PLOS ONE

RESEARCH ARTICLE

Cytokine profiles in the aqueous humor and serum of patients with dry and treated wet age-related macular degeneration

Jan Spindler[1,2], Souska Zandi[1], Isabel B. Pfister[1,2], Christin Gerhardt[1,2], Justus G. Garweg[1,2]*

1 Swiss Eye Institute and Berner Augenklinik am Lindenhofspital, Bern, Switzerland, **2** University of Bern, Bern, Switzerland

* garweg@swiss-eye-institute.com

OPEN ACCESS

Citation: Spindler J, Zandi S, Pfister IB, Gerhardt C, Garweg JG (2018) Cytokine profiles in the aqueous humor and serum of patients with dry and treated wet age-related macular degeneration. PLoS ONE 13(8): e0203337. https://doi.org/10.1371/journal.pone.0203337

Editor: Monica M. Jablonski, University of Tennessee Health Science Center, UNITED STATES

Received: June 4, 2018

Accepted: August 17, 2018

Published: August 29, 2018

Data Availability Statement: All relevant data are within the paper.

Funding: The manuscript was partially funded by a grant from the Scientific Funds of the Lindenhof Foundation without commercial interests. There was no additional external funding received for this study. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have the following interests: JGG acts as an advisor for

Abstract

Purpose

To identify disease-specific cytokine profile differences in the aqueous humor (AH) (other than the vascular endothelial growth factor) between patients with dry and treated wet age-related macular degeneration (AMD) and healthy controls.

Methods

This retrospective study drew on a case-series of patients diagnosed with dry AMD ($n = 25$) and treated wet AMD ($n = 19$), as well as on healthy controls (no systemic therapy; $n = 20$) undergoing phacoemulsification or vitrectomy. Samples of AH and serum were collected in parallel at the beginning of surgery. The levels of 43 cytokines were simultaneously determined using the Bio-Plex® multiplex beads system. Differences between the three groups were statistically compared using the Kruskal-Wallis H-Test after applying the Bonferroni correction for multiple comparisons ($p<0.0012$).

Results

The concentrations of three cytokines were elevated in the AH of patients with dry AMD (CXCL6; $p = 0.00067$) and treated wet AMD (CXCL5, CXCL6, MIG/XCXL; all $p<0.001$) relative to those in the healthy controls. No other differences between the three groups were identified. The AH levels of seven cytokines (16%), including CXCL6, ranged below the lower limit of quantitation of the assay. Without the correction for multiple comparisons ($p<0.05$), the levels of 31 of the 43 cytokines in the AH of patients with AMD would have differed significantly from those in the control. The systemic cytokine profiles (serum) were similar in all three groups.

Conclusions

No systematic differences in the AH cytokine environment were identified between patients with dry AMD and those with treated wet AMD. This finding might indicate that AMD is either the result of a persistent imbalance in the physiological tissue milieu, or that the localized

several pharmaceutical companies (Novartis, Bayer, Allergan, Alcon, AbbVie) and contributes to a number of industry-sponsored international multicenter studies. There are no patents, products in development or marketed products to declare. This does not alter our adherence to all the PLOS ONE policies on sharing data and materials.

process induces no significant change in the cytokine environment of the anterior ocular segment.

Introduction

Age-related macular degeneration (AMD), particularly advanced stages of the disease, such as choroidal neovascularization (CNV) and geographic atrophy (GA), is the most common cause of irreversible vision loss in elderly individuals [1, 2, 3].

In clinical terms, the distinction between "wet" and "dry" forms of the disease is based upon the manifestation of either CNV or atrophy of the retinal pigment epithelium (RPE), the choriocapillaris and the overlying photoreceptors, respectively [4, 5]. Despite its convenience, this dichotomization does not reflect the continuum of the underlying progressive pathology of the macula with advanced age. Whereas wet AMD can be impeded (to a certain degree) by intravitreal treatment with the anti-vascular endothelial growth factor (anti-VEGF), the dry AMD component of the disease cannot be therapeutically influenced which indeed seems to be somewhat clinically distinct [1, 4, 6, 7]. However, the underlying reason for the switch towards either the atrophic, dry or the neovascular, wet form of the disease is not fully understood [8]. Since wet AMD is typically preceded by more or less prominent changes that are attributed to dry AMD, its presence could be considered as a risk factor [7]. Furthermore, advanced manifestations of both dry and wet AMD may coexist in the same eye, which argues in favor of a continuous rather than a dichotomatous process [7, 9]. Correspondingly, there exist significant overlaps in the mechanisms that underlie these seemingly disparate clinical conditions [4, 10], which is not a surprising finding for such a multifactorial disease [2] that is influenced by aging, oxidative stress, mitochondrial dysfunction, environmental factors and chronic, age-related low-grade inflammation [1]. Close correlations between AMD and various immunological/inflammatory gene polymorphisms have been reported, thereby suggesting the involvement of immune mediated processes (e.g., complement activation) and inflammation [2]. Changes in the cytokine and chemokine concentrations at both the local and the systemic levels, predominantly in patients with wet AMD, have been also documented [1, 11–17]. These cytokines appear to play an integral role in the initiation, perpetuation or subsequent down-regulation of the immune response, eventually leading to wound healing by the formation of a fibrotic scar [18, 19]. Correspondingly, histological investigations in eyes with early AMD (e.g., drusen) have revealed chronic inflammation at the RPE/choroidal interface [20]. Furthermore immunocompetent cells, such as lymphocytes and macrophages [3, 21, 22], have been observed in chorioretinal tissue that had been derived from eyes with wet AMD [2]. Generally, the molecular mechanisms that underline the development and progression of CNV, the hallmark of wet AMD, are better understood than those that are involved in the evolution of the dry form of the disease [23, 24].

It has been postulated that in the aging eye, the dysregulation of reparative (para-)inflammatory mechanisms, particularly the down-regulation of pro-inflammatory cytokines and the up-regulation of anti-inflammatory cytokines by the RPE [e.g., in response to stimulation by the deposition of advanced glycation end products (AGEs)] [25], might induce and perpetuate the low-grade chronic inflammatory process that contributes to the progression of AMD [1]. However, whether these factors are the cause or the effect of the low-grade inflammation that is associated with the progression of AMD remains to be determined [2, 6, 26].

In an attempt to understand the pathological process, changes in many different cytokines compared to healthy controls have been reported [6, 27]. Since the role of the abundancy of a

single agent in the pathogenesis of AMD is difficult to estimate, we monitored and compared cytokine environmental changes by a maximally broad panel of 43 inflammatory and pro-fibrotic biomarkers in the aqueous humor (AH) and sera of patients with dry and treated wet AMD and in healthy controls. By implementing the Bonferroni correction for multiple comparisons, we endeavored to identify the most relevant beyond all significant intergroup changes in this context.

Patients and methods

Patients

This retrospective case series included patients with either dry or treated wet AMD or healthy controls without any relevant systemic or ocular disease (apart from senile cataract or macular hole (MH)), who were scheduled for phacoemulsification surgery and/or vitrectomy. Clinical data regarding ophthalmologic and systemic diagnoses and findings, systemic and local medications, as well as duration of the ocular symptoms (e.g., visual distortion, if manifested) were collected. For the purpose of this study, the preoperative Snellen's best-corrected visual acuity (BCVA) was converted into Early Treatment Diabetic Retinopathy Study (ETDRS)-letter scores (with 85 letters representing a BCVA of 1.0). Samples of blood serum and AH were collected at the beginning of ocular surgery at the Berner Augenklinik am Lindenhofspital, between August 2013 and January 2016. The grading of macular changes was based upon clinical findings and OCT diagnostics in dependence on the Clinical Age-Related Maculopathy Staging System (CARMS) [28]. The following stages were distinguished: healthy controls (no chorioretinal changes), dry AMD ($\geq$15 intermediate drusen or any large drusen, no intra- or sub-retinal fluid or hemorrhages), and treated wet AMD (signs of exudative AMD, such as serous retinal detachments, non-drusenoid RPE detachments, CNV with sub-RPE or subretinal exudations or fibrosis prior to the onset of anti-VEGF therapy, or the presence of scars consistent with AMD-treatment) [28].

Exclusion criteria included a history of systemic malignant, vascular or inflammatory comorbidities, namely, diabetes mellitus or rheumatic diseases; a history of any previous intraocular surgery or ocular trauma in the affected eye or of intraocular inflammation; the presence or history of vitreal/(sub-)retinal hemorrhage; any ocular vascular occlusive disease; or myopia of more than 6 diopters.

The informed and written consent of all individuals concerned was obtained, in strict accordance with the tenets of the Declaration of Helsinki. The present study was approved by the local Ethics Commission of the University of Bern in Switzerland (reference number: 152/08).

Collection of aqueous humor

Samples of aqueous humor were collected at the onset of phacoemulsification surgery. About 150 to 200 microliters of undiluted aqueous humor was obtained via aspiration through a 30-gauge needle. The samples were stored within 4 hours at -20˚C for maximally 2 months and thereafter at -80˚C until the time of the analysis.

Cytokine analysis

Within four hours of collection, the aliquots of AH and serum were frozen at -20˚C and stored at this temperature for up to two months, thereafter at -80˚C until the time of analysis, which was conducted simultaneously for all samples.

The Bio-Plex® multiplex immunoassay beads system (Bio-Plex 100 array reader and Bio-Plex Manager software, version 6.1, Bio-Rad, Hercules, CA, USA) was used to simultaneously quantify the concentrations of 43 cytokines and chemokines according to the manufacturer instructions, as previously described [18]. A concentration standard was run in parallel on each test plate. It represented the average of triplicate standard dilutions of each corresponding chemokine/cytokine. A standard curve was generated and the sample concentrations were determined by curve-fitting. The assays were performed in a blinded manner by an experienced technician [29].

Statistical analysis

Quantitative data are presented as mean values together with the standard deviation (SD). According to the standard curve, the lower limit of quantitation (LLOQ) of the assay working range was typically about 1 pg/ml (http://www.biorad.com). The concentrations of several cytokines ranged below the curve fit of the standards (out of range). To avoid a bias that would have been introduced by excluding these values, they were set at half of the lowest quantified level for the particular cytokine in question. Outliers were identified by a box-plot analysis (box-whisker plot). Extreme outliers (viz., values that lay 3 box-lengths beyond the box-edges) were excluded from the statistical analysis.

To ascertain whether or not the data were normally distributed the Shapiro-Wilk test was applied. Since the data did not meet the criteria of a normal distribution, the non-parametric Kruskal-Wallis H-Test was applied for the intergroup comparisons, using the level of statistical significance of $p \leq 0.05$. To counteract the Type I error that was attributable to the multiple comparisons, the Bonferroni correction was implemented to the level of significance, which resulted in a critical value for significance of $p < 0.0012$ [30]. The statistical analyses were performed using the open source software R (Version 3.3.2–2016 RStudio, Inc.; psych package) and SPSS (version 23.0; IBM SPSS Statistics, Armonk, NY, USA) [18, 29].

Results

Patients

The analysis included 64 eyes from 64 patients. They were allocated to one of three groups: healthy controls ($n = 20$); dry AMD ($n = 25$); treated wet AMD ($n = 19$; Table 1). The patients with dry AMD and those with treated wet AMD were of similar age ($p > 0.05$), whereas healthy controls were younger ($p = 0.0005$). The proportion of females was higher than that of males in each group (63.2% to 76.0%; chi-square test: $p = 0.65$).

The BCVAs of the healthy controls and of the patients with dry AMD differed from those of the individuals with treated wet AMD ($p = 0.002$ and $p = 0.02$, respectively). The central retinal thickness (CRT) and choroidal thickness were similar in all groups ($p > 0.10$ for all comparisons).

Table 1. Demographics: Patient characteristics and clinical data for the corresponding groups.

Baseline characteristics	Healthy controls		Dry AMD		Treated wet AMD	
Number of participants, *n* (%)	20	(31.2)	25	(39.1)	19	(29.7)
Number of females, *n* (%)	14	(70.0)	19	(76.0)	12	(63.2)
Age at sample collection, *y* mean (SD)	74.7	(5.6)	83.5	(6.9)	84.9	(5.1)
Best-corrected visual acuity, ETDRS-letter score, mean (SD)	68.2	(11.2)	61.8	(19.7)	46.9	(22.0)
Central retinal thickness, μm, mean (SD)	237.8	(30.3)	226.2	(46.3)	236.5	(63.9)
Choroidal thickness, μm, mean (SD)	172.5	(59.3)	142.4	(56.7)	147.8	(61.2)

https://doi.org/10.1371/journal.pone.0203337.t001

In patients with treated wet AMD, the mean time that elapsed between the last anti-VEGF injection and the collection of the samples was 14.9 ± 20.9 months. No differences in the cytokine levels were observed between patients who had received the last anti-VEGF injection within 6 months prior of the collection of the samples and those who had received the injection heretofore.

Cytokine analysis

The concentrations of the different cytokines in the AH (pg/ml) spanned a broad range (Tables 2 and 3).

After application of the Bonferroni correction, the AH-concentrations of most of the cytokines ($n = 40$) (in particular CCL21, CXCL13, CCL27, CCL11, CCL24, CCL26, CX3CL1, GM-CSF, CXCL1, CXCL2, CCL1, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8/CXCL8, IL-10, Il-16, CXCL10, CXCL11, CCL2, CCL8, CCL7, CCL13, CCL22, MIF, CCL3, CCL15, CCL20, CCL19, CCL23, CXCL16, CXCL12, CCL17, CCL25, TNF-α, TGF-β1, TGF-β2, and TGF-β3) were similar in all three groups [healthy controls, dry AMD and treated wet AMD ($p>0.0012$)].

Eyes with treated wet AMD exhibited the highest absolute AH-concentrations in 31 of the 43 analyzed cytokines (72%). In the dry AMD group, the AH-concentrations of CXCL5, CCL11, CCL24, GM-CSF, IL-4, CCL2, CCL13, MIF, CCL19, CCL17, TGF-β2, and TGF-β3 were higher than those in either the healthy controls or the eyes with treated wet AMD (Fig 1, Table 2).

The concentrations of three cytokines were 1.7 to 72.8-fold higher ($p<0.0012$) in patients with either dry AMD [CXCL6 ($p = 0.00067$)] and/or treated wet AMD [CXCL5 ($p = 0.00099$), CXCL6 ($p = 0.00067$), MIG/CXCL9 ($p = 0.00019$)] than in the healthy controls, with significant intergroup differences being registered after the application of the Bonferroni correction (Fig 2, Table 3).

No intergroup differences in the AH-concentrations of the cytokines were observed between eyes with dry AMD and those with treated wet AMD (all $p>0.01$).

CXCL6 and CCL7 were the only cytokines whose AH-concentrations increased with progression of age ($p = 0.0006439$ and $p = 0.0006956$, respectively; other cytokines: $p>0.0012$).

In no instance were any intergroup differences observed in the serum concentrations of the monitored cytokines [$p>0.0012$ (Tables 4 and 5)]. The cytokine concentrations were 1.2 to 5775.6-fold higher in the serum than in the corresponding AH samples with the following exceptions: CXCL5 (dry AMD only), GM-CSF, CCL2, TGF-β1 (healthy controls only), TGF-β2 and TGF-β3. No age-correlated changes in any of the cytokines were identified in the serum samples ($p>0.0012$).

Discussion

Our results revealed an upregulation of CXCL5, CXCL6 and MIG/CXCL9 ($p<0.0012$) in the AH of eyes with dry and stable treated wet AMD, when compared to healthy controls. These were the only cytokines whose up-regulation remained significant after the application of the Bonferroni correction for multiple comparison. Heretofore, an additional 31 cytokines had qualified for this designation when considering a level of significance of $p<0.05$. These findings indicate that an analysis of the level of a single cytokine in clinical samples may suffer from the weakness of attempting to detect and interpret a single point change in the complex pathomechanism of AMD. Furthermore, it is challenging to estimate modulations in the local cytokine environment/milieu at the lesion site by specimens taken from the anterior ocular compartment rather than from where the pathology actually takes place. It remains a matter of speculation whether or not the AH is indeed representative of the relatively small volume of

Table 2. Mean concentrations (pg/ml) and standard deviations (SD) of the 43 monitored cytokines in the aqueous humor of healthy controls and of patients with either dry or treated wet age-related macular degeneration (AMD).

Cytokine	Healthy controls		Dry AMD		Treated wet AMD		Assay Working Ranges	
	Mean (pg/ml)	SD	Mean (pg/ml)	SD	Mean (pg/ml)	SD	LLOQ (pg/ml)	ULOQ (pg/ml)
CCL21	976.6	931.2	1'617.3	1'100.1	2'014.8	1'627.1	21.9	3'923
CXCL13	0.6	1.9	0.3	0.5	0.9	1.8	0.7	1'200
CCL27	0.3	1.3	0.4	1.3	1.9	3.4	1.2	5'000
CXCL5	47.0	74.6	3'419.0	14'656.2	635.9	2'162.5	7.3	120'000
CCL11	5.8	4.4	8.9	8.9	8.2	3.9	1.5	3'859
CCL24	11.8	11.4	42.3	93.8	18.4	9.9	6.2	4'073
CCL26	4.1	4.6	7.7	6.7	10.9	10.2	0.9	12'109
CX3CL1	33.8	20.7	51.7	38.2	55.5	34.9	4.0	11'463
CXCL6	0.1	0.0	0.3	0.3	0.7	1.2	0.8	11'135
GM-CSF	49.2	25.9	93.7	87.1	67.8	33.7	5.3	35'000
CXCL1	28.4	21.1	45.8	35.7	48.3	16.5	3.1	7'024
CXCL2	1.8	1.0	3.1	2.9	3.7	2.5	4.6	13'257
CCL1	9.4	7.7	16.6	8.8	19.1	10.9	1.8	1'015
IFN-γ	3.0	3.4	7.7	7.7	8.7	7.3	2.3	20'236
IL-1β	0.5	0.4	0.8	0.6	1.1	0.7	0.4	7'000
IL-2	0.9	1.1	1.1	1.2	1.3	0.9	0.8	13'000
IL-4	0.6	1.4	15.4	58.7	6.3	16.2	1.2	4'804
IL-6	4.7	7.1	3.4	1.8	4.9	3.8	0.7	12'000
IL-8/CXCL8	3.6	2.9	4.5	2.5	6.1	3.0	0.5	7'640
IL-10	2.3	3.9	4.6	7.2	6.2	6.8	1.3	18'708
IL-16	4.3	5.7	9.2	9.8	14.3	10.7	2.1	34'000
CXCL10	41.0	40.9	58.9	72.2	122.0	112.2	1.6	7'714
CXCL11	1.3	2.0	2.6	2.7	4.5	4.6	0.1	2'298
CCL2	300.0	102.6	351.0	336.9	340.9	87.3	0.3	4'812
CCL8	2.3	2.2	4.5	4.7	5.7	3.5	0.3	4'056
CCL7	3.1	4.6	7.7	13.4	12.5	15.0	1.9	20'133
CCL13	0.5	0.4	1.8	3.6	1.2	0.8	0.2	3'368
CCL22	6.8	4.6	12.3	13.2	13.4	5.9	0.9	14'649
MIF	50'207.1	96'380.3	128'907.2	363'701.7	112'391.1	116'068.9	23.1	377'721
MIG/CXCL9	7.5	12.3	32.1	59.2	81.0	119.5	1.8	19'600
CCL3	0.8	0.7	1.3	0.9	1.6	1.1	0.4	1'543
CCL15	296.3	182.7	409.9	358.1	554.0	396.6	1.7	9'100
CCL20	2.9	3.1	3.2	4.4	4.6	5.7	0.3	4'675
CCL19	2.4	2.6	5.7	10.9	5.2	5.1	3.0	48'494
CCL23	5.8	5.1	13.0	12.0	13.5	10.2	1.0	14'450
CXCL16	490.7	198.4	459.1	266.2	539.9	221.7	0.5	2'867
CXCL12	82.0	60.1	107.9	107.9	162.6	148.0	8.3	115'730
CCL17	0.2	0.3	1.4	5.2	0.4	1.3	1.7	430
CCL25	117.2	153.3	243.5	444.7	260.2	196.6	20.6	114'493
TNF-α	5.0	3.3	9.5	7.0	10.6	6.5	0.9	13'879
TGF-β1	80.6	132.4	129.1	215.8	165.5	135.4	1.7	27'616
TGF-β2	1'288.5	738.0	1'761.9	450.0	1'742.4	831.7	14.7	30'080
TGF-β3	6.0	11.9	9.7	14.2	7.5	8.5	2.8	15'031

LLOQ: Lower limit of quantitation; ULOQ: Upper limit of quantitation

https://doi.org/10.1371/journal.pone.0203337.t002

Table 3. P-values appertaining to the 43 monitored cytokines in the aqueous humor of healthy controls and of patients with either dry or treated wet age-related macular degeneration (AMD).

Cytokine	Kruskal-Wallis H-Test	Kruskal-Wallis H-Test	Kruskal-Wallis H-Test
	Healthy controls vs.	Healthy controls vs	Dry AMD vs.
	Dry AMD	Treated wet AMD	Treated wet AMD
CCL21	$p = 0.034$	$p = 0.034$	$p = 0.69$
CXCL13	$p = 0.1546$	$p = 0.0082$	$p = 0.0113$
CCL27	$p = 0.075$	$p = 0.029$	$p = 0.118$
CXCL5	$p = 0.00265$	$\mathbf{p = 0.00099}$	$p = 0.26924$
CCL11	$p = 0.13$	$p = 0.11$	$p = 0.57$
CCL24	$p = 0.0035$	$p = 0.0609$	$p = 0.0609$
CCL26	$p = 0.0079$	$p = 0.0079$	$p = 0.2915$
CX3CL1	$p = 0.041$	$p = 0.041$	$p = 0.67$
CXCL6	$\mathbf{p = 0.00067}$	$\mathbf{p = 0.00067}$	$p = 0.10159$
GM-CSF	$p = 0.0014$	$p = 0.0866$	$p = 0.1096$
CXCL1	$p = 0.084$	$p = 0.015$	$p = 0.245$
CXCL2	$p = 0.0036$	$p = 0.0025$	$p = 0.0812$
CCL1	$p = 0.0093$	$p = 0.0093$	$p = 0.5295$
IFN-γ	$p = 0.02$	$p = 0.019$	$p = 0.529$
IL-1β	$p = 0.059$	$p = 0.008$	$p = 0.126$
IL-2	$p = 0.533$	$p = 0.067$	$p = 0.261$
IL-4	$p = 0.0091$	$p = 0.0093$	$p = 0.9518$
IL-6	$p = 0.39$	$p = 0.31$	$p = 0.68$
IL-8/CXCL8	$p = 0.082$	$p = 0.019$	$p = 0.082$
IL-10	$p = 0.086$	$p = 0.016$	$p = 0.129$
IL-16	$p = 0.0829$	$p = 0.0023$	$p = 0.0773$
CXCL10	$p = 0.784$	$p = 0.0078$	$p = 0.022$
CXCL11	$p = 0.0127$	$p = 0.0031$	$p = 0.169$
CCL2	$p = 0.66$	$p = 0.17$	$p = 0.1$
CCL8	$p = 0.0424$	$p = 0.0026$	$p = 0.0698$
CCL7	$p = 0.012$	$p = 0.002$	$p = 0.135$
CCL13	$p = 0.0048$	$p = 0.0014$	$p = 0.5613$
CCL22	$p = 0.0607$	$p = 0.0028$	$p = 0.0773$
MIF	$p = 0.222$	$p = 0.023$	$p = 0.222$
MIG/CXCL9	$p = 0.06656$	$\mathbf{p = 0.00019}$	$p = 0.02358$
CCL3	$p = 0.0218$	$p = 0.0048$	$p = 0.1441$
CCL15	$p = 0.081$	$p = 0.023$	$p = 0.081$
CCL20	$p = 0.91$	$p = 0.65$	$p = 0.65$
CCL19	$p = 0.72$	$p = 0.61$	$p = 0.61$
CCL23	$p = 0.014$	$p = 0.0059$	$p = 0.4767$
CXCL16	$p = 0.38$	$p = 0.56$	$p = 0.36$
CXCL12	$p = 0.982$	$p = 0.024$	$p = 0.056$
CCL17	$p = 0.2$	$p = 0.2$	$p = 0.62$
CCL25	$p = 0.0869$	$p = 0.0051$	$p = 0.0972$
TNF-α	$p = 0.0057$	$p = 0.0057$	$p = 0.5613$
TGF-β1	$p = 0.16$	$p = 0.04$	$p = 0.16$
TGF-β2	$p = 0.0037$	$p = 0.1705$	$p = 0.6544$
TGF-β3	$p = 0.16$	$p = 0.16$	$p = 0.81$

https://doi.org/10.1371/journal.pone.0203337.t003

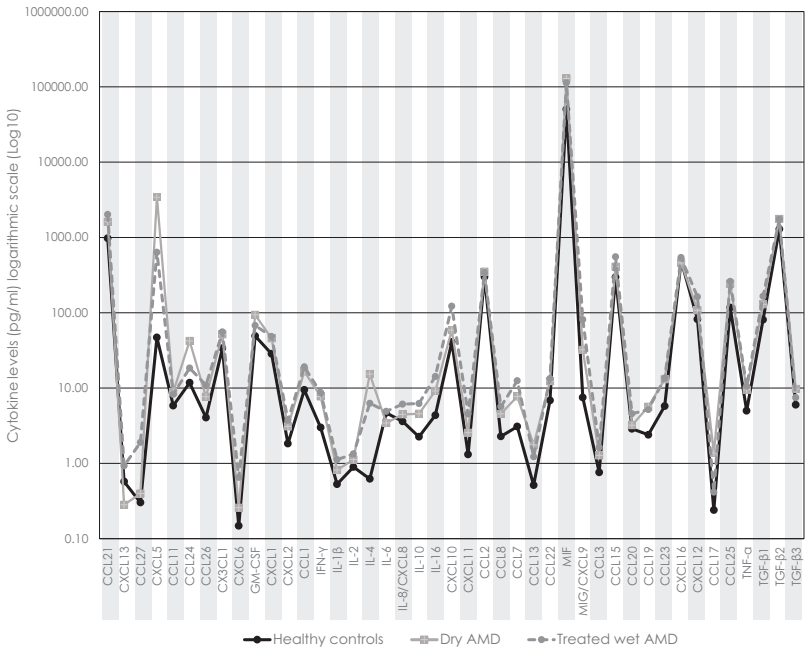

Fig 1. Mean concentrations of the 43 monitored cytokines in the aqueous humor of healthy controls (continuous black trace) and of patients with either dry age-related macular degeneration (AMD; continuous grey trace) or treated wet AMD (dashed grey trace), represented on a logarithmic scale. Note: The presentation of non-continuous data as a line graph permits an improved estimation of concentration changes not only for individual cytokines (points) but also for the cytokine environment as a whole.

https://doi.org/10.1371/journal.pone.0203337.g001

the macular RPE/Bruch's membrane complex. Likely, only the most abundant changes in the cytokine concentrations at the lesion site will be detectable in the constantly renewing AH. This circumstance may partially explain why the number of cytokines for which intergroup differences were detected was not larger than it was. The three that qualified for this distinction, namely, CXCL5, CXCL6 and MIG/CXCL9, probably figure in a true disease-associated effect. Interestingly, the differences in their concentrations were between the healthy controls and the two pathological sub-groups, *not* between the two forms of the AMD pathology. This finding and the fact that no systematic change in the cytokine environment was detected between dry AMD and treated stable wet AMD (Fig 1) suggests that a persistent imbalance in the local milieu might exist in this disease, with smooth transitions occurring between dry and wet AMD pathogenesis [4]. However, the fact that we investigated samples of treated stable wet AMD leads to the speculation that we looked at an inactive state of the disease with possible cytokine downregulation. Nevertheless, CNV reactivation is possible at any time so that it might be at least partially similar to the situation prior to the primary activation of the lesion.

Inflammatory activity at a subclinical level could indeed figure in AMD, in analogy to the situation that is observed in the pseudoexfoliation syndrome, which is another age-dependent degenerative disease with an inflammatory background. This postulate fits well with the concept of inflammaging or immunosenescence, an age-related inflammatory response to aging changes, found in many organs [31–37]. Since aging is the strongest risk factor for the development and progression of AMD, the existence of a link between the pathogenesis of the disease and local immunosenescence is very likely [2, 38].

In the ocular tissue of patients with AMD, an accumulation of T-cells has been observed. Consequently, it has been proposed that lymphocytes may play a pivotal role in the breakdown of Bruch's membrane, in RPE-atrophy and in the onset CNV in early and late stages of AMD

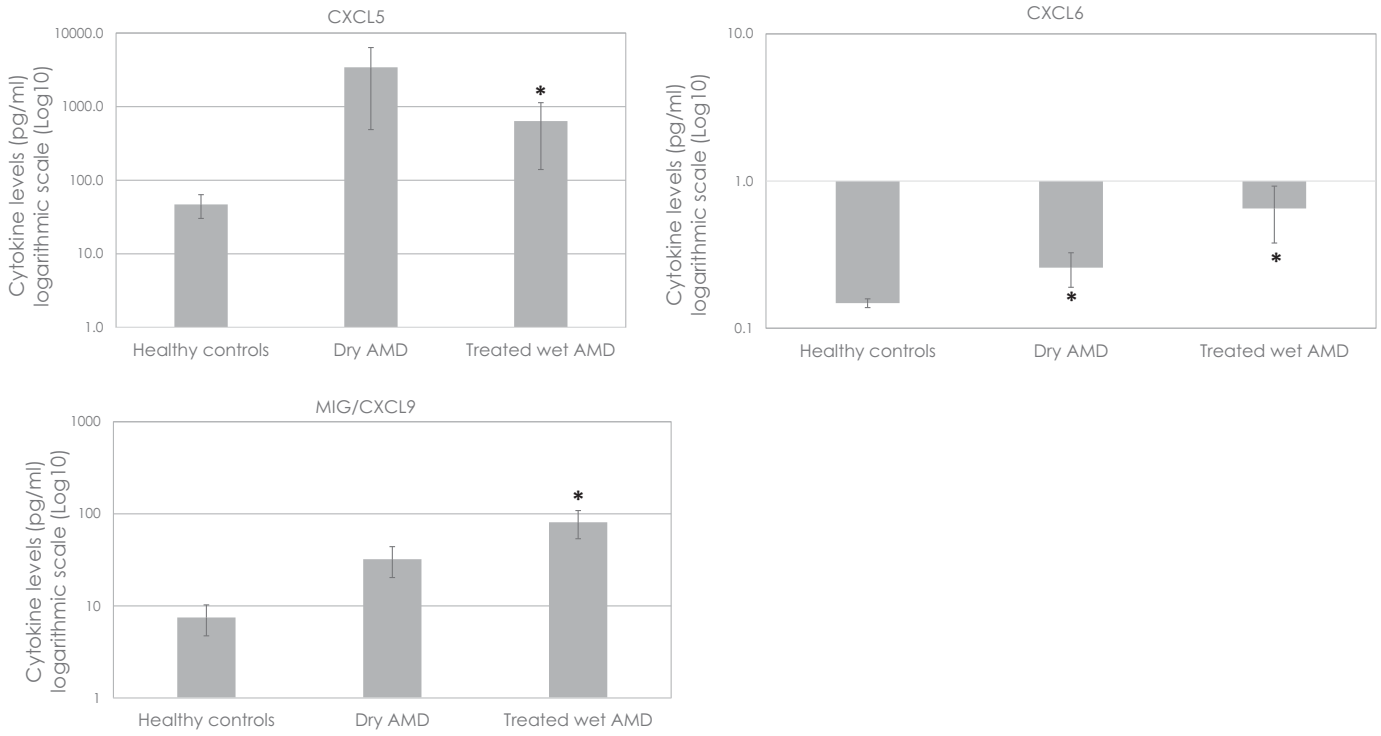

Fig 2. Mean concentrations (pg/ml) of the three cytokines/chemokines CXCL5, CXCL6 and MIG/CXCL9 in the aqueous humor of patients with either dry age-related macular degeneration (AMD) or treated wet AMD showing relevant differences (*, $p<0.0012$) in their levels when compared to healthy controls. Whiskers represent the standard error of the mean. Values are represented on a logarithmic scale.

https://doi.org/10.1371/journal.pone.0203337.g002

[39, 40]. Monokine induced by interferon-gamma (-γ) (MIG/CXCL9), which was up-regulated in treated wet AMD, is known to be a crucial chemokine in many inflammatory processes, particularly in those that are mediated by T-cells [41]. It is specific for T-cell chemotaxis and cell attraction and was found to be overexpressed in the RPE/Bruch's membrane/choriocapillaris complex of aging mice [42]. Although our sample number may have been too small to detect age-related changes in in the concentrations of MIG/CXCL9 within the AH or the serum, Torres and coworkers recently described serum MIG/CXCL9 levels to increase with progression of age by using a large population-based cohort study [43, 44]. Shi and colleagues reported that MIG/CXCL9, interferon-γ inducible protein 10-kDa (IP-10, CXCL10), and interferon-γ inducible T-cell alpha (α) chemoattractant (I-TAC, CXCL11) are expressed in RPE [45]. However, the specific functions of these chemokines in angiogenesis remain to be determined [45–48]. As an indicator of its clinical relevance, Jonas et al. reported an association between the severity of retinal edema and elevated concentrations of MIG/CXCL9 [13]. CNV, on the other hand, does not appear to be driven primarily by inflammatory activity, since a relevant effect was not detected in AMD-patients who had undergone treatment with corticosteroids in addition to anti-VEGF therapy in a prospective randomized clinical trial [49].

The finding that elevated levels of MIG/CXCL9 were apparent only in treated wet AMD could suggests that this change is not only age-related [43, 44], but also a marker of retinal diseases with inflammatory components such as AMD [50] at a late, steady-state stage, which deserves further attention.

Table 4. Mean concentrations (pg/ml) and standard deviations (SD) of the 43 monitored cytokines in the sera of healthy controls and of patients with either dry or treated wet age-related macular degeneration (AMD).

Cytokine	Healthy controls		Dry AMD		Treated wet AMD		Assay Working Ranges	
	Mean (pg/ml)	SD	Mean (pg/ml)	SD	Mean (pg/ml)	SD	LLOQ (pg/ml)	ULOQ (pg/ml)
CCL21	7'971.5	2'008.0	14'357.7	17'653.8	19'630.5	18'509.2	21.9	3'923
CXCL13	23.3	13.1	33.1	25.4	35.1	25.3	0.7	1'200
CCL27	1'612.3	428.2	2'293.4	1'188.6	2'587.4	1'177.7	1.2	5'000
CXCL5	724.7	274.1	1'217.4	1'155.9	1'374.2	1'039.9	7.3	120'000
CCL11	48.4	9.5	57.5	24.1	76.2	36.6	1.5	3'859
CCL24	371.6	196.3	584.0	560.2	433.0	250.9	6.2	4'073
CCL26	42.0	14.1	49.9	44.6	63.9	49.4	0.9	12'109
CX3CL1	166.2	46.7	242.0	152.0	271.7	179.3	4.0	11'463
CXCL6	32.1	10.1	34.6	18.4	45.8	24.1	0.8	11'135
GM-CSF	22.9	18.4	32.9	30.0	43.5	41.3	5.3	35'000
CXCL1	318.0	63.1	328.5	117.4	365.1	98.9	3.1	7'024
CXCL2	494.4	265.4	678.3	726.3	1'083.1	982.7	4.6	13'257
CCL1	66.7	10.3	75.5	40.7	87.9	45.8	1.8	1'015
IFN-γ	63.0	22.4	74.8	46.9	93.4	50.3	2.3	20'236
IL-1β	5.2	1.7	5.0	2.2	5.3	1.9	0.4	7'000
IL-2	10.9	4.2	11.7	6.1	13.6	5.9	0.8	13'000
IL-4	44.0	6.7	52.4	25.7	56.9	28.7	1.2	4'804
IL-6	6.5	2.9	8.8	3.7	10.3	4.1	0.7	12'000
IL-8/CXCL8	10.9	7.0	20.1	27.2	37.0	45.4	0.5	7'640
IL-10	70.8	25.5	88.1	63.4	105.5	79.0	1.3	18'708
IL-16	285.1	73.9	458.9	246.4	714.5	589.4	2.1	34'000
CXCL10	191.5	116.2	483.0	1124.9	313.1	203.7	1.6	7'714
CXCL11	38.8	9.1	108.0	189.1	84.6	65.0	0.1	2'298
CCL2	60.1	14.7	81.6	51.6	95.4	56.2	0.3	4'812
CCL8	88.7	21.6	151.7	143.5	160.1	131.0	0.3	4'056
CCL7	128.3	44.5	154.6	109.4	184.0	117.8	1.9	20'133
CCL13	81.8	24.8	115.4	80.2	125.3	78.0	0.2	3'368
CCL22	1010.5	1063.4	641.5	330.4	1022.7	598.4	0.9	14'649
MIF	68'484.2	78'856.9	166'888.4	259'605.2	254'498.5	239'185.4	23.1	377'721
MIG/CXCL9	432.0	228.1	2'200.1	6'308.5	1'221.1	1'222.3	1.8	19'600
CCL3	8.1	2.7	9.9	5.9	11.2	3.6	0.4	1'543
CCL15	5'487.2	2'218.7	9'693.6	6'064.3	10'720.8	7'705.4	1.7	9'100
CCL20	18.0	12.0	19.0	13.1	18.8	9.3	0.3	4'675
CCL19	512.1	344.9	679.1	648.3	802.2	857.2	3.0	48'494
CCL23	350.4	116.7	399.1	192.6	427.0	226.1	1.0	14'450
CXCL16	580.9	155.1	689.7	277.5	857.2	394.3	0.5	2'867
CXCL12	929.8	298.2	1172.3	774.1	1'598.1	1'319.1	8.3	115'730
CCL17	206.9	160.7	275.3	283.3	344.4	421.4	1.7	430
CCL25	810.8	286.0	1'063.3	713.7	1'531.1	913.4	20.6	114'493
TNF-α	53.4	11.2	72.6	53.4	87.7	61.5	0.9	13'879
TGF-β1	13.6	0.0	205.2	376.9	273.0	548.5	1.7	27'616
TGF-β2	3.1	0.0	107.6	423.6	3.1	0.0	14.7	30'080
TGF-β3	1.5	1.9	4.6	7.1	5.0	9.2	2.8	15'031

LLOQ: Lower limit of quantitation; ULOQ: Upper limit of quantitation

https://doi.org/10.1371/journal.pone.0203337.t004

Table 5. P-values appertaining to the 43 monitored cytokines in the sera of healthy controls and of patients with either dry or treated wet age-related macular degeneration (AMD).

Cytokine	Kruskal-Wallis H-Test Healthy controls vs. Dry AMD	Kruskal-Wallis H-Test Healthy controls vs Treated wet AMD	Kruskal-Wallis H-Test Dry AMD vs. Treated wet AMD
CCL21	$p = 0.6$	$p = 0.6$	$p = 0.65$
CXCL13	$p = 0.36$	$p = 0.36$	$p = 0.99$
CCL27	$p = 0.17$	$p = 0.12$	$p = 0.51$
CXCL5	$p = 0.45$	$p = 0.45$	$p = 0.45$
CCL11	$p = 0.27$	$p = 0.27$	$p = 0.27$
CCL24	$p = 0.7$	$p = 0.7$	$p = 0.7$
CCL26	$p = 0.77$	$p = 0.77$	$p = 0.77$
CX3CL1	$p = 0.2$	$p = 0.2$	$p = 0.85$
CXCL6	$p = 1.0$	$p = 0.27$	$p = 0.27$
GM-CSF	$p = 0.64$	$p = 0.64$	$p = 0.64$
CXCL1	$p = 0.69$	$p = 0.54$	$p = 0.54$
CXCL2	$p = 1.0$	$p = 0.6$	$p = 0.6$
CCL1	$p = 0.74$	$p = 0.74$	$p = 0.74$
IFN-γ	$p = 0.54$	$p = 0.28$	$p = 0.36$
IL-1β	$p = 0.9$	$p = 0.9$	$p = 0.9$
IL-2	$p = 0.77$	$p = 0.59$	$p = 0.61$
IL-4	$p = 0.5$	$p = 0.5$	$p = 0.71$
IL-6	$p = 0.174$	$p = 0.076$	$p = 0.259$
IL-8/CXCL8	$p = 0.29$	$p = 0.12$	$p = 0.12$
IL-10	$p = 0.74$	$p = 0.74$	$p = 0.74$
IL-16	$p = 0.076$	$p = 0.076$	$p = 0.381$
CXCL10	$p = 0.47$	$p = 0.3$	$p = 0.47$
CXCL11	$p = 0.27$	$p = 0.12$	$p = 0.51$
CCL2	$p = 0.41$	$p = 0.22$	$p = 0.41$
CCL8	$p = 0.27$	$p = 0.27$	$p = 0.68$
CCL7	$p = 0.79$	$p = 0.65$	$p = 0.65$
CCL13	$p = 0.58$	$p = 0.58$	$p = 0.61$
CCL22	$p = 0.48$	$p = 0.46$	$p = 0.05$
MIF	$p = 0.213$	$p = 0.057$	$p = 0.141$
MIG/CXCL9	$p = 0.18$	$p = 0.12$	$p = 0.72$
CCL3	$p = 0.4$	$p = 0.14$	$p = 0.22$
CCL15	$p = 0.037$	$p = 0.037$	$p = 0.745$
CCL20	$p = 0.88$	$p = 0.88$	$p = 0.88$
CCL19	$p = 0.97$	$p = 0.97$	$p = 0.97$
CCL23	$p = 0.87$	$p = 0.87$	$p = 0.87$
CXCL16	$p = 0.3$	$p = 0.14$	$p = 0.3$
CXCL12	$p = 0.48$	$p = 0.48$	$p = 0.48$
CCL17	$p = 0.97$	$p = 0.97$	$p = 0.97$
CCL25	$p = 0.6$	$p = 0.14$	$p = 0.16$
TNF-α	$p = 0.41$	$p = 0.41$	$p = 0.41$
TGF-β1	$p = 0.086$	$p = 0.086$	$p = 0.915$
TGF-β2	$p = 0.21$	-	$p = 0.21$
TGF-β3	$p = 0.66$	$p = 0.66$	$p = 0.96$

https://doi.org/10.1371/journal.pone.0203337.t005

In the vast majority of our patients with wet AMD, an interval of more than 6 months had elapsed between the last anti-VEGF injection and the time at which the samples were collected. Hence, the influence of intravitreal anti-VEGF treatment should be negligible [51, 52].

In the context of ocular diseases, data appertaining to the neutrophil-specific pro-inflammatory and chemoattractive cytokines/chemokines CXCL5 (ENA-78) and CXCL6 (GCP-2) are sparse. Both chemokines show a high homology in primary structure (chemotactic potency: CXCL6 > CXCL5 [53]) and they both interact with CXCR-2 (CXC chemokine receptor 2) [54]. The present study is one of the first to identify noticeably elevated levels of these cytokines/chemokines in the eyes of patients with dry and/or treated wet AMD relative to the situation in the healthy controls, which remained statistically significant even after the correction for multiple comparisons. The extent to which these increases contribute to the pathogenesis and/or persistence of AMD remains to be addressed.

A similar up-regulation has been reported also for late forms of the pseudoexfoliation syndrome with luxation of the intraocular lens [29] and in eyes with epiretinal membranes [18]. Parmar et al. reported increases in the levels of several cytokines, including CXCL5, which have been identified as an acute stress response to intense light in immunodeficient mice with a disrupted visual cycle and as pivotal factors in the development of retinal degeneration [55]. An association between chronic light-induced damage and AMD has been reported, and this circumstance could account for the almost 14-fold up-regulation of CXCL5 [56]. In patients with dry AMD and in those with treated wet AMD, CXCL6 was up-regulated relative to the situation in the healthy controls. Since the AH-levels of CXCL6 (min. 0.1 pg/ml in healthy controls; max. 0.7 pg/ml in treated wet AMD) ranged below the LLOQ for the used immunoassay system (0.8 pg/ml) the detected differences may reflect not only a true disease-based change, but also partially measurement inaccuracies. Until this latter possibility can be excluded, an interpretation of this finding is not possible.

Moreover, we did not observe any difference in serum cytokine profiles neither between the three compared groups nor with progression of age within the analyzed pool of patients. This might indicate that AMD is a local ocular process without measurable systemic cytokine environmental changes which has already been discussed controversially (e.g., TNF-α) [57, 58]. However, an age-related low-grade systemic/generalized inflammation within a similar degree in healthy controls and AMD patients cannot be excluded.

Beyond the limitations of this study, the healthy controls were approximately 10 years younger than the patients with dry or treated wet AMD. A possible influence of age on the results cannot therefore be excluded. However, since a correlation between age and the AH-levels of the cytokines was detected in only two instances [CXCL6 ($p = 0.0006$) and CCL7 ($p = 0.0007$)] and not at all between age and the serum concentrations, any effect, if it existed, would be marginal. Nevertheless, in future studies, an age-matched group of healthy controls would be included to enhance the power of the intergroup differences. Given that no age-related differences between dry and treated wet AMD were identified, the absence of intergroup differences is probably a reliable finding. Finally, since a very robust level of significance was employed in the present study (Bonferroni correction), only differences of high significance ($p<0.0012$) were considered. Hence, we cannot exclude the possibility that some relevant results might have been thereby dismissed. Nevertheless, in terms of biological relevance, we believe that the application of such a high level of significance contributed to the strength of our findings.

For 40 of the 43 monitored cytokines, we could identify no intergroup differences in concentration. Our findings respecting some of these cytokines conflict with existing data, which reveal specific associations between their levels and the pathogenesis of wet AMD [1, 11, 13, 17, 25, 26, 48, 59–63] as well as aging [64–66], either locally in the AH or systemically in the

blood serum. Of particular note are our findings respecting IL-4 and IL-10, which, in contrast to existing data, were not up-regulated. An up-regulation of these two cytokines would indeed be reasonable, since both appear to be involved in the pathogenesis of AMD [3, 11, 21, 22, 26, 59, 67–70] and the process of aging [22, 25, 64, 43]. The discrepancies between our own and existing data may be partially explained by the circumstance that we chose to evaluate not only individual cytokines but also the cytokine environment as a whole, which necessitated a correction for multiple comparisons. As a consequence, the concentrations of 31 of the monitored cytokines fell below the level of statistical significance ($p>0.0012$). The heterogeneity of the AMD-stages that were investigated in various published studies may also have contributed to the different outcomes [67].

In conclusion, three of the 43 monitored cytokines (7%) were up-regulated in the AH of AMD-patients relative to the situation in the healthy controls. No differences between dry and treated wet AMD were identified. Our data support existing evidence that inflammatory/immunological processes play a role in the pathogenesis of AMD. The finding that 31 of the 43 monitored cytokines (72%) were dysregulated in patients with wet AMD relative to the situation in the healthy controls at a significance level of $p<0.05$ affords strong evidence that data appertaining to the concentrations of individual cytokines are barely interpretable on this statistical basis. The circumstance that the AH-levels of seven of the 43 tested cytokines (16%) hovered below the LLOQ for the immunoassay contributes to the difficulty of interpretation. Consequently, at the present time, estimating the specific role of these cytokines in the pathogenesis of AMD is challenging, since a relevant effect at the tissue level of the RPE/Bruch's membrane/choriocapillaris complex cannot excluded in the face of existing data.

Acknowledgments

We gratefully acknowledge the assistance of Alain Despont in conducting the immunoassay analysis as well as the support of Robert Rieben and Natalie Reichen in performing this study.

Author Contributions

Conceptualization: Justus G. Garweg.

Data curation: Jan Spindler, Souska Zandi, Isabel B. Pfister, Christin Gerhardt, Justus G. Garweg.

Formal analysis: Jan Spindler, Souska Zandi, Isabel B. Pfister, Christin Gerhardt.

Funding acquisition: Justus G. Garweg.

Investigation: Justus G. Garweg.

Methodology: Souska Zandi, Justus G. Garweg.

Project administration: Justus G. Garweg.

Resources: Justus G. Garweg.

Software: Isabel B. Pfister.

Supervision: Souska Zandi, Justus G. Garweg.

Visualization: Jan Spindler, Isabel B. Pfister, Christin Gerhardt.

Writing – original draft: Jan Spindler.

Writing – review & editing: Souska Zandi, Justus G. Garweg.

References

1. Knickelbein JE, Chan CC, Sen HN, Ferris FL, Nussenblatt RB. Inflammatory mechanisms of age-related macular degeneration. Int Ophthalmol Clin. 2015; 55(3): 63–78. https://doi.org/10.1097/IIO.0000000000000073 PMID: 26035762
2. Gallenga CE, Parmeggiani F, Costagliola C, Sebastiani A, Gallenga PE. Inflammaging: should this term be suitable for age related macular degeneration too? Inflamm Res. 2014 Feb; 63(2):105–7. https://doi.org/10.1007/s00011-013-0684-2 PMID: 24202618
3. Kelly J, Khan AA, Yin J, Ferguson TA, Apte RS. Senescence regulates macrophage activation and angiogenic fate at sites of tissue injury in mice. J Clin Invest. 2007 Nov 1; 117(11): 3421–3426. https://doi.org/10.1172/JCI32430 PMID: 17975672
4. Ambati J, Fowler BJ. Mechanisms of age-related macular degeneration. Neuron. 2012 July 12; 75(1): 26–39. https://doi.org/10.1016/j.neuron.2012.06.018 PMID: 22794258
5. Bird AC, Phillips RL, Hageman GS. Geographic atrophy: a histopathological assessment. JAMA Ophthalmol. 2014 Mar; 132(3):338–45. https://doi.org/10.1001/jamaophthalmol.2013.5799 PMID: 24626824
6. Kauppinen A, Paterno JJ, Blasiak J, Salminen A, Kaarniranta K. Inflammation and its role in age-related macular degeneration. Cell. Mol. Life Sci. (2016) 73:1765–1786. https://doi.org/10.1007/s00018-016-2147-8 PMID: 26852158
7. van Lookeren Campagne M, LeCouter J, Yaspan BL, Ye W. Mechanisms of age-related macular degeneration and therapeutic opportunities. J Pathol. 2014 Jan; 232(2):151–64. https://doi.org/10.1002/path.4266 PMID: 24105633
8. Zhu D, Deng X, Xu J, Hinton DR. What determines the switch between atrophic and neovascular forms of age related macular degeneration?—the role of BMP4 induced senescence. Aging (Albany NY). 2009 Aug 12; 1(8):740–5.
9. Danis RP, Lavine JA, Domalpally A. Geographic atrophy in patients with advanced dry age-related macular degeneration: current challenges and future prospects. Clin Ophthalmol. 2015 Nov 20; 9:2159–74. https://doi.org/10.2147/OPTH.S92359 PMID: 26640366
10. Sheu SJ, Ger LP, Kuo NW, Liu NC, Wu TT, Lin MC. Association of IL-4 gene polymorphism and age-related macular degeneration in Taiwanese adults. Taiwan Journal of Ophthalmology 2 (2012) 51–55.
11. Cha DM, Woo SJ, Kim HJ, Lee C, Park KH. Comparative Analysis of Aqueous Humor Cytokine Levels Between Patients With Exudative Age-Related Macular Degeneration and Normal Controls. Invest Ophthalmol Vis Sci. 2013; 54:7038–7044. https://doi.org/10.1167/iovs.13-12730 PMID: 24106111
12. Miao H, Tao Y, Li XX. Inflammatory cytokines in aqueous humor of patients with choroidal neovascularization. Mol Vis. 2012; 18:574–80. PMID: 22419849
13. Jonas JB, Tao Y, Neumaier M, Findeisen P. Cytokine concentration in aqueous humour of eyes with exudative age-related macular degeneration. Acta Ophthalmol. 2012 Aug; 90(5):e381–8. https://doi.org/10.1111/j.1755-3768.2012.02414.x PMID: 22490043
14. Anand A, Sharma NK, Gupta A, Prabhakar S, Sharma SK, Singh R, et al. Single Nucleotide Polymorphisms in MCP-1 and Its Receptor Are Associated with the Risk of Age Related Macular Degeneration. PLoS One. 2012; 7(11): e49905. https://doi.org/10.1371/journal.pone.0049905 PMID: 23185481
15. Jonas JB, Neumaier M. Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor in Exudative Age-Related Macular Degeneration and Diffuse Diabetic Macular Edema. Ophthalmic Res. 2007; 39(3):139–42. https://doi.org/10.1159/000102935 PMID: 17505145
16. Kawai M, Inoue T, Inatani M, Tsuboi N, Shobayashi K, Matsukawa A, et al. Elevated Levels of Monocyte Chemoattractant Protein-1 in the Aqueous Humor after Phacoemulsification. Invest Ophthalmol Vis Sci. 2012 Dec 3; 53(13):7951–60. https://doi.org/10.1167/iovs.12-10231 PMID: 23132797
17. Liu F, Ding X, Yang Y, Li J, Tang M, Yuan M, et al. Aqueous humor cytokine profiling in patients with wet AMD. Mol Vis. 2016; 22: 352–361. PMID: 27122966
18. Zandi S, Tappeiner C, Pfister IB, Despont A, Rieben R, Garweg JG. Vitreal Cytokine Profile Differences Between Eyes With Epiretinal Membranes or Macular Holes. Invest Ophthalmol Vis Sci. 2016 Nov 1; 57 (14):6320–6326. https://doi.org/10.1167/iovs.16-20657 PMID: 27893098
19. Opal SM, DePalo VA. Anti-inflammatory cytokines. Chest. 2000 Apr; 117(4):1162–72. PMID: 10767254
20. Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. Prog Retin Eye Res. 2001 Nov; 20 (6):705–32. PMID: 11587915
21. Dace DS, Khan AA, Kelly J, Apte RS. Interleukin-10 Promotes Pathological Angiogenesis by Regulating Macrophage Response to Hypoxia during Development. PLoS One. 2008; 3(10):e3381. https://doi.org/10.1371/journal.pone.0003381 PMID: 18852882

22. Zandi S, Nakao S, Chun KH, Fiorina P, Sun D, Arita R, et al. ROCK-Isoform Specific Polarization of Macrophages Associated with Age-Related Macular Degeneration. Cell Rep. 2015 Feb 24; 10 (7):1173–86. https://doi.org/10.1016/j.celrep.2015.01.050 PMID: 25704819

23. Folkman J. Endogenous angiogenesis inhibitors. APMIS. 2004 Jul-Aug; 112(7–8):496–507. https://doi.org/10.1111/j.1600-0463.2004.apm11207-0809.x PMID: 15563312

24. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev. 2004 Aug; 25(4):581–611. https://doi.org/10.1210/er.2003-0027 PMID: 15294883

25. Lin T, Walker GB, Kurji K, Fang E, Law G, Prasad SS, et al. Parainflammation associated with advanced glycation endproduct stimulation of RPE in vitro: Implications for agerelated degenerative diseases of the eye. Cytokine. 2013 Jun; 62(3):369–81. https://doi.org/10.1016/j.cyto.2013.03.027 PMID: 23601964

26. Nassar K, Grisanti S, Elfar E, Lüke J, Lüke M, Grisanti S. Serum cytokines as biomarkers for age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol (2015) 253:699–704. https://doi.org/10.1007/s00417-014-2738-8 PMID: 25056526

27. Rutar M., Provis J. Role of Chemokines in Shaping Macrophage Activity in AMD. In: Bowes Rickman C., LaVail M., Anderson R., Grimm C., Hollyfield J., Ash J. Retinal Degenerative Diseases. Adv Exp Med Biol. 2016;854:11–6.

28. Seddon JM, Sharma S, Adelman RA. Evaluation of the clinical age-related maculopathy staging system. Ophthalmology. 2006 Feb; 113(2):260–6. https://doi.org/10.1016/j.ophtha.2005.11.001 PMID: 16458093

29. Garweg JG, Zandi S, Pfister IB, Skowronska M, Gerhardt C. Comparison of cytokine profiles in the aqueous humor of eyes with pseudoexfoliation syndrome and glaucoma. PLoS One. 2017 Aug 10; 12 (8):e0182571. https://doi.org/10.1371/journal.pone.0182571 PMID: 28797085

30. Miller RG. Simultaneous statistical inference. Springer-Verlag, New York.1981 pp. 67–70.

31. Ventura MT, Casciaro M, Gangemi S, Buquicchio R. Immunosenescence in aging: between immune cells depletion and cytokines up-regulation. Clin Mol Allergy. 2017 Dec 14; 15:21. https://doi.org/10.1186/s12948-017-0077-0 PMID: 29259496

32. Ma Y, Mouton AJ, Lindsey ML. Cardiac macrophage biology in the steady-state heart, the aging heart, and following myocardial infarction. Transl Res. 2018 Jan; 191:15–28. https://doi.org/10.1016/j.trsl.2017.10.001 PMID: 29106912

33. Martínez-Cengotitabengoa M, Carrascón L, O'Brien JT, Díaz-Gutiérrez MJ, Bermúdez-Ampudia C, Sanada K, et al. Peripheral Inflammatory Parameters in Late-Life Depression: A Systematic Review. Int J Mol Sci. 2016 Dec 2; 17(12). pii: E2022.

34. van der Kraan P, Matta C, Mobasheri A. Age-Related Alterations in Signaling Pathways in Articular Chondrocytes: Implications for the Pathogenesis and Progression of Osteoarthritis—A Mini-Review. Gerontology. 2017; 63(1):29–35. https://doi.org/10.1159/000448711 PMID: 27595269

35. Bauer ME, Fuente M de L. The role of oxidative and inflammatory stress and persistent viral infections in immunosenescence. Mech Ageing Dev. 2016 Sep; 158:27–37. https://doi.org/10.1016/j.mad.2016.01.001 PMID: 26773975

36. Minciullo PL, Catalano A, Mandraffino G, Casciaro M, Crucitti A, Maltese G, et al. Inflammaging and Anti-Inflammaging: The Role of Cytokines in Extreme Longevity. Arch Immunol Ther Exp (Warsz). 2016 Apr; 64(2):111–26.

37. Frasca D, Blomberg BB. Inflammaging decreases adaptive and innate immune responses in mice and humans. Biogerontology. 2016 Feb; 17(1):7–19. https://doi.org/10.1007/s10522-015-9578-8 PMID: 25921609

38. Klein R, Klein BE, Tomany SC, Meuer SM, Huang GH. Ten-year incidence and progression of age-related maculopathy: The Beaver Dam eye study. Ophthalmology. 2002 Oct; 109(10):1767–79. PMID: 12359593

39. Penfold P, Killingsworth M, Sarks S. An ultrastructural study of the role of leucocytes and fibroblasts in the breakdown of Bruch's membrane. Aust J Ophthalmol. 1984 Feb; 12(1):23–31. PMID: 6732655

40. Penfold PL, Killingsworth MC, Sarks SH. Senile macular degeneration: the involvement of immunocompetent cells. Graefes Arch Clin Exp Ophthalmol. 1985; 223(2):69–76. PMID: 2408968

41. Marshall A, Celentano A, Cirillo N, McCullough M, Porter S. Tissue-specific regulation of CXCL9/10/11 chemokines in keratinocytes: Implications for oral inflammatory disease. PLoS One. 2017; 12(3): e0172821. https://doi.org/10.1371/journal.pone.0172821 PMID: 28253295

42. Camelo Serge. Potential Sources and Roles of Adaptive Immunity in Age-Related Macular Degeneration: Shall We Rename AMD into Autoimmune Macular Disease? Autoimmune Dis. 2014; 2014: 532487. https://doi.org/10.1155/2014/532487 PMID: 24876950

43. Torres KCL, Rezende VB, Lima-Silva ML, Santos LJS, Costa CG, Mambrini JVM, et al. Immune senescence and biomarkers profile of Bambuí aged population-based cohort. Exp Gerontol. 2018 Mar; 103:47–56. https://doi.org/10.1016/j.exger.2017.12.006 PMID: 29247791

44. Shurin GV, Yurkovetsky ZR, Chatta GS, Tourkova IL, Shurin MR, Lokshin AE. Dynamic alteration of soluble serum biomarkers in healthy aging. Cytokine. 2007 Aug; 39(2):123–9. Epub 2007 Aug 8. https://doi.org/10.1016/j.cyto.2007.06.006 PMID: 17689975

45. Shi G, Maminishkis A, Banzon T, Jalickee S, Li R, Hammer J, et al. Control of chemokine gradients by the retinal pigment epithelium. Invest Ophthalmol Vis Sci. 2008 Oct; 49(10):4620–30. https://doi.org/10.1167/iovs.08-1816 PMID: 18450597

46. Fujimura S, Takahashi H, Yuda K, Ueta T, Iriyama A, Inoue T, et al. Angiostatic Effect of CXCR3 Expressed on Choroidal Neovascularization. Invest Ophthalmol Vis Sci. 2012 Apr 18; 53(4):1999–2006. https://doi.org/10.1167/iovs.11-8232 PMID: 22408007

47. Falk MK, Singh A, Faber C, Nissen MH, Hviid T, Sørensen TL. Dysregulation of CXCR3 expression on peripheral blood leukocytes in patients with neovascular age-related macular degeneration. Invest Ophthalmol Vis Sci. 2014 May 8; 55(7):4050–6. https://doi.org/10.1167/iovs.14-14107 PMID: 24812555

48. Juel HB, Faber C, Udsen MS, Folkersen L, Nissen MH. Chemokine expression in retinal pigment epithelial ARPE-19 cells in response to coculture with activated T cells. Invest Ophthalmol Vis Sci. 2012 Dec 19; 53(13):8472–80. https://doi.org/10.1167/iovs.12-9963 PMID: 23150618

49. Chaudhary V, Barbosa J, Lam WC, Mak M, Mavrikakis E, Mohaghegh P SM. Ozurdex in age-related macular degeneration as adjunct to ranibizumab (The OARA Study). Can J Ophthalmol. 2016 Aug; 51 (4):302–305. https://doi.org/10.1016/j.jcjo.2016.04.020 PMID: 27521672

50. Hooks JJ, Nagineni CN, Hooper LC, Hayashi K, Detrick B. IFN-beta provides immuno-protection in the retina by inhibiting ICAM-1 and CXCL9 in retinal pigment epithelial cells. J Immunol. 2008 Mar 15; 180 (6):3789–96. PMID: 18322185

51. Fauser S, Muether PS. Clinical correlation to differences in ranibizumab and aflibercept vascular endothelial growth factor suppression times. Br J Ophthalmol. 2016 Nov; 100(11):1494–1498. https://doi.org/10.1136/bjophthalmol-2015-308264 PMID: 26888975

52. Miao H, Tao Y, Li XX. Inflammatory cytokines in aqueous humor of patients with choroidal neovascularization. Mol Vis. 2012; 18:574–80. PMID: 22419849

53. Fox SE, Lu W, Maheshwari A, Christensen RD, Calhoun DA. The effects and comparative differences of neutrophil specific chemokines on neutrophil chemotaxis of the neonate. Cytokine. 2005 Feb 7; 29 (3):135–40. Epub 2004 Dec 8. https://doi.org/10.1016/j.cyto.2004.10.007 PMID: 15613281

54. Wuyts A, Schutyser E, Menten P, Struyf S, D'Haese A, Bult H, et al. Biochemical and biological characterization of neutrophil chemotactic protein, a novel rabbit CXC chemokine from alveolar macrophages. Biochemistry. 2000 Nov 28; 39(47):14549–57. PMID: 11087410

55. Parmar T, Parmar VM, Arai E, Sahu B, Perusek L, Maeda A. Acute Stress Responses Are Early Molecular Events of Retinal Degeneration in Abca4-/-Rdh8-/- Mice After Light Exposure. Invest Ophthalmol Vis Sci. 2016 Jun 1; 57(7):3257–67. https://doi.org/10.1167/iovs.15-18993 PMID: 27315541

56. Marquioni-Ramella MD, Suburo AM. Photo-damage, photo-protection and age-related macular degeneration. Photochem Photobiol Sci. 2015 Sep 26; 14(9):1560–77. https://doi.org/10.1039/c5pp00188a PMID: 26198091

57. Zehetner C1, Kirchmair R, Neururer SB, Kralinger MT, Bechrakis NE, Kieselbach GF. Systemic upregulation of PDGF-B in patients with neovascular AMD. Invest Ophthalmol Vis Sci. 2014 Jan 20; 55 (1):337–44. https://doi.org/10.1167/iovs.13-12978 PMID: 24334449

58. Cao S, Ko A, Partanen M, Pakzad-Vaezi K, Merkur AB, Albiani DA et al. Relationship between systemic cytokines and complement factor H Y402H polymorphism in patients with dry age-related macular degeneration. Am J Ophthalmol. 2013 Dec; 156(6):1176–83. https://doi.org/10.1016/j.ajo.2013.08.003 PMID: 24083687

59. Hong HS, Kim S, Nam S, Um J, Kim YH, Son Y. Effect of substance P on recovery from laser-induced retinal degeneration. Wound Repair Regen. 2015 Mar-Apr; 23(2):268–77. https://doi.org/10.1111/wrr.12264 PMID: 25682893

60. Huang H, Liu Y, Wang L, Li W. Age-related macular degeneration phenotypes are associated with increased tumor necrosis-alpha and subretinal immune cells in aged Cxcr5 knockout mice. PLoS One. 2017 Mar 10; 12(3):e0173716. https://doi.org/10.1371/journal.pone.0173716 PMID: 28282423

61. Kutty RK, Samuel W, Abay R, Cherukuri A, Nagineni CN, Duncan T, et al. Resveratrol attenuates CXCL11 expression induced by proinflammatory cytokines in retinal pigment epithelial cells. Cytokine. 2015 Aug; 74(2):335–8. https://doi.org/10.1016/j.cyto.2015.03.016 PMID: 25890876

62. Shevchenko AV, Prokof'ev VF, Konenkov VI. [Cytokine gene polymorphisms in patients with age-related macular degeneration]. Vestn Oftalmol. 2016 Mar-Apr; 132(2):8–13. https://doi.org/10.17116/oftalma201613228-13 PMID: 27213791

63. Shen D, Cao X, Zhao L, Tuo J, Wong WT, Chan CC. Naloxone ameliorates retinal lesions in Ccl2/Cx3cr1 double-deficient mice via modulation of microglia. Invest Ophthalmol Vis Sci. 2011 May 2; 52 (6):2897–904. https://doi.org/10.1167/iovs.10-6114 PMID: 21245403

64. Sharma R, Kapila R, Kapasiya M, Saliganti V, Dass G, Kapila S. Dietary supplementation of milk fermented with probiotic Lactobacillus fermentum enhances systemic immune response and antioxidant capacity in aging mice. Nutr Res. 2014 Nov; 34(11):968–81. https://doi.org/10.1016/j.nutres.2014.09.006 PMID: 25311611

65. Machado-Silva W, Henriques AD, Souza GD, Gomes L, Ferreira AP, Brito CJ, et al. Serum Immune Mediators Independently Associate with Atherosclerosis in the Left (But Not Right) Carotid Territory of Older Individuals. J Stroke Cerebrovasc Dis. 2016 Dec; 25(12):2851–2858. https://doi.org/10.1016/j.jstrokecerebrovasdis.2016.07.047 PMID: 27554076

66. Bartlett DB, Firth CM, Phillips AC, Moss P, Baylis D, Syddall H, et al. The age-related increase in low-grade systemic inflammation (Inflammaging) is not driven by cytomegalovirus infection. Aging Cell. 2012 Oct; 11(5):912–5. https://doi.org/10.1111/j.1474-9726.2012.00849.x PMID: 22708923

67. Sakamoto S, Takahashi H, Tan X, Inoue Y, Nomura Y, Arai Y, et al. Changes in multiple cytokine oncentrations in the aqueous humour of neovascular age-related macular degeneration after 2 months of ranibizumab therapy. Br J Ophthalmol. 2017 Aug 1. pii: bjophthalmol-2017-310284

68. Apte RS, Richter J, Herndon J, Ferguson TA. Macrophages Inhibit Neovascularization in a Murine Model of Age-Related Macular Degeneration. PLoS Med. 2006 Aug; 3(8):e310. https://doi.org/10.1371/journal.pmed.0030310 PMID: 16903779

69. Nakamura R, Sene A, Santeford A, Gdoura A, Kubota S, Zapata N, et al. IL10-driven STAT3 signalling in senescent macrophages promotes pathological eye angiogenesis. Nat Commun. 2015 Aug 11; 6:7847. https://doi.org/10.1038/ncomms8847 PMID: 26260587

70. Silvestre JS, Mallat Z, Duriez M, Tamarat R, Bureau MF, Scherman D, et al. Antiangiogenic effect of interleukin-10 in ischemia-induced angiogenesis in mice hindlimb. Circ Res. 2000 Sep 15; 87(6):448–52. PMID: 10988235