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Corresponding Author: Ms. Natasha Spiteri, M.D., FRCOphth

Corresponding Author's Institution:

First Author: Natasha Spiteri, M.D., FRCOphth

Order of Authors: Natasha Spiteri, M.D., FRCOphth; Vito Romano, MD; Yalin Zheng, PhD; Sohraab Yadav, MBChB; Rahul Dwivedi, BSc(Hons), MBChB; Jern Chen, MD; Sajjad Ahmad, PhD, FRCOphth; Colin Willoughby, MD, FRCOphth; Stephen Kaye, MD, FRCOphth

Abstract: Corneal angiography for guiding and evaluating fine needle diathermy treatment of corneal neovascularization

Natasha Spiteri MD1, FRCOphth, Vito Romano1 MD, Yalin Zheng PhD 2, Sohraab Yadav MBChB 1, Rahul Dwivedi BSc(Hons), MBChB1, Jern Chen1 MD, Sajjad Ahmad PhD, FRCOphth 1,2, Colin Willoughby MD, FRCOphth1, Stephen B Kaye MD, FRCOphth1,2

1. St Paul's Eye Unit, Royal Liverpool University Hospital, Liverpool, L7 8XP, UK
2. Department of Eye and Vision Science, University of Liverpool, UK.

Tel: +44 1517062000

Corresponding Author:

Natasha Spiteri

St Paul's Eye Unit, 8Z link, Royal Liverpool University Hospital, Liverpool, L7 8XP, UK  
nax024@gmail.com

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Running head: Targeted diathermy for corneal new vessels

Address for reprints:

St Paul's Eye Unit, 8Z link, Royal Liverpool University Hospital, Liverpool, L7 8XP, UK

This article contains one video as additional online-only material.

## Abstract

**Purpose:** To investigate the outcome of selective occlusion of the afferent vessel of corneal neovascular complexes (CoNV), using angiographically guided fine needle diathermy (FND).

**Design:** Retrospective interventional case series

**Subjects:** Patients with CoNV unresponsive to topical steroid therapy.

**Methods:** Visual acuity, color images, and fluorescein angiography (FA) and indocyanine green angiography (ICGA) were measured before and following FND with a minimum of three months follow-up. The number of afferent vessels crossing the limbus, time to fluorescein leakage, area and geometric properties of the CoNV were determined using an in-house automated program written in numerical computing language (MatLab R14; The MathWorks Inc., Natick, MA). The location of the afferent vessel was identified from the angiographic images and marked at the slit lamp using a needle to make a cut to the depth of the vessel. FND was then applied using an electrolysis needle.

**Main Outcome Measure:** Area of CoNV

**Results:** 30 patients underwent FND for CoNV that had not responded to treatment with topical steroids. The CoNV was associated with previous microbial keratitis (26), intrastromal corneal ring segments (2), ectodermal dysplasia (1) and corneal choristoma (1). Duration of CoNV was over six months in the 23 patients (77%), between three and six months in 3 patients (10%), and less than three months in 5 patients (13%). The number of afferent vessels per CoNV complex ranged from 1 to 3 with a mean diameter of 40 $\mu$ m (SD 10 $\mu$ m) and mean time to leakage from apical vessels was 44.22 seconds (min: 27.43s max: 63.59s). The number of episodes of FND treatments that were required was one for 20 patients (66.6%), two for 8 patients (26.6%) and three for 2 patients (6.6%). Following FND, the area of CoNV reduced by 1.80mm<sup>2</sup> (SD: 1.40); from 2.42mm<sup>2</sup> (SD: 1.59) to 0.62mm<sup>2</sup> (SD: 0.73) up to 12 weeks post-operatively (p<0.01).

**Conclusions:** The differentiation of afferent and efferent vessels using corneal angiography enables treatment to be selectively applied to the afferent vessels of which, there are usually 1 to 2 for each CoNV complex.

**POINT-BY-POINT RESPONSE FORM**

Please list the editor’s, reviewer(s), and editorial office’s comments in the left-hand column, spacing them so that you can insert the relevant response in the center column and the respective point(s) in the text (and tables or legends, if appropriate) in the right-hand column. Adding line numbers to the manuscript file and referring to specific line numbers will be useful in determining which parts of the manuscript changed.

Manuscript #: 2014-2033R1

Manuscript title: **Corneal angiography for guiding and evaluating fine needle diathermy treatment of corneal neovascularization**

<b>Suggestion, Question, or Comment from the Editor</b>	<b>Author’s Response</b>	<b>Change in the Manuscript</b>
Please clarify the length of follow-up in the abstract, precis, and table(s), e.g. in the precis: "...appears effective with 4 months of follow-up	Minimum 3 months follow-up specified	Lines 39-40, 106
Please review, discuss and cite the paper by Trikha et al. as suggested by Reviewer #2. This paper had a mean follow-up of almost 19 months	This paper which had a range of follow-up of 1 -56months, has been cited and discussed in both introduction and discussion	Lines 78, 80-2, 194-8
Please add details about the length of steroid treatment prior to considering diathermy, and whether steroid treatment was continued beyond the first 4 weeks after treatment in any cases	Details regarding steroid treatment have now been included. Steroids post-treatment limited to 4 weeks in all. Pre-treatment steroid ranged between 6 and 10 weeks	Lines 101-2, 153

<b>Suggestion, Question, or Comment from Reviewer #1</b>	<b>Author’s Response</b>	<b>Change in the Manuscript</b>
The authors need to acknowledge clearly that the afferent vs. efferent nature of the neovessels in the cornea can often be identified easily at the slit lamp and does	Whilst we respect the reviewer’s comment “the afferent vs. efferent nature of the neovessels in the cornea can often be identified easily	Lines 89 - 90  Although in some cases it may be

<p>not require imaging in all cases.</p> <p>The authors make the erroneous assertion in their discussion (in citing reference 17) that the central epithelium of the cornea is entirely devoid of Langerhans cells. This misconception was laid to rest many years ago; the central epithelium of the cornea DOES contain a few LCs most are not functional. Thus, the authors need to modify their assertion and simply state that FND can increase the numbers of these cells.</p>	<p>at the slit lamp”, we would also offer evidence to the contrary (References 11-14).</p> <p>We do however, agree that Identifying and distinguishing afferent and efferent vessels may be possible in some cases but this is not the general situation. We have shown that not all the vessels can be seen or easily distinguished on slit lamp biomicroscopy even aided by the patients pulse. We hope the reviewer will accept our comment that ‘once the afferent vessels or vessels have been identified on angiography, it then makes their identification on slit lamp biomicroscopy more evident and reliable’ and that this is a fair reflection of the literature.</p> <p>Thank you. Statement regarding absence of LCs in central cornea deleted</p>	<p>possible to differentiate afferent and efferent vessels on slit lamp biomicroscopy, aided for example by the patient’s pulse, this can be difficult. Once the afferent vessels or vessels have been identified on angiography, however, it then makes the identification on slit lamp biomicroscopy more evident and reliable.</p> <p>Line 218</p>
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Suggestion, Question,	Author’s Response	Change in the
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or Comment from Reviewer #2		Manuscript
<p>The technique described in the text lines (137-149) appear to show a bipolar approach to fine needle diathermy whereas the original description by Pillai et al (ref 7) used a unipolar approach. In the methods it is not clear whether the approach is bipolar or unipolar. A unipolar approach uses one electrode (suture needle) allowing a current to pass through the vascular complex (the patient is 'earthed'), allowing tissue to be coagulated. In a bipolar diathermy approach the current passes at the point of the electrode and superficial heating of tissue (vaporization effect) is created. This difference may not be that clinically significant but the authors could consider adding this to the discussion and indicating in the methods which type of diathermy was used.</p>	<p>Thank you. It was unipolar and this has been corrected.</p>	<p>Line 149</p>
<p>The table data include BCVA pre- and post-op but there is no statistical analysis on whether the improvement in BCVA is significant with this sample number.</p>	<p>Thank you. Statistical test result has been added. There was a significant difference between pre and post operative BCVA</p>	<p>Lines 156-7 176-7</p>
<p>The discussion mentions that long term results are poorly understood (lines 218-19), however, Trikha et al BJO "Long-term outcomes of Fine Needle Diathermy for established corneal neovascularization" have already shown a positive effect in the long term with this method.</p>	<p>Thank you. We have acknowledged and added this to the manuscript. Trikha et al have shown a improvement in the reduction in CoNV using FND.</p>	<p>Lines 78, 80-2, 194-8</p>

Suggestion, Question, or Comment from the Editorial Office	Author's Response	Change in the Manuscript
<p>Please upload the Author Contributorship Form. If you have more than 8 co-authors on the byline, please use additional forms. The form can be found at <a href="http://cdn.elsevier.com/promis_misc/OPHTHA_Contributorship.pdf">http://cdn.elsevier.com/promis_misc/OPHTHA_Contributorship.pdf</a>.</p> <p>Please be sure your references conform to OPTHALMOLOGY's style at this stage. Journal abbreviations should conform to those used by the National Library of Medicine. For more information, please visit the "References" section of our Guide for Authors at <a href="http://cdn.elsevier.com/promis_misc/oph%20gfa%2018%20mar%202014.pdf">http://cdn.elsevier.com/promis_misc/oph%20gfa%2018%20mar%202014.pdf</a>.</p>	<p>Author contributorship form uploaded.</p> <p>References checked and conform to Ophthalmology's style and NLM abbreviations.</p>	

Fine needle diathermy is an easy and effective treatment for corneal neovascularization, however since its cellular effects are poorly understood, diathermy should be used sparingly. This can be achieved by angiographically-guided targeting of afferent vessels.

1 **Corneal angiography for guiding and evaluating fine needle diathermy treatment of corneal**  
2 **neovascularization**

3

4 Natasha Spiteri MD<sup>1</sup>, FRCOphth, Vito Romano<sup>1</sup> MD, Yalin Zheng PhD<sup>2</sup>, Sohraab Yadav MBChB<sup>1</sup>, Rahul  
5 Dwivedi BSc(Hons), MBChB<sup>1</sup>, Jern Chen<sup>1</sup> MD, Sajjad Ahmad PhD, FRCOphth<sup>1,2</sup>, Colin Willoughby MD,  
6 FRCOphth<sup>1</sup>, Stephen B Kaye MD, FRCOphth<sup>1,2</sup>

7

8 1. St Paul's Eye Unit, Royal Liverpool University Hospital, Liverpool, L7 8XP, UK

9 2. Department of Eye and Vision Science, University of Liverpool, UK.

10

11 Tel: +44 1517062000

12

13 Corresponding Author:

14 Natasha Spiteri

15 St Paul's Eye Unit, 8Z link, Royal Liverpool University Hospital, Liverpool, L7 8XP, UK

16 nax024@gmail.com

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35 neovascular complexes (CoNV), using angiographically guided fine needle diathermy (FND).

36 **Design:** Retrospective interventional case series

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38 **Methods:** Visual acuity, color images, and fluorescein angiography (FA) and indocyanine green  
39 angiography (ICGA) were measured before and following FND [with a minimum of three months](#)  
40 [follow-up](#). The number of afferent vessels crossing the limbus, time to fluorescein leakage, area and  
41 geometric properties of the CoNV were determined using an in-house automated program written in  
42 numerical computing language (MatLab R14; The MathWorks Inc., Natick, MA). The location of the  
43 afferent vessel was identified from the angiographic images and marked at the slit lamp using a  
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47 steroids. The CoNV was associated with previous microbial keratitis (26), intrastromal corneal ring  
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49 months in the 23 patients (77%), between three and six months in 3 patients (10%), and less than  
50 three months in 5 patients (13%). The number of afferent vessels per CoNV complex ranged from 1  
51 to 3 with a mean diameter of 40 $\mu$ m (SD 10 $\mu$ m) and mean time to leakage from apical vessels was  
52 44.22 seconds (min: 27.43s max: 63.59s). The number of episodes of FND treatments that were  
53 required was one for 20 patients (66.6%), two for 8 patients (26.6%) and three for 2 patients (6.6%).  
54 Following FND, the area of CoNV reduced by 1.80mm<sup>2</sup> (SD: 1.40); from 2.42mm<sup>2</sup> (SD: 1.59) to  
55 0.62mm<sup>2</sup> (SD: 0.73) up to 12 weeks post-operatively (p<0.01).

56 **Conclusions:** The differentiation of afferent and efferent vessels using corneal angiography enables  
57 treatment to be selectively applied to the afferent vessels of which there are usually 1 to 2 for each  
58 CoNV complex.

59

60 **Introduction**

61

62 Avascularity of the cornea is important for its homeostasis and function. Corneal neovascularization  
63 (CoNV), however, may develop in response to hypoxia or inflammations, as infectious, allergic, toxic  
64 and traumatic injuries.<sup>1</sup> The presence of CoNV reflects an imbalance between anti-angiogenic  
65 factors, such as pigment-epithelium-derived factor (PEDF), and angiogenic factors, such as fibroblast  
66 and vascular endothelial growth factors (VEGF).<sup>2,3</sup> CoNV is however, part of the wound healing  
67 response and may be useful in the acute phase for the transport of, humoral and cellular elements  
68 involved in immune response, materials required for repair and regeneration, removal of toxic  
69 substances and drugs to the site of inflammation. Chronic up-regulation of the angiogenic response,  
70 however, results in the persistence of pathological new vessels, which have increased vascular  
71 permeability resulting in corneal oedema, lipid exudation, chronic or recurrent inflammation and  
72 scarring.<sup>4</sup> There is also the potential establishment of lymphatics, normally absent from the cornea,  
73 which may further disrupt the “immune privilege” status of the cornea.<sup>4</sup>

74

75 Various techniques have been employed to treat CoNV, including topical steroids, anti-VEGF,  
76 metalloproteinase inhibitors, photodynamic therapy, Argon laser, yellow dye laser, radiation,  
77 cryotherapy and conjunctival resection.<sup>4-6</sup> Fine-needle diathermy (FND) has been described by  
78 several groups for the treatment of CoNV.<sup>5,7-9</sup> It involves the application of a coagulating current  
79 through a unipolar diathermy unit or thermal cautery, usually delivered through a needle such as a  
80 cutting or an electrolysis needle. ~~Although~~ FND has shown promise with the largest retrospective  
81 study reporting a series of 56 eyes, showing regression of CoNV in 89.3% of patients following two or  
82 less treatments.<sup>9</sup> It is, however not known what long-term effects diathermy has on the cornea at a  
83 cellular level, particularly if applied to the multitude of vessels in the CoNV complexes. Reducing the  
84 number of vessels that need to be closed may reduce the potential risks associated with FND and  
85 improve the efficacy of the procedure. It is questionable whether both the afferent (presumed  
86 arteriole) and efferent (presumed venule) systems of CoNV require treatment and selective  
87 treatment to the afferent system may be sufficient. It is of note, that Cursiefen et al found that on  
88 histology, that arterioles tend to comprise less than 1% of CoNV.<sup>10</sup> One option therefore, would be  
89 to only treat the afferent vessel(s) of the CoNV complex.<sup>11</sup> While it is sometimes possible to  
90 distinguish afferent from efferent CoNV using slit lamp biomicroscopy at the slit lamp, it has been  
91 shown that the full extent and origin of the CoNV complex is not apparent often difficult to identify  
92 on color images.<sup>12-14</sup> We have, however, recently shown that corneal angiography (fluorescein  
93 angiography, FA and indocyanine green angiography, ICGA) are particularly useful in identifying

94 vessels not seen on color images<sup>12-14</sup>, especially in the presence of corneal scars or inflammation and  
95 [facilitate](#) identification and differentiation of afferent and efferent vessels and vessel leakage.<sup>13</sup> In  
96 order therefore, to minimise the amount of diathermy applied to the cornea, we describe the use of  
97 angiography to identify and specifically treat the afferent vessels using FND.

98

## 99 **Methods**

100 Patients who were undergoing FND for CoNV associated with previous corneal disease such as  
101 microbial keratitis and that had not responded to topical steroid therapy were included. [Topical](#)  
102 [steroids had been used given for between 66 and 12 weeks duration.](#) Inclusion criteria were CoNV  
103 extending more than 3mm into cornea with varying degrees of lipid keratopathy and no active  
104 keratitis or corneal ulceration. Patients were followed up for a period of at least four months.  
105 Patients were examined with slit lamp biomicroscopy, and color and red free images, and FA and  
106 ICGA were obtained pre-treatment and [three months](#) post-treatment. IRB approval and informed  
107 consent were obtained and the Tenets of the Declaration of Helsinki were adhered to.

108

109 Color, FA and ICGA images.

110 Color images were captured using a slit lamp mounted digital system (Topcon SL-D Digital Slit Lamp,  
111 Tokyo, Japan) with 10 to 25 times magnification. An HRA2 Scanning Laser Ophthalmoscope  
112 (Heidelberg Engineering, Heidelberg, Germany) with a 20 degree imaging lens set at 34 diopter (D),  
113 was used for ICGA and FA as previously described.<sup>12, 13</sup> After injection of 5ml of indocyanine green  
114 dye (5mg/ml) (Pulsion Medical Systems, Germany) videography was recorded for 25 seconds. Single  
115 frame ICGA images of the whole cornea capturing corneal blood vessel fluorescence were taken  
116 every 3-5 seconds for 3 minutes followed by late images at 5 and 10 minutes. An intravenous  
117 injection of 3mL of 20% Sodium fluorescein (Martindale Pharmaceuticals, Essex, UK) was then given  
118 and the videography repeated. During the acquisition of single frame ICGA and FA images hi-  
119 resolution mode with eye tracking automatic real-time (ART) software was used.

120

121 Image analysis

122 The region of interest (ROI) was defined pre-operatively as the area of the cornea containing the  
123 CoNV and was used to compare pre and post-operative images. Images of pre- and final post-  
124 operative angiograms of grade 3 or 4 were selected for analysis as previously described by two  
125 independent observers (SY and RD).<sup>12</sup> The number of afferent vessels crossing the limbus and time  
126 to leakage of fluorescein were recorded. The area and geometric properties of the vessels were  
127 determined on the selected images using an in-house automated program developed in Matlab R14

128 (The MathWorks Inc., Natick, MA).<sup>13</sup> In brief, the major steps of the program are as follows. For  
129 each image the pixel resolution (mm/pixel) was first defined as the ratio between the diameter of  
130 the cornea (mm) and the number of pixels measured manually from the image. A ROI containing all  
131 the corneal vessels was then defined by hand. The CoNV in the ROI was detected by applying  
132 Gaussian enhancement, selective vessel enhancement and thresholding techniques to the ROI in a  
133 sequential order. In the resulting binary image (1 indicating vessel pixels while 0 indicating  
134 background pixels) the area of CoNV (mm<sup>2</sup>) was computed by multiplying the number of CoNV pixels  
135 and the area (mm<sup>2</sup>) occupied by a pixel. The centrelines of the vessels were identified by a 'thinning'  
136 operation so that the branch points and terminal points can be identified. The branching and  
137 terminal points were then used to divide the vascular tree into individual vessel segments. The  
138 mean diameter (mm) of each segment can be computed so as to characterise the CoNV complex in  
139 the image.

140

141 FND technique (Video)

142 Afferent and efferent vessels were identified on videography (Figure 1) and the former labelled. The  
143 annotated image was used as a reference to mark the afferent vessel(s) with an inked needle. Using  
144 slit lamp biomicroscopy, under topical anaesthesia, a partial thickness incision was made using a 25  
145 gauge needle on a 1 ml syringe into the posterior stroma over the identified afferent vessel(s) either  
146 at the limbus or at the level of marginal corneal arcades.<sup>15</sup> If the afferent vessel was transected, an  
147 interruption of flow was evident in the visible vessels carrying red blood cells (Video). Under the  
148 operating microscope, an Ellman MH-EL-A2D fine wire electrolysis needle was then applied into the  
149 incision. Energy was delivered [using a unipolar approach](#) by a Surgitron® Dual RF™ machine (Ellman  
150 International Inc, Oceanside, NY, USA) using the lowest setting (1 joule per second) until a visible  
151 blanching of the cornea around the afferent vessel was seen and segmentation of the blood flow in  
152 the larger efferent vessels if not already present. Patients received guttae prednisolone 1% qds and  
153 chloramphenicol 0.5% qds post-operatively for 4 weeks [only](#).

154

155 Statistical analysis

156 A Mann Whitney test was used to compare pre- and post-operative area of CoNV [and a paired](#)  
157 [samples t-test for change in visual acuity](#) (SPSS Statistics 21).

158

159 **Results**

160 30 patients (mean age 56 years, range: 23 to 95 years, male:female::1:1) undergoing FND for CoNV  
161 were included. Demographic details as well as diagnoses and previous treatments are provided in

162 Table 1. The causes of the CoNV were previous herpes simplex keratitis (HSK) (13), clinically  
163 suspected bacterial keratitis (13), vascularization associated with intrastromal corneal ring segments  
164 (2), ectodermal dysplasia (1) and corneal choristoma (1). The duration of CoNV at time of FND was  
165 over six months in the 23 patients (77%), between three and six months in 3 patients (10%), and less  
166 than three months in 5 patients (13%). The number of FND treatments performed was single  
167 treatment for 20 patients (66.6%), two for 8 patients (26.6%) and three for 2 patients (6.6%) (Table  
168 1).

169 The number of afferent vessels per CoNV complex ranged from 1 to 2 with a mean diameter of  
170  $40\mu\text{m}$  (SD:  $10\mu\text{m}$ ) (Table 2). The mean time to leakage from the apical vessels on fluorescein  
171 angiography was 44.22 seconds (min: 27.43s, max: 63.59s). The area of CoNV reduced from  
172  $2.42\text{mm}^2$  (SD: 1.59) to  $0.62\text{mm}^2$  (SD: 0.73) post-operatively ( $p < 0.01$ ), a mean reduction of  $1.80\text{mm}^2$   
173 (SD: 1.40). The percentage reduction in area of CoNV for each patient is shown in Table 2. Although  
174 the area of CoNV reduced post-treatment (Figure 2), FND did not lead to a complete closure of the  
175 entire CoNV complexes in all patients with some residual vessels in the periphery of the cornea.

176 There was [statistically significant](#) improvement in BCVA from 0.59 (SD: 0.71) to 0.40 (SD: 0.42) post-  
177 treatment, [t\(29\) = 2.32, two tail \(p = 0.027\) with, and](#) a mean change of 0.17 LogMAR (Table 1).

178 Adverse events and reactions included peripheral corneal thinning (Patient 6), intrastromal  
179 haemorrhage, which cleared spontaneously (Patient 11), recurrence of herpes simplex keratitis  
180 (Patients 9 and 11) and a change in refractive error (Patient 1).

181

## 182 Discussion

183 The presence of CoNV threatens the functional integrity of the cornea with the risk of lipid  
184 exudation, loss of transparency, loss of visual acuity, increased inflammatory response and an  
185 increased risk of graft failure. To date, there is no universally accepted treatment strategy to  
186 successfully treat CoNV although topical steroids are perhaps the most commonly employed  
187 treatment.<sup>4</sup> Topical and injected anti-VEGF has been utilised in the treatment of CoNV, however, it  
188 has been suggested that once vessels mature and acquire a pericyte-covered wall, they are no  
189 longer dependent on VEGF for growth and are thus unresponsive to anti-VEGF.<sup>16,17</sup> Argon laser has  
190 been shown to have limited efficacy and with a risk of complications such as iris atrophy.<sup>4,7</sup> The  
191 speed of red blood cell movement in afferent vessels and their usual deeper location in the cornea  
192 makes them relatively insensitive to Argon laser.

193

194 FND has shown promising [short-term](#) results for treatment of CoNV. [Trikha et al reported FND to be](#)  
195 [a safe and effective in the medium to long term, with mean follow-up of 18.9 months \(range 1 - 56](#)

196 months). They reported only one complication, that of corneal and subconjunctival haemorrhage in  
197 their large series of patients, and also demonstrated a significant improvement in LogMAR VA from  
198 0.82 pre-treatment to 0.72 post-treatment.<sup>9</sup> FND, ~~has however, has~~ been applied without  
199 distinction to afferent or efferent vessels, usually to the larger vessels identified on color images.<sup>18</sup>  
200 The larger vessels and more numerous vessels that are identified on color images, slit lamp  
201 biomicroscopy are efferent vessels so that FND applied to these vessels may not be as effective as if  
202 applied to the afferent vessels, which are narrower and slightly less tortuous. ~~In particular, the~~  
203 ~~differentiation of afferent and efferent vessels aided by corneal angiography enables such treatment~~  
204 ~~to be applied to the afferent vessels of which, there are usually only 1 to 2 for each CoNV complex.~~  
205 ~~Although in some cases it may be possible to differentiate afferent and efferent vessels on slit lamp~~  
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207 ~~vessel or vessels have been identified on angiography, however, it then makes the identification on~~  
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209 ~~efferent vessels using corneal angiography enables such treatment to be applied to the afferent~~  
210 ~~vessels of which there are usually only 1 to 2 for each CoNV complex.~~

Comment [NS1]: Have changed back to American spelling for Ophthalmology

211  
212 FND is a relatively easy procedure to perform with only a few minor complications reported,  
213 including transient whitening of the cornea and intrastromal haemorrhage, which usually resolves  
214 without sequelae,<sup>5,7</sup> recurrent HSK and localised thinning and ectasia.<sup>7</sup> The cellular changes that  
215 occur following FND, however, are less well understood. In rats, a significant increase in the number  
216 of B7+ MHC II<sup>+</sup> Langerhans cells in the limbal surface epithelium ~~occurs within hours of cautery, and~~  
217 ~~later throughout the entire corneal epithelium, suggesting an inflammatory reaction as well as of~~  
218 ~~these cells the central cornea, where they are normally absent.~~<sup>19</sup> ~~Langerhans cells are the~~  
219 ~~professional antigen-presenting cells of the corneal epithelium, and their absence from the central~~  
220 ~~cornea plays an important role in maintaining the immune privilege of the cornea. In addition,~~  
221 ~~Feldman et al, in an experimental study on rabbits showed that radial thermokeratoplasty caused~~  
222 ~~significant damage to the corneal endothelium beneath and surrounding the coagulation site.~~<sup>20</sup>  
223 ~~Furthermore corneal heating reduces corneal curvature, with therapeutic potential for correction of~~  
224 ~~myopia and as a treatment for corneal ectasia.~~<sup>21,22</sup> ~~However at a molecular level, however, it~~  
225 ~~results in shrinkage of corneal stromal collagen.~~<sup>22</sup> It is also unclear what the long-term effects of  
226 diathermy to the cornea may be. The process of corneal diathermy itself may be a stimulus for  
227 further CoNV. It would therefore seem reasonable, to try and minimise the application of FND to the  
228 cornea. ICGA or FA offers the ability to identify the afferent or feeder vessels for FND, and  
229 application of anti-angiogenic factors such as anti-VEGF may be better applied to less mature or

230 immature vessels,<sup>17</sup> which can be identified using time to leakage on FA.<sup>12,13</sup> In particular topically  
231 applied pazopanib, a selective multi-targeted receptor tyrosine kinase inhibitor of VEGF receptor and  
232 platelet derived growth factor (PDGF) receptor has shown promise in the treatment of CoNV.<sup>15</sup>

233

234 | Our results suggest that angiographically-guided FND, targeting the afferent vessels may be effective  
235 | in reducing the area of CoNV. In our series, although around a third of patients required  
236 | retreatment, ~~this may reflect identification of vessels on angiography that are not apparent on~~  
237 | ~~colour images.~~<sup>11-14</sup> Similar to ~~Trikha et al<sup>9</sup>~~, adverse events were uncommon and mostly transient, and  
238 | there was a ~~n-small~~ improvement in visual acuity. ~~In those patients undergoing corneal~~  
239 | ~~transplantation, the reduction in CoNV may be of benefit.~~ Since the long-term effects of FND are  
240 | poorly understood, it is the authors' opinion that diathermy should be used sparingly. The  
241 | differentiation of afferent and efferent vessels ~~aided by using~~ corneal angiography, enables such  
242 | treatment to be applied to the afferent vessels, of which there are usually only 1 to 2 for each CoNV  
243 | complex.

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328 **Figure Captions**

329

330 **Figure 1. Identification of afferent and efferent vessels.**

331 The 3 afferent vessels (two superiorly and one inferiorly) are identified as the first to fluoresce in the  
332 angiogram (arrows in A). As the angiogram is continued (B and C) the efferent vessels fill and  
333 become prominent (arrows). Note the larger efferent vessels partially overlying and obscuring the  
334 narrower afferent vessels (D).

335

336 **Figure 2. Treatment of CoNV effect following FND.**

337 Pre (A) and post-treatment closure of CoNV (B) following occlusion of the afferent vessel with FND.  
338 Note the narrower and less tortuous afferent vessel compared to the more obvious efferent vessels  
339 draining the complex.

340

341 **Video**

342 Transection of afferent vessel using a 25 gauge needle. Note the stagnation of flow in the larger  
343 efferent vessels.

1 **Corneal angiography for guiding and evaluating fine needle diathermy treatment of corneal**  
2 **neovascularization**

3

4 Natasha Spiteri MD<sup>1</sup>, FRCOphth, Vito Romano<sup>1</sup> MD, Yalin Zheng PhD<sup>2</sup>, Sohraab Yadav MBChB<sup>1</sup>, Rahul  
5 Dwivedi BSc(Hons), MBChB<sup>1</sup>, Jern Chen<sup>1</sup> MD, Sajjad Ahmad PhD, FRCOphth<sup>1,2</sup>, Colin Willoughby MD,  
6 FRCOphth<sup>1</sup>, Stephen B Kaye MD, FRCOphth<sup>1,2</sup>

7

8 1. St Paul's Eye Unit, Royal Liverpool University Hospital, Liverpool, L7 8XP, UK

9 2. Department of Eye and Vision Science, University of Liverpool, UK.

10

11 Tel: +44 1517062000

12

13 Corresponding Author:

14 Natasha Spiteri

15 St Paul's Eye Unit, 8Z link, Royal Liverpool University Hospital, Liverpool, L7 8XP, UK

16 nax024@gmail.com

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27 Address for reprints:

28 St Paul's Eye Unit, 8Z link, Royal Liverpool University Hospital, Liverpool, L7 8XP, UK

29

30 This article contains one video as additional online-only material.

31

32

33 **Abstract**

34 **Purpose:** To investigate the outcome of selective occlusion of the afferent vessel of corneal  
35 neovascular complexes (CoNV), using angiographically guided fine needle diathermy (FND).

36 **Design:** Retrospective interventional case series

37 **Subjects:** Patients with CoNV unresponsive to topical steroid therapy.

38 **Methods:** Visual acuity, color images, and fluorescein angiography (FA) and indocyanine green  
39 angiography (ICGA) were measured before and following FND with a minimum of three months  
40 follow-up. The number of afferent vessels crossing the limbus, time to fluorescein leakage, area and  
41 geometric properties of the CoNV were determined using an in-house automated program written in  
42 numerical computing language (MatLab R14; The MathWorks Inc., Natick, MA). The location of the  
43 afferent vessel was identified from the angiographic images and marked at the slit lamp using a  
44 needle to make a cut to the depth of the vessel. FND was then applied using an electrolysis needle.

45 **Main Outcome Measure:** Area of CoNV

46 **Results:** 30 patients underwent FND for CoNV that had not responded to treatment with topical  
47 steroids. The CoNV was associated with previous microbial keratitis (26), intrastromal corneal ring  
48 segments (2), ectodermal dysplasia (1) and corneal choristoma (1). Duration of CoNV was over six  
49 months in the 23 patients (77%), between three and six months in 3 patients (10%), and less than  
50 three months in 5 patients (13%). The number of afferent vessels per CoNV complex ranged from 1  
51 to 3 with a mean diameter of 40 $\mu$ m (SD 10 $\mu$ m) and mean time to leakage from apical vessels was  
52 44.22 seconds (min: 27.43s max: 63.59s). The number of episodes of FND treatments that were  
53 required was one for 20 patients (66.6%), two for 8 patients (26.6%) and three for 2 patients (6.6%).  
54 Following FND, the area of CoNV reduced by 1.80mm<sup>2</sup> (SD: 1.40); from 2.42mm<sup>2</sup> (SD: 1.59) to  
55 0.62mm<sup>2</sup> (SD: 0.73) up to 12 weeks post-operatively (p<0.01).

56 **Conclusions:** The differentiation of afferent and efferent vessels using corneal angiography enables  
57 treatment to be selectively applied to the afferent vessels of which, there are usually 1 to 2 for each  
58 CoNV complex.

59

## 60 Introduction

61

62 Avascularity of the cornea is important for its homeostasis and function. Corneal neovascularization  
63 (CoNV), however, may develop in response to hypoxia or inflammations, as infectious, allergic, toxic  
64 and traumatic injuries.<sup>1</sup> The presence of CoNV reflects an imbalance between anti-angiogenic  
65 factors, such as pigment-epithelium-derived factor (PEDF), and angiogenic factors, such as fibroblast  
66 and vascular endothelial growth factors (VEGF).<sup>2,3</sup> CoNV is however, part of the wound healing  
67 response and may be useful in the acute phase for the transport of, humoral and cellular elements  
68 involved in immune response, materials required for repair and regeneration, removal of toxic  
69 substances and drugs to the site of inflammation. Chronic up-regulation of the angiogenic response,  
70 however, results in the persistence of pathological new vessels, which have increased vascular  
71 permeability resulting in corneal oedema, lipid exudation, chronic or recurrent inflammation and  
72 scarring.<sup>4</sup> There is also the potential establishment of lymphatics, normally absent from the cornea,  
73 which may further disrupt the “immune privilege” status of the cornea.<sup>4</sup>

74

75 Various techniques have been employed to treat CoNV, including topical steroids, anti-VEGF,  
76 metalloproteinase inhibitors, photodynamic therapy, Argon laser, yellow dye laser, radiation,  
77 cryotherapy and conjunctival resection.<sup>4-6</sup> Fine-needle diathermy (FND) has been described by  
78 several groups for the treatment of CoNV.<sup>5,7-9</sup> It involves the application of a coagulating current  
79 through a unipolar diathermy unit or thermal cautery, usually delivered through a needle such as a  
80 cutting or an electrolysis needle. FND has shown promise with the largest retrospective study  
81 reporting a series of 56 eyes, showing regression of CoNV in 89.3% of patients following two or less  
82 treatments.<sup>9</sup> It is, however not known what effects diathermy has on the cornea at a cellular level,  
83 particularly if applied to the multitude of vessels in the CoNV complexes. Reducing the number of  
84 vessels that need to be closed may reduce the potential risks associated with FND and improve the  
85 efficacy of the procedure. It is questionable whether both the afferent (presumed arteriole) and  
86 efferent (presumed venule) systems of CoNV require treatment and selective treatment to the  
87 afferent system may be sufficient. It is of note, that Cursiefen et al found that on histology, that  
88 arterioles tend to comprise less than 1% of CoNV.<sup>10</sup> One option therefore, would be to only treat the  
89 afferent vessel(s) of the CoNV complex.<sup>11</sup> While it is sometimes possible to distinguish afferent from  
90 efferent CoNV using slit lamp biomicroscopy, it has been shown that the full extent and origin of the  
91 CoNV complex is not apparent on color images.<sup>12-14</sup> We have, however, recently shown that corneal  
92 angiography (fluorescein angiography, FA and indocyanine green angiography, ICGA) are particularly  
93 useful in identifying vessels not seen on color images<sup>12-14</sup>, especially in the presence of corneal scars

94 or inflammation and facilitate identification and differentiation of afferent and efferent vessels and  
95 vessel leakage.<sup>13</sup> In order therefore, to minimise the amount of diathermy applied to the cornea, we  
96 describe the use of angiography to identify and specifically treat the afferent vessels using FND.

97

## 98 **Methods**

99 Patients who were undergoing FND for CoNV associated with previous corneal disease such as  
100 microbial keratitis and that had not responded to topical steroid therapy were included. Topical  
101 steroids had been used for between 6 and 12 weeks duration. Inclusion criteria were CoNV  
102 extending more than 3mm into cornea with varying degrees of lipid keratopathy and no active  
103 keratitis or corneal ulceration. Patients were followed up for a period of at least four months.  
104 Patients were examined with slit lamp biomicroscopy, and color and red free images, and FA and  
105 ICGA were obtained pre-treatment and three months post-treatment. IRB approval and informed  
106 consent were obtained and the Tenets of the Declaration of Helsinki were adhered to.

107

108 Color, FA and ICGA images.

109 Color images were captured using a slit lamp mounted digital system (Topcon SL-D Digital Slit Lamp,  
110 Tokyo, Japan) with 10 to 25 times magnification. An HRA2 Scanning Laser Ophthalmoscope  
111 (Heidelberg Engineering, Heidelberg, Germany) with a 20 degree imaging lens set at 34 diopter (D),  
112 was used for ICGA and FA as previously described.<sup>12,13</sup> After injection of 5ml of indocyanine green  
113 dye (5mg/ml) (Pulsion Medical Systems, Germany) videography was recorded for 25 seconds. Single  
114 frame ICGA images of the whole cornea capturing corneal blood vessel fluorescence were taken  
115 every 3-5 seconds for 3 minutes followed by late images at 5 and 10 minutes. An intravenous  
116 injection of 3mL of 20% Sodium fluorescein (Martindale Pharmaceuticals, Essex, UK) was then given  
117 and the videography repeated. During the acquisition of single frame ICGA and FA images hi-  
118 resolution mode with eye tracking automatic real-time (ART) software was used.

119

120 Image analysis

121 The region of interest (ROI) was defined pre-operatively as the area of the cornea containing the  
122 CoNV and was used to compare pre and post-operative images. Images of pre- and final post-  
123 operative angiograms of grade 3 or 4 were selected for analysis as previously described by two  
124 independent observers (SY and RD).<sup>12</sup> The number of afferent vessels crossing the limbus and time  
125 to leakage of fluorescein were recorded. The area and geometric properties of the vessels were  
126 determined on the selected images using an in-house automated program developed in Matlab R14  
127 (The MathWorks Inc., Natick, MA).<sup>13</sup> In brief, the major steps of the program are as follows. For

128 each image the pixel resolution (mm/pixel) was first defined as the ratio between the diameter of  
129 the cornea (mm) and the number of pixels measured manually from the image. A ROI containing all  
130 the corneal vessels was then defined by hand. The CoNV in the ROI was detected by applying  
131 Gaussian enhancement, selective vessel enhancement and thresholding techniques to the ROI in a  
132 sequential order. In the resulting binary image (1 indicating vessel pixels while 0 indicating  
133 background pixels) the area of CoNV (mm<sup>2</sup>) was computed by multiplying the number of CoNV pixels  
134 and the area (mm<sup>2</sup>) occupied by a pixel. The centrelines of the vessels were identified by a 'thinning'  
135 operation so that the branch points and terminal points can be identified. The branching and  
136 terminal points were then used to divide the vascular tree into individual vessel segments. The  
137 mean diameter (mm) of each segment can be computed so as to characterise the CoNV complex in  
138 the image.

139

#### 140 FND technique (Video)

141 Afferent and efferent vessels were identified on videography (Figure 1) and the former labelled. The  
142 annotated image was used as a reference to mark the afferent vessel(s) with an inked needle. Using  
143 slit lamp biomicroscopy, under topical anaesthesia, a partial thickness incision was made using a 25  
144 gauge needle on a 1 ml syringe into the posterior stroma over the identified afferent vessel(s) either  
145 at the limbus or at the level of marginal corneal arcades.<sup>15</sup> If the afferent vessel was transected, an  
146 interruption of flow was evident in the visible vessels carrying red blood cells (Video). Under the  
147 operating microscope, an Ellman MH-EL-A2D fine wire electrolysis needle was then applied into the  
148 incision. Energy was delivered using a unipolar approach by a Surgitron® Dual RF™ machine (Ellman  
149 International Inc, Oceanside, NY, USA) using the lowest setting (1 joule per second) until a visible  
150 blanching of the cornea around the afferent vessel was seen and segmentation of the blood flow in  
151 the larger efferent vessels if not already present. Patients received guttae prednisolone 1% qds and  
152 chloramphenicol 0.5% qds post-operatively for 4 weeks only.

153

#### 154 Statistical analysis

155 A Mann Whitney test was used to compare pre- and post-operative area of CoNV and a paired  
156 samples t-test for change in visual acuity (SPSS Statistics 21).

157

#### 158 **Results**

159 30 patients (mean age 56 years, range: 23 to 95 years, male:female::1:1) undergoing FND for CoNV  
160 were included. Demographic details as well as diagnoses and previous treatments are provided in  
161 Table 1. The causes of the CoNV were previous herpes simplex keratitis (HSK) (13), clinically

162 suspected bacterial keratitis (13), vascularization associated with intrastromal corneal ring segments  
163 (2), ectodermal dysplasia (1) and corneal choristoma (1). The duration of CoNV at time of FND was  
164 over six months in the 23 patients (77%), between three and six months in 3 patients (10%), and less  
165 than three months in 5 patients (13%). The number of FND treatments performed was single  
166 treatment for 20 patients (66.6%), two for 8 patients (26.6%) and three for 2 patients (6.6%) (Table  
167 1).

168 The number of afferent vessels per CoNV complex ranged from 1 to 2 with a mean diameter of  
169  $40\mu\text{m}$  (SD:  $10\mu\text{m}$ ) (Table 2). The mean time to leakage from the apical vessels on fluorescein  
170 angiography was 44.22 seconds (min: 27.43s, max: 63.59s). The area of CoNV reduced from  
171  $2.42\text{mm}^2$  (SD: 1.59) to  $0.62\text{mm}^2$  (SD: 0.73) post-operatively ( $p < 0.01$ ), a mean reduction of  $1.80\text{mm}^2$   
172 (SD: 1.40). The percentage reduction in area of CoNV for each patient is shown in Table 2. Although  
173 the area of CoNV reduced post-treatment (Figure 2), FND did not lead to a complete closure of the  
174 entire CoNV complexes in all patients with some residual vessels in the periphery of the cornea.  
175 There was significant improvement in BCVA from 0.59 (SD: 0.71) to 0.40 (SD: 0.42) post-treatment ( $p$   
176  $= 0.027$ ) with a mean change of 0.17 LogMAR (Table 1). Adverse events and reactions included  
177 peripheral corneal thinning (Patient 6), intrastromal haemorrhage, which cleared spontaneously  
178 (Patient 11), recurrence of herpes simplex keratitis (Patients 9 and 11) and a change in refractive  
179 error (Patient 1).

180

## 181 Discussion

182 The presence of CoNV threatens the functional integrity of the cornea with the risk of lipid  
183 exudation, loss of transparency, loss of visual acuity, increased inflammatory response and an  
184 increased risk of graft failure. To date, there is no universally accepted treatment strategy to  
185 successfully treat CoNV although topical steroids are perhaps the most commonly employed  
186 treatment.<sup>4</sup> Topical and injected anti-VEGF has been utilised in the treatment of CoNV, however, it  
187 has been suggested that once vessels mature and acquire a pericyte-covered wall, they are no  
188 longer dependent on VEGF for growth and are thus unresponsive to anti-VEGF.<sup>16,17</sup> Argon laser has  
189 been shown to have limited efficacy and with a risk of complications such as iris atrophy.<sup>4,7</sup> The  
190 speed of red blood cell movement in afferent vessels and their usual deeper location in the cornea  
191 makes them relatively insensitive to Argon laser.

192

193 FND has shown promising results for treatment of CoNV. Trikha et al reported FND to be safe and  
194 effective in the medium to long term, with mean follow-up of 18.9 months (range 1 - 56 months).  
195 They reported only one complication, that of corneal and subconjunctival hemorrhage and also



196 demonstrated a significant improvement in LogMAR VA from 0.82 pre-treatment to 0.72 post-  
197 treatment.<sup>9</sup> FND, however, has been applied without distinction to afferent or efferent vessels,  
198 usually to the larger vessels identified on color images.<sup>18</sup> The larger vessels and more numerous  
199 vessels that are identified on color images, slit lamp biomicroscopy are efferent vessels so that FND  
200 applied to these vessels may not be as effective as if applied to the afferent vessels, which are  
201 narrower and slightly less tortuous. In particular, the differentiation of afferent and efferent vessels  
202 aided by corneal angiography enables such treatment to be applied to the afferent vessels of which,  
203 there are usually only 1 to 2 for each CoNV complex. Although in some cases it may be possible to  
204 differentiate afferent and efferent vessels on slit lamp biomicroscopy, aided for example by the  
205 patient's pulse, this can be difficult. Once the afferent vessel or vessels have been identified on  
206 angiography, however, it then makes the identification on slit lamp biomicroscopy more apparent  
207 and reliable.

208

209 FND is a relatively easy procedure to perform with only a few minor complications reported,  
210 including transient whitening of the cornea and intrastromal haemorrhage, which usually resolves  
211 without sequelae,<sup>5,7</sup> recurrent HSK and localised thinning and ectasia.<sup>7</sup> The cellular changes that  
212 occur following FND, however, are less well understood. In rats, a significant increase in the number  
213 of B7+ MHC II<sup>+</sup> Langerhans cells in the limbal surface epithelium occurs within hours of cautery, and  
214 later throughout the entire corneal epithelium, suggesting an inflammatory reaction.<sup>19</sup> In addition,  
215 Feldman et al, showed that radial thermokeratoplasty caused significant damage to the corneal  
216 endothelium beneath and surrounding the coagulation site.<sup>20</sup> Furthermore corneal heating reduces  
217 corneal curvature, with therapeutic potential for correction of myopia and as a treatment for  
218 corneal ectasia.<sup>21,22</sup> At a molecular level, however, it results in shrinkage of corneal stromal  
219 collagen.<sup>22</sup> It is also unclear what the long-term effects of diathermy to the cornea may be. The  
220 process of corneal diathermy itself may be a stimulus for further CoNV. It would therefore seem  
221 reasonable, to try and minimise the application of FND to the cornea. ICGA or FA offers the ability to  
222 identify the afferent or feeder vessels for FND, and application of anti-angiogenic factors such as  
223 anti-VEGF may be better applied to less mature or immature vessels,<sup>17</sup> which can be identified using  
224 time to leakage on FA.<sup>12,13</sup> In particular topically applied pazopanib, a selective multi-targeted  
225 receptor tyrosine kinase inhibitor of VEGF receptor and platelet derived growth factor (PDGF)  
226 receptor has shown promise in the treatment of CoNV.<sup>15</sup>

227

228 Our results suggest that angiographically-guided FND, targeting the afferent vessels may be effective  
229 in reducing the area of CoNV. In our series, although around a third of patients required

230 retreatment, this may reflect identification of vessels on angiography that are not apparent on  
231 colour images.<sup>11-14</sup> Similar to Trikha et al<sup>9</sup>, adverse events were uncommon and mostly transient, and  
232 there was an improvement in visual acuity. Since the long-term effects of FND are poorly  
233 understood, it is the authors' opinion that diathermy should be used sparingly. The differentiation of  
234 afferent and efferent vessels aided by corneal angiography, enables such treatment to be applied to  
235 the afferent vessels, of which there are usually only 1 to 2 for each CoNV complex.

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315 **Figure Captions**

316

317 **Figure 1. Identification of afferent and efferent vessels.**

318 The 3 afferent vessels (two superiorly and one inferiorly are identified as the first to fluoresce in the  
319 angiogram (arrows in A). As the angiogram is continued (B and C) the efferent vessels fill and  
320 become prominent (arrows). Note the larger efferent vessels partially overlying and obscuring the  
321 narrower afferent vessels (D).

322

323 **Figure 2. Treatment of CoNV effect following FND.**

324 Pre (A) and post-treatment closure of CoNV (B) following occlusion of the afferent vessel with FND.  
325 Note the narrower and less tortuous afferent vessel compared to the more obvious efferent vessels  
326 draining the complex.

327

328 **Video**

329 Transection of afferent vessel using a 25 gauge needle. Note the stagnation of flow in the larger  
330 efferent vessels.

Table 1

Table 1 Patient demographics, diagnosis, treatment and outcomes.

Patient	Age	Diagnosis**	Sex	Duration (months) of CoNV	Treatment prior to FND *	N°. of FND	Adverse reactions	Pre-op BCVA (logMAR)	Post-op BCVA (logMAR)
1	74	HSK	F	>6	Aciclovir	1	None	0.18	0
2	56	BK ( <i>S.aureus</i> )	F	3-6	Antimicrobial	1	None	0	0.2
3	23	Corneal choristoma	F	>6	Bevacizumab	1	None	0	0
4	54	HSK	M	<3	Aciclovir	1	None	0.6	0.6
5	70	BK ( <i>S.aureus</i> )	M	>6	Aciclovir, Antimicrobial	1	None	0.18	0.18
6	70	HSK	M	>6	Aciclovir	1	Peripheral thinning	0.6	0.48
7	79	BK ( <i>S. aureus</i> )	M	>6	Antimicrobial	3	None	0.3	0.3
8	78	BK ( <i>S. aureus</i> )	F	>6	Antimicrobial	1	None	0.3	0.5
9	70	HSK	F	>6	Aciclovir	2	Recurrence of HSK	0.18	0.18
10	54	BK	M	>6	Antimicrobial	1	None	1	0.78
11	47	HSK	M	>6	Aciclovir	2	Recurrence of HSK , intrastromal hemorrhage	0.18	0.18
12	70	BK ( <i>S.aureus</i> )	M	>6	Antimicrobial	1	None	0.3	0.3
13	22	BK ( <i>P. aeruginosa</i> )	M	>6	Antimicrobial	1	None	-0.2	-0.2
14	84	BK ( <i>S. aureus</i> )	M	3-6	Antimicrobial	1	None	0.18	0.18
15	52	HSK	M	>6	Aciclovir, Antimicrobial	1	Refractive error	0.18	0.18
16	39	BK ( <i>S.aureus</i> )	F	3-6	Antimicrobial	2	None	0.3	0.18
17	34	KC (Intacs)	M	<3		1	None	3	2
18	34	HSK	M	>6	Aciclovir, Foscarnet	1	None	0.18	0
19	59	BK ( <i>S.aureus</i> )	M	>6	Antimicrobial, Argon laser	1	None	0.5	0.6
20	67	HSK	F	>6	Aciclovir	2	None	1.3	0.67
21	47	Ectodermal dysplasia	F	>6	Retinoic acid	3	None	0.48	0.48
22	69	HSK	F	<3	Aciclovir	1	None	0.18	0.18
23	44	BK ( <i>S.aureus</i> )	M	<3	Antimicrobial	2	None	1	0.3
24	71	BK ( <i>P. aeruginosa</i> )	M	>6	Antimicrobial	2	None	0.6	1
25	95	HSK	F	>6	Argon laser	2	None	1	0.8
26	25	HSK	F	<3	Aciclovir	1	None	2	0.48
27	35	KC (Intacs)	F	>6		1	None	0.18	0.18
28	62	HSK	F	>6	Aciclovir	1	None	2	0.6
29	24	HSK	F	>6	Aciclovir	2	None	0	0
30	62	BK ( <i>S. aureus</i> )	F	>6	Antimicrobial	1	None	1	0.78
Mean	56							0.59	0.40
SD	19.72							0.71	0.42

Footnotes: \*All the patient had received topical steroids before and after treatment.; HSK: Herpes Simplex Keratitis, KC: keratoconus; BK: bacterial keratitis, LSCD: Limbal stem cell deficiency, ACV: aciclovir, AM: antimicrobials. Diagnosis\*\* type of bacteria if isolated

Table 2. Data on pre- and post-operative outcomes

Patient number	Area of CoNV (mm <sup>2</sup> )			Percentage change	Afferent vessels		Time to leakage on pre-op FFA (s)
	Pre-op	Post-op	Change		Diameter (mm)	Number on pre-op angiogram	
1	0.89	0	-0.89	100	0.04	1	27.43
2	2.89	0	-2.89	100	0.04	2.5	28.28
3	0.5	0	-0.5	100	0.06	1	38.26
4	3.13	0	-3.13	100	0.03	1	30.83
5	0.25	0	-0.25	100	0.04	3	63.59
6	0.98	0	-0.98	100	0.07	2	48.9
7	2.42	0	-2.42	100	0.05	2	41.7
8	0.96	0	-0.96	100	0.04	1.5	33.91
9	4.04	0.12	-3.92	97	0.03	1	30
10	5.09	0.44	-4.65	91	0.05	2	41.82
11	3.81	0.36	-3.45	91	0.03	2	32.37
12	5.67	0.61	-5.06	89	0.05	2.5	41
13	2.85	0.41	-2.44	86	0.04	1	27.98
14	2.84	0.44	-2.4	85	0.05	2.5	34.79
15	0.95	0.19	-0.75	79	0.05	1	35.71
16	0.13	0.04	-0.1	77	0.03	1	43.18
17	0.5	0.12	-0.38	76	0.03	2	33.41
18	2.52	0.61	-1.9	75	0.04	2	42.31
19	2.11	0.61	-1.5	71	0.08	3	55.8
20	5.54	1.62	-3.91	71	0.05	2	32.01
21	4.6	1.48	-3.11	68	0.04	3	50.91
22	3.17	1.11	-2.07	65	0.04	1	38.19
23	3.02	1.16	-1.86	62	0.03	1	33.64
24	2.1	1.02	-1.08	51	0.04	2	28.15
25	2.49	1.46	-1.03	41	0.05	1	28.01
26	0.77	0.51	-0.25	32	0.03	1	30.5
27	0.6	0.41	-0.19	32	0.04	1	27.87
28	1.82	1.26	-0.56	31	0.05	3	40.1
29	1.52	1.13	-0.39	26	0.05	3	49.19
30	4.37	3.46	-0.91	21	0.05	2	36.85
Mean	2.42	0.62	-1.80	74	0.04	1.80	37.55
SD	1.59	0.73	1.40		0.01	0.75	9.06

**Figure 1**  
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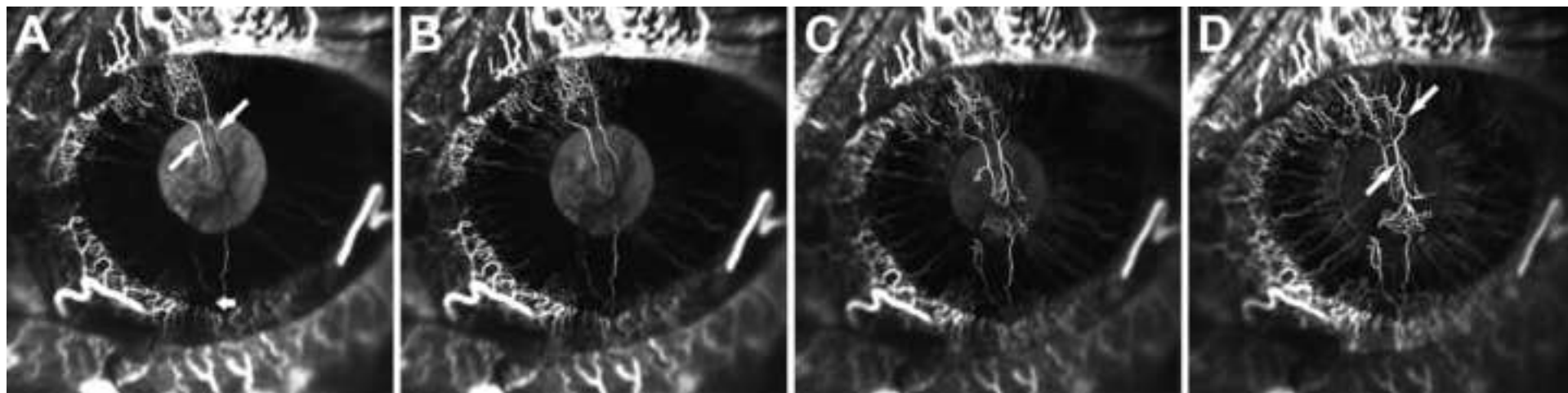
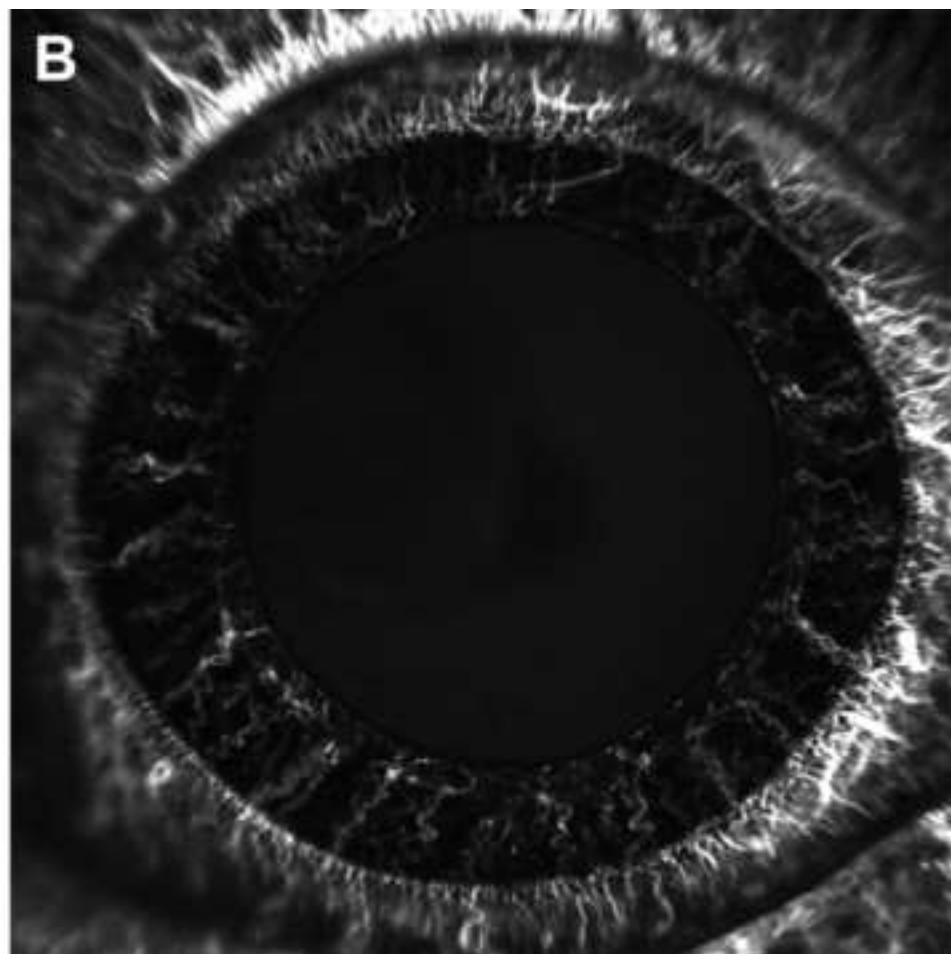
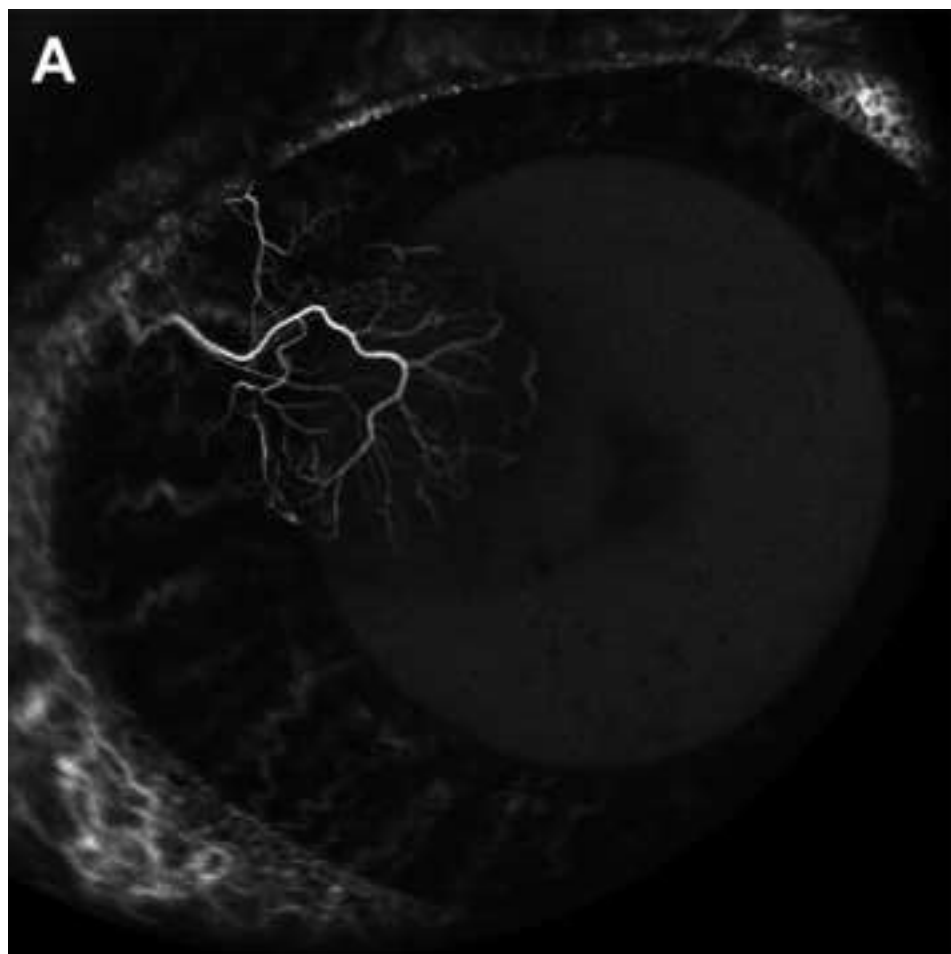




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