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(Article begins on next page)

## **CD93 as a potential target in neovascular age-related macular degeneration**

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### **Running head**

CD93 expression and shedding in AMD

### **Choroidal neovascularization**

#### **Angiogenesis**

#### **C1qRp**

#### **Endothelial cell.**

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## **Abstract**

In patients with age-related macular degeneration (AMD), choroidal neovascularization is the major cause of severe visual loss. In these patients, the persistence of neovascular growth despite vascular endothelial growth factor-A blockage needs the discovery of new endothelial cell targets. The glycoprotein CD93, highly expressed in activated endothelial cells, has been recently involved in the regulation of the angiogenic process both as transmembrane and soluble protein.

Choroidal neovascular membranes from patients affected by AMD were examined by immunofluorescence using anti-CD93 and anti-von Willebrand factor antibodies. Blood vessels within intraocular and extraocular neoplasias were used as controls for CD93 expression. All choroidal neovascular membranes displayed strong CD93 staining in the von Willebrand factor-positive endothelial cells, consistently with the analyses showing a high colocalization coefficient in the blood vessels. Intraocular and extraocular tumor vessels showed similar results, whereas the normal choroid displayed blood vessels with only faint CD93 staining. Additionally, the concentration of soluble CD93 was determined in the aqueous humor of patients affected by naïve neovascular AMD by enzyme-linked immunosorbent assays. Age-matched cataract patients served as controls. Soluble CD93 was significantly increased in the aqueous humor of naïve neovascular AMD patients and tended to decrease after treatment with an antiangiogenic drug. In conclusion, both transmembrane and soluble CD93 are overexpressed in patients with neovascular AMD, indicating that CD93 may represent a potential new antiangiogenic target in the treatment of choroidal neovascularization.

## Introduction

Age-related macular degeneration (AMD) is a leading cause of adult blindness in the developed world. Severe visual loss in AMD patients is caused by subfoveal geographic atrophy and choroidal neovascularization (CNV) (Schmidt-Erfurth et al., 2014). Although neovascular AMD accounts for approximately 10-20 % of the overall incidence of AMD, this subtype is the major cause of severe visual loss among AMD patients (Balasubramanian et al., 2014).

Antigens involved in ocular angiogenesis and suitable for antibody-based therapeutics are usually proteins that support the formation of new vasculature, such as the growth factors belonging to the vascular endothelial growth factor (VEGF) family (Campochiaro, 2015; Dong et al., 2014; Gao et al., 2016; Miller, 2016). However, although VEGF antagonists have revolutionized the treatment of neovascular AMD, the long-term results of anti-VEGF therapy are poor in one third of the treated patients (Schmidt-Erfurth et al., 2014). The persistence of CNV despite VEGF blockage, as well as the resumption of neovascular growth and leakage during treatment lapses, leave considerable needs unmet. An alternative strategy could be to target molecules which are specifically expressed by proliferating endothelial cells but not by quiescent endothelial cells (Umeda et al., 2006).

Human CD93 is a cell surface glycoprotein composed of 652 amino acids. Structural domain analysis within CD93 from the N- to C-terminus reveals the presence of a C-type lectin-like domain, five epidermal growth factor epidermal growth factor-like repeats, a mucin-like domain, a transmembrane domain, and a cytoplasmic domain (Nepomuceno et al., 1997). Although human CD93 is expressed in different cellular types, its predominant site of expression is the vascular endothelium (Fonseca et al., 2001; McGreal et al., 2002). Much evidence suggests that CD93 may be involved in the regulation of endothelial cell function and that it plays a role in the activated endothelium rather than in the quiescent one. Consistently with an angiogenic role for CD93, it has been shown that the mouse homologue of CD93, AA4, is expressed in vascular endothelial cells within the developing embryo, during the remodeling of blood vessels (Petrenko et al., 1999). Furthermore, human CD93 has been showed to be involved in tumor angiogenesis, being highly expressed in the endothelial cells of tumor blood vessels and faintly expressed in the non-proliferating endothelium (Bao et al., 2016; Langenkamp et al., 2015; Masiero et al., 2013; Olsen et al., 2015). As shown by our group, CD93 promotes endothelial cell migration, survival and tube formation through a cooperative interaction with dystroglycan, an extracellular matrix adhesion molecule (Galvagni et al., 2016). Moreover, we have shown that an anti-CD93 monoclonal antibody was able to neutralize the formation of new blood vessels, both in vitro and in vivo without affecting endothelial cell survival, thus suggesting a possible inhibitory effect on tumor angiogenesis (Orlandini et al., 2014). Lastly, it has been reported that soluble

CD93 fragments are released during inflammation (Greenlee et al., 2009; Mälärstig et al., 2011) and that these soluble forms exhibit proangiogenic effects on the endothelium, acting as epidermal growth factor receptor agonists (Kao et al., 2012).

Here, we investigated the expression of CD93 in the blood vessels of choroidal neovascular membranes (CNVMs) of AMD patients and we also measured the concentration of soluble CD93 in the aqueous humor of naïve neovascular AMD patients at baseline and after antiangiogenic treatment.

## **Materials and Methods**

### *Subjects*

Institutional Review Board/Ethics Committee approval was obtained for this multi-center prospective study. The described research adhered to the tenets of the Declaration of Helsinki. Patients were treated after being informed of the nature of the treatment being offered, the potential risks, benefits, adverse effects, possible treatment outcomes, and after having signed a consent form. The included patients presented with CNV secondary to AMD. The diagnosis was confirmed by fluorescein angiography, indocyanine green angiography, and spectral domain optical coherence tomography. CNVMs were collected from nine AMD patients (Table 1) during submacular surgery for retinal pigment epithelium-choroid graft transplantation. All surgical procedures were performed between May 2015 and January 2016 by the same surgeon (BP). Briefly, following complete vitrectomy and internal limiting membrane peeling, iatrogenic retinal detachment and a 180° retinotomy were performed to gain access to the subretinal space. CNVMs were then removed and the retinal pigment epithelium-choroid patch transplantation performed. Laser retinopexy was performed to reattach the retina. Globes enucleated due to primitive intraocular neoplasias (two retinoblastomas and two malignant choroidal melanomas) and to an extraocular epithelial neoplasia with ocular involvement were used as controls. Anterior chamber taps were performed from March to July 2016 at the Ophthalmology Unit of the Department of Medicine, in order to obtain aqueous samples for the measurement of soluble CD93 in the aqueous humor. This procedure involved twenty-two patients affected by naïve neovascular AMD (Table 2). Exclusion criteria were considered to be previous treatment for neovascular AMD or any previous ophthalmic surgery, except cataract removal. Any cataract surgery was performed at least 9 months prior to inclusion. The controls were age-matched patients undergoing cataract surgery (Table 2). The exclusion criteria for controls were any ocular disease except cataracts and any previous ophthalmic surgery. Diabetes mellitus, the use of immunosuppressive drugs, and malignant tumors at any location were considered exclusion criteria for both patients and controls. All patients with neovascular AMD received 3 consecutive monthly intravitreal injections of 2 mg aflibercept. Aqueous samples were

collected at baseline (day of the first injection), month 1 (day of the second injection), and month 2 (day of the third injection). Anterior chamber taps were performed in the operating room prior to each intravitreal injection (patients) or before cataract surgery (controls). A 30-gauge needle was inserted in the anterior chamber, and 0.1 mL of aqueous was collected, centrifuged to remove cells and debris, aliquoted, and frozen at -80°C until analysis.

#### *Immunofluorescence and immunohistochemistry*

CNVM samples were formalin-fixed, embedded in paraffin, sectioned and treated as previously described (Galvagni et al., 2012). Briefly, after heat induced antigen retrieval in pH 6.0 citrate buffer, specimens were incubated with mouse anti-CD93 antibodies (LSBio, #LS-B3777, Seattle, WA, USA) and rabbit antibodies to von Willebrand factor glycoprotein (Dako, Glostrup, Denmark), which is a commonly used endothelial cell marker (Pusztaszeri et al., 2006). Alexa Fluor-488 or -568 (Thermo Fisher Scientific, Waltham, MA, USA) secondary antibodies were used. Fluorescent images were captured using a Leica TCS SP2 laser-scanning confocal microscope and overlaid images were produced. Because of the combined contributions of green and red fluorescence, in the merged images colocalization structures appear yellow (Dunn et al., 2011). To better visualize the colocalization events, the same merged images were processed using ImageJ and the Colocalization plug-in (threshold channel green and red 90%; ratio setting value 50%), which highlights the colocalization points of green and red images with white dots. The quantitative colocalization analyses of CD93 and von Willebrand factor signals were performed on optical sections by limiting the analyzed area to around blood vessels and using ImageJ and the JACoP plug-in to determine Manders' coefficient  $M_1$  (Manders et al., 1992), representing the percentage of CD93 pixels that overlap with von Willebrand factor pixels.

Immunohistochemistry analyses were performed using anti-CD93 antibodies (LSBio) as previously described (Galvagni et al., 2007). Sections were counterstained with hematoxylin. Images were observed under a polarized light microscope (Zeiss Axio Lab.A1).

#### *Cell culture and immunoblotting analysis*

Retinal pigment epithelial cells (ARPE-19) were kindly provided by Dr. Mario Chiariello (CNR, Siena, Italy). Cells were grown in 1:1 DMEM:F12 culture medium containing 10% fetal bovine serum. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords collected from consenting healthy patients according to institutional guidelines. Cells were grown on gelatin-coated dishes as previously described (Galvagni et al., 2016). Immunoblotting experiments were performed as previously described (Orlandini et al., 2008).

#### *Enzyme-linked immunosorbent assay (ELISA)*

The concentration of soluble CD93 in the patients' aqueous humor was measured using an ELISA kit for human C1qR1/CD93 (Quantikine ELISA kit #DCD930; R&D Systems, Minneapolis, MN). The assay was performed according to the manufacturer's instructions.

### *Statistical analysis*

Data analysis was performed using Prism6 statistical software (GraphPad Software Inc., San Diego, CA). The ELISA data are presented as box and whisker plot displaying median, lower and upper quartiles, and minimum-maximum. The data were evaluated using ANOVA. Differences in the protein concentrations among the groups were estimated using the Student *t* test and the nonparametric Mann-Whitney U test. Two-tailed probabilities of less than 0.05 were considered significant.

## **Results**

### *Analysis of CD93 expression in blood vessels within CNVMs*

In order to assess whether CD93 was expressed in the new blood vessels originating from the choroid, we collected CNVMs from AMD patients. The patients' characteristics are reported in Table 1. Five patients underwent submacular surgery without any prior intravitreal anti-VEGF injection, while four patients received intravitreal anti-VEGF injections before surgery. The interval between the last intravitreal injection and surgery varied from 3 to 5 months. CNVMs were analyzed by immunofluorescence using an anti-CD93 monoclonal antibody (Orlandini et al., 2014). Anti-von Willebrand factor antibodies were used to stain blood vessels. Since in the CNVM sections there were no areas containing quiescent blood vessels, we employed ocular neoplasias to show differences in CD93 staining between quiescent and growing blood vessels. Consistently with recent work on CD93 expression in tumor vessels (Bao et al., 2016; Langenkamp et al., 2015; Olsen et al., 2015), we observed that, compared to normal choroidal tissue, CD93 was highly expressed in blood vessels within ocular and extraocular neoplasias (Fig. 1A). It should be stressed that normal choroidal blood vessels were analyzed in the same tumor sections but far from the tumor area. Moreover, as shown in Figure 1B, colocalization analyses between CD93 and von Willebrand factor confirmed a higher expression of CD93 in both ocular and extraocular tumor blood vessels (colocalization index, mean  $0.479 \pm 0.023$ ) compared to normal choroidal blood vessels (colocalization index, mean  $0.098 \pm 0.014$ ). This differential expression of CD93 between normal and tumor vessels was confirmed by immunohistochemistry analyses (Fig. 2A). It is noteworthy that blood vessels within the CNVMs from all nine AMD patients displayed strong CD93 immunoreactivity (Fig. 2B and 3A) and colocalization indexes between CD93 and von Willebrand factor ( $0.440 \pm 0.031$ ) (Fig. 3B) comparable to those observed in tumor vessels, indicating that not only tumor but also

CNVM proliferating endothelial cells express high levels of CD93. In the CNVMs, CD93 expression resulted confined to endothelial cells, because in spite of its autofluorescence, the retinal pigment epithelium did not express CD93 (Fig. 4).

#### *Analysis of soluble CD93 in the aqueous humor of AMD patients*

Since soluble CD93 has an angiogenic activity (Kao et al., 2012), we used ELISA to measure CD93 concentration in the aqueous humor of twenty-two patients with naïve exudative AMD who were undergoing antiangiogenic treatment. Twenty-two age-matched cataract patients constituted the control group. The mean age was  $80.68 \pm 6.7$  years among the patients affected by neovascular AMD and  $78.18 \pm 5.39$  years among the controls ( $P = 0.18$ ). The male-to-female ratio was 0.68:1 and 1:1 in the AMD patients and controls, respectively. Aqueous samples were collected from all patients before each intravitreal injection and in all controls at the time of cataract surgery. As reported in Figure 5, the concentration of soluble CD93 was significantly higher in the naïve neovascular AMD patients (baseline) as compared to the controls (AMD median = 471.5 pg/mL [range of variation = 95-1248], controls median = 233.0 pg/mL [110-930];  $P = 0.0039$ ). After the first injection, month 1 CD93 levels were lower (median = 386.5 pg/mL [170-1109]), although not significantly lower compared to baseline ( $P = 0.8374$ ), and still significantly higher than in controls ( $P = 0.0059$ ). After the second injection, month 2 CD93 levels (median 342.5 pg/mL [53-982]) were further reduced compared to baseline, although again not significantly ( $P = 0.4389$ ); this further reduction took the month 2 values of CD93 closer to the control levels ( $P = 0.0529$ ).

#### **Discussion**

In the present study, we have shown a strong expression of CD93 in the endothelial cells of CNVMs using immunofluorescence. In contrast, CD93 staining was faint in normal and quiescent choroidal endothelial cells. Patients were affected by different disease stages of neovascular AMD: some of them were not undergoing intravitreal treatment, while others were undergoing intravitreal treatment with different anti-VEGF drugs. CD93 staining resulted uniform in all of the CNVMs and CD93 expression in the CNVM endothelial cells did not seem to be influenced by disease stage or anti-VEGF treatment. Moreover, we observed a significant increase in soluble CD93 concentration in the aqueous humor of patients with naïve CNV compared to controls. In response to intravitreal treatment, soluble CD93 concentration decreased after injections. However, this post-treatment reduction was moderate, suggesting that the intravitreal antiangiogenic treatment was not able to completely neutralize proangiogenic activity (Kao et al., 2012).

Here, for the first time to our knowledge, we reported that CD93 was highly expressed not only in blood vessels within different ocular and extraocular tumors, but also in proliferating



endothelial cells arising in the context of a degenerative ocular disease, such as AMD. The glycoprotein CD93 is predominantly expressed at the cell surface of endothelial cells and in some hematopoietic subsets (Fonseca et al., 2001; McGreal et al., 2002). Previously, we demonstrated that CD93 activated angiogenesis by promoting the adhesion of endothelial cells (Orlandini et al., 2014). Along with our findings, a good amount of evidence suggests that CD93 plays a role in the activated endothelium rather than in the quiescent one. In the developing embryo, the mouse homologue of CD93 is expressed during the remodeling of blood vessels (Petrenko et al., 1999). More importantly, the role of CD93 in tumor angiogenesis is becoming increasingly clear. Indeed, CD93 gene has been identified as one of the top 20 genes of a core human primary tumor angiogenesis signature highly expressed in head and neck squamous cell carcinomas, breast cancers, and clear cell carcinomas (Masiero et al., 2013). Additionally, CD93 gene polymorphism has been associated with disseminated colorectal cancer and elevated expression of CD93 has been seen to promote angiogenesis in nasopharyngeal carcinoma (Bao et al., 2016; Olsen et al., 2015). Recently, Langenkamp et al. showed that elevated expression of CD93 in the glioblastoma vasculature was associated with enhanced vessel function, identifying CD93 as a key regulator of glioblastoma angiogenesis acting via cytoskeletal rearrangements required for cell-cell and cell-matrix adhesion (Langenkamp et al., 2015). These results are consistent with our previous findings, which identified cooperative interactions between CD93 and the adhesion molecule dystroglycan and demonstrated that this relationship promoted endothelial cell migration, suggesting a role for CD93 in proliferating but not in quiescent endothelial cells (Galvagni et al., 2016).

The role of CD93 in CNV was further confirmed by the significantly increased aqueous concentration of soluble CD93 in naïve AMD patients compared to controls. Soluble CD93 fragments are released during inflammation and exhibit proangiogenic effects on the endothelium acting as epidermal growth factor receptor agonists (Kao et al., 2012). Soluble CD93 could be the product of ectodomain cleavage (shedding) of transmembrane CD93, mediated by matrix metalloproteinases, which are known to be key factors in vascular remodeling, thus creating a self-support for the neovascular process (Bohlson et al., 2005). Moreover, as during diabetic retinopathy VEGF is able to induce metalloproteinase activation (Behl and Kotwani, 2015), VEGF inhibition could account for soluble CD93 decrease in patients receiving intravitreal treatment, whereas CD93 expression as a transmembrane protein remains unchanged. CD93 antagonism could interrupt this vicious circle.

The rationale behind intraocular CD93 antagonism in neovascular AMD patients could be dual. The first is suggested in this paper, as we have shown the overexpression of transmembrane CD93 in endothelial cells and not in other cells of both intravitreally treated and untreated AMD patients. This was confirmed by the soluble CD93 data, which showed

only a partial and not significant reduction following anti-VEGF treatment. VEGF neutralization after intravitreal treatment suppresses leakage and stops the growth of neovascular lesions, but does not usually cause regression of CNV (Campochiaro, 2013; Kuehlewein et al., 2015), which might continue to express/shed CD93. The second rationale is suggested by our results on extraocular CD93 antagonism. In fact, we have shown that 4E1, a monoclonal antibody to CD93, was able to neutralize the formation of new blood vessels both in vitro and in vivo without affecting endothelial cell survival, demonstrating that antiangiogenic therapy targeting CD93 is feasible (Orlandini et al., 2014).

Our data show the involvement of CD93 in neovascular AMD, confirming the similarities between ocular and tumor angiogenesis. Indeed, tumor angiogenesis has been proven to share common molecular pathways with ocular neoangiogenesis, as testified by the fundamental role of VEGF in the genesis and maintenance of both. However, one third of patients with CNV show a poor response to anti-VEGF therapy (Yang et al., 2016). A more efficient strategy could involve the multi-targeting of parallel proangiogenic signaling pathways. The overexpression of transmembrane CD93 in CNVM endothelial cells and the increased concentration of soluble CD93 in the aqueous humor of neovascular AMD patients suggest a potential new target for CNV treatment.

### **Acknowledgments**

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### Figure legends

Figure 1. CD93 is localized predominantly in growing tumor blood vessels. A: fixed paraffin-embedded human eye sections containing both tumor and normal choroidal blood vessels were analyzed by immunofluorescence using anti-CD93 (green) and anti-von Willebrand factor (red) antibodies. Overlaid stained sections are shown (left-side image in each row). To better visualize colocalization structures, white dot colocalization images are shown (right-side image in each row). Normal choroidal blood vessels were analyzed in the same sections of the tumors but far from the tumor area. Arrowheads indicate blood vessels. The arrow in the normal choroid indicates the retinal pigment epithelium, which exhibits autofluorescence. Scale bar, 28  $\mu\text{m}$ . B: Plot shows quantification (using Manders' coefficient; mean  $\pm$  SD; vessels = 23; sections = 3) of CD93 colocalization with von Willebrand factor in blood vessels of normal choroid (chor) or ocular tumors (RB, retinoblastoma; MCM, choroidal melanoma; ET, extraocular epithelial tumor with ocular involvement). The means  $\pm$  SD reported in the chart were obtained by analyzing eye sections from: five patients for normal choroid, two patients for retinoblastoma and choroidal melanoma, and one patient for extraocular epithelial tumor with ocular involvement.

Figure 2. Immunohistochemical staining of CD93. Fixed paraffin-embedded sections were stained using anti-CD93 antibodies. A: human eye sections containing both tumor and normal choroidal blood vessels from retinoblastoma and extraocular epithelial tumor with ocular involvement. Normal choroidal blood vessels were analyzed in the same sections of the tumors but far from the tumor area. B: CNVM section from AMD patient number 4. Arrowheads indicate blood vessels. Scale bars, 100  $\mu\text{m}$  (A), 60  $\mu\text{m}$  (B).

Figure 3. CD93 is expressed in blood vessels located in the CNVMs of AMD patients. A: fixed paraffin-embedded CNVM sections from AMD patients were analyzed by immunofluorescence using anti-CD93 (green) and anti-von Willebrand factor (red) antibodies. Overlaid stained sections are shown (left-side image in each row). To better visualize colocalization structures, white dot colocalization images are shown (right-side image in each row). Arrowheads indicate blood vessels. Arrows indicate the retinal pigment epithelium, which exhibits autofluorescence (CNVMs, patient #7 and #8). Scale bar, 40 $\mu\text{m}$ .

B: Plot shows quantification for each AMD patient (using Manders' coefficient; mean  $\pm$  SD; vessels = 21; sections = 3) of CD93 colocalization with von Willebrand factor in blood vessels of CNVMs.

Figure 4. CD93 is not expressed in the retinal pigment epithelium. Cell lysates from retinal pigment epithelial cells (RPEC) and endothelial cells (EC) were analyzed by Western blotting with anti-CD93 antibodies. Anti- $\beta$ -actin antibodies were used to confirm equal loading.

Figure 5. Soluble CD93 is increased in the aqueous humor of AMD patients. The CD93 concentration in the aqueous humor of patients and controls was determined by ELISA. Data are presented as box and whisker plot displaying median, lower and upper quartiles (boxes), and minimum–maximum (whiskers). N = number of subjects. *P*-values were calculated by comparing the baseline (just before the first intravitreal injection – Naïve), month 1 (after 1 intravitreal injection – Treat. 1), and month 2 (after two intravitreal injections – Treat. 2) values of neovascular AMD patients with control group (Contr.). The *P*-value calculated by comparing Naïve with Treat. 2 sample groups is indicated beside the bracket. The overall *P*-value obtained by ANOVA is indicated above the graph.

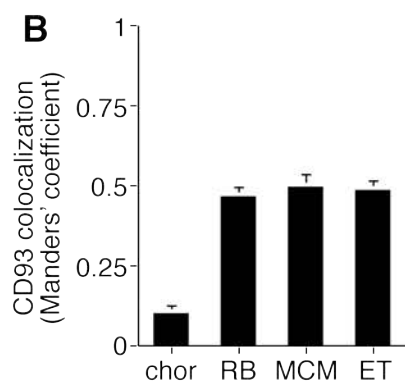
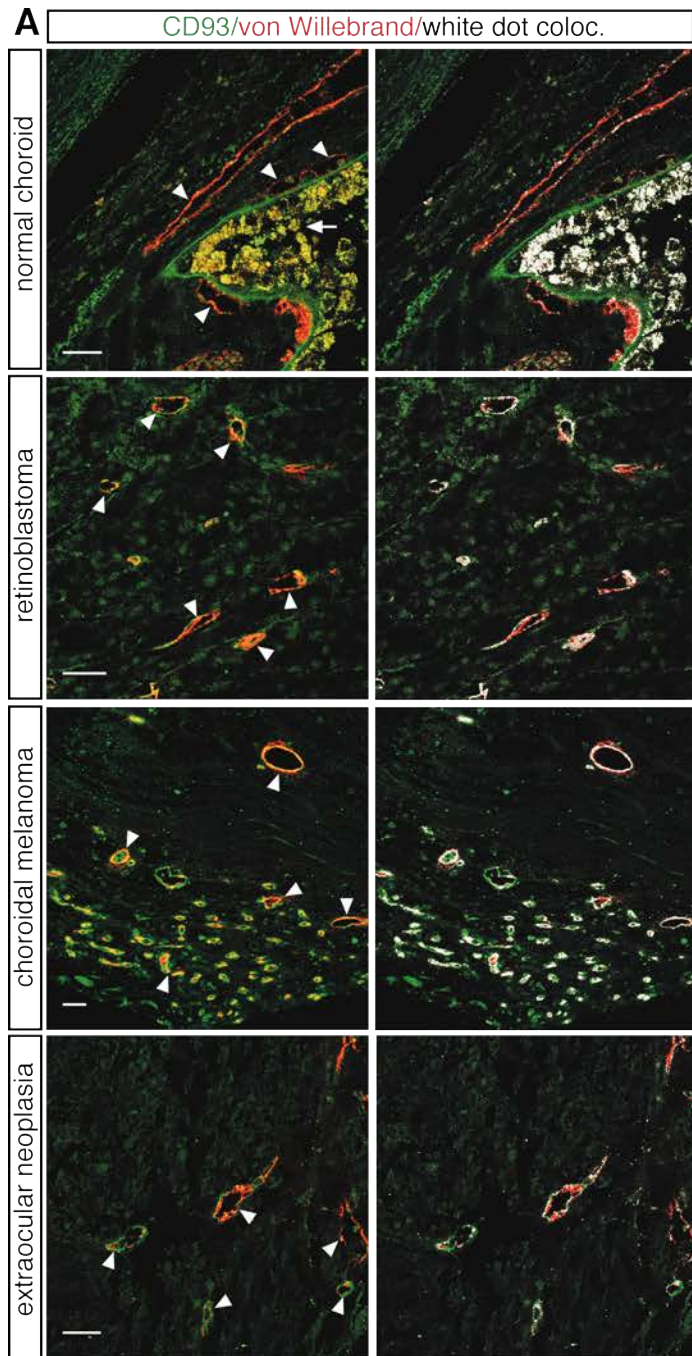


Figure 1

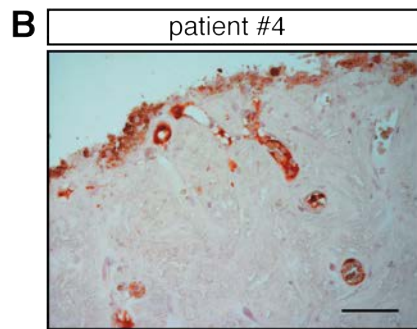
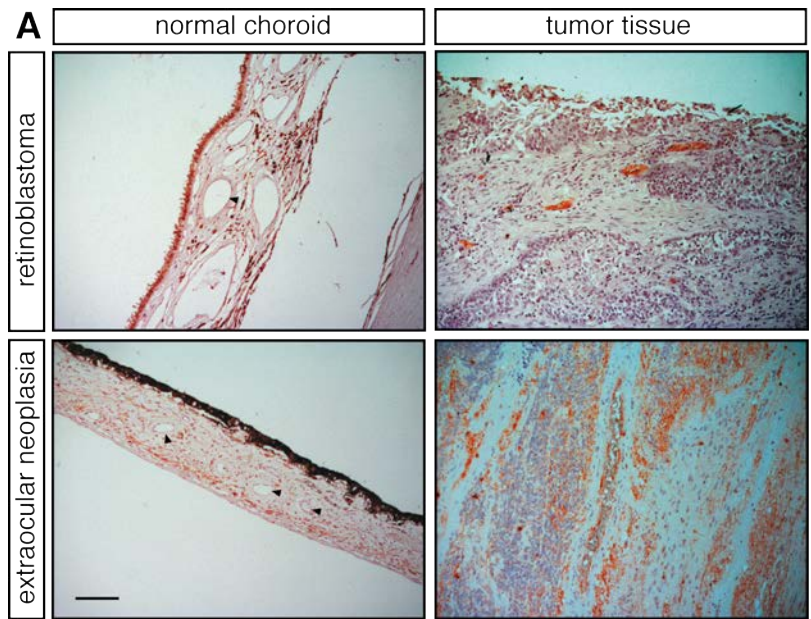


Figure 2



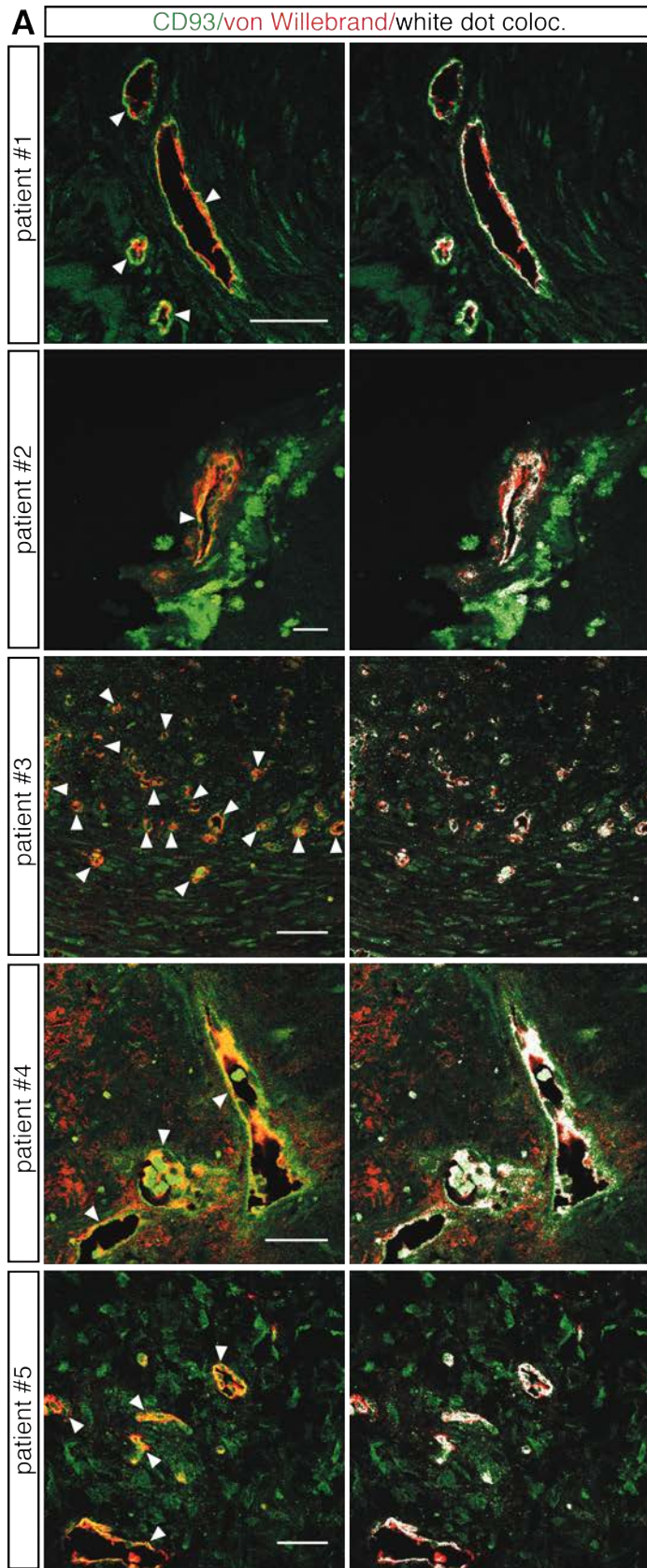


Figure 3A

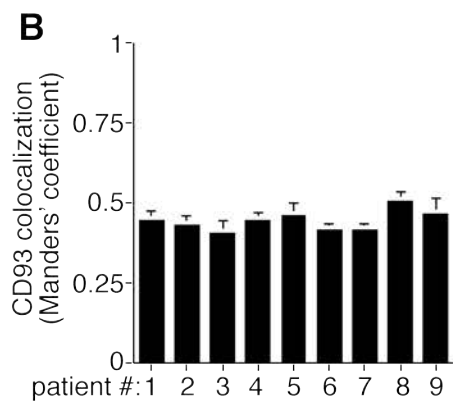
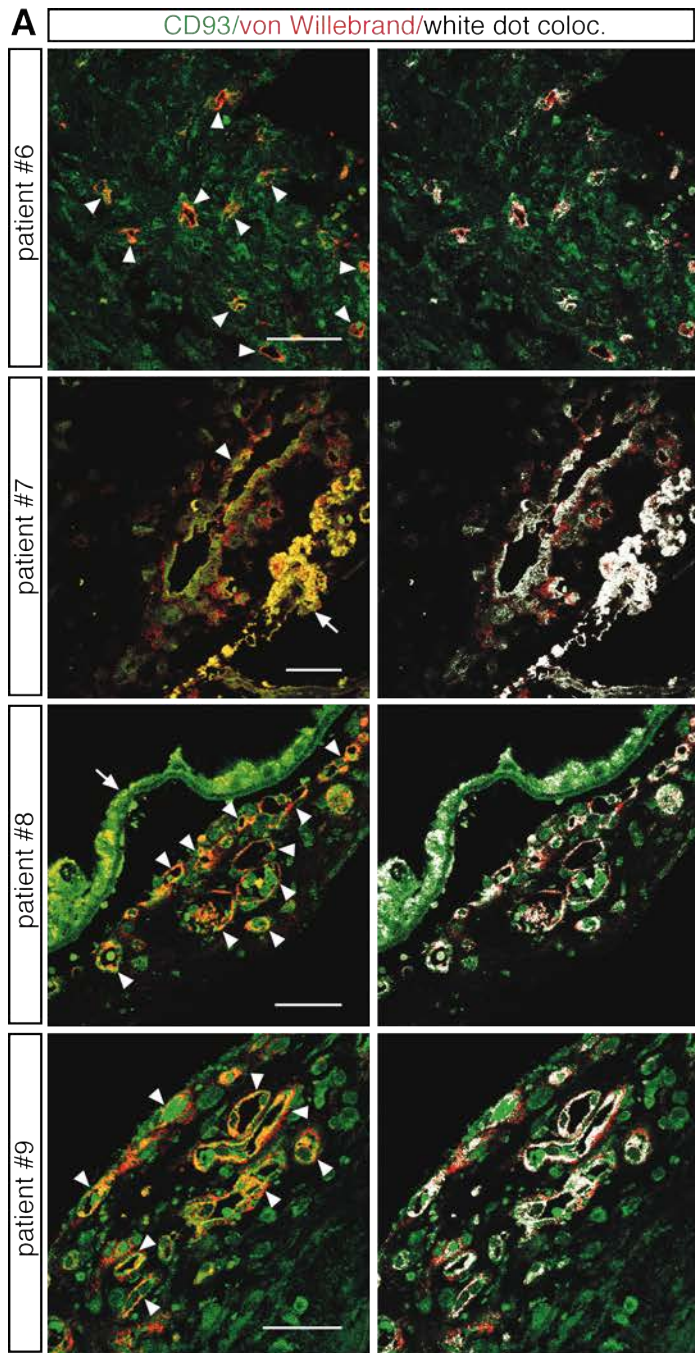


Figure 3AB

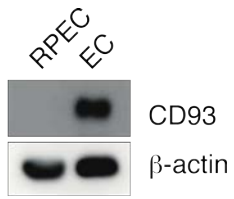


Figure 4

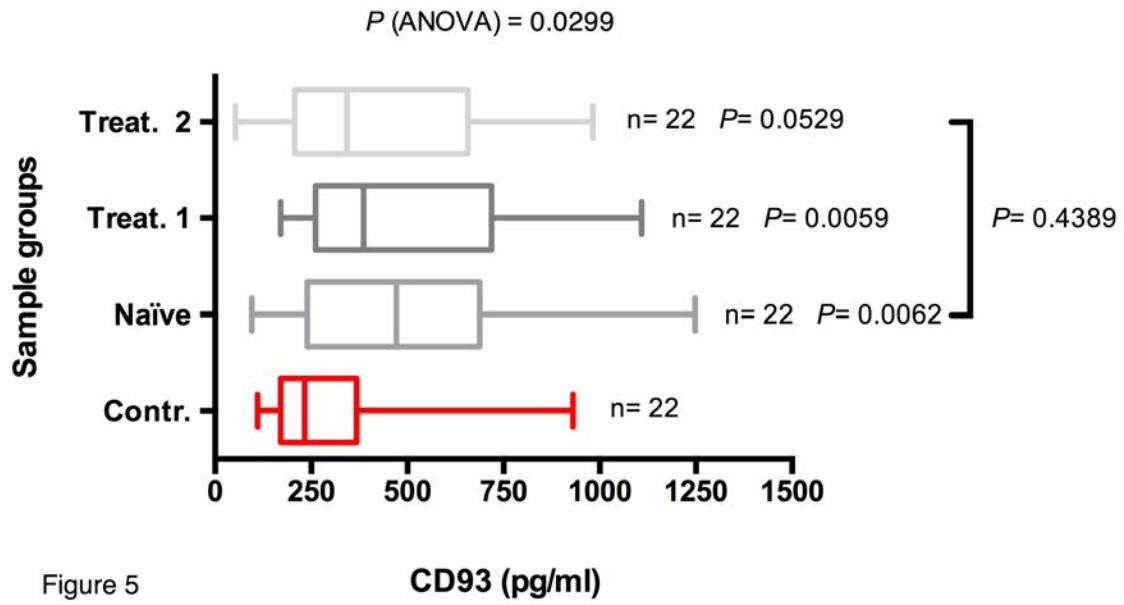


Figure 5

Table 1. Demographics and clinical characteristics of patients subjected to submacular surgery and CNVM collection.

Patient	Age	Sex	Anti-VEGF treatment			Time since last injection (months)	CNV Type
			Bevacizumab	Ranibizumab	Aflibercept		
# 1	74	M	0	2	0	4	2
# 2	70	M	0	0	0	-	1
# 3	80	F	0	0	0	-	1
# 4	68	M	0	0	0	-	1
# 5	77	F	0	0	0	-	1
# 6	67	M	0	0	0	-	2
# 7	85	M	0	2	0	5	PCV*
# 8	66	M	0	2	4	3	1
# 9	69	F	3	4	1	3	1

\*PCV: Polypoidal Choroidal Vasculopathy.

Table 2. Comparison of patients with exudative AMD and control group and clinical characteristics of patients.

	Exudative AMD Group, n=22	Control Group, n=22
Age, y, mean $\pm$ SD	80.68 $\pm$ 6.7	78.18 $\pm$ 5.39
Sex, M:F	0.68:1	1:1
Eyes	22 eyes; 50% right	22 eyes; 54,5% right
Hypertension, n		
Yes	13	10
No	9	12
Type of choroidal neovascularization	13 type 1 8 type 2 1 PCV	N/A
Central Retinal Thickness day 0 ( $\mu$ m)	307.33 $\pm$ 70.44	N/A
Central Retinal Thickness after 3 treatments ( $\mu$ m)	278.05 $\pm$ 100.56	N/A

PCV: Polypoidal Choroidal Vasculopathy.

N/A: not applicable