

RESEARCH ARTICLE

Increased Expression of Angiogenic and Inflammatory Proteins in the Vitreous of Patients with Ischemic Central Retinal Vein Occlusion

Christoph Ehlken^{1*}, Bastian Grundel¹, Daniel Michels¹, Bernd Junker^{1,2}, Andreas Stahl¹, Günther Schlunck¹, Lutz L. Hansen¹, Nicolas Feltgen³, Gottfried Martin¹, Hansjürgen T. Agostini¹, Amelie Pielen^{1,2}

1 Klinik für Augenheilkunde, Universitätsklinikum Freiburg, Freiburg, Germany, **2** Hannover Medical School, Eye Hospital, Hannover, Germany, **3** Augenklinik, Universitätsklinikum Göttingen, Göttingen, Germany

* christoph.ehlken@uniklinik-freiburg.de



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Abstract

Background

Central retinal vein occlusion (CRVO) is a common disease characterized by a disrupted retinal blood supply and a high risk of subsequent vision loss due to retinal edema and neovascular disease. This study was designed to assess the concentrations of selected signaling proteins in the vitreous and blood of patients with ischemic CRVO.

Methods

Vitreous and blood samples were collected from patients undergoing surgery for ischemic CRVO (radial optic neurotomy (RON), n = 13), epiretinal gliosis or macular hole (control group, n = 13). Concentrations of 40 different proteins were determined by an ELISA-type antibody microarray.

Results

Expression of proteins enriched in the vitreous (CCL2, IGFBP2, MMP10, HGF, TNFRSF11B (OPG)) was localized by immunohistochemistry in eyes of patients with severe ischemic CRVO followed by secondary glaucoma. Vitreal expression levels were higher in CRVO patients than in the control group (CRVO / control; p < 0.05) for ADIPOQ (13.6), ANGPT2 (20.5), CCL2 (MCP1) (3.2), HGF (4.7), IFNG (13.9), IGFBP1 (14.7), IGFBP2 (1.8), IGFBP3 (4.1), IGFBP4 (1.7), IL6 (10.8), LEP (3.4), MMP3 (4.3), MMP9 (3.6), MMP10 (5.4), PPBP (CXCL7 or NAP2) (11.8), TIMP4 (3.8), and VEGFA (85.3). In CRVO patients, vitreal levels of CCL2 (4.2), HGF (23.3), IGFBP2 (1.23), MMP10 (2.47), TNFRSF11B (2.96), and VEGFA (29.2) were higher than the blood levels (vitreous / blood, p < 0.05). Expression of CCL2, IGFBP2, MMP10, HGF, and TNFRSF11B was preferentially localized to the retina and the retinal pigment epithelium (RPE).

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Conclusion

Proteins related to hypoxia, angiogenesis, and inflammation were significantly elevated in the vitreous of CRVO patients. Moreover, some markers known to indicate atherosclerosis may be related to a basic vascular disease underlying RVO. This would imply that local therapeutic targeting might not be sufficient for a long term therapy in a systemic disease but hypothetically reduce local changes as an initial therapeutic approach.

Introduction

Retinal vein occlusion is the second most common vascular eye disease and causes vision loss due to macular edema, retinal bleeding and ischemia [1]. The worldwide prevalence is estimated at 1:1250 [2]. Central retinal vein occlusion (CRVO) is less frequent than branch retinal vein occlusion (BRVO) but results in greater retinal damage.

Visual acuity (VA) prognosis in CRVO is significantly improved by treatment of macular edema either with intravitreal steroids or anti-VEGF therapeutics that address inflammatory and VEGF-driven ocular changes [3]. Intravitreal anti-VEGF treatment leads to significant visual gain of 15 letters or more in up to 60% of the patients (47% ranibizumab [4], 55% aflibercept [5], 60% bevacizumab [6]) at one year. However, final VA of $\geq 20/40$, sufficient to allow for driving and reading, is only reached in every second patient (47% ranibizumab [4]). This underlines the need for a detailed characterization of risk factors and further improvement of treatment strategies.

Known risk factors for RVO are advanced age [1], glaucoma and systemic diseases, especially components of the metabolic syndrome such as diabetes mellitus, hypertension and hyperlipidemia [7]. Regarding diabetes, patients with end-organ damage from diabetes have a significantly increased risk of CRVO, while those without do not [7]. Hyperlipidemia leads to atherosclerosis, which represents a later state of the disease. Atherosclerosis of the central retinal artery was found in association with CRVO [8]. The hypothesis that atherosclerosis is associated with a higher risk of CRVO is supported by the finding that history of stroke and peripheral arterial disease are associated with higher incidence of CRVO [7,9,10].

Inflammatory cytokines, chemokines and neurotrophic factors have been investigated in the vitreous of patients with retinal vascular diseases due to diabetes or retinal vein occlusion. VEGF is upon the most investigated as anti-VEGF is implemented in therapy [3,11]. Elevated levels of inflammatory immune mediators such as IL-6, IL-8, CCL2 were reported in central and branch RVO, diabetic macular edema, proliferative diabetic retinopathy and retinal detachment [12]. Others found significantly higher levels of IL-1 β , IL-2, IL-5, IL-8, IL-9, IL-10, IL-12, IL-13, CCL11, G-CSF, IFN- γ , CXCL10, CCL2, CCL4, TNF, and VEGF specifically in CRVO [13]. An association between the expression of inflammatory factors and the severity of macular edema was observed in CRVO [14]. Levels of VEGF, IL-6, sICAM-1 and PEDF correlated independently with vascular permeability. These factors were higher in CRVO than in controls, higher in ischemic versus non-ischemic CRVO and correlated with macular edema in optic coherence tomography [14].

Analysis of plasma levels of atherosclerotic and thrombophilic risk factors demonstrated that arterial hypertension, hypercholesterolemia, hyperhomocysteinemia and elevated factor VIII were associated with an increased risk for ischemic versus non-ischemic CRVO [15]. We set out to simultaneously investigate the expression of 40 proteins associated with inflammation, hypoxia, angiogenesis and atherosclerosis in vitreous and blood samples of patients

undergoing RON (radial optic neurotomy) for clinically defined ischemic CRVO and compared it to a control group of patients receiving surgery for epiretinal gliosis or a macular hole. Criteria for the selection of the proteins to measure were solubility in the cytoplasm (as we did not expect cells or cell membranes in the vitreous), a context with angiogenesis and inflammation, and availability from the provider of the array. Our data suggest that distinct chemokines (CCL2) and growth factors (HGF) may represent valuable targets for novel therapeutic approaches to treat or prevent ischemic complications in CRVO patients. The observations also support epidemiologic data regarding risk factors such as atherosclerosis.

Materials and Methods

Ethics statement

All patients gave their written informed consent prior to their inclusion in the study. The study was registered as experimental laboratory investigation at the Center of Clinical Trials and approved by the Institutional Review Board of the University Freiburg (No 215/08) and performed in accordance with the IRB's requirements, with the ethical standards laid down in the 1964 Declaration of Helsinki and with the federal laws in Germany.

Patients and study design

Patients with ischemic CRVO were recruited between 2005 and 2006. At the time of sample acquisition, radial optic neurotomy was thought a valuable surgical approach for ischemic CRVO. However, this technique did not fulfil expectations [16]. In recent years, intravitreal anti-VEGF treatment has been introduced to treat macular edema secondary to CRVO. It is currently the new standard of treatment for either non-ischemic and ischemic CRVO and surgical approaches are left to rare severe cases. CRVO patients with ischemic occlusive disease, indicated either by nonperfusion in fluorescence angiography (> 10 disc diameters), visual acuity > 1.0 log MAR, and/or clinical findings such as dark hemorrhages, a high number of cotton wool spots, or massive leakage of the vessels and papilledema [17], were selected for vitrectomy and radial optic neurotomy (n = 13). Duration of CRVO was defined as time from onset of symptoms until surgery. Neovascularizations of the iris were found in 2/13 patients. Control specimens were collected from 13 patients undergoing vitrectomy for macular pucker and macular hole. Patient data is presented in Table 1. CRVO patients did not show differences compared to control regarding age and the risk factors arterial hypertension, diabetes, and history of stroke. Significantly more CRVO patients presented with hyperlipidemia, history of smoking, glaucoma and use of anticoagulants (aspirin or phenprocoumon), indicating a higher prevalence of cardiovascular diseases known as risk factors for CRVO.

A standard 3-port vitrectomy was performed during surgery. Sample acquisition was achieved as the first step of the surgery avoiding dilution by the infusion. Depending on clinical findings, additional procedures such as laser photocoagulation, intravitreal administration of triamcinolone or bevacizumab could be included at the end of surgery. Samples (200–400 μ l each) were immediately stored at -80°C until further investigation.

Patients with other proliferative eye diseases, such as uveitis or diabetic retinopathy, or patients with intraocular surgery within the last 6 months, or history of vitrectomy, were excluded from the study.

Measurement of proteins

Concentrations of various proteins from vitreous and blood samples were measured with an ELISA-type antibody microarray (Quantibody, Raybiotech Inc., Norcross, GA) following the

Table 1. Patient characteristics.

	CRVO	Control
Number of patients (male)	13 (5)	13 (4)
Age (mean ± SD, years)	74.6 ± 9.7	69.5 ± 9.8
Duration of CRVO (weeks)	9.4 ± 5.9	not applicable
Neovascularisation of the iris (at time of surgery)	2/13 (15%)	0 (0%)
Neovascularisation of the disc or elsewhere in the retina	0 (0%)	0 (0%)
Mean visual acuity (log MAR +/- SD)	1.6 +/- 0.48	not applicable
Risk factors for CRVO (%):		
Glaucoma	6/13 (46%)	0 (0%)
Arterial hypertension	10/13 (77%)	11/13 (85%)
Diabetes	0 (0%)	0 (0%)
Hyperlipidemia	2/13 (15%)	0 (0%)
History of stroke	1/13 (8%)	1/13 (8%)
Smoking	2/13 (15%)	0 (0%)
Anticoagulation	5/ 13 aspirin 2/ 13 marcumar	2/ 13 aspirin 0/ 13 marcumar

SD = standard deviation
 BCVA = best corrected visual acuity
 CRVO = central retinal vein occlusion
 Control = epiretinal gliosis or macular hole.

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manufacturer’s instructions. Antibodies for each protein were arrayed in quadruplicates per array. 80 µl of vitreous or blood was used for each sample. The detection antibodies were labelled with biotin which was detected with Alexa Fluor 555-conjugated streptavidin. The signals were read with a G2565 microarray reader (Agilent Technologies, Santa Clara, CA). TM4 Spotfinder (<http://www.tm4.org>, [18]) was used for quantification of the spots. The concentrations of the proteins were calculated from the median intensities of the spots using standard curves obtained with a mix of the 40 peptide standards. Detection limits were calculated from the standard curves with DINTEST (<http://www.luw.ethz.ch/computer/software/>) according to DIN 32645. Protein concentrations were determined using the Bradford assay with BSA as a standard [19] as the BCA (Bicinchoninic acid) test resulted in erroneously high values if the proteins were not precipitated.

Mean concentrations for CRVO and control groups were compared by nonparametric comparisons (R package nparcomp, <http://www.r-project.org/>) with Tukey’s correction for multiple comparisons. Correlation between concentrations and the time after CRVO was determined by the Pearson product-moment correlation coefficient. $p < 0.05$ was considered significant. Correlations among proteins as well as among patients were tested in R with corr (psych package) using the Spearman coefficient and Holm adjustment for multiple comparison. Biochemical pathways were analyzed by enrichment analysis (EnrichmentBrowser and gage in Bioconductor) against the KEGG pathways database (<http://www.genome.jp/kegg/>) and the gene ontology database (<http://geneontology.org/>) for the CRVO patients and vitreous samples with 24 genes showing expression above background. A complete list of factors tested, gene symbol, gene ID and gene name is provided as supplementary [S1 Table](#).

Immunohistochemistry

Enucleated eyes from two female patients (88 and 61 years old) with severe ischemic CRVO followed by neovascular glaucoma were investigated for expression of the proteins that showed

Table 2. Antibodies used for immunohistochemistry.

Antibody against	Company	Product No.	Type	Host	Dilution
ADIPOQ	Acris	SP2182P	Polyclonal	rabbit	1:50
CCL2	Acris	PP1044P1	Polyclonal	rabbit	1:400
COL IV	Abcam	ab6586	Polyclonal	rabbit	1:500
GFAP	Dako	Z0334	Polyclonal	rabbit	1:1000
HGF	Aviva	ARP44317_P050	Polyclonal	rabbit	1:400
IBA1	Wako	019-19741	Polyclonal	rabbit	1:500
IGFBP2	GeneTex	GTX113471	Polyclonal	rabbit	1:400
MMP10	Abcam	ab38930	Polyclonal	rabbit	1:1000
PPBP	PeptoTech	500-P03	Polyclonal	rabbit	1:50
TNFRSF11B	Acris	SM7070P	Monoclonal	mouse	1:50

COL IV: collagen IV

GFAP: glial fibrillary acidic protein

IBA1: allograft inflammatory factor 1 (AIF1).

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higher concentrations in the vitreous than in blood. Sections (5 µm) of paraffin embedded eyes were dewaxed and demasked for 20 min in 100 mM sodium citrate, pH 6.0, in a steamer. After transfer to TBST (50 mM Tris / HCl pH 7.6, 0.9% NaCl, 0.02% Tween 20), sections were blocked with Ultra V Block (Lab Vision at medac GmbH, Wedel, Germany) and incubated with antibodies as listed in Table 2 for 3 h. After washing in TBST, an AP-labelled goat anti-mouse secondary antibody (A3562, Sigma-Aldrich, Taufkirchen, Germany) was applied for 1 h, or a biotin-labelled goat anti-rabbit secondary antibody (71-00-30, KPL, Gaithersburg, MD, USA) was applied for 1 h followed by streptavidin-coupled AP (71-00-45, KPL) for 1 h. Slides were washed and AP was made visible by the Vector Red AP Substrate Kit I (SK-5100, Vector Labs at Axxora, Lörrach, Germany). Sections were counter-stained with hematoxylin.

Results

Concentration of various proteins in the vitreous or blood

The blood concentrations of the proteins investigated in this study were similar in CRVO patients compared to control patients (range of the ratios between 0.22 and 1.93, median 1.04, Table 3). In contrast, the concentration of total protein in the vitreous of CRVO patients was 6.4 fold elevated compared to that of control patients (Table 3). The vitreous concentrations of proteins ADIPOQ, ANGPT2, CCL2, HGF, IFNG, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IL6, LEP, MMP3, MMP9, MMP10, PPBP, TIMP4, and VEGFA were elevated in CRVO patients compared to control patients (range of the ratios between 0.75 and 85.3, median 1.82, Table 3). Proteins like FGF6, FGF7, MMP1, TIMP1, and TIMP2 did not show enhanced vitreous concentrations in CRVO patients compared to controls. This indicates that there was not only a break-down of the blood retina barrier but also a local production within the eye or a selective transport of proteins. The total increase of the proteins measured in this study was 1.1 µg/ml in the vitreous (mainly contributed by PPBP), while the increase in total vitreal protein was 2.8 mg/ml indicating a 2500 fold impact of blood retina barrier break-down as compared to ocular protein expression. Taking into account the ocular expression of proteins not measured in this study, the factor will be somewhat smaller than 2500.

In CRVO patients, most of the proteins investigated had significantly higher concentrations in blood than in the vitreous. However, protein concentrations (ratio vitreous / blood) of CCL2

Table 3. Concentration of various factors in vitreous fluid and blood serum of CRVO patients and controls.

Gene	Unit	CRVO			Control			Vitreous CRVO / Control	Detection limit	Correlation with time after CRVO	
		Blood ± SD	Vitreous ± SD	V / B	Blood ± SD	Vitreous ± SD	V / B			Blood	Vitreous
ADIPOQ	ng/ml	151 ± 23	61 ± 24	0.40 *	135 ± 25	4.5 ± 3.9	0.03 *	13.6 *	1.0	-0.22	-0.28
ANGPT2	pg/ml	1268 ± 1258	1619 ± 1120	1.28	1120 ± 554	79 ± 26	0.07 *	20.5 *	63	0.01	-0.33
CCL2	pg/ml	193 ± 85	809 ± 118	4.20 *	218 ± 92	252 ± 113	1.15	3.2 *	17	-0.13	-0.55 *
HGF	ng/ml	0.50 ± 0.26	11.7 ± 6.4	23.3 *	0.55 ± 0.32	2.5 ± 1.5	4.55 *	4.7 *	0.13	-0.33	-0.28
IFNG	pg/ml	725 ± 273	97 ± 110	0.13 *	998 ± 559	7 ± 12	0.01 *	13.9 *	43	-0.21	-0.33
IGFBP1	ng/ml	7.8 ± 2.8	3.0 ± 1.8	0.38 *	6.1 ± 1.7	0.20 ± 0.12	0.03 *	14.7 *	0.13	0.38	-0.06
IGFBP2	ng/ml	9.3 ± 1.5	11.4 ± 1.6	1.23 *	8.8 ± 1.1	6.4 ± 2.8	0.73 *	1.8 *	0.44	-0.09	-0.41
IGFBP3	ng/ml	51 ± 14	6.8 ± 3.4	0.13 *	36 ± 11	1.7 ± 0.69	0.05 *	4.1 *	1.4	-0.08	-0.29
IGFBP4	ng/ml	68 ± 24	9.4 ± 2.6	0.14 *	50 ± 23	5.4 ± 2.2	0.11 *	1.7 *	4.0	0.22	-0.28
IL6	pg/ml	94 ± 31	43 ± 38	0.46 *	118 ± 60	4 ± 3	0.03 *	10.8 *	6	-0.19	-0.42
LEP	ng/ml	10 ± 10	0.98 ± 0.96	0.10 *	33 ± 41	0.29 ± 0.12	0.01 *	3.4 *	0.40	0.26	0.67 *
MMP3	ng/ml	12.0 ± 6.4	1.02 ± 0.62	0.08 *	9.3 ± 5.1	0.24 ± 0.29	0.03 *	4.3 *	0.40	-0.36	-0.40
MMP9	pg/ml	13039 ± 6806	142 ± 63	0.01 *	11886 ± 7446	39 ± 14	0.00 *	3.6 *	43	-0.47	-0.29
MMP10	pg/ml	198 ± 306	488 ± 350	2.47 *	339 ± 236	90 ± 58	0.26 *	5.4 *	47	-0.42	-0.27
PPBP	ng/ml	4867 ± 1522	1105 ± 442	0.23 *	3091 ± 1488	94 ± 80	0.03 *	11.8 *	9.0	0.03	-0.73 *
TIMP4	ng/ml	3.9 ± 1.7	1.1 ± 0.39	0.28 *	2.70 ± 0.87	0.29 ± 0.10	0.11 *	3.8 *	0.17	0.46	-0.43
TNFRSF11B	ng/ml	1.42 ± 0.76	4.2 ± 1.6	2.96 *	2.1 ± 2.1	2.7 ± 1.7	1.30	1.5	0.19	0.09	0.40
VEGFA	pg/ml	202 ± 278	5883 ± 4503	29.2 *	398 ± 413	69 ± 27	0.17 *	85.3 *	136	-0.24	-0.46
ANGPT1	ng/ml	13 ± 12	0 ± 0	0.00 *	25 ± 15	0 ± 0	0.00 *	-	1.5	-0.44	-
CCL7	pg/ml	74 ± 22	4 ± 2	0.06 *	55 ± 29	2 ± 1	0.04 *	2.0 *	14	0.17	-0.38
CXCL11	pg/ml	70 ± 38	3 ± 2	0.04 *	49 ± 29	4 ± 3	0.08 *	0.75	6	0.25	0.09
EGF	pg/ml	854 ± 721	0 ± 1	0.00 *	1867 ± 1246	0 ± 1	0.00 *	-	12	-0.41	0.11
FGF2	pg/ml	361 ± 160	139 ± 26	0.39 *	338 ± 129	151 ± 42	0.45 *	0.92	209	-0.03	0.69 *
IGF1	pg/ml	3963 ± 1873	85 ± 167	0.02 *	3417 ± 2076	97 ± 236	0.03 *	0.88	2078	0.06	-0.44
IGFBP5	ng/ml	6.9 ± 4.1	3.0 ± 3.7	0.43	6.6 ± 5.7	1.6 ± 1.2	0.24 *	1.9	3.8	0.17	-0.11

(Continued)

Table 3. (Continued)

Gene	Unit	CRVO			Control			Vitreous		Correlation with time after CRVO	
		Blood ± SD	Vitreous ± SD	V / B	Blood ± SD	Vitreous ± SD	V / B	CRVO / Control	Detection limit	Blood	Vitreous
<i>IL1B</i>	pg/ml	14 ± 6	7 ± 2	0.51 *	13 ± 7	8 ± 2	0.63	0.88	11	0.17	0.26
<i>IL4</i>	pg/ml	47 ± 23	10 ± 6	0.21 *	46 ± 29	7 ± 4	0.15 *	1.4	10	-0.17	-0.36
<i>IL13</i>	pg/ml	53 ± 18	7 ± 8	0.13 *	81 ± 51	0 ± 0	0.00 *	- *	13	-0.41	-0.21
<i>IL18BP</i>	pg/ml	592 ± 263	110 ± 56	0.19 *	584 ± 290	65 ± 28	0.11 *	1.7 *	133	-0.24	-0.49
<i>MMP2</i>	pg/ml	838 ± 350	142 ± 52	0.17 *	453 ± 520	150 ± 96	0.33	0.95	179	0.04	0.30
<i>MMP8</i>	pg/ml	87 ± 95	9 ± 14	0.10 *	76 ± 78	4 ± 5	0.05 *	2.25	22	-0.30	-0.34
<i>TNF</i>	pg/ml	197 ± 117	11 ± 33	0.06 *	887 ± 971	0 ± 0	0.00 *	-	65	-0.31	-0.20
<i>TNFRSF18</i>	pg/ml	210 ± 132	16 ± 9	0.07 *	263 ± 140	11 ± 9	0.04 *	1.5	76	-0.36	-0.34
<i>FGF6</i>	pg/ml	174 ± 99	43 ± 6	0.25 *	166 ± 75	40 ± 7	0.24 *	1.08	38	-0.02	0.46
<i>FGF7</i>	pg/ml	143 ± 90	23 ± 8	0.16 *	198 ± 181	25 ± 12	0.13 *	0.92	15	-0.24	-0.47
<i>MMP1</i>	ng/ml	1.95 ± 2.6	0.33 ± 0.10	0.17 *	4.7 ± 4.5	0.27 ± 0.12	0.06 *	1.2	0.20	-0.45	0.23
<i>TIMP1</i>	ng/ml	34 ± 19	5.4 ± 1.6	0.16 *	33 ± 18	4.4 ± 2.5	0.13 *	1.2	0.22	-0.47	-0.33
<i>TIMP2</i>	ng/ml	15.0 ± 5.4	9.6 ± 1.4	0.64 *	7.7 ± 3.1	8.0 ± 2.6	1.03	1.2	0.32	-0.39	-0.40
Protein	mg/ml	46 ± 12	3.3 ± 1.7	0.07 *	56 ± 12	0.51 ± 0.58	0.01 *	6.4 *	0.48		

*significant difference as determined by nonparametric comparisons (p < 0.05)

Criteria for selected factors labeled in bold: vitreous concentration higher than blood values; or vitreous concentration of CRVO and control significantly different, and at least one value for vitreous above the detection limit. Italic factors: Vitreous values below detection limit.

All values for FGF4 and MMP13 were below the detection limit, and the values for concentrations of ANGPT1, CCL7, CXCL11, EGF, FGF2, IGF1, IGFBP5, IL1B, IL4, IL13, IL18BP, MMP2, MMP8, TNF, and TNFRSF18 in the vitreous of both CRVO patients and controls were below the detection limit. The column "Correlation with time after occlusion" shows the Pearson product-moment correlation coefficient that is a measure of the linear correlation between the protein concentration in the vitreous or blood and the time after occlusion. * indicates statistical significance (p < 0.05). Note that the significance of LEP is lost if the highest value is omitted. The time after occlusion is the time between the CRVO and vitrectomy.

SD = standard deviation

V / B = vitreous / blood

CRVO = central retinal vein occlusion.

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(4.2), HGF (23.3), IGFBP2 (1.23), MMP10 (2.47), TNFRSF11B (2.96), and VEGFA (29.2) were significantly higher in the vitreous than in the blood of the same patient (p < 0.05, Table 3). This indicates that the proteins showing higher concentrations in the vitreous were, at least partially, produced within the eye or actively transported there. In control patients, only HGF showed significantly higher concentrations in the vitreous than in blood. Statistical analysis of the correlations among proteins or patients were not conclusive, most probably because of the

small number of proteins and patients. The same was true for the biochemical pathway analyses.

Dependence of the protein concentrations from the time after occlusion

The time between the onset of symptoms due to CRVO and the time point at which the vitreous specimen was taken was different for each patient (time after occlusion, mean: 9.4 ± 5.9 weeks). As surgery was performed once in every patient, specimens could not be taken at different time points which limits the interpretation of the results. We compared the concentrations of the proteins measured to the time after occlusion (Table 3). PPBP (-0.73, $p < 0.05$) and CCL2 (-0.55, $p < 0.05$) showed a negative correlation (Fig 1) that may reflect an increased selective permeability for certain small proteins or an increased inflammatory or angiogenic state shortly after CRVO that is repaired with time. Similar tendencies, though not statistically significant, were found for IGFBP2, IL6, MMP3, TIMP4, and VEGFA. In contrast, LEP showed a positive correlation (0.67, $p < 0.05$).

Localization of selected proteins in the human eye

Ocular localization of the proteins that showed significantly higher expression in the vitreous than in blood (CCL2, IGFBP2, MMP10, HGF, TNFRSF11B) was investigated in histological specimens of eyes from patients with painful blindness due to secondary glaucoma after CRVO (Fig 2). Staining for all these factors was found preferentially in the retina and in the retinal pigment epithelium (RPE) but to a much lesser extent in the optic nerve head or extrascleral nerves. Staining intensity was higher in ocular areas affected with inflammation. HGF was additionally found in the endothelium and media of some but not all extrascleral vessels. Staining for GFAP (glial marker), IBA1 (microglial and macrophage marker), and COL IV (marker for basement membranes of vessels) was used for comparison.

Discussion

Retinal vein occlusion and subsequent ischemia are followed by the release of cytokines, growth factors and enzymes which contribute to severe vision loss due to retinal edema and neovascularization. Previous studies in patients suffering from retinal vein occlusions detected a range of proteins in the vitreous [20,21]. Vitreous VEGFA concentrations were also determined earlier [11,22,23] and used as a reference in this study. In contrast to previous reports, we had the opportunity to assess distinct protein levels in the vitreous as well as in blood samples of patients following ischemic CRVO and compare them to unrelated controls. This allowed us to further characterize CRVO-specific changes in vitreous protein expression patterns and to gather evidence for ischemia-induced localized expression of distinct proteins as opposed to a release from blood.

Most of the proteins that were found to be more prevalent in the vitreous of CRVO patients than in controls appear to be strongly related to hypoxia, inflammation or angiogenesis. VEGFA, ADIPOQ, ANGPT2, CCL2, IGFBP1, or LEP share a common hypoxia-response element (HRE) at their promoter or intron [24–27] indicating that they are regulated by HIF1A or HIF2A. In addition, IGFBP2 and IGFBP3 are known to be up-regulated upon hypoxia [28,29], but it is currently unclear if they are upregulated by HIF or by one of his target genes. Three of these factors (VEGFA, CCL2 and IGFBP2) showed significantly higher levels in the vitreous than in the blood of CRVO patients. These data are clearly consistent with the activation of hypoxia-induced gene networks and a localized intraocular expression of specific proteins due to a hypoxic state.

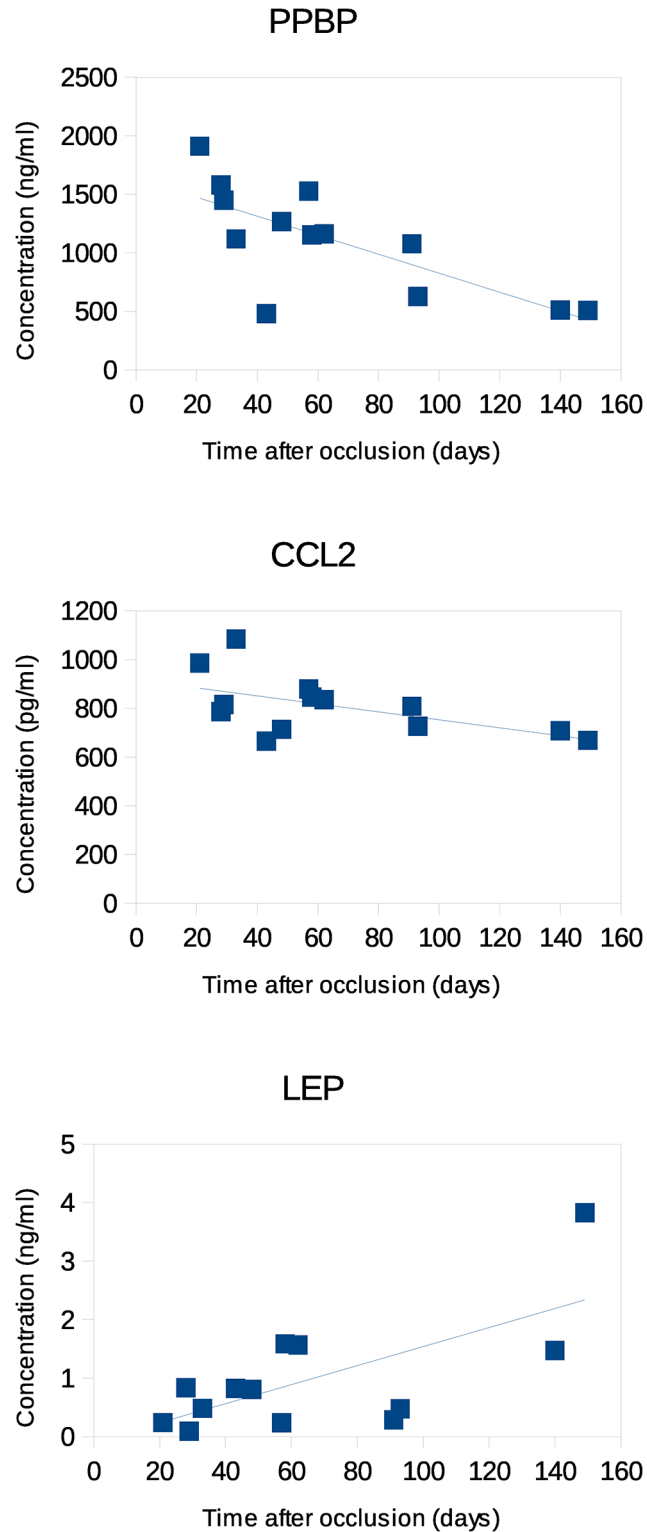


Fig 1. Correlation of the vitreal concentration of PPBP, CCL2, and LEP with the time after occlusion (time between onset of symptoms due to CRVO and surgery). Each data point represents a pair of data from an individual patient. Note that the significance of LEP is lost if the highest value is omitted.

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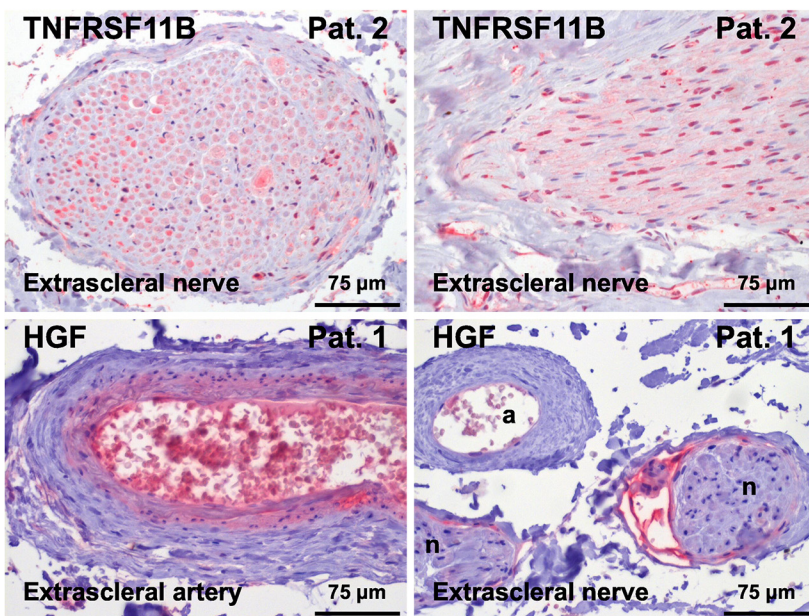
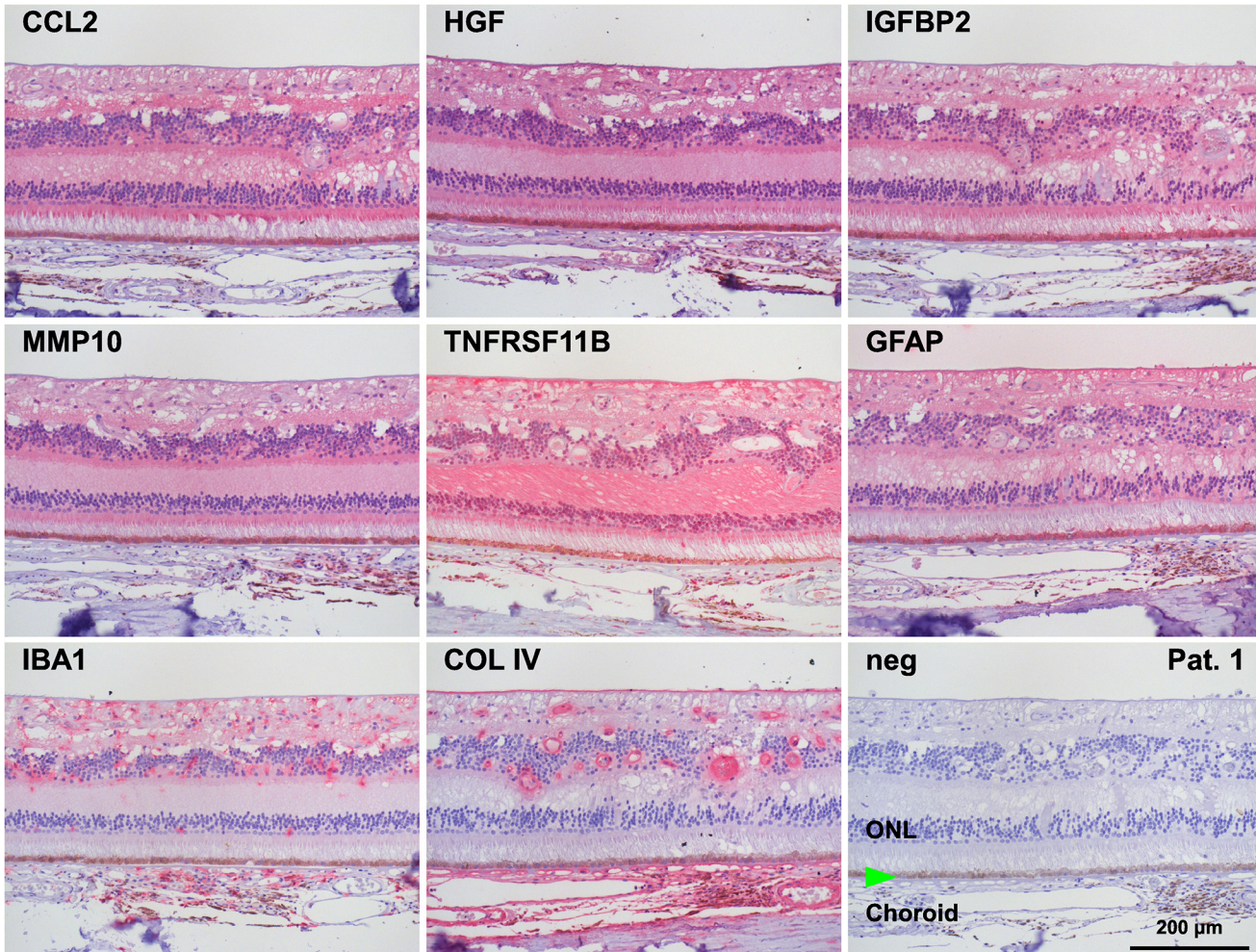


Fig 2. Immunohistochemical staining (alkaline phosphatase, red; blue counter staining: hematoxylin) for CCL2, HGF, IGFBP2, MMP10, and TNFRSF11B in ocular samples of various patients. These factors were found preferentially in the retina and additionally in nerves and in the RPE. TNFRSF11B was found in the axons and some nuclei of extrascleral nerves. HGF was additionally found in the endothelium and media of some but not all extrascleral vessels. GFAP (glia cell marker), IBA1 (microglia and macrophage marker, and COL IV (basement membrane marker, e.g. in the basement membrane of vessels and in the membrana limitans interna) are shown for comparison. Note that GFAP is not expressed in the outer segments of the photoreceptors as is the case for CCL2, HGF, IGFBP2, MMP10, and TNFRSF11B. neg: negative control without primary antibody, a: artery, n: nerve, ONL: outer nuclear layer, green arrow: RPE.

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The transcription factor NF κ B is upregulated by hypoxia. It has a central role in inflammation as it induces IFN γ [30] and IL6 [31]. Both were detected in the vitreous of CRVO patients and significantly increased compared to controls. In atherosclerosis, CCL2 (also named MCP-1) is involved in initial steps of inflammation by attracting monocytes, T-cells and dendritic cells [32,33]. These data strongly support the notion that CRVO is inducing an inflammatory response in the vitreous.

VEGFA, HGF, MMP3 and MMP9 share common ETS1 binding sites in their promoter region [34–36]. The transcription factor ETS1 is expressed in endothelial cells and upregulates genes involved in angiogenesis. ETS1 itself is induced by angiogenic factors like VEGF, HGF, or FGF2 resulting in a positive feed-back loop [37,38]. Moreover, expression of ETS1 is induced by HIF1A [39] linking angiogenesis to hypoxia in addition to the up-regulation of VEGFA by HIF. The metalloprotease MMP10 is induced by the transcription factor MEF2 in response to VEGFA [40,41]. This indicates that significant vitreal levels of angiogenic signaling factors are present in ischemic CRVO before neovascular changes are clinically apparent. In addition, these findings point towards a set of angiogenic target proteins including HGF and selected MMPs which may be amenable to pharmacological intervention.

HGF was found to be increased in vitreous samples of patients with proliferative diabetic retinopathy and was higher in vitreous than in blood similar to our results [42]. The intraocular expression of CCL2, HGF, IGFBP2, MMP10, and TNFRSF11B was confirmed by immunohistochemistry in eyes of patients with secondary glaucoma after RVO. This validates some of our earlier results and provides strong evidence that these proteins are expressed in ocular tissues. For most of them, expression within the eye has been reported earlier: CCL2, HGF [43,44], IGFBP2, MMP10, TNFRSF11B, and VEGFA [45] demonstrating that at least one cell type in the eye can produce these proteins under certain conditions.

Current ocular treatment is focused on anti-VEGF agents and anti-inflammatory steroids. Our data may add therapeutic targets to improve current anti-VEGF therapy in ischemic CRVO. Further investigation in the factors associated with hypoxia, inflammation and angiogenesis in ischemic CRVO may also lead to new therapeutic approaches to prevent conversion from non-ischemic to ischemic CRVO.

We also asked, whether our limited sample reflects known risk factors for retinal vein occlusion such as metabolic syndrome (diabetes, hypertension, hyperlipidemia (> 1 factor)), atherosclerosis of central retinal artery, history of stroke, and peripheral artery disease. More CRVO patients presented with one or more risk factors compared to controls (most pronounced differences in hyperlipidemia, smoking and use of anticoagulation). This is in line with previous findings: Analysis of plasma levels of atherosclerotic and thrombophilic risk factors demonstrated that arterial hypertension, hypercholesterolemia, hyperhomocysteinemia, elevated factor VIII were associated with an increased risk for ischemic versus non ischemic CRVO [15]. Our findings stress the need for careful work-up of ischemic CRVO patients to detect risk factors and adequately treat all the patient's diseases.

Pathophysiology of CRVO is not yet completely clear, but it is agreed that atherosclerotic changes of the retinal arteries contribute to the disease [46]. With regard to the vitreal proteins

detected in CRVO patients, TNFRSF11B is a marker of atherosclerosis [47], though its pathophysiological role is yet unclear [48]. Similarly, serum concentration of TIMP4 is increased in systemic sclerosis [49]. TIMP4 is the major MMP inhibitor in platelets and is released upon platelet aggregation induced by collagen and thrombin [50,51]. MMP10 is upregulated by thrombin in endothelial cells and enhances fibrinolysis [52,53]. PPBP is expressed upon platelet activation during thrombus formation [54]. PPBP expression is induced by MMP3 [55]. Both MMP9 and CCL2 are associated with atherosclerosis [56] where CCL2 attracts monocytes that mature into macrophages and produce MMP9. This cleaves components of the extracellular matrix within the atherosclerotic plaques. Thus, several of the vitreal proteins we detected are consistent with an atherosclerotic phenotype. Since data on the vitreal protein expression patterns preceding the retinal vein occlusion are not available, it remains challenging to dissect which vitreal proteins reflect an underlying chronic condition rather than an acute occlusion response.

In summary, ischemic CRVO is characterized by increased vitreal levels of a distinct set of proteins, some of them locally expressed, which may serve as targets for novel therapeutic approaches to augment current anti-inflammatory and anti-angiogenic treatments.

Supporting Information

S1 Table. Factors tested. V / B = vitreous / blood. CRVO = central retinal vein occlusion. Corr. = correlation. (DOCX)

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Author Contributions

Conceived and designed the experiments: LLH GM HTA NF AP. Performed the experiments: CE BG DM GM AS. Analyzed the data: CE BG DM BJ AS GS LLH GM HTA AP. Contributed reagents/materials/analysis tools: BJ NF AP. Wrote the paper: CE AS GS NF GM LLH HTA AP.

References

1. Cugati S, Wang JJ, Rochtchina E, Mitchell P. Ten-year incidence of retinal vein occlusion in an older population: the Blue Mountains Eye Study. *Arch Ophthalmol*. 2006; 124: 726–732. doi: [10.1001/archophth.124.5.726](https://doi.org/10.1001/archophth.124.5.726) PMID: [16682596](https://pubmed.ncbi.nlm.nih.gov/16682596/)
2. Rogers S, McIntosh RL, Cheung N, Lim L, Wang JJ, Mitchell P, et al. The prevalence of retinal vein occlusion: pooled data from population studies from the United States, Europe, Asia, and Australia. *Ophthalmology*. 2010; 117: 313–319.e1. doi: [10.1016/j.ophtha.2009.07.017](https://doi.org/10.1016/j.ophtha.2009.07.017) PMID: [20022117](https://pubmed.ncbi.nlm.nih.gov/20022117/)
3. Pielon A, Feltgen N, Isserstedt C, Callizo J, Junker B, Schmucker C. Efficacy and safety of intravitreal therapy in macular edema due to branch and central retinal vein occlusion: a systematic review. *PLoS One*. 2013; 8: e78538. doi: [10.1371/journal.pone.0078538](https://doi.org/10.1371/journal.pone.0078538) PMID: [24205253](https://pubmed.ncbi.nlm.nih.gov/24205253/)
4. Campochiaro PA, Brown DM, Awh CC, Lee SY, Gray S, Saroj N, et al. Sustained benefits from ranibizumab for macular edema following central retinal vein occlusion: twelve-month outcomes of a phase III study. *Ophthalmology*. 2011; 118: 2041–2049. doi: [10.1016/j.ophtha.2011.02.038](https://doi.org/10.1016/j.ophtha.2011.02.038) PMID: [21715011](https://pubmed.ncbi.nlm.nih.gov/21715011/)
5. Brown DM, Heier JS, Clark WL, Boyer DS, Vittori R, Berliner AJ, et al. Intravitreal aflibercept injection for macular edema secondary to central retinal vein occlusion: 1-year results from the phase 3 COPERNICUS study. *Am J Ophthalmol*. 2013; 155: 429–437.e7. doi: [10.1016/j.ajo.2012.09.026](https://doi.org/10.1016/j.ajo.2012.09.026) PMID: [23218699](https://pubmed.ncbi.nlm.nih.gov/23218699/)
6. Epstein DL, Algvere PV, von Wendt G, Seregard S, Kvanta A. Benefit from bevacizumab for macular edema in central retinal vein occlusion: twelve-month results of a prospective, randomized study. *Ophthalmology*. 2012; 119: 2587–2591. doi: [10.1016/j.ophtha.2012.06.037](https://doi.org/10.1016/j.ophtha.2012.06.037) PMID: [22902212](https://pubmed.ncbi.nlm.nih.gov/22902212/)

7. Stem MS, Talwar N, Comer GM, Stein JD. A longitudinal analysis of risk factors associated with central retinal vein occlusion. *Ophthalmology*. 2013; 120: 362–370. doi: [10.1016/j.ophtha.2012.07.080](https://doi.org/10.1016/j.ophtha.2012.07.080) PMID: [23177364](https://pubmed.ncbi.nlm.nih.gov/23177364/)
8. Prisco D, Marcucci R, Bertini L, Gori AM. Cardiovascular and thrombophilic risk factors for central retinal vein occlusion. *Eur J Intern Med*. 2002; 13: 163–169. PMID: [12020623](https://pubmed.ncbi.nlm.nih.gov/12020623/)
9. Mitchell P, Smith W, Chang A. Prevalence and associations of retinal vein occlusion in Australia. The Blue Mountains Eye Study. *Arch Ophthalmol*. 1996; 114: 1243–1247. PMID: [8859084](https://pubmed.ncbi.nlm.nih.gov/8859084/)
10. Zhou JQ, Xu L, Wang S, Wang YX, You QS, Tu Y, et al. The 10-year incidence and risk factors of retinal vein occlusion: the Beijing eye study. *Ophthalmology*. 2013; 120: 803–808. doi: [10.1016/j.ophtha.2012.09.033](https://doi.org/10.1016/j.ophtha.2012.09.033) PMID: [23352194](https://pubmed.ncbi.nlm.nih.gov/23352194/)
11. Ehlken C, Rennel ES, Michels D, Grundel B, Pielen A, Junker B, et al. Levels of VEGF but not VEGF (165b) are increased in the vitreous of patients with retinal vein occlusion. *Am J Ophthalmol*. 2011; 152: 298–303.e1. doi: [10.1016/j.ajo.2011.01.040](https://doi.org/10.1016/j.ajo.2011.01.040) PMID: [21621189](https://pubmed.ncbi.nlm.nih.gov/21621189/)
12. Yoshimura T, Sonoda K, Sugahara M, Mochizuki Y, Enaida H, Oshima Y, et al. Comprehensive analysis of inflammatory immune mediators in vitreoretinal diseases. *PLoS One*. 2009; 4: e8158. doi: [10.1371/journal.pone.0008158](https://doi.org/10.1371/journal.pone.0008158) PMID: [19997642](https://pubmed.ncbi.nlm.nih.gov/19997642/)
13. Suzuki Y, Nakazawa M, Suzuki K, Yamazaki H, Miyagawa Y. Expression profiles of cytokines and chemokines in vitreous fluid in diabetic retinopathy and central retinal vein occlusion. *Jpn J Ophthalmol*. 2011; 55: 256–263. doi: [10.1007/s10384-011-0004-8](https://doi.org/10.1007/s10384-011-0004-8) PMID: [21538000](https://pubmed.ncbi.nlm.nih.gov/21538000/)
14. Noma H, Mimura T, Masahara H, Shimada K. Pentraxin 3 and other inflammatory factors in central retinal vein occlusion and macular edema. *Retina Phila Pa*. 2014; 34: 352–359. doi: [10.1097/IAE.0b013e3182993d74](https://doi.org/10.1097/IAE.0b013e3182993d74) PMID: [23842103](https://pubmed.ncbi.nlm.nih.gov/23842103/)
15. Sodi A, Giambene B, Marcucci R, Sofi F, Fedi S, Abbate R, et al. Atherosclerotic and thrombophilic risk factors in patients with ischemic central retinal vein occlusion. *Retina Phila Pa*. 2011; 31: 724–729. doi: [10.1097/IAE.0b013e3181eef419](https://doi.org/10.1097/IAE.0b013e3181eef419) PMID: [21178660](https://pubmed.ncbi.nlm.nih.gov/21178660/)
16. Hasselbach HC, Ruefer F, Feltgen N, Schneider U, Bopp S, Hansen LL, et al. Treatment of central retinal vein occlusion by radial optic neurotomy in 107 cases. *Graefes Arch Clin Exp Ophthalmol Albrecht Von Graefes Arch Für Klin Exp Ophthalmol*. 2007; 245: 1145–1156. doi: [10.1007/s00417-006-0501-5](https://doi.org/10.1007/s00417-006-0501-5)
17. Hayreh SS. Ocular vascular occlusive disorders: natural history of visual outcome. *Prog Retin Eye Res*. 2014; 41: 1–25. doi: [10.1016/j.preteyeres.2014.04.001](https://doi.org/10.1016/j.preteyeres.2014.04.001) PMID: [24769221](https://pubmed.ncbi.nlm.nih.gov/24769221/)
18. Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, et al. TM4: a free, open-source system for microarray data management and analysis. *BioTechniques*. 2003; 34: 374–378. PMID: [12613259](https://pubmed.ncbi.nlm.nih.gov/12613259/)
19. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72: 248–254. PMID: [942051](https://pubmed.ncbi.nlm.nih.gov/942051/)
20. Koss MJ, Pfister M, Rothweiler F, Michaelis M, Cinatl J, Schubert R, et al. Comparison of cytokine levels from undiluted vitreous of untreated patients with retinal vein occlusion. *Acta Ophthalmol (Copenh)*. 2012; 90: e98–e103. doi: [10.1111/j.1755-3768.2011.02292.x](https://doi.org/10.1111/j.1755-3768.2011.02292.x) PMID: [22066978](https://pubmed.ncbi.nlm.nih.gov/22066978/)
21. Noma H, Funatsu H, Mimura T, Eguchi S. Vitreous inflammatory factors and serous retinal detachment in central retinal vein occlusion: a case control series. *J Inflamm Lond Engl*. 2011; 8: 38. doi: [10.1186/1476-9255-8-38](https://doi.org/10.1186/1476-9255-8-38) PMID: [22152024](https://pubmed.ncbi.nlm.nih.gov/22152024/)
22. Koss M, Pfister M, Rothweiler F, Rejdak R, Ribeiro R, Cinatl J, et al. Correlation from undiluted vitreous cytokines of untreated central retinal vein occlusion with spectral domain optical coherence tomography. *Open Ophthalmol J*. 2013; 7: 11–17. doi: [10.2174/1874364101307010011](https://doi.org/10.2174/1874364101307010011) PMID: [23560031](https://pubmed.ncbi.nlm.nih.gov/23560031/)
23. Noma H, Funatsu H, Mimura T, Harino S, Hori S. Aqueous humor levels of vasoactive molecules correlate with vitreous levels and macular edema in central retinal vein occlusion. *Eur J Ophthalmol*. 2010; 20: 402–409. PMID: [19967679](https://pubmed.ncbi.nlm.nih.gov/19967679/)
24. Wenger RH, Stiehl DP, Camenisch G. Integration of oxygen signaling at the consensus HRE. *Sci STKE Signal Transduct Knowl Environ*. 2005; 2005: re12. doi: [10.1126/stke.3062005re12](https://doi.org/10.1126/stke.3062005re12) PMID: [16234508](https://pubmed.ncbi.nlm.nih.gov/16234508/)
25. Mojsilovic-Petrovic J, Callaghan D, Cui H, Dean C, Stanimirovic DB, Zhang W. Hypoxia-inducible factor-1 (HIF-1) is involved in the regulation of hypoxia-stimulated expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) and MCP-5 (Ccl12) in astrocytes. *J Neuroinflammation*. 2007; 4: 12. doi: [10.1186/1742-2094-4-12](https://doi.org/10.1186/1742-2094-4-12) PMID: [17474992](https://pubmed.ncbi.nlm.nih.gov/17474992/)
26. Natarajan R, Salloum FN, Fisher BJ, Kukreja RC, Fowler AA 3rd. Hypoxia inducible factor-1 upregulates adiponectin in diabetic mouse hearts and attenuates post-ischemic injury. *J Cardiovasc Pharmacol*. 2008; 51: 178–187. doi: [10.1097/FJC.0b013e31815f248d](https://doi.org/10.1097/FJC.0b013e31815f248d) PMID: [18287886](https://pubmed.ncbi.nlm.nih.gov/18287886/)
27. Simon M-P, Tournaire R, Pouyssegur J. The angiopoietin-2 gene of endothelial cells is up-regulated in hypoxia by a HIF binding site located in its first intron and by the central factors GATA-2 and Ets-1. *J Cell Physiol*. 2008; 217: 809–818. doi: [10.1002/jcp.21558](https://doi.org/10.1002/jcp.21558) PMID: [18720385](https://pubmed.ncbi.nlm.nih.gov/18720385/)

28. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*. 2003; 3: 721–732. doi: [10.1038/nrc1187](https://doi.org/10.1038/nrc1187) PMID: [13130303](https://pubmed.ncbi.nlm.nih.gov/13130303/)
29. Benita Y, Kikuchi H, Smith AD, Zhang MQ, Chung DC, Xavier RJ. An integrative genomics approach identifies Hypoxia Inducible Factor-1 (HIF-1)-target genes that form the core response to hypoxia. *Nucleic Acids Res*. 2009; 37: 4587–4602. doi: [10.1093/nar/gkp425](https://doi.org/10.1093/nar/gkp425) PMID: [19491311](https://pubmed.ncbi.nlm.nih.gov/19491311/)
30. Balasubramani A, Mukasa R, Hatton RD, Weaver CT. Regulation of the *lfn* locus in the context of T-lineage specification and plasticity. *Immunol Rev*. 2010; 238: 216–232. doi: [10.1111/j.1600-065X.2010.00961.x](https://doi.org/10.1111/j.1600-065X.2010.00961.x) PMID: [20969595](https://pubmed.ncbi.nlm.nih.gov/20969595/)
31. Cummins EP, Taylor CT. Hypoxia-responsive transcription factors. *Pflüg Arch Eur J Physiol*. 2005; 450: 363–371. doi: [10.1007/s00424-005-1413-7](https://doi.org/10.1007/s00424-005-1413-7)
32. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2^{-/-} mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature*. 1998; 394: 894–897. doi: [10.1038/29788](https://doi.org/10.1038/29788) PMID: [9732872](https://pubmed.ncbi.nlm.nih.gov/9732872/)
33. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, et al. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell*. 1998; 2: 275–281. PMID: [9734366](https://pubmed.ncbi.nlm.nih.gov/9734366/)
34. Ghosh S, Basu M, Roy SS. ETS-1 protein regulates vascular endothelial growth factor-induced matrix metalloproteinase-9 and matrix metalloproteinase-13 expression in human ovarian carcinoma cell line SKOV-3. *J Biol Chem*. 2012; 287: 15001–15015. doi: [10.1074/jbc.M111.284034](https://doi.org/10.1074/jbc.M111.284034) PMID: [22270366](https://pubmed.ncbi.nlm.nih.gov/22270366/)
35. Hashiya N, Jo N, Aoki M, Matsumoto K, Nakamura T, Sato Y, et al. In vivo evidence of angiogenesis induced by transcription factor Ets-1: Ets-1 is located upstream of angiogenesis cascade. *Circulation*. 2004; 109: 3035–3041. doi: [10.1161/01.CIR.0000130643.41587.DB](https://doi.org/10.1161/01.CIR.0000130643.41587.DB) PMID: [15173033](https://pubmed.ncbi.nlm.nih.gov/15173033/)
36. Ishida S, Usui T, Yamashiro K, Kaji Y, Amano S, Ogura Y, et al. VEGF164-mediated inflammation is required for pathological, but not physiological, ischemia-induced retinal neovascularization. *J Exp Med*. 2003; 198: 483–489. doi: [10.1084/jem.20022027](https://doi.org/10.1084/jem.20022027) PMID: [12900522](https://pubmed.ncbi.nlm.nih.gov/12900522/)
37. Sato Y, Teruyama K, Nakano T, Oda N, Abe M, Tanaka K, et al. Role of transcription factors in angiogenesis: Ets-1 promotes angiogenesis as well as endothelial apoptosis. *Ann N Y Acad Sci*. 2001; 947: 117–123. PMID: [11795259](https://pubmed.ncbi.nlm.nih.gov/11795259/)
38. Paumelle R, Tulasne D, Kherrouche Z, Plaza S, Leroy C, Reveneau S, et al. Hepatocyte growth factor/scatter factor activates the ETS1 transcription factor by a RAS-RAF-MEK-ERK signaling pathway. *Oncogene*. 2002; 21: 2309–2319. doi: [10.1038/sj.onc.1205297](https://doi.org/10.1038/sj.onc.1205297) PMID: [11948414](https://pubmed.ncbi.nlm.nih.gov/11948414/)
39. Oikawa M, Abe M, Kurosawa H, Hida W, Shirato K, Sato Y. Hypoxia induces transcription factor ETS-1 via the activity of hypoxia-inducible factor-1. *Biochem Biophys Res Commun*. 2001; 289: 39–43. doi: [10.1006/bbrc.2001.5927](https://doi.org/10.1006/bbrc.2001.5927) PMID: [11708773](https://pubmed.ncbi.nlm.nih.gov/11708773/)
40. Chang S, Young BD, Li S, Qi X, Richardson JA, Olson EN. Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. *Cell*. 2006; 126: 321–334. doi: [10.1016/j.cell.2006.05.040](https://doi.org/10.1016/j.cell.2006.05.040) PMID: [16873063](https://pubmed.ncbi.nlm.nih.gov/16873063/)
41. Ha CH, Jhun BS, Kao H-Y, Jin Z-G. VEGF stimulates HDAC7 phosphorylation and cytoplasmic accumulation modulating matrix metalloproteinase expression and angiogenesis. *Arterioscler Thromb Vasc Biol*. 2008; 28: 1782–1788. doi: [10.1161/ATVBAHA.108.172528](https://doi.org/10.1161/ATVBAHA.108.172528) PMID: [18617643](https://pubmed.ncbi.nlm.nih.gov/18617643/)
42. Cantón A, Burgos R, Hernández C, Mateo C, Segura RM, Mesa J, et al. Hepatocyte growth factor in vitreous and serum from patients with proliferative diabetic retinopathy. *Br J Ophthalmol*. 2000; 84: 732–735. PMID: [10873984](https://pubmed.ncbi.nlm.nih.gov/10873984/)
43. Cui JZ, Chiu A, Maberley D, Ma P, Samad A, Matsubara JA. Stage specificity of novel growth factor expression during development of proliferative vitreoretinopathy. *Eye Lond Engl*. 2007; 21: 200–208. doi: [10.1038/sj.eye.6702169](https://doi.org/10.1038/sj.eye.6702169)
44. Sun W, Funakoshi H, Nakamura T. Differential expression of hepatocyte growth factor and its receptor, c-Met in the rat retina during development. *Brain Res*. 1999; 851: 46–53. PMID: [10642827](https://pubmed.ncbi.nlm.nih.gov/10642827/)
45. Kinnunen K, Puustjärvi T, Teräsvirta M, Nurmenniemi P, Heikura T, Laidinen S, et al. Differences in retinal neovascular tissue and vitreous humour in patients with type 1 and type 2 diabetes. *Br J Ophthalmol*. 2009; 93: 1109–1115. doi: [10.1136/bjo.2008.148841](https://doi.org/10.1136/bjo.2008.148841) PMID: [19304585](https://pubmed.ncbi.nlm.nih.gov/19304585/)
46. Seitz R. Die Netzhautgefäße: vergleichende ophthalmoskopische und histologische Studien an gesunden und kranken Augen. Stuttgart: Enke; 1962.
47. Augoulea A, Vrachnis N, Lambrinoukaki I, Dafopoulos K, Iliodromiti Z, Daniilidis A, et al. Osteoprotegerin as a marker of atherosclerosis in diabetic patients. *Int J Endocrinol*. 2013; 2013: 182060. doi: [10.1155/2013/182060](https://doi.org/10.1155/2013/182060) PMID: [23401681](https://pubmed.ncbi.nlm.nih.gov/23401681/)
48. Van Campenhout A, Golledge J. Osteoprotegerin, vascular calcification and atherosclerosis. *Atherosclerosis*. 2009; 204: 321–329. doi: [10.1016/j.atherosclerosis.2008.09.033](https://doi.org/10.1016/j.atherosclerosis.2008.09.033) PMID: [19007931](https://pubmed.ncbi.nlm.nih.gov/19007931/)

49. Elias GJ, Ioannis M, Theodora P, Dimitrios PP, Despoina P, Kostantinos V, et al. Circulating tissue inhibitor of matrix metalloproteinase-4 (TIMP-4) in systemic sclerosis patients with elevated pulmonary arterial pressure. *Mediators Inflamm*. 2008; 2008: 164134. doi: [10.1155/2008/164134](https://doi.org/10.1155/2008/164134) PMID: [19190762](https://pubmed.ncbi.nlm.nih.gov/19190762/)
50. Radomski A, Jurasz P, Sanders EJ, Overall CM, Bigg HF, Edwards DR, et al. Identification, regulation and role of tissue inhibitor of metalloproteinases-4 (TIMP-4) in human platelets. *Br J Pharmacol*. 2002; 137: 1330–1338. doi: [10.1038/sj.bjp.0704936](https://doi.org/10.1038/sj.bjp.0704936) PMID: [12466243](https://pubmed.ncbi.nlm.nih.gov/12466243/)
51. Santos-Martínez MJ, Medina C, Jurasz P, Radomski MW. Role of metalloproteinases in platelet function. *Thromb Res*. 2008; 121: 535–542. doi: [10.1016/j.thromres.2007.06.002](https://doi.org/10.1016/j.thromres.2007.06.002) PMID: [17681591](https://pubmed.ncbi.nlm.nih.gov/17681591/)
52. Orbe J, Rodríguez JA, Calvayrac O, Rodríguez-Calvo R, Rodríguez C, Roncal C, et al. Matrix metalloproteinase-10 is upregulated by thrombin in endothelial cells and increased in patients with enhanced thrombin generation. *Arterioscler Thromb Vasc Biol*. 2009; 29: 2109–2116. doi: [10.1161/ATVBAHA.109.194589](https://doi.org/10.1161/ATVBAHA.109.194589) PMID: [19762781](https://pubmed.ncbi.nlm.nih.gov/19762781/)
53. Orbe J, Barrenetxe J, Rodríguez JA, Vivien D, Orset C, Parks WC, et al. Matrix metalloproteinase-10 effectively reduces infarct size in experimental stroke by enhancing fibrinolysis via a thrombin-activatable fibrinolysis inhibitor-mediated mechanism. *Circulation*. 2011; 124: 2909–2919. doi: [10.1161/CIRCULATIONAHA.111.047100](https://doi.org/10.1161/CIRCULATIONAHA.111.047100) PMID: [22104553](https://pubmed.ncbi.nlm.nih.gov/22104553/)
54. Brandt E, Petersen F, Ludwig A, Ehlert JE, Bock L, Flad HD. The beta-thromboglobulins and platelet factor 4: blood platelet-derived CXC chemokines with divergent roles in early neutrophil regulation. *J Leukoc Biol*. 2000; 67: 471–478. PMID: [10770278](https://pubmed.ncbi.nlm.nih.gov/10770278/)
55. Kruidenier L, MacDonald TT, Collins JE, Pender SLF, Sanderson IR. Myofibroblast matrix metalloproteinases activate the neutrophil chemoattractant CXCL7 from intestinal epithelial cells. *Gastroenterology*. 2006; 130: 127–136. doi: [10.1053/j.gastro.2005.09.032](https://doi.org/10.1053/j.gastro.2005.09.032) PMID: [16401476](https://pubmed.ncbi.nlm.nih.gov/16401476/)
56. Tan C, Liu Y, Li W, Deng F, Liu X, Wang X, et al. Associations of matrix metalloproteinase-9 and monocyte chemoattractant protein-1 concentrations with carotid atherosclerosis, based on measurements of plaque and intima-media thickness. *Atherosclerosis*. 2014; 232: 199–203. doi: [10.1016/j.atherosclerosis.2013.11.040](https://doi.org/10.1016/j.atherosclerosis.2013.11.040) PMID: [24401238](https://pubmed.ncbi.nlm.nih.gov/24401238/)