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Lymphatic corneal neovascularisation affects graft survival in high-risk corneal transplantation

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ABSTRACT

Objectives Corneal neovascularisation (CoNV) is a major risk factor for corneal allograft rejection and failure. This study assessed the impact of preoperative lymphatic and haematic vascularisation of the graft bed on graft survival in a clinical setting.

Methods and analysis This retrospective study included patients with histologically confirmed CoNV (positive staining for CD-31) who underwent penetrating keratoplasty (PK) between 2008 and 2023 at the Medical University of Innsbruck, Austria. Cases were divided into two groups depending on the presence or absence of lymphatic CoNV (podoplanin staining). Follow-up was 2 years or until graft failure. Outcome parameters included the risk of graft failure and leakage patterns in a subgroup with preoperative indocyanine green (ICG) angiography.

Results Of 17 included patients, lymphatic CoNV was identified in the excised corneal buttons of 10 cases (group 1). Seven cases stained only for haematic CoNV (group 2). Group 1 had a shorter age of CoNV (0.6±0.4 vs 2.3±0.8 years, p<0.001) and a higher rate of graft failure (6/10 vs 0/7, p=0.005). Lymphatic CoNV was only present in the age of CoNV less than 12 months. ICG leakage was associated with a younger age of CoNV (p=0.0338), corresponding to the presence of lymphatic CoNV at a younger age of CoNV.

Conclusion Lymphatic CoNV in haemvascularised corneal stromal beds increases the risk of graft failure within 2 years. Lymphatic CoNV regression occurs within the first year of an inciting event. This time period or the presence of ICG dye leakage indicates a very high risk for corneal transplantation.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Corneal neovascularisation (CoNV) is a major risk factor for corneal allograft rejection and failure.

WHAT THIS STUDY ADDS

⇒ The presence of lymphatic vessels significantly raises the risk of graft failure compared with corneal stromal beds with only haematic CoNV. Lymphatic CoNV was associated with a younger age of CoNV and found in cases aged<1 year.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ In the absence of clinically feasible diagnostic tools to identify lymphatic CoNV, the identification of a high-risk period for lymphatic CoNV as well as the potential use of indocyanine green angiography can be of additional value in the patient's medical and surgical management.

INTRODUCTION

Corneal transplantation is the most commonly and successfully performed tissue transplantation worldwide and has been proven as an effective procedure in restoring sight to those affected by visual-impairing corneal diseases.[1 2]

The corneal immune privilege is one of the main contributing factors to its success in reversing blindness. Anatomical, cellular and molecular barriers in the cornea as well as tolerance related to anterior chamber-associated immune deviation and regulatory T cells and the immunosuppressive intraocular microenvironment play a critical role in maintaining its privilege.[3] A balance between proangiogenic and antiangiogenic factors is essential in maintaining the avascularity of the cornea as part of it. During infection, trauma or idiopathic limbal stem cell insufficiency, haematic and lymphatic vessels from the adjacent limbal vascular arcade can overcome those barriers and invade corneal tissue. Corneal neovascularisation (CoNV) leads to a loss of the corneal immune privilege and is a well-established risk factor for graft failure after corneal transplantation leading to high-risk status.[2–7]

Clinical experience out of the Australian Corneal Graft Registry report from 2018 has shown that the survival rate of avascular grafts at 4 years was 83%, compared with 73%, 66%, 63% and 50% for 1-quadrant, 2-quadrant, 3-quadrant and 4-quadrant neovascularisation, respectively. For recipients with CoNV, graft failure is still very relevant.[8]

The introduction of markers for lymphatic endothelium in the last decades, such as podoplanin, LYVE-1 and Prox1, allowed for

advanced research of the immunological mechanisms of otherwise biomicroscopically invisible lymphatic CoNV. Among them, podoplanin has been confirmed as a reliable marker in the identification of lymphatic vessels in human corneal buttons and other organs.[9–11]

Experimental studies were able to provide us with valuable insights into the interaction of both vessel types (haematic and lymphatic)—confirming that CoNV initially occurs as a combined outgrowth of both, followed by an earlier and more pronounced regression of lymphatic compared with haematic CoNV.[12 13] Cursiefen *et al* were able to demonstrate that lymphatic CoNV as afferent and haematic CoNV as efferent components together with the associated regional lymph nodes form an 'immune reflex arc', which is primarily responsible for recognising foreign tissue and triggering immune response.[4 12 14–17]

Murine studies suggest that not the haematic but lymphatic CoNV are the most important promotors for graft failure. However, there is a lack of clinical studies due to small case numbers and difficulties in verifying lymphatic CoNV in humans.[12 18]

The present study aims to close this gap by assessing graft survival after penetrating keratoplasty (PK) depending on the presence of lymphatic CoNV in the recipient's corneal graft bed.

MATERIALS AND METHODS

This investigation was designed as a retrospective chart review, including patients independent of age or gender, who underwent PK with evident CoNV in colour photograph between 1 January 2008 and 31 December 2022, within the Department of Ophthalmology, Medical University of Innsbruck (MUI), Austria. The obtained corneal material from transplantation was routinely stained for CD-31 as for vascular endothelium and for podoplanin as for lymphatic vessels. Only cases with confirmed CD-31-positive haematic CoNV in the excised corneal buttons were included. These cases were divided into two groups depending on the presence or absence of lymphatic markers (group 1: podoplanin positive; group 2: podoplanin negative). Follow-up was for a minimum of 2 years or until graft failure with the need for re-transplantation. Graft complications were recorded as noted in the patient chart and classified as either severe immunological (including primary graft failure, epithelial and endothelial rejection resulting in a need for re-transplantation), mild immunological (rejection that could be resolved with intensive topical and systemic treatment) and non-immunological complication (microbial keratitis). A subset of patients had received indocyanine green angiography (ICGA) previously. For this subgroup, the presence of dye leakage was correlated with the age of CoNV.

Transplantation and postoperative treatment

Corneal transplantation was performed by one of two surgeons (BS and CP). Standard postoperative topical treatment consisted of dexamethasone 0.1% eye drops five times daily, cyclosporine 2% eye drops five times daily, ofloxacin 3 mg/mL eye drops four times daily, artificial tears and timolol 0.5% eye drops two times daily. In uneventful follow-up examinations, topical treatment was tapered according to the following regimen:

- Dexamethasone: months 1–4: five times daily, months 5–8: four times daily, months 9–12: two times daily, months 13–18: once daily
- Cyclosporine: months 1–18: five times daily.
- Ofloxacin: months 1–4: four times daily, months 4–18: two times daily
- Artificial tears: month 1: hourly, months 2–18: reduction individually
- Timolol: months 1–18: two times daily.

In case of rejection, topical dexamethasone was increased to hourly application and the patient was hospitalised for systemic treatment.

Systemic postoperative immunosuppressive or immunomodulatory treatment (cyclosporine, mycophenolate mofetil or methylprednisolone) was given in cases with more than two quadrants of CoNV or after a first rejection episode, between systemic regarding on the patient's comorbidities.

Age of CoNV

Age of CoNV was defined as the time elapsed from the last active inflammatory or infectious episode to keratoplasty, associated with documented corneal angiogenesis. The date of the last active inflammatory or infectious episode was determined by reviewing the patient's chart. Episodes were rated as active when both criteria were fulfilled: (1) topical antibiotic, antiherpetic or corticosteroid treatment was initiated or increased and (2) CoNV was clinically graded 'active' according to a modified CoNV grading score by Palme *et al*.[19]

In the context of ICGA, the age of CoNV was calculated as the period from the last active episode regarding the same criteria, until performed angiography.

Colour photography

Corneal colour photography was obtained using a slit-lamp mounted digital system (SL-D Digital Slit Lamp; Topcon, Tokyo, Japan).

Immunohistochemistry/immunofluorescence staining

For immunohistochemical staining, paraffin-embedded tissue samples were cut into 3 µm sections according to a mark placed on the excised tissue by the surgeon to specify the region affected by CoNV. After heat-mediated antigen retrieval with sodium-citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0), samples were blocked with 3% H202 (8070.2, Roth) and Ultravision Protein Block (TL-015-HD, Epredia) at room temperature for 10 respectively 30 min. Slides then were incubated with CD31 (M0823, Dako) 1:25 or podoplanin (M3619, Dako) 1:150 overnight at 4°C diluted in SignalStain Antibody Diluent (8112, Cell Signaling). For signal detection,

Ultravision LP Detection System HRP DAB (TL-015-HD, Epredia) and Large Volume AEC Chromogen Single Solution (TA-125-SA Thermo Shandon Limited) were used in accordance with the manufacturer's user instructions. Haematoxilin (T865.2, Roth) staining was performed for 4 min. Imaging was performed with an upright light microscope (Olympus BX53, Olympus Life Science Solutions, Tokyo, Japan).

For immunofluorescence staining samples were mounted horizontally with Tissue Tek (Dormagen, Germany) OCT compound (Sakura cat 4583) on a specimen disc. The mounted samples were cut parallel to the surface at a thickness of 100 µm and transferred in Tris-buffered saline for immunofluorescence staining to identify blood and lymphatic CoNV. All staining steps were done free floating at 4°C for 24 hours. After blocking and permeabilisation with 5% donkey serum (Abcam, ab7475) in TBS-T 0,1% buffer, the samples were incubated with podoplanin (1:300, Dako cat M3619) and CD-31 (1:50, Abcam cat ab28364). The detection of the primary antibodies was undertaken with donkey anti-mouse Alexa Fluor 555 (Invitrogen cat A-31570) and donkey anti-rabbit Alexa Fluor 488 (Abcam cat ab150111) as secondary antibodies. After washing, the slides were mounted carefully on microscope slides with antifading mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI, Dianova cat SCR-038448). Controls were prepared by omitting one of the two antibodies. Microphotographs were taken on a Zeiss Axio Imager Z2 fluorescence microscope.

ICGA

Corneal angiography was performed as previously described:[19] ICGA was performed using a scanning laser ophthalmoscope (HRA2; Heidelberg Engineering, Heidelberg, Germany. 5 mL of 5 mg/mL ICG dye (Pulsion Medical Systems, Munich, Germany, ICG) were injected into a peripheral arm vein followed immediately by videography for 20 s. Single-frame ICGA photographs of the whole cornea capturing corneal vessel fluorescence every 5 s were taken for 3 min in high-resolution mode incorporating automatic real-time software. Late ICGA images were taken every minute between 5 and 10 min. Video pictures taken immediately after the dye injection were analysed for dye appearance by two independent observers (NF and CP). Haziness and increasing fluorescence of corneal stroma adjacent to CoNV on ICGA images were considered as evidence of leakage. The relationship between leakage of ICG dye and the age of CoNV was examined.

Statistical analysis

Two-tailored Student's t-test, Welch's t-test and Pearson's χ^2 test were performed using Jamovi 2.3.21 (www.jamovi.org). Mantel-Cox test for survival analysis was performed using GraphPad Prism (V.10.1.2; 2023; GraphPad Software, Boston). Results are presented as mean±SD with p values<0.05 considered significant. Figures and tables were designed using GraphPad Prism (V.10.1.2; 2023; GraphPad Software, Boston), Excel and Word (Microsoft; Redmond, Washington, USA).

RESULTS

17 patients were included in this study, 7 of them were female and 10 were male. All patients stained positive for haematic CoNV and were divided into two groups depending on the immunohistochemical presence of lymphatic CoNV: Group 1 included 10 patients who stained positive for CD-31 and podoplanin and group 2 included 7 patients who stained positive for CD-31 but negative for podoplanin. Patients' baseline characteristics are summarised in table 1.

Significant differences in the patient's baseline examination were found for the patient's age and age of CoNV. Mean recipient's age at surgery was 69.5±15.5 years in group 1 and 49.4±21.1 years in group 2 (p=0.039). The age of CoNV was 0.6±0.4 years for group 1 (range 0.06–0.99 years) and 2.3±0.8 years for group 2 (range 0.61–2.91 years; p<0.001). Corneal lymphatic vessels were exclusively identified for the age of CoNV of less than 1 year.

Gender (female/male: 2/10 vs 4/7; p=0.13) and preoperative visual acuity (2.5±0.6 vs 1.8±0.9 logmar, p=0.07) were not significantly different.

The area of CoNV was described as 1-quadrant, 2-quadrant, 3-quadrant or 4-quadrant CoNV. Comparing groups 1 and 2, we found the following distribution: 4-quadrant CoNV (4/10 vs 2/7; p=0.653), 3-quadrant CoNV (0/10 vs 2/7; p=0.172), 2-quadrant CoNV (5/10 vs 2/7; p=0.409) and 1-quadrant CoNV (1/10 vs 1/7; p=0.803).

4 of 10 cases in group 1 and 5 of 7 cases in group 2 were first corneal grafts. The remaining cases were re-grafts (p=0.226).

1 of 10 patients in group 1 and 0 of 7 patients of group 2 were pretreated with systemic immunosuppressive medication (cyclosporine=1) (p=0.42), 7 of 10 patients in group 1 and 4 of 7 patients in group 2 were pretreated with topical corticosteroids (p=0.612) until PK. The mean duration of topical steroids was 1.11 years for group 1 (range 0.12–3.26 years) and 1.11 years for group 2 (range 0.44–2.2 years; p=0.996). Type and dosage were individually chosen for each patient.

Aetiology of CoNV were microbial keratitis (n=9), dystrophy (n=6) and trauma (n=2). There were no significant differences for both groups (microbial keratitis: 6/10 vs 3/7, p=0.517; dystrophy: 3/10 vs 3/7, p=0.612; trauma: 1/10 vs 1/7, p=0.803).

Postoperative treatment, clinical graft outcome and graft complications

7 of 10 patients in group 1 received systemic immunosuppressive or immunomodulatory treatment after surgery (cyclosporine=4, mycophenolate mofetil=1, methylprednisolone=2), compared with 5 of 7 patients in group 2

Table 1 Patients' characteristics

	Group 1 (CD-31 +/ podoplanin +) n=10	Group 2 (CD-31 +/podoplanin –) n=7	
Age at surgery (years)	69.5±15.5	49.4±21.1	p=0.039*
Gender (female, male)	2, 8	4, 3	p=0.13
Preoperative visual acuity (logmar)	2.5±0.6	1.8±0.9	p=0.07
Age of CoNV (years)	0.6±0.4	2.3±0.8	p<0.001***
Quadrants of CoNV (n)			
4 quadrants	4	2	p=0.653
3 quadrants	0	2	p=0.172
2 quadrants	5	2	p=0.409
1 quadrant	1	1	p=0.803
Number of grafts (first graft, re-graft)	4, 6	5, 2	p=0.226
Preoperative systemic immunosuppressive therapy (yes, no)	1, 9	0, 7	p=0.42
Preoperative topical steroids (yes, no)	7, 3	4, 3	p=0.612
Aetiology of CoNV (n)			
Microbial keratitis	6	3	p=0.517
Dystrophy	3	3	p=0.612
Trauma	1	1	p=0.803

Continuous data are presented as mean±SD.
*p<0.05, ***p<0.001.
CoNV, corneal neovascularisation.

(cyclosporine=3, mycophenolate mofetil=1, methylprednisolone=1; p=0.953).

Both groups received identical postoperative topical treatment. Tapering was applied for both groups as described with only minor individual changes from protocol. At the time of rejection, there were no major differences in topical treatment regarding corticosteroids and cyclosporine between the two groups.

A higher rate of graft failure was observed in group 1 versus group 2 (6/10 vs 0/7, p=0.005). The age of CoNV was significantly decreased in cases with graft failure compared with cases without graft failure (0.56±0.448 vs 1.68±1.04 years, p=0.008). No significant association between graft failure and male gender (5/11 vs 1/6, p=0.232) and graft failure and increased patient's age (72.5±12.1 vs 55.1±21.4 years; p=0.088) was found.

Of six failed cases, failure occurred within 1 year in four cases, within 2 years in one case and after 2 years in one case. Hence, 1-year and 2-year graft survival in group 1 were 60.0% and 50.0%, respectively, compared with a 100% 2-year graft survival for patients in group 2 (p=0.037) (figure 1).

Regarding on type of graft complication, we found a significantly higher rate of severe immunological complications with a need for re-transplantation in group 1 versus group 2 (5/10 vs 0/7; p=0.015). Mild immunological complications were not significantly different for both groups (0/10 vs 2/7; p=0.172), as were other complications (2/10 vs 0/7; p=0.168). The mean period from transplantation to beginning rejection was 6 months for both groups (0.518 years, range 0.05–1.31 vs 0.54 years, range 0.5–0.58; p=0.964). The type of complication was illustrated by reflecting on vessel type and quadrants of CoNV (figure 1). Recipients with CoNV>2 quadrants did not show more overall (5/8 vs 4/9; p=0.488) or immunological (4/8 vs 3/9; p=0.517) complications compared with recipients with a lower-quadrant CoNV.

Group 1 presented with more re-grafts than group 2 (6/10 vs 2/7; p=0.226), five of the eight re-grafts failed. All failed re-grafts were from group 1, hence stained positive for lymphatic CoNV. Age of CoNV for lymph-positive primary versus re-grafts (0.65±0.37 vs. 0.56±0.45 years; p=0.756), was not significantly different. Overall graft failure was significantly increased in re-grafts compared with primary grafts (5/8 vs 1/9; p=0.034). Severe immunological complications were not significantly increased comparing re-grafts to primary grafts (4/8 vs 1/9; p=0.103), but severe immunological complications were significantly increased when comparing lymphatic-positive to lymphatic-negative re-grafts (4/6 vs 0/2; p=0.025). No graft failure was found in lymphatic-negative primary grafts as well as lymphatic-negative re-grafts.

ICGA subgroup

Subgroup analysis was performed for 6 out of 17 patients, who had received ICGA for staging purposes prior to corneal transplantation. Angiography was performed

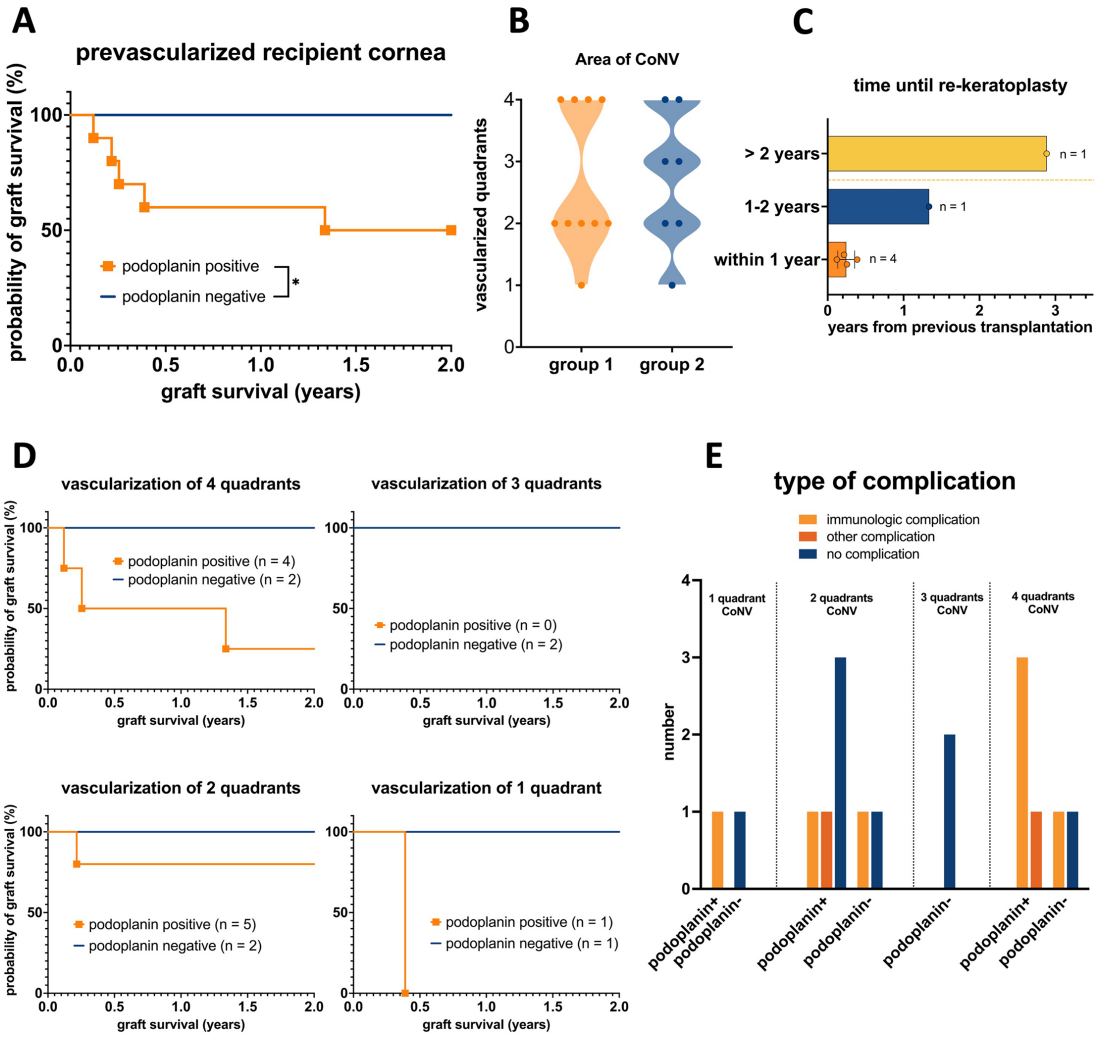

Figure 1 Additional lymphatic CoNV is associated with worse clinical outcomes compared with haematic CoNV only. (A) 2-year outcome analysis after high-risk corneal transplantation for patients in group 1 (podoplanin positive) versus group 2 (podoplanin negative), (B) distribution for both groups regarding on quadrants of CoNV, (C) time until graft failure for group 1, (D) 2-year outcome analysis for group 1 and group 2 regarding on vascularised quadrants, (E) type of complications listed for group 1 (podoplanin+) and group 2 (podoplanin–) with special regard to quadrant CoNV. Results are presented as mean±SD. Statistical comparison: Mantel-Cox test: *p<0.05. CoNV, corneal neovascularisation.

between 0.79 and 3.26 years before PK. Gender and patient's age are shown in table 2.

The mean age of CoNV at the time of ICG angiography was 252 days (range 42–555, SD±194 days). ICG dye leakage was observed in 2 of 6 cases (33%). The time to ICG dye leakage was 25 to 253 s. The mean age of CoNV for leakage-positive cases was 56 days (range 42–70, SD±19.8 days) and for leakage-negative cases was 351 days (range 182–555, SD±156 days, p=0.0338). All cases with the age of CoNV of less than 10 weeks showed ICG leakage, while no case with the age of CoNV of more than 26 weeks showed leakage. There was no statistically significant difference regarding affected quadrants of CoNV, graft number or topical corticosteroid pretreatment (table 2).

Leakage patterns and immunohistochemical staining were correlated in 4 of 6 cases. 2 of 6 had to be excluded because of an inflammatory event in the time period between ICGA and PK—angiography was performed 1.2 and 3.26 years prior to surgery in these cases. 1 of 2 cases with ICG dye leakage stained positive for podoplanin. In the case with positive staining, angiography was performed 0.79 years before PK, in the case with negative staining, angiography was performed 2.34 years before PK. No lymphatic CoNV was identified in cases without angiographic dye leakage (0 of 2) (figure 2).

DISCUSSION

The state of corneal vascularity has drawn major interest in recent research as it implies individual risk assessment scores for the ideal time point of surgery as well as possible clinical preventive measures.[20] Clinical studies investigating the role of the different types of CoNV have been scarce due to difficulties in confirming lymphatic CoNV in humans and overall low patient numbers and heterogenous baseline situations.

Table 2 Subgroup analysis: ICG angiography

	ICGA: leakage positive n=2	ICGA: leakage negative n=4	
Patient's age at the time of angiography (years)	57.6±3.19	61.0±9.67	p=0.557
Age at CoNV at the time of angiography (years)	0.15±0.05	0.96±0.43	p=0.0338*
Gender (female, male)	0, 2	1, 3	p=0.541
Time to ICG leakage min–max (sec)	25–253	–	
Quadrants of CoNV (n)			
4 quadrants	1	1	
3 quadrants	0	0	
2 quadrants	1	2	
1 quadrant	0	1	
Number of grafts (first graft, re-graft)	0, 2	2, 2	p=0.182
Topical steroids prior to angiography (yes, no)	1, 1	0, 4	p=0.500

Continuous data are presented as mean±SD.
*p<0.05.
CoNV, corneal neovascularisation; ICGA, indocyanine green angiography.

Previous animal studies have shown that lymphatic rather than haematic vessels are primarily responsible for corneal graft failure resulting in significantly worse survival rates for combined haemvascularised and lymphvascularised recipients. Recipients with haemvascularisation only showed comparably high survival rates to recipients with avascular graft beds.[18] The present study was conducted in a clinical setting in humans and could confirm these results. The presence of lymphatic was associated with a significantly higher risk of graft failure due to more severe immunological graft complications. Furthermore, this study has identified a high-risk time period of 12 months for lymphatic CoNV that can be determined by clinicians from previous examinations and patient history. This exact time period varies slightly from previous studies but can be explained by a yet inconsistent definition of 'duration'/'age' of CoNV and a potentially altered angiogenesis/angioregression in recipients who are not treatment-naïve regarding immunomodulatory therapy.[12 21]

Potential contributing risk factors for poor graft outcome

In the literature, other risk factors associated with poorer graft survival are mentioned that need to be addressed:

Regarding recipient's age, Maguire *et al*,[5] Musch and Meyer[22] and Boisjoly *et al*[23] found that younger patients are at increased risk for adverse outcomes. In these studies, not all keratoplasties were performed in vascularised situations. Hos *et al*[24] have demonstrated in a recent murine study that inflammation-induced corneal lymphangiogenesis significantly decreases with ageing. This could not be confirmed in our study, as lymphatic

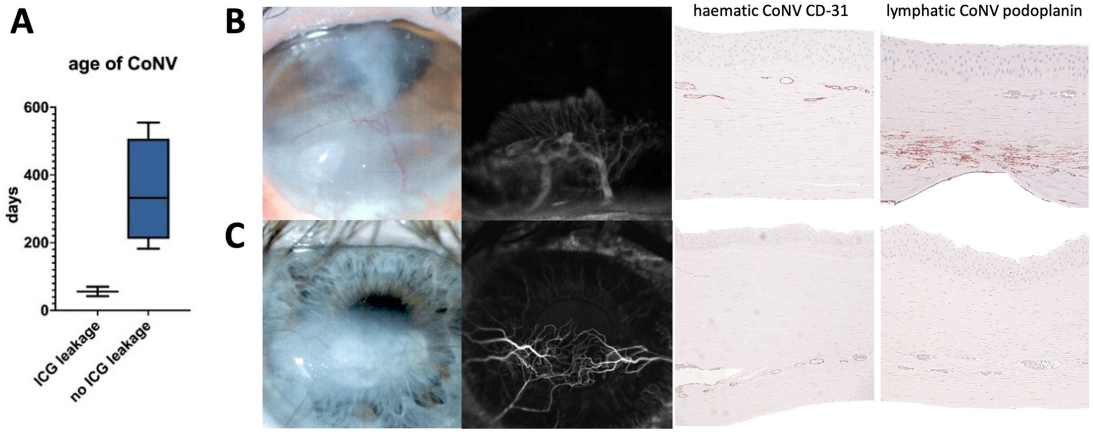

Figure 2 Association between age of CoNV, ICGA and immunohistochemistry. (A) ICG dye leakage is present in patients with the age of CoNV≤70 days. (B) Case with positive lymphatic CoNV: slit lamp image of CoNV and corresponding leakage in ICG angiography; positive staining for both CD-31 and podoplanin. (C) Case with negative lymphatic CoNV: slit lamp image of CoNV and corresponding ICG angiography with absent leakage; positive CD-31 and negative podoplanin staining. Results are presented as mean±SD and range. CoNV, corneal neovascularisation; ICGA, indocyanine green angiography.

vessels were more evident in older recipients (p=0.039). Bachmann *et al*[25] suggest in a meta-analysis that increased recipient age could be an independent risk factor in recipients with CoNV. In our study, we found a significantly higher presence of lymphvascularisation in older recipients but when comparing patient's ages for failed and non-failed grafts, age was not significant (p=0.088). Still, it could be an additional risk factor that was not confirmed significant due to limited patient numbers. Overall, the controversial findings in the literature let us suggest that recipient's ages may not be the most prominent promotor for adverse outcomes.

Male gender, number of quadrants affected by CoNV and former grafts have been addressed as risk factors for graft failure.[5 25 26] Male gender was not identified to be a significant cofactor, but overall, more men were included in this study. The number of quadrants affected by CoNV was not a relevant cofactor, as overall (p=0.488) and immunological complications (p=0.517) were not significantly increased in recipients with CoNV>2 quadrants. The majority of cases in the lymphatic group received repeat corneal transplantation (6/10 vs 2/7), which can be considered to reflect on our results. Since all failed grafts stained positive for lymphatic CoNV but not all were re-grafts and likewise, all re-graft with negative lymphatic CoNV survived, lymphatic CoNV seems to outweigh the risks of failure associated with repeat transplantation in our study. This could even indicate that lymphatic vessels might be a cofactor in the worse outcome of corneal re-grafts, as severe immunological complications were increased in lymphatic re-grafts compared with haemvascularised re-grafts supported by the findings of Diamond *et al*, who found lymphatic CoNV in all failed vascularised transplants in a series of nine cases.[10] Larger patient numbers are required to further assess a potential relation.

ICGA

In vivo identification of lymphatic vessels remains challenging due to their anatomical features and fluid transparency. As various anti-(lymph-)angiogenic treatment methods are emerging, reliable in vivo methods to identify lymphatic neovascularisation are needed to determine which patient should be selected for pretreatment.[27]

Angiography using sodium fluorescein (FA) and ICG is a well-established diagnostic examination in ophthalmology with a low risk for serious adverse events and is available in most centres. As for corneal diseases affected by CoNV, it gives us excellent details of vessel topography and depth also in situations with extensive corneal opacity and scarring—mainly due to high plasma protein binding of ICG (98 %). Both FA and ICGA can provide us with functional information on vessel maturity and disease activity. Corneal angiography has successfully been used in guidance and monitoring medical and surgical management of CoNV, mainly in fine-needle diathermy as it aids to distinguish between afferent (arteriole) and efferent (venule) vessels.[28–36]

Various methods have been presented that aim to verify lymphatic CoNV in vivo. Romano *et al* have published a method using digital subtraction analysis (DSA) of a two-step FA and ICGA to visualise lymphatic vessels, presuming an uptake of dye in lymphatic CoNV after leakage. Results were correlated to confocal microscopy. As the author discussed himself, the method for DSA currently available is difficult to perform.[37] Le *et al* presented a method of intrastromal injection of fluorescein in mice to identify lymphatic vessels based on the same presumption. Corneal colour photographs were used for matching and LYVE-1 staining confirmed lymphatic CoNV. Matching with colour photograph may be an issue since generally not all CoNV can be well visualised with this approach.[38–40] Following optical coherence tomography (OCT)-based methods were examined: Microscopic OCT (mOCT) allowed non-invasive imaging of lymphatic CoNV in murine. The technology of mOCT has recently been implanted into a slit lamp-based imaging system, further results are awaited.[27 41] OCT angiography is currently not comparable to ICGA in quality of characterising CoNV especially as it is unable to detect vessels without red blood cell flow.[42]

In the current study, we present a potential additional use of ICGA to indirectly indicate the presence and absence of lymphatic CoNV based on the findings that both lymphatic CoNV and leakage of ICG are associated with younger age of CoNV.

We found that leakage in ICGA is present in patients with younger age of CoNV (<10 weeks), whereas leakage was absent in CoNV>26 weeks and therefore suggest ICG leakage to be a risk indicator for active CoNV more likely accompanied by lymphatic vessels. Our staining results however cannot be exactly correlated to the leakage pattern, since there was a variable period between ICGA and PK that is likely confounding our results. However, a correlation between leakage positivity and presence can be presumed in one case with the age of CoNV of 0.19 years and staining for positive staining for lymphatic CoNV 0.79 years later, thus within the determined risk period for lymphatic CoNV<1 year. Due to our small dataset, we cannot refer to a specific leakage pattern for the time period from 10 to 26 weeks of CoNV. Our results however are consistent with current literature: Cursiefen *et al* found lymphatic CoNV in all excised human corneas with CoNV aged<3 months, while no excised human corneas with CoNV aged>60 months contained lymphatic CoNV. Palme *et al* demonstrated that ICG dye leakage was only observed in cases with active CoNV.[19 21] Our findings offer a combined clinical and angiographic assessment on this behalf. The exact added value as well as sensitivity and specificity of ICGA as indirect indicators have yet to be determined in larger-scale prospective studies.

In conclusion, this clinical study confirms previous experimental evidence of a strong negative correlation between lymphatic CoNV and graft survival. In the

absence of clinically feasible diagnostic tools to identify lymphatic CoNV, the determined risk period for lymphatic CoNV and the use of ICG angiography may be of additional value in the determination of very high-risk situations.

Limitations of our study are low patient numbers and its retrospective nature. The value-add of ICGA remains uncertain due to the limited use and unstandardised time of performance making it almost impossible to correlate with our immunohistochemical evaluation. ICGA might have led to selection bias as not all included patients have routinely received the examination.

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