Purpose: Corneal neovascularization (CoNV) is a major risk factor for corneal graft rejection and other corneal conditions. The maturity of CoNV is important to guide treatment. This study investigated associations between clinical and angiographic characteristics of CoNV.

Methods: In a prospective cross-sectional study patients with CoNV of variable but known duration and etiology were included. All cases were clinically staged according to a simplified three-grade scale as active, inactive, and regressed and assessed using color photography, anterior-segment optical coherence tomography, and fluorescein and indocyanine green (ICG) angiography. Outcome parameters included age and depth of CoNV, perfusion times and time to leakage of fluorescein and ICG.

Results: Forty eyes of 39 patients with CoNV were included, active (14), inactive (22), and regressed CoNV (4). There were significant associations between the time to fluorescein or ICG leakage and clinical staging of CoNV ($R^2 = 0.24; P = 0.0011$, and $R^2 = 0.3; P = 0.0001$). In addition, there was a significant association between the time to fluorescein leakage and the age of CoNV ($R^2 = 0.32; P = 0.0002$). ICG leakage within 10 minutes was observed significantly more frequently in active than the inactive group and was not observed in regressed cases ($P < 0.0001$).

Conclusions: Simplification of the staging of CoNV to active, inactive, and regressed to is significantly associated with the time to extravascular leakage of fluorescein and indocyanine and may be useful to guide the selection of appropriate treatments.

Translational Relevance: The association between clinical and angiographic characteristics of CoNV may provide guidance to the treatment approaches.
to reduce mature CoNV while carrying less potential
to damage to the limbal epithelial stem cell niche and
the peripheral corneal stroma.\textsuperscript{10,11}

Selective inhibitors of vascular endothelial growth
factor (VEGF) and steroid eye drops are used
successfully to reduce young active CoNV, targeting
predominantly immature vessels.\textsuperscript{12} In an attempt
to estimate the activity and maturity of CoNV, a five-
stage clinical grading system has been proposed.\textsuperscript{13,14}
It is, however, extremely difficult if not impossible to
reliably assess these parameters based solely on the
biomicroscopic appearance of CoNV. Additionally,
not all of the CoNV is clinically evident, especially in
the presence of stromal scarring.\textsuperscript{15} Consequently,
color photographic assessment may underestimate the
area of CoNV (AOC) and relative risk to a planned
corneal transplant. As shown by our group, the
combined use of fluorescein and indocyanine green
angiography (FA/ICGA) has demonstrated better
vessel delineation than that obtained with color
images alone, particularly for blood vessels located
in deeper layers of the cornea, beneath areas of
corneal scarring and for inactive vessels with
decreased or absent red blood cell traffic.\textsuperscript{16,17} We
have also shown, that ICGA in conjunction with
objective computer-assisted image analysis provides a
reliable method for assessing quantitative changes of
CoNV by measuring the vessel diameter, distribution
of vessel diameters, origin, and vessel tortuosity.\textsuperscript{18,19}

We provided preliminary evidence, that the time and
extent of leakage of fluorescein dye may reflect the
activity of CoNV, and therefore offer the potential to
functionally stage CoNV.\textsuperscript{16,17} This study was per-
formed in order to test for an association between
angiographic features of perfusion and leakage with
the clinical stage of CoNV. The aim of this study was
to determine objective parameters of vessel maturity,
which may be useful to guide the selection of the most
appropriate type of treatment of CoNV.

Methods

This investigation was designed as a prospective
cross-sectional observational study, including patients
between 18 and 85 years of age with clinically visible
CoNV of variable but specified etiology and duration,
extending at least 3 mm onto the cornea on slit-lamp
biomicroscopy. Consecutive patients were recruited,
who presented for specialist assessment at the cornea
clinic at the Department of Ophthalmology of the
Medical University of Innsbruck (MUI), Austria. All
patients with CoNV of documented onset and age
and who were fit to have FA/ICGA were invited to
join the study.

Exclusion criteria included an undocumented age
and onset of CoNV, recurrent episode(s) of inflam-
mation after the documented onset of CoNV, a
history of allergy to fluorescein, iodides, and shellfish,
or the inability to fixate a target.

After inclusion, an ophthalmic history was collect-
ed in each patient on a standardized data collection
form. This included best-corrected visual acuity (BCVA) recorded on a decimal scale with visual
acuities of counting finger, hand movements, and
projection of light converted according to a validated
scale\textsuperscript{20} patient demographics (age, sex), diagnosis,
duration of symptoms, previous treatment (medical
and surgical), BCVA, and history of allergies. A
clinical examination using slit-lamp biomicroscopy
was undertaken and a diagram of CoNV and
opacification was recorded. Particular note was made
of any conjunctival, limbal, or corneal abnormalities.
For each case, clinical staging was initially attempted
according to a biomicroscopic five-point scale for
CoNV, briefly defined as follows\textsuperscript{13}:

Stage 1. Active young vessels: newly formed and
progressing vessels with bright-red appearance and
a well-defined fine capillary network. Signs of
leakage and edema in the surrounding corneal
stroma.

Stage 2. Active old vessels: less bright vessels that
reach the offending lesion, not progressing.

Stage 3. Partially regressed vessels: vessels with
slow blood circulation and reduced visibility.
Abated corneal pathology and edema in response
to treatment.

Stage 4. Mature vessels: relatively large vessels,
with minimal arborization and regressed or absent
capillary networks, persisting in scar tissue or in the
corneal stroma, with maintained red blood cell
perfusion.

Stage 5. Regressed (ghost vessels): fine white lines
mirroring the morphology of the original vessels
without visible red blood cell circulation.

It was found, however, that the differentiation
between stages 1 and 2, as well as between stages 3
and 4 according to the published criteria, was
unreliable and too subjective, as also acknowledged
by the authors.\textsuperscript{13} Therefore, for the no distinction was
made between stages 1 and 2, and between 3 and 4.
Three subgroups of clinical stages of CoNV were
therefore formed, ’Active CoNV’ (stages 1 or 2),
'Inactive CoNV' (stages 3 or 4), and 'Regressed CoNV' (ghost vessels, stage 5).

The presence of corneal scarring, corneal edema, exudative keratopathy, corneal ulceration, and relative depth of corneal melting was assessed using slit-lamp biomicroscopy.

Additional assessment included color, red-free and autofluorescence photography, anterior-segment optical coherence tomography (AS-OCT), and FA/ICGA of the AOC and adjacent limbus and conjunctiva, which is the region of interest (ROI). The limbus and adjacent conjunctiva are imaged to identify the main afferent and efferent vessels of the CoNV, which are situated in the limbus and adjacent conjunctiva. The depth of CoNV and minimal corneal thickness in the AOC was calculated from AS-OCT sections.

Informed consent was obtained and the study was conducted according to the tenets of the Declaration of Helsinki. The Ethics committee of MUI, carrying the identification number AN2015 0287 356/4.8, approved this study. The occurrence of adverse events and reactions (anaphylaxis, microbial keratitis, uveitis, loss of 2 or more lines of visual acuity) was documented on a designated and approved adverse events and reactions form.

**Color Photography**

Color photographs of the cornea were recorded using a slit-lamp mounted digital system (SL-D Digital Slit Lamp; Topcon, Tokyo, Japan). The entire cornea was imaged from limbus to limbus using 10-fold magnification of the ROI. Illumination was from a 45° angled beam on slit-lamp biomicroscopy with a diffuser filter and a variable flash intensity. An anterior-posterior image with diffuse beam and no filter was also obtained. Images with green filter and open beam at magnifications of 10-fold were recorded. A total of 18 color images were obtained for each patient.

**Fluorescein and Indocyanine Green Angiography (ICGA/FA)**

Both ICGA and FA were acquired using a scanning laser ophthalmoscope (HRA2; Heidelberg Engineering, Heidelberg, Germany). Five milliliters of 5 mg/mL indocyanine green dye (Pulsion Medical Systems, Munich, Germany) were injected into a peripheral arm followed immediately by videography for 20 seconds. Single-frame ICGA photographs of the whole cornea capturing corneal blood vessel fluorescence every 5 seconds were taken for 3 minutes in high-resolution mode incorporating automatic real-time (ART) software. This was followed by an intravenous injection of 3 mL of 10% sodium fluorescein (Martindale Pharmaceuticals, Essex, UK). The photographs were taken up to 3 minutes, similar to ICGA. Late ICGA and FA images were taken every minute between 5 and 10 minutes.

**Anterior Segment Optical Coherence Tomography (AS-OCT)**

Using the Heidelberg Spectralis HRA-OCT scanning laser ophthalmoscope (Heidelberg Engineering, Heidelberg, Germany) in conjunction with the AS module, corneal AS-OCT scans of the AOC were performed to calculate the depth of CoNV and corneal thickness in the AOC with the system-integrated software.

**Image Analysis**

Video pictures taken immediately after the injection of fluorescein or ICG were analyzed independently by two observers (BS and CP) for appearance of dye. Color images were analyzed for quality and clarity of ROI. The best ICGA, FA, and color images were independently selected by same two observers based on the following qualitative subjective parameters: quality grading for ICGA and FA was categorized as 0 to 4 (0, no vessel discernible; 1, poor vessel delineation; 2, good vessel delineation; 3, very good vessel delineation; 4, excellent vessel delineation). The images with good vessel delineation would have at least 50% of vessels clearly evident with distinct boundaries and hyperfluorescence. Quality grading for color images was categorized as 0 to 3 (0, no focus on ROI; 1, poor focus on ROI, no details of corneal scar/vessel; 2, acceptable focus on ROI, identifiable corneal scar/vessel; 3, very good or excellent focus on ROI, details of corneal scar and vessels evident). Only patients with images of quality score 3 or more for both ICGA and FA and quality score 2 or more for color were included for further analysis. Haziness and increasing fluorescence of corneal stroma adjacent to the blood vessels on FA images was considered as evidence of leakage. The relationship between leakage of fluorescein dye and duration of CNV was also examined. The best available ICGA, FA, and color image of the study eye of each patient was exported in TIFF format for the purpose of further analysis.
Table 1. Clinical Patient Characteristics in the Three Groups

<table>
<thead>
<tr>
<th></th>
<th>Active CoNV</th>
<th>Inactive CoNV</th>
<th>Regressed CoNV</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>Age (mean, y)</td>
<td>65.2 ± 15.4</td>
<td>55.4 ± 23.1</td>
<td>68.3 ± 10.1</td>
</tr>
<tr>
<td>(minimum [min] 43,</td>
<td></td>
<td>(min 14, max 85)</td>
<td>(min 56, max 78)</td>
</tr>
<tr>
<td>maximum [max] 87)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% female</td>
<td>14.3 (n = 2)</td>
<td>18.2 (n = 4)</td>
<td>50.0 (n = 2)</td>
</tr>
<tr>
<td>BCVA (mean)</td>
<td>0.22 ± 0.30</td>
<td>0.25 ± 0.36</td>
<td>0.31 ± 0.34</td>
</tr>
<tr>
<td>% topical steroids</td>
<td>28.6 (n = 4)</td>
<td>31.2 (n = 7)</td>
<td>0.0</td>
</tr>
<tr>
<td>% topical antimicrobials</td>
<td>85.7 (n = 12)</td>
<td>18.2 (n = 4)</td>
<td>0.0</td>
</tr>
<tr>
<td>% exudative keratopathy</td>
<td>42.9 (n = 6)</td>
<td>18.2 (n = 4)</td>
<td>0</td>
</tr>
<tr>
<td>% corneal edema</td>
<td>71.4 (n = 10)</td>
<td>4.5 (n = 1)</td>
<td>0</td>
</tr>
<tr>
<td>% epithelial defect</td>
<td>42.9 (n = 6)</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>% corneal scar</td>
<td>64.3 (n = 9)</td>
<td>100 (n = 22)</td>
<td>100 (n = 3)</td>
</tr>
<tr>
<td>% corneal infiltrate</td>
<td>35.7 (n = 5)</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Relative loss of corneal thickness</td>
<td>18.3 ± 22.01</td>
<td>22.7 ± 20.6</td>
<td>3.4 ± 5.6</td>
</tr>
<tr>
<td>(% melting)</td>
<td>(min 0, max 80)</td>
<td>(min 0, max 80)</td>
<td>(min 0, max 10)</td>
</tr>
<tr>
<td>Age of CoNV (median, wk)</td>
<td>4 ± 7</td>
<td>203 ± 381</td>
<td>3104 ± 1418</td>
</tr>
<tr>
<td></td>
<td>(min 1, max 28)</td>
<td>(min 23, max 1720)</td>
<td>(min 435, max 3432)</td>
</tr>
</tbody>
</table>

Outcome Parameters and Statistical Analysis

Outcome measures included the time to dye leakage, perfusion velocity, and depth of CoNV. The primary outcomes were tested for normal distribution, and if the normality was not satisfied, normality transformations, such as log or sort, were used. A Student t-test was used for if the data followed a normal distribution and a Mann-Whitney U test for nonparametric distribution. The associations between outcomes were tested using general linear models. A level of probability of less than 0.05 was considered statistically significant. A Bonferroni correction was made for multiple tests. Mean values with standard deviation are given for normally distributed data, and medians when the normality was not satisfied.

Results

Forty eyes of 39 consecutive patients with CoNV fulfilling the inclusion criteria were included. There were 31 men and 8 women, with a mean overall age of 60.1 ± 20.4 years. The most common diagnoses were herpetic keratitis (n = 15), trophic ulcer (n = 8), and bacterial keratitis (n = 6). Other diagnoses included graft rejection (n = 4), interstitial keratitis (n = 3), Acanthamoeba keratitis (n = 2), exposure keratitis (n = 1), and chemical burn (n = 1). The median of the age of CoNV was 96 weeks (minimum 1, maximum 3432).

Eleven cases (27.5%) were treated with steroid eye drops, and 16 cases (40.0%) treated with topical antimicrobial treatments at the time of the study examination.

Patients were classified as cases with active CoNV (stages 1 or 2; 14 cases), inactive CoNV (stages 3 or 4; 22 cases), or regressed CoNV (stage 5; 4 cases). Clinical characteristics of included patients in these three groups are given in Table 1, OCT-based and angiographic characteristics in Table 2.

The patients in the three groups did not differ significantly with regard to age, sex, diagnoses, and BCVA. Topical steroids were used in 28.6% of cases in the active and 31.2% in the inactive group and in none of the regressed CoNV group. Within these groups, steroid use was not associated with a longer time to fluorescein leakage (34.2 ± 5.7 vs. 30.7 ± 5.8, P = 0.450 in the active group; 45.7 ± 27.3 vs. 62.9 ± 33.4, P = 0.677 in the inactive group). Time to fluorescein leakage was significantly associated with the clinical staging of CoNV, with longer time to leakage in more advanced stages (R² = 0.24; P = 0.0011 and R² = 0.22; P = 0.0018). Likewise, there was a significant association between the time to ICG leakage and clinical staging (R² = 0.37; P = 0.0001 and R² = 0.31; P = 0.0002). Additionally, there was a significant association between the time to fluorescein leakage and the age of CoNV (R² = 0.32; P = 0.0002, Fig. 1). ICG leakage within 10 minutes was observed significantly more frequently in active than the inactive group and was not observed in regressed cases (P <
0.0001). ICG leakage was observed in 100% of the active group, 9.1% of cases in the inactive group and no cases in the regressed group. The mean time to ICG leakage was 316 ± 175.4 sec. ICG leakage was not observed in cases with CoNV older than 28 weeks (Fig. 1). Representative cases of all three subgroups are shown in Figure 2, including color photographs, ICG, and fluorescein angiograms.

Table 2. Angiographic and OCT-Based Characteristics of CoNV

<table>
<thead>
<tr>
<th></th>
<th>Active CoNV</th>
<th>Inactive CoNV</th>
<th>Regressed CoNV</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>22</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Time to fluorescein leakage, s</td>
<td>31.7 ± 5.8</td>
<td>57.4 ± 31.9</td>
<td>95.8 ± 89.7</td>
<td>0.007*</td>
</tr>
<tr>
<td>(min 23, max 42)</td>
<td>(min 25, max 130)</td>
<td>(min 45, max 230)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of ICG leakage, %</td>
<td>100.0 (13/13)a</td>
<td>9.1 (2/22)</td>
<td>0.0 (0/4)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>FA perfusion time, s</td>
<td>29.3 ± 7.2</td>
<td>44.0 ± 34.3</td>
<td>60.0 ± 23.8</td>
<td>0.102</td>
</tr>
<tr>
<td>(min 17, max 40)</td>
<td>(min 21, max 185)</td>
<td>(min 36, max 86)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICGA Perfusion time, s</td>
<td>28.8 ± 7.3</td>
<td>50.7 ± 48.5</td>
<td>63.2 ± 24.8</td>
<td>0.146</td>
</tr>
<tr>
<td>(min 17, max 43)</td>
<td>(min 22, max 255)</td>
<td>(min 38, max 85)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depth of CoNV, µm</td>
<td>271.9 ± 129.2</td>
<td>256.1 ± 105.4</td>
<td>258.3 ± 117.9</td>
<td>0.921</td>
</tr>
<tr>
<td>(n = 28)</td>
<td>(min 120, max 528)</td>
<td>(min 150, max 400)</td>
<td>(min 133, max 367)</td>
<td></td>
</tr>
<tr>
<td>Posterior depth of scar/infiltrate, µm</td>
<td>226.0 ± 88.3</td>
<td>330.5 ± 158.5</td>
<td>486.3 ± 178.9</td>
<td>0.056</td>
</tr>
<tr>
<td>(n = 28)</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

* Significance set at P < 0.0083 (Bonferroni correction).

a One case excluded due to lacking ICG angiographic data.

Figure 1. Association between time to fluorescein leakage and age of CoNV. The clinical stage of CoNV is indicated as follows: + active CoNV, ◊ inactive CoNV, × regressed CoNV. All cases with observed ICG leakage are contained in the marked square.
Discussion

It is known that the angioregressive potential of locally applied corticosteroids or inhibiting agents of VEGF receptor (anti-VEGF) decrease with the maturity of the targeted CoNV.\textsuperscript{7,14} Therefore, surgical methods, such as FND, are then recommended to treat mature CoNV.\textsuperscript{7,10,11} During vessel maturation in corneal angiogenesis, pericyte coverage is a histologic indicator of decreasing VEGF dependence. Pericyte recruitment is a fairly rapid process occurring in the majority of CoNV between its onset and keratoplasty.\textsuperscript{21} It is however, difficult to estimate the level of vessel maturity in vivo. The proposed clinical staging scales of CoNV lack objective measures of vessel maturity and the biomicroscopic characteristics have not been correlated with histologic evidence of vessel maturity.\textsuperscript{13}

In this study, we identified functional angiographic parameters that were significantly associated with clinically evident stages of CoNV. Based on previous preliminary evidence supporting the hypothesis of changing fluorescein leakage behavior during maturation of CoNV,\textsuperscript{16} our results confirm that the time to both fluorescein and ICG leakage increase with clinical staging of CoNV. Specifically, ICG leakage was predominately found in young and active CoNV, which strongly suggests this parameter as an indicator of vessel immaturity and thus potential responsiveness to anti-VEGF treatment. While the time to leakage is useful for FA, ICG leakage is best described as either present or absent.

The use of a previously proposed five-grade biomicroscopic staging scale of CoNV\textsuperscript{13} was attempted but found to be of limited clinical feasibility due to unreliable differentiation between stages 1 and 2 or

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Figure 2. Fluorescein and ICG angiograms of representative cases from the three subgroups of clinical stages of CoNV, showing significant ICG leakage from active but not inactive or regressed CoNV.
CoNV.13 ICG leakage was observed to a maximum stromal bleeding in areas of actively progressing agreement with previously described localized corneal proteins, intravenous dyes, and even erythrocytes, in formation may serve as possible exit points of plasma more than 80% findings reporting at least partial pericyte coverage of equal in the active (29% regressed group, the proportion of steroid use was may be a potential confounder not being used in the groups. Although steroid usage increased time to fluorescein leakage was observed in observed dye leakage behavior, no significantly in- of current topical medication potentially affecting the inclusion. Although patients were included irrespective ocular surface inflammation at the time of study heterogeneous study population with varying levels of feasibility of ICGA to 10 minutes. decreases over time, which in our experience limits quality of ICG angiograms, however, gradually a later time may therefore have been missed. The minutes after injection. Any occurrence of leakage at 7 TVST.

Endothelial sprouting by individual endothelial tip cells is preceded by a decrease in cell junction integrity and degradation of the endothelial lamina by matrix metalloproteinases.23 The mechanism of transvascular ICG leakage from unfenestrated CoNV may be explained by ultrastructural changes during corneal angiogenesis. It is known, that new corneal vessels form from existing limbal blood vessels.15 Endothelial sprouting by individual endothelial tip cells is preceded by a decrease in cell junction integrity and degradation of the endothelial lamina by matrix metalloproteinases.24,25 Thus, intercellular gaps during tube formation may serve as possible exit points of plasma proteins, intravenous dyes, and even erythrocytes, in agreement with previously described localized corneal stromal bleeding in areas of actively progressing CoNV.13 ICG leakage was observed to a maximum age of CoNV of 28 weeks. This is in line with histologic findings reporting at least partial pericyte coverage of more than 80% of all vessels after 3 months.21

In this study, we performed ICGA imaging for 10 minutes after injection. Any occurrence of leakage at a later time may therefore have been missed. The quality of ICG angiograms, however, gradually decreases over time, which in our experience limits the feasibility of ICGA to 10 minutes.

This study is cross-sectional and limited by a heterogeneous study population with varying levels of ocular surface inflammation at the time of study inclusion. Although patients were included irrespective of current topical medication potentially affecting the observed dye leakage behavior, no significantly increased time to fluorescein leakage was observed in steroid users within the groups. Although steroid usage may be a potential confounder not being used in the regressed group, the proportion of steroid use was equal in the active (29%) and inactive groups (31%).

Our study underlines the importance of intravenous angiographic analysis of CoNV despite the increasing use of noninvasive optical coherence-based angiography (OCTA). Ang and co-worker26 found OCTA comparable to ICGA for assessment of the area of CoNV. In a comparative analysis by our group, OCTA provided lower-quality images and was less precise in capturing small vessels of neovascular trees compared with ICGA.27 Current OCTA systems do not provide information on the afferent or efferent quality of CoNV, perfusion velocity, and leakage behavior or maturity of corneal vessels, as does FA/ICGA.16 Despite its current limitations in image quality OCTA is a feasible tool to assess quantitative information on CoNV. It cannot, however, currently replace intravenous angiography for qualitative assessment of CoNV. In the future, a more thorough understanding of changes of vessel architecture, arborization patterns and the capillary bed of CoNV over time may potentially allow deductions on vessel maturity from OCTA angiograms.

The results of this study connect biomicroscopic clinical assessment of CoNV with functional angiographic properties of CoNV. Intravenous dye leakage may be associated with incomplete pericyte coverage of CoNV as a promising predictor of the CoNV treatment response to anti-VEGF agents and serve as a clinically useful guide to the most appropriate therapeutic approach in the preconditioning treat- ment of CoNV, specifically between surgical (e.g., fine needle diathermy)11 and medical options.28

In conclusion, we found simplification of the staging of CoNV to active, inactive, and regressed to be clinically feasible and to be significantly associated with the time to extravascular leakage of fluorescein and indocyanine. Although a longitudinal study is needed, these features as features of vessel maturity may be useful to guide the selection of appropriate treatments of CoNV.

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References


