by increased VEGF and VEGF-R increase based on the inflammatory stimulus. Hos et al. (2016) showed that IL-10 modulates corneal lymphangiogenesis and resolution of inflammation. IL-10 was not expressed in healthy corneas but was up-regulated in inflamed corneas by infiltrating macrophages. Lymphangiogenesis plays therefore an unexpectedly beneficial role in the regulation of corneal oedema and transparency (Hos et al., 2017). Thus, the ingrowths of pathologic lymphatic vessels into the cornea not only reduce its transparency and thereby visual acuity, but also significantly increases the rate of graft rejections after subsequent corneal transplantation (Bock et al., 2013). Lymphangiogenesis also occur due to malignant tumours and these tumours can invade these newly built lymphatic vessels (Heindl et al., 2010; Hos et al., 2015).

Or, conversely, are such changes the basis for a loss of retention function, which leads to dehydration of the conjunctiva? Is this also true for the age-related "dry eye"? Age changes in conjunctiva-associated lymphoid tissue (Cain and Phillips, 2008) and in lymphangiogenesis (Hos et al., 2008) have been confirmed in animal experiments, but are they also true for internal structures? Zolla et al. (2015) described aging-related anatomical and biochemical changes in lymphatic collectors impairing lymphatic transport, fluid homeostasis, and pathogen clearance. However, there are no indications of changes in the area of interior structures. Only the old anatomical ideas of the valves appear again, "*which close in coordination with the lymphatic muscle contractile activity, prevent lymphatic backflow at the end of each contraction*".

Lymphangiogenesis is also a disturbing factor in transplantation surgery. In cardiac allografts, most often, lymphangiogenesis increases the chances of rejection in cardiac transplantation (Abouelkheir et al., 2017), or corneal transplantation (Dietrich et al., 2010).

New intravital imaging techniques such as two-photon microscopy (Steven et al., 2011), microscopical optical coherence tomography (Horstmann et al., 2017), or more invasively by injection of fluorescin into the corneal stroma (Le et al., 2018), increase the spectrum of methods and by doing so in-vivo new and promising results have to be expected.

Because of the accessible position, the eye offers itself like no other organ for the study of the lymphatic vessels, but let us not forget the admonition of the Swiss anatomist His, who wrote in his introduction in 1863:

"*If one wants to make a theory about the formation of the lymph and of the forces acting on its locomotion, which one desires, this, as an indispensable basis, demands a precise idea of the anatomical behaviour of the first roots of the system, with this idea either sprouted from observation or only assumed hypothetically.*"

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