solution combined with $H_2O_2$. For CK 18 staining, the chromogen was replaced with AEC high sensitive substrate chromogen (Cytomation, Code K3461, Dako). Immunohistochemical staining for VEGF was performed by the alkaline-phosphatase method according to the manufacturer's instructions (ChemMate Detection Kit, Alkaline Phosphatase/RED, Rabbit/Mouse, K5005; Dako). A monoclonal mouse anti-human VEGF antibody (clone C-1; Santa Cruz Biotechnology, Santa Cruz, CA), non-crossreactive with VEGF-C, VEGF-D or PlGF (Placental Growth Factor) but specific for VEGF-A, was used. The biotinylated goat antimouse secondary antibody was followed by Streptavidin conjugated to alkaline phosphatase and Chromogen Red. Levamisole was applied to inhibit endogenous alkaline phosphatase activity. Hematoxylin III according to Gill (Merck, Darmstadt, Germany) was used as a counterstain. For negative controls, the primary antibodies were substituted by appropriate normal sera or obmitted.

**Analysis**

**Two serial sections from a specimen were analyzed three times and independently by two masked observers (OT, SG) by light microscopy.**

Vascularization was calculated by counting the number of CD34 and CD105 positive vascular-like patterns in the most vascularized area under x200 magnification

**Ki-67 positive nuclei in RPE, EC and stromal cells were counted separately in each specimen totally. The percentage of the Ki-67 expressing RPE, EC and stromal cells with regard to the total number of proliferating cells in the groups of treated and nontreated CNV membranes were calculated. Quantitative analysis of proliferative**

**activity (nuclei/mm$^2$) in each specimen was determined by calculating the ratio of the total number of Ki-67 positive nuclei in CNV to the area of the membrane (mm$^2$).**

Immunoreactivity for VEGF was analyzed separately in RPE, endothelial cells and stroma. A grading scheme indicating the degree was used: 3,2,1,0 were assigned to indicate intense (70-100% positive cells); moderate (40-69% positive cells); weak VEGF labelling (1-39% positive cells) and and absence (-) of any staining respectively.

**Based on previous studies,[9,10] the CNV membranes were classified semiquantitatively as "inflammatory active" when inflammatory cells were more dominant than fibrosis or "inflammatory inactive" when fibrosis was dominant with minor or absent inflammatory response.**

**The proliferative activity and intensity of VEGF immunostaining between groups were analysed with Mann-Whitney U test. $P \leq 0.05$ was considered significant.**

## RESULTS

### Angiographic Classification and Characterization

The angiographic features classified according to the TAP and VIP reports differed depending on the post-PDT time interval (Table 1). In all of the 4 membranes extracted three days after PDT, a hypofluorescence suggesting nonperfusion of the irradiated area and the CNV was seen in early phases of angiography at the day of surgery (Figure 1A). Late phases FA revealed hyperfluorescence and leakage at the fovea consistent with choroidal ischemia (Figure 1B). In CNV membranes extracted at longer post-PDT intervals, FA on the day of surgery disclosed a membrane (Figure 1C) with leakage in late phases (Figure 1D).

### Histologic Characterization, CD 34, CD 105, Ki-67 Labeling

All but one membrane in each group of treated and untreated cases were vascularized as evidenced by CD 34 positive vessels (Figure 2A). In CNV membranes devoid of PDT, all vessels stained positively for CD 34 (Figure 3A) but stained only partially for CD 105 (Figure 3A and B). In membranes extracted 3 days after PDT, immunohistology with CD 34 and CD 105, disclosed not only many collapsed vessels (mean 81.1 %) but also patent ones (Figure 3D). The endothelial cells lining the patent vessels appeared damaged. In contrast, vessels in CNV membranes extracted at longer post-PDT intervals were all patent with endothelial cells displaying prominent nuclei. The specimens were hypercellular and highly vascularized. The vessels strongly expressed CD 105 (Figure 3E) reflecting very vital and active endothelial cells.

**In non-treated CNV membranes, a differing number of Ki-67 positive proliferating cells could be detected (Figure 2A). Ki-67 positive cells (n=544) were rarely endothelial cells (n=31, 5.7%) or RPE cells (n=15, 2.8%) but appeared to belong to stromal cells (n=498, 91.5%), especially to inflammatory infiltrate (Figure 3C). In fact, specimens classified as inflammatory active (IA) (n=20) disclosed significantly higher proliferative activity (median proliferative activity : 59.846 nuclei/mm2, range:0-514,08) than inflammatory inactive (II) specimens (n=10, median proliferative activity : 6.815 nuclei/mm2, range:0-113.69) (p=0.0018). In specimens treated with PDT, a higher number of Ki-67 positive nuclei (n=1222) were detected which were rarely RPE cells (n=74, 6%) or EC (n=51, 4.2%) but mostly stromal cells (n=1097, 89.8 %). However, in membranes extracted 3 days after PDT, Ki-67 expressing cells (n=37) were completely negative in 2 cases and relatively high only in one specimen (n=36) (Figure 2A). The proliferative activity in specimens extracted 3 days after PDT (median:4.85 nuclei/mm$^2$, range:0-9.7101) was smaller than that in the CNV membranes without prior PDT (median:53.203 nuclei/mm$^2$, range:0-514.08), but this difference did not reach statistical significance (p=0.1339). However, at longer intervals following PDT, proliferative activity increased significantly (median :78.2755 nuclei/ mm$^2$, range:0-829.29) (p=0,0469) (Figure 2A and 3F).**

**Expression of VEGF**

In CNV membranes untreated by PDT, VEGF staining was absent in the RPE cells of 60% (18 of 30) of the specimens (Figure 2B). In the rest (12 of 30), VEGF staining was mostly weak to moderate (Figure 4A and B). A moderate VEGF expression was found in 53.3% (16 of 30) of vascular endothelial cells (EC). Only 4 of 30 specimens, however, displayed an intense staining (Figure 2B). VEGF staining within the stroma

appeared to be present both in fibroblast like and inflammatory cells (Figures 2B, 4A and B).

In all membranes (n=4) extracted three days after PDT, CK 18 positive RPE cells (Figure 4C) displayed an intense staining for VEGF (Figures 2B, 4C and 4D). At longer post-treatment intervals, VEGF staining in RPE persisted to different degrees (Figures 2B and 4F) being absent in only 2 cases. Three days after PDT, only one membrane disclosed endothelial cells with a moderate expression of VEGF. The other three specimens were either negative (n=1) or just weakly (n=2) stained. At greater time intervals (n=16) VEGF staining in EC appeared to increase, with only one case of negative EC, but 62,5% of specimens with moderate to intense staining at the vessels (Figure 2B and 4E and F). VEGF staining was significantly increased in RPE ($p<0.0001$) and EC ($p=0.0076$) and stromal cells ($p=0.0194$) after PDT. (Figure 2B and 4E and F). VEGF staining intensity showed no predilection according to the localization in the CNV.

## COMMENT

Lately, photodynamic therapy has gained an important role in the treatment of the neovascular AMD. The potential and benefit of this therapy, however, is compromised by high recurrence rates and a reduced functional prognosis. To promote this treatment concept and to reduce its limitations, knowledge of the biological effects of PDT in CNV is, therefore, crucial.

CNV are most likely the result of neoangiogenesis and VEGF is supposed to play a pivotal role within this process.[9-15] VEGF has been shown to be required for normal vascular development[16], survival and morphology of choriocapillaris[17] as well as retinal neovascularization[18] in experimental animal models. It plays a key role in the pathogenesis of ischemia-associated retinal neovascularization,[19] and is a major stimulator of CNV development and growth.[14] VEGF is present in both surgically excised CNV membranes from human AMD eyes[9-12] and experimental CNV membranes.[13] VEGF can induce CNV in animal models[14] and CNV has been suppressed by anti-VEGF therapy in primates.[15]

In order to understand the changes that might be related to PDT, we first examined CNV membranes that did not receive PDT before surgery. Herein, VEGF expression by RPE cells could be detected in less than 50% of the cases with an intense expression in only 13% of the membranes. In contrast expression by stromal cells was encountered in 90% of the cases. These results confirm the findings of Kvanta and colleagues[10] who detected VEGF staining and VEGF mRNA expression particularly in fibroblastlike cells but only occasionally in RPE of the human CNV membranes. Lopez and colleagues[12] reported VEGF production by RPE cells, EC, fibroblasts, macrophages,

and monocytes in CNV membranes. In our membranes, VEGF staining in EC, RPE and stromal cells at different intensities was in concordance with their findings.

In our series of 20 patients receiving presurgical PDT, four membranes were extracted 3 days after PDT. **FA on the day of surgery revealed nonperfusion of the CNV within the area of the laser treatment, as demonstrated by the occlusion of most of the vessels both within the CNV and in the surrounding normal choroid.** [20,21] **Histology of PDT treated human eyes supported these findings by identifying an occluded choriocapillary layer within the spot produced by laser.**[3,22,23] We detected as an early change many collapsed vessels as well as several patent vessels with presumably damaged EC. The proliferative activity within these specimens was low.[20] Interestingly, in all of these membranes, intense VEGF staining was extremely prominent in RPE cells whereas it varied in EC and stroma. **VEGF is known to be strongly induced by hypoxia in RPE cells** [24] **as well as reactive oxygen intermediates (ROI).** [25] **Either aggravated hypoperfusion of the choroid** [21,26-28] **and/or ROI released by the photochemical effect of PDT might be the angiogenic stimuli inducing VEGF secretion by RPE after PDT.**

**Whatever reason might be responsible, enhanced VEGF expression by RPE cells, even when temporary, leads to increased vascular leakage and development of CNV.** [29] In fact, CNV membranes extracted at longer time intervals after PDT disclosed patent vessels lined by healthy EC that were highly positive for CD 34 and CD 105. None of these 16 specimens disclosed occluded or collapsed vessels. The proliferative activity was significantly increased and associated with a cell-rich fibrocellular stroma and an inflammatory response. The VEGF expression by RPE cells persisted in most of these cases. **In contrast, the VEGF expression in EC and stromal cells appeared to**

**be enhanced compared to the early post-PDT cases. The high inflammatory activity appearing in the longer post-PDT intervals may also be a factor in sustaining the increased VEGF expression.**

Bula and colleagues examined VEGF expression in six CNV extracted 3 months after PDT.[30] Four CNV without prior PDT composed their control group. Contrary to our results, no significant difference in the VEGF expression was detected between the treated and untreated CNV. The small number of specimens as well as the long post-PDT interval in their work might be responsible for their controversial results.

The number of re-treatments did not seem to affect the chronology of the process that is induced by the last treatment. PDT, though being a mild and selective modality, is still a traumatizing event inducing a wound healing cascade with the initial characteristics of angiogenesis within an inflammatory setting. In fact, though all membranes appear to be inflammatory inactive and non-proliferative three days after PDT, there seems to be a rebound activity thereafter.

PDT, when applied to normal chorioretinal structures, increased VEGF expression in EC of the choriocapillaris, but not at the level of RPE.[31] However, in our study, PDT applied to CNV induced an early VEGF response by RPE.

Our results may explain the clinical observation of a decreased retreatment rate and an increased visual improvement when PDT is combined with anti-VEGF treatment.[32-33] Intravitreal injections of anti-VEGF should interrupt the **vicious cycle** induced by PDT. In the Phase II study [33], anti-VEGF aptamer was injected intravitreally 5 to 10 days

after PDT. Prominent VEGF expression in RPE cells already 3 days after PDT in our samples, however, suggest an earlier anti-VEGF intervention.

To our knowledge, this is the first clinicopathological correlation of changes regarding VEGF expression, proliferative activity and vascularization in CNV membranes treated by PDT. The proper interpretation of this study, however, is limited by the fact that our cases may represent a negative selection. Though the histopathologic findings in patients who profit from Verteporfin PDT might differ, it is conceivable that PDT causes a trauma followed by an enhanced VEGF expression and angiogenesis associated with an inflammatory wound healing process. With regard to the re-initiation of the angiogenic cascade, the need for a counteracting adjunctive therapy started on the proper time becomes more and more obvious.[34]

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**Table 1:** Clinical characteristics of patients treated with PDT before surgical removal of the CNV.

| Case | Eye | Age/ Sex | CNV type | Visual acuity | PDT treatments | Time to surgery from the first PDT/last PDT |
|---|---|---|---|---|---|---|
| **1** | L | 76/m | classic | 0.025 | 1 | **3** days |
| **2** | R | 78/f | classic | 0.02 | 1 | **3** days |
| **3** | L | 54/m | predominantly classic | 0.063 | 2 | 113/**3** days |
| **4** | L | 84/m | classic | 0.025 | 1 | **3** days |
| **5** | L | 83/m | classic | 0.03 | 1 | **34** days |
| **6** | L | 85/f | classic | 0.03 | 1 | **37** |
| **7** | R | 73/f | occult | 0.1 | 3 | 208/138/**40** days |
| **8** | L | 79/m | classic | 0.1 | 1 | **55** days |
| **9** | R | 80/f | classic | 0.1 | 2 | 172/**69** days |
| **10** | L | 77/m | minimally classic | 0.25 | 1 | **84** days |
| **11** | R | 93/m | classic | 0.16 | 2 | 154/**95** days |
| **12** | L | 76 /f | occult | 0.6 | 1 | **105** days |
| **13** | L | 81/m | classic | 0.08 | 2 | 213/**131** days |
| **14** | R | 70/f | classic | 0.05 | 2 | 151/**132** days |
| **15** | L | 78/f | classic | 0.05 | 3 | 344/222/**146** days |
| **16** | L | 77/m | classic | 1/30 MV | 3 | 329/245/**147** days |
| **17** | L | 72/m | predominantly classic | 0.08 | 2 | 232/**156** days |
| **18** | R | 74/f | hemorrhagic | 0.3 | 1 | **246** days |
| **19** | L | 73/f | classic | HM | 4 | 677/558/467/**383** days |
| **20** | L | 77/f | predominantly classic | 1/15 MV | 7 | unknown*/772/**655** days |

CNV = choroidal neovascularization; PDT = photodynamic therapy
MV= meter vision; HM= hand movement; * Time of 1-5$^{th}$ PDT session unknown

**Figure 1:** Fluorescein angiography 3 days (A, B) and 34 days (C and D) after PDT taken on the day of surgical extraction of the CNV. A and C depict the early and B and C the late phases of the fluorescein angiography in cases 4 (A, B) and 5 (C, D), respectively.

**Figure 2**: Graphs showing the distribution (%) of endothelial and proliferation marker (A) and of VEGF (B) in CNV without pretreatment, 3 days after PDT and 1 or more months after PDT. Vascularization was calculated by counting the number of CD34 and CD105 positive vascular-like patterns in the most vascularized area under x200 magnification and results were classified as: >10 (❑), 5-10 (▒), 1-5 (■) or 0 (■).
Proliferative activity was evaluated by counting the absolute number of Ki-67 positive nuclei within the specimen and shown as: >20 (❑), 11-20 (▒), 1-10 (■) or absent (■).
VEGF immunostaining in retinal pigment epithelium (RPE), endothelial (EC) and stromal cells were evaluated separately. This semiquantitative evaluation was represented as intense (❑), moderate (▒), mild (■) or absent (■).

**Figure 3:** Photomicrographs of a surgically excised CNV. A-C depicts a case devoid of PDT treatment. The specimen was probed with antibodies against CD 34 (A) and CD 105 (B), stained with 3-Diaminobenzidine resulting in a brown chromogen and counterstained with hematoxylin. The endothelial cell markers CD 34 and CD 105 are selectively expressed in vascular structures (e.g. arrows). Some endothelial cells do not stain for endoglin (B; arrow head). (C) Several cell nuclei express the proliferation marker Ki-67 (e.g. arrows). The brown chromogen can be distinguished from the melanin granula (asterisk) contained in pigmented cells. D) CNV membrane from case 2 (Table 1), extracted 3 days after PDT, was probed with CD 34. Some of the vessels depicted by the brown chromogen are still patent (arrow) others appear collapsed (arrow heads). The brown chromogen can be distinguished from the melanin granula (asterisk) contained in pigmented cells. E an F depict a CNV membrane (case 7; Table 1) extracted 40 days after PDT. The specimen stained (brown chromogen CD 105 (E), and Ki-67 (F) discloses patent and vital looking vessels (E) and several proliferating cells (F; brown nuclei). Scale bars: 50 μm.

**Figure 4** Photomicrographs of two distinct specimens devoid of PDT (A and B) treatment stained for VEGF (red chromogen). Both membranes disclose a RPE cell layer (asterisk), vascularization (arrows), fibroblastic stroma cells and different degrees of an inflammatory infiltration (arrow head). VEGF staining can be detected within inflammatory cells (A), endothelial cells and stromal cells (A, B), whereas RPE cells are negative or very faintly stained (B). C and D depict case 2 (Table 1), a CNV extracted 3 days after PDT and stained (red chromogen) for CK 18 (C) and VEGF (D). Compared to A and B, the RPE cells are strongly positive for VEGF (arrows). E and F depict specimens from cases 7 and 11 (Table 1), respectively, stained for VEGF (red chromogen). VEGF is expressed by endothelial cells (arrows) stromal cells (arrow heads) and RPE (E; asterisk). Scale bars: 50 µm.

Letter to the Editor - Clinical Case Notes

# Microcirculation at Optic Disc Rim is Correlated with Visual Field Defects in Cases of Anterior Ischemic Optic Neuropathy

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**Running title:** Optic nerve head blood flow in AION

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