



Tear cytokines as potential biomarkers in non-infectious uveitis: post hoc analysis of a randomised clinical trial

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Abstract

Purpose The study aimed to evaluate cytokines in tears as potential biomarkers in non-infectious uveitis.

Methods Tear samples were obtained using Schirmer strips from 50 patients enrolled in a randomised controlled study of simvastatin in non-infectious uveitis and from a control group of 30 healthy participants. The concentrations of IL-6, IL-8, IL-10, and IFN- γ in tears were measured at the study's baseline and again after 8 weeks of treatment using commercial enzyme-linked immunosorbent assay kits.

Results The concentrations of tears IL-6 and IL-10 were increased in patients with non-infectious uveitis in comparison with the healthy participants. Longer disease duration was associated with elevated levels of IL-6. The concentrations of the studied cytokines in tears did not correlate with the extent of eye inflammation at baseline. No link between the changes in cytokine levels and changes in clinical parameters during treatment was observed. Baseline IL-10 concentrations independently predicted the development of the clinical response to treatment at weeks 4 and 8.

Conclusion Although elevated in non-infectious uveitis patients, tear cytokine levels do not correlate with eye inflammation and are not sensitive to change after treatment. However, the level of IL-10 may be a predictive biomarker of response to the treatment of uveitis.

Trial registration NCT04183387

Keywords Uveitis · Autoimmune · Non-infectious · Hydroxymethylglutaryl-CoA reductase inhibitors · Statins · Cytokines · Tears

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Introduction

Non-infectious uveitis is a significant health problem that contributes up to 10% of cases of total blindness [1] and has a socioeconomic impact at least as pronounced as that of diabetic retinopathy [2].

Given the methods for assessing uveitis activity are primarily subjective and lack discrimination and reliability, there is a need for biomarkers reflecting changes in the extent of eye inflammation in patients with uveitis [3]. Finding predictive biomarkers of uveitis would allow identification of patients who are less likely to respond to conventional treatments and who would benefit from early, aggressive treatment.

It is known that cytokines play a substantial role in inflammation in general and in local eye inflammation in non-infectious uveitis. In many studies, eye inflammation has been associated with an increase in pro-inflammatory cytokines in the serum and aqueous humour [4, 5]. Thus, cytokines might be potential biomarkers for non-infectious uveitis. However,

the poor acceptability of cytokines in the aqueous humour limits this approach. Compared with the aqueous humour or vitreous samples, tear samples are more conveniently collected and so tear cytokines may be feasible biomarkers for uveitis. There is a lack of studies evaluating tear cytokines as biomarkers of eye inflammation or as predictive biomarkers of response to treatment in non-infectious uveitis patients.

The aims of the present study were to (a) compare the pro-inflammatory and anti-inflammatory cytokine levels in the tears of non-infectious uveitis patients and healthy participants; (b) evaluate clinical and demographic factors associated with the concentrations of cytokines in tears; (c) report changes in the cytokine levels of tears following uveitis treatment; and d) evaluate the possibility of using tear cytokines to predict clinical responses to uveitis treatment.

Methods

Healthy subjects

A group of 30 healthy medical students was used for this study. The exclusion criterion was any inflammatory disease of the eye.

Patients

The patients with uveitis were from the study evaluating the effects of simvastatin on eye inflammation in patients receiving conventional treatment. The conventional treatment consisted of local (one or both of eye drops and periorbital injections) and systemic corticosteroids, prescribed at the physician's discretion. The study was registered on the [Clinicaltrials.gov](https://clinicaltrials.gov) database (NCT04183387).

Details of the study protocol and the clinical results of the study are reported elsewhere [6]. Briefly, in this open-label study, 50 patients with acute non-infectious uveitis were randomised to receive, for 2 months, either conventional treatment alone or conventional treatment plus 40 mg/day of simvastatin. The clinical parameters used were slit-lamp anterior chamber cells, anterior chamber flare, vitreous haze grade, intraocular pressure, and estimation of best-corrected visual acuity in logMAR format. In the case of bilateral uveitis, the most affected eye was selected for the assessments.

The response to treatment was defined as follows:

- (1) ≤ 0.5 + anterior chamber cells, ≤ 0.5 + vitreous haze, and no active retinal/choroidal lesions; and
- (2) ≤ 10 mg of oral prednisone daily and ≤ 2 drops of prednisolone acetate 1% (or equivalent) a day.

The study was approved by the Independent Ethics Committee of Municipal City Hospital No. 1, Novosibirsk,

and was conducted in accordance with the Declaration of Helsinki; all subjects provided written informed consent.

Tear sample collection

The tear samples were collected as previously described. The investigator, with gloves, placed a Schirmer strip over the lid margin at the junction of the lateral and middle thirds of the lower eyelids; it was kept in place for 5 min while subjects closed their eyes without an anaesthetic. The Schirmer strips were removed with gloves and tear volume was recorded in millimetres. Each Schirmer strip was placed into a sterile 2-mL centrifuge tube, stored on ice for between 20 min and 1 h, and then stored at minus 20 °C until processed [7].

Tear extraction from Schirmer strips

The method described previously was used to extract tears from the Schirmer strips [7]. In brief, a standard curve was first developed to calculate millimetres of wetting from the volume of tears collected. A known volume of phosphate-buffered saline (PBS) was placed on a Schirmer strip and wetting in millimetres was recorded after 1 min. This process was repeated a total of 3 times for each volume tested, and a standard curve for volume to Schirmer strip reading was generated.

Tubes containing a strip with a tear sample were warmed to ambient temperature and were incubated with an assay buffer (200 μ L) for 3 h. Before discarding the Schirmer strips, residual liquid was removed and combined with the stored extraction buffer.

Detection of cytokines using an enzyme-linked immunosorbent assay

The multiple-antigen detection method developed by Wakefield et al. [8] was used to detect cytokines in the samples. Following this method, 110 μ L of the diluted tears was placed on an antigen-capture enzyme-linked immunosorbent assay (ELISA) plate, where specific antigen from the sample were captured while other cytokines and chemokines remained in the solution. After incubation, assuming that approximately 5 μ L of sample volume would be lost during transfer, the samples (105 μ L) were retrieved from the ELISA plate and placed on the next antigen-capture ELISA plate. The ELISA analyses were performed using commercial ELISA kits (IL-6, IL-8, IL-10, TNF α , and IFN- γ ELISA-Best, Vector-Best, Russia). Based on the manufacturer's instructions, the detection limits for IL-6, IL-8, IL-10, TNF α , and IFN- γ were 0.5, 2, 2.5, 2, and 5 pg/mL. Concentrations below these thresholds were considered non-detects. The concentrations of the detected cytokines in tear samples were then calculated based on the volume of tears in the sample and the

volume of added assay buffer (200 μ L) used for the cytokine extraction.

Statistical analysis

As is often the case for immunological data, cytokine values featured a substantial proportion of non-detects. For a given cytokine, concentrations below the reporting threshold were considered non-detectable and therefore left-censored. Tobit regression, a recommended statistical approach for left-censored data [9], was performed for each cytokine separately. This method allows adjustment for the effects of potential confounders such as age and sex. Summary statistics and regression equations for the left-censored data were computed using maximum likelihood estimation (MLE) [10]. Using MLE allows data to be analysed with up to 80% of censored values [11]. Using R package censReg version 0.5-26 [12], both the Student *t* test and censored regressions with and without potential confounders were performed to test the differences in cytokine levels between the control group and the patients. Multiple Tobit regression models were used to evaluate factors associated with baseline concentrations of cytokines in tears.

The changes before and after treatment were assessed using paired Student's *t* test. The correlations between changes in tear cytokines and changes in clinical parameters were assessed using Spearman correlation test. The differences in baseline tear cytokine concentrations between patients who responded and who did not respond to treatment were assessed using both the Student *t* test as well as adjusted and non-adjusted censored regression models.

When adjusting for confounders, the usual rule of thumb of ten was not adhered to because it mostly concerns logistic regression analysis; in linear regression, the number of participants for one confounder may be significantly lower [13].

All statistical analyses were performed using the R software (version 3.5.1).

Results

Baseline demographic and clinical characteristics of the patients are presented in Table 1. Most patients had anterior uveitis. Almost half of the patients were HLA-B27-positive. More than 50% of patients had a diagnosis of underlying systemic disease, primarily ankylosing spondylitis. The mean age of the control group was 20.7 (1.2) years; 17 (57%) were male and 13 (43%) were female. The patients did not receive any systemic treatment at the time of baseline tear collection. There were no instances of decreased tear production in either the patient or control group.

Overall, baseline percentages of censored values for IL-6, IL-8, IL-10, TNF α , and IFN- γ were 51.25%, 1.25%, 73.75%,

Table 1 Baseline characteristics of patients

	<i>n</i> = 50
Age (years)	43.98 (14.90)
Gender	
Male	28 (56.0)
Female	22 (44.0)
Duration of uveitis (years)	3.90 (2.18)
Bilateral uveitis	3 (6.0)
Anatomical location of uveitis	
Anterior uveitis	45 (90.0)
Intermediate uveitis	2 (4.0)
Posterior uveitis or panuveitis	3 (6.0)
HLA B27	24 (48)
Underlying diagnosis	
Ankylosing spondylitis	23 (46)
Behcet's disease	1 (2)
Juvenile idiopathic arthritis	4 (8)
Psoriatic arthritis	1 (2)
Reiter disease	3 (6)
Intraocular pressure (mm Hg)	17.82 (3.29)
Schirmer test (mm)	20.74 (6.64)
Visual acuity, logMAR	1.09 (0.86)
Visual field loss	13 (26.0)
Anterior chamber cells	
0+	1 (2.0)
0.5+	1 (2.0)
1+	13 (26.0)
2+	17 (34.0)
3+ or more	18 (36.0)
Anterior chamber flare	
0+	2 (4.0)
1+	2 (4.0)
2+	15 (30.0)
3+ or more	31 (62.0)
Posterior synechia	48 (96.0)
Lens opacities	1 (2.0)
Vitreous haze	
0+	13 (26.0)
1+	28 (56.0)
2+ or more	9 (18.0)
Involvement of the retina and choroidea	3 (6.0)

Continuous variables are presented as mean (SD), categorical variables are presented as number (%)

97.5%, and 100%, respectively. The percentages of censored values on the last visit for IL-6, IL-8, IL-10, TNF α , and IFN- γ were 36%, 0%, 80%, 94%, and 96%. Therefore, data from TNF α and IFN- γ were excluded from all further analyses.

Table 2 presents a comparison of tear cytokine concentrations between the patients with uveitis and the healthy

Table 2 Baseline tear cytokines in patients with uveitis in comparison with healthy controls

Cytokine	Patients with uveitis (<i>n</i> = 50)	Healthy controls (<i>n</i> = 30)	<i>t</i> ^a	<i>P</i> value ^a	<i>t</i> ^b	<i>P</i> value ^b	<i>t</i> ^c	<i>P</i> value ^c
IL-6 (pg/mL)	114.80 (178.03)	16.52 (85.97)	3.31	0.001	4.24	< 0.001	2.58	0.01
IL-8 (pg/mL)	916.22 (1329.86)	424.34 (578.32)	2.28	0.03	2.02	0.044	0.72	0.47
IL-10 (pg/mL)	45.43 (71.53)	8.60 (27.10)	3.27	0.002	2.6	0.01	2.88	0.004

Cytokine concentrations are presented as mean (SD)

^a Student's *t* test

^b Tobit regression taking censoring into account

^c Tobit regression taking censoring into account and adjusting for sex and age

participants. Uveitis patients showed a sevenfold increase in concentration for IL-6, a twofold increase for tear IL-8, and a fivefold increase for IL-10 using both the Student *t* test and Tobit regression modelling adjusting for left-censoring. However, after adjustment for demographics, only the differences in IL-6 and IL-10 concentrations remained statistically significant.

Neither demographic characteristics nor signs of ocular inflammation were linked with tear cytokine concentrations. Among the chosen predictor variables, only the duration of uveitis was associated with higher concentrations of IL-6 and IL-8 at baseline. The β -coefficients 49 and 235 indicated that every year spent with uveitis was associated with a 49-pg/mL average increase in the concentration of IL-6 and a 235-pg/mL average increase in IL-8 (Table 3).

The treatment of uveitis did not result in significant changes in IL-6, IL-8, and IL-10 concentrations. After stratification for simvastatin use, the patient control group showed a significant decrease in IL-6 levels (Table 4). No correlations were found between changes in cytokine concentrations and changes in clinical measures (Table 5).

Table 6 presents the differences in baseline tear cytokine levels between patients who responded and those who did not respond to the treatment. The responders at week 4 and week 8 showed fourfold and ninefold higher baseline concentrations

of IL-10 in the tear samples. These associations remained after adjustments for left-censoring and clinical covariates (Table 6).

Discussion

This study found increased concentrations of IL-6 and IL-10 in the tears of patients with uveitis compared with healthy control subjects. Increases in IL-6 levels were associated with longer disease duration. No link between changes in tear cytokine concentrations and changes in eye inflammation during the treatment was found. The baseline IL-10 was a significant and independent predictor of clinical response to treatment.

Most studies evaluating cytokines in uveitis have sampled from one or both of the aqueous humour and serum. Carreno et al., in a cross-sectional study investigating tear cytokines in uveitis, measured the levels of 21 molecules using multiplex bead-based assays [14]. This study's observations match some of those made by Carreno et al. While the percentages of IL-8 and IL-10 detects were similar in both studies, this study detected IL-6 in more samples (50% compared with 20%). In both studies, TNF α and IFN- γ were not detected in almost 100% of the tear samples. Similar to Carreno et al., we found a significant increase in tear IL-8 levels in uveitis patients in

Table 3 Factors associated with tear concentrations of cytokines. Results from multiple censored regression (Tobit) models

	IL-6		IL-8		IL-10	
	β -Coefficient	<i>P</i> value	β -Coefficient	<i>P</i> value	β -Coefficient	<i>P</i> value
Age (years)	0.79	0.73	13.68	0.39	-3.46	0.15
Gender	70.38	0.23	310.90	0.47	-59.72	0.31
Duration of uveitis (years)	49.43	<i>0.001</i>	235.20	<i>0.04</i>	2.76	0.86
Intermediate uveitis	-799.48	0.87	-383.93	0.71	-572.84	0.91
Posterior uveitis	-41.83	0.75	236.61	0.78	-14.59	0.91
Intraocular pressure (mmHg)	1.68	0.86	-13.51	0.83	-8.19	0.43
Anterior chamber cells	91.53	0.07	-3.14	0.99	-49.80	0.37
Anterior chamber flare	-42.47	0.41	-3.71	0.99	11.45	0.83

Italicized *P*-values are statistically significant (*P*<0.05)

Table 4 Changes in tear cytokine concentrations before and after treatment

Whole group, <i>n</i> = 50			
	Before treatment	After treatment	<i>P</i> value
IL6 (pg/mL)	115.42 (177.63)	186.60 (802.86)	0.54
IL8 (pg/mL)	916.22 (1329.86)	876.45 (1139.62)	0.88
IL10 (pg/mL)	59.45 (66.78)	52.53 (97.81)	0.68
Control group, <i>n</i> = 25			
	Before treatment	After treatment	<i>P</i> value
IL6 (pg/mL)	150.71 (227.84)	42.74 (44.77)	0.03
IL8 (pg/mL)	1229.43 (1777.26)	827.80 (879.33)	0.33
IL10 (pg/mL)	42.20 (44.99)	49.03 (93.22)	0.74
Simvastatin group, <i>n</i> = 25			
	Before treatment	After treatment	<i>P</i> value
IL6 (pg/mL)	80.13 (99.57)	330.47 (1127.34)	0.28
IL8 (pg/mL)	603.01 (497.74)	925.11 (1368.74)	0.3
IL10 (pg/mL)	76.71 (80.37)	56.03 (104.00)	0.44

comparison with healthy control subjects using non-adjusted models, but this was not confirmed in models adjusted for age and sex. In contrast with Carreno et al., this study used statistical techniques accounting for censoring and was able to perform further analyses on IL-10.

This study and that of Carreno et al. may differ in findings because almost half of the patients in the latter study received immunomodulatory agents at the time of the assessment, while patients in this study were treatment-free at the baseline.

The results of this study also concur with the findings of Türkçüoğlu et al., showing no association between pro-inflammatory cytokine IL-2 concentrations in tears and disease activity in patients with Behcet disease [15]. In another study, several cytokines in the tears of paediatric patients with non-infectious uveitis were found [16]. The detected cytokines differed from those in this study; this can be explained by the differences in cytokine profiles in adult patients with non-infectious uveitis and paediatric patients with non-infectious uveitis [17].

The findings indicate that duration of chronic eye inflammation rather than intensity is associated with higher pro-

inflammatory tear cytokine levels. Therefore, IL-6 and IL-8 concentrations may be biomarkers of the cumulative burden of eye inflammation. On the other hand, IL-6 contributes to the transition from acute to chronic inflammation by changing the nature of leucocyte infiltrate and by stimulating T and B cells [18]; it may, therefore, be a marker of uveitis chronicity. The absence of changes in cytokine concentrations after treatment and the lack of association between changes in clinical parameters indicate that the tear cytokines studied may not be useful as disease activity biomarkers. These findings accord with the results of Carreno et al. who did not find any difference in tear cytokines between patients with active and non-active uveitis [14]. This can also be explained by the underdiagnosis of inflammation in patients with uveitis described by clinical information as inactive.

Numerous studies have assessed cytokine levels in the serum and aqueous humour of patients with uveitis. Similar to this study, higher IL-6 concentrations were found in the vitreous fluid of chronic uveitis patients than in that of patients without uveitis [19]. In addition, targeting IL-6 seems to be a promising strategy for the treatment of autoimmune uveitis [20]. In one study, serum IL-8 levels were higher in patients with non-infectious uveitis and associated with disease activity [21]. A study demonstrated that serum and aqueous humour IL-10 concentrations were increased in uveitis patients [22]. This study's findings suggest that tear IL-6 and IL-10 levels may parallel the concentrations of these cytokines in the aqueous humour and serum.

TNF α plays an important role in the pathogenesis of uveitis, as evidenced by the efficacy of the anti-TNF blockade. Increased TNF α concentrations were found in both the serum and aqueous humour of patients with uveitis [23]—but were rarely detected in tear samples—in both this study and that of Carreno et al.—indicating that tear TNF α levels do not reflect the concentrations within the eye.

In another previous study, IFN- γ concentrations were found to be higher in the aqueous humour and serum of uveitis patients. Moreover, elevated serum IFN-gamma levels seemed to predispose the patient to a more serious loss of vision [22]. The low frequency of IFN- γ detection in this study indicates there might be no link between the concentration of IFN- γ in tears and the intraocular levels.

Table 5 Correlations between changes in tear cytokines and changes in clinical parameters

	Anterior chamber cells		Anterior chamber flare		Intraocular pressure		Vitreous haze		Visual acuity, logMAR	
	Spearman <i>r</i>	<i>P</i> value	Spearman <i>r</i>	<i>P</i> value	Spearman <i>r</i>	<i>P</i> value	Spearman <i>r</i>	<i>P</i> value	Spearman <i>r</i>	<i>P</i> value
IL6	0.19	0.19	0.16	0.28	−0.06	0.69	−0.18	0.21	−0.05	0.75
IL8	0.08	0.57	0.11	0.45	−0.01	0.95	−0.01	0.92	0.03	0.82
IL10	−0.07	0.64	0.01	0.96	−0.22	0.12	0	1.00	0.01	0.96

Table 6 Relationship between baseline tear cytokine concentrations and response to treatment

Cytokine (pg/mL)	Baseline tear cytokine concentrations		<i>t</i> ^a	<i>P</i> value ^a	<i>t</i> ^b	<i>P</i> value ^b	<i>t</i> ^c	<i>P</i> value ^c
	Week 4							
	Non-responders (<i>n</i> = 23)	Responders (<i>n</i> = 27)						
IL6	102.67 (176.57)	125.13 (181.96)	0.44	0.66	0.78	0.43	1.12	0.26
IL8	1016.05 (1416.08)	831.18 (1272.76)	0.48	0.63	0.49	0.63	0.39	0.7
IL10	17.80 (42.05)	68.96 (83.04)	2.8	<i>0.01</i>	2.56	<i>0.009</i>	2.55	<i>0.01</i>
	Week 8							
	Non-responders (<i>n</i> = 15)	Responders (<i>n</i> = 35)						
IL6	125.55 (210.44)	110.19 (165.43)	0.25	0.783	0.005	0.99	1.15	0.25
IL8	1186.38 (1661.34)	800.44 (1168.88)	0.81	0.352	0.94	0.35	0.33	0.74
IL10	6.38 (24.72)	62.16 (78.49)	3.8	<i>0.01</i>	2.67	<i>0.008</i>	2.4	<i>0.017</i>

Italicized *P*-values are statistically significant (*P*<0.05)

Cytokine concentrations are presented as mean (SD)

^a Student's *t* test

^b Tobit regression taking censoring into account

^c Tobit regression taking censoring into account and adjusting for sex, age, anterior chamber cells, and anterior chamber flare

Also, matching this study's findings, another study observed no changes in IL-6 and IL-10 levels in the aqueous humour during treatment of uveitis with the anti-TNF blocker adalimumab [24]. This suggests that cytokines have low sensitivity to change and therefore might not be a useful biomarker of changes in uveitis activity.

This study found the IL-10 level in tears could potentially predict responses to simvastatin treatment. This finding can be explained by the immunoregulatory effects of statins: statins were shown to induce immune tolerance [25], while IL-10 is necessary for the development of induced immune tolerance in experimental uveitis [26]. Therefore, it can be speculated that statins can exhibit anti-inflammatory effects and induce immunological tolerance in the presence of increased concentrations of IL-10 in patients with uveitis.

The main limitation of this study is the low number of cytokines studied.

The strength of this study was its longitudinal design and the use of statistical methods accounting for censoring inherent in the measurement of cytokine concentrations in biological fluids. The baseline evaluation of treatment-free patients allowed the exclusion of a potentially substantial confounder, the influence of immunosuppressive treatment on cytokine production.

Conclusion

The cytokines studied in tears do not appear to be potential biomarkers of disease activity in non-infectious uveitis and are not sensitive to change. The level of IL-10 in tears has potential utility as a predictive biomarker of response to treatment.

Author contributions All authors contributed to the study conception and design. Material preparation and data collection were performed by Anastasia Biruukova and Natalia Kalinovskaya; analyses were performed by Ivan Shirinsky and Valery Shirinsky. The first draft of the manuscript was written by Ivan Shirinsky, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the LEC attached to Municipal City Hospital No. 1, Novosibirsk, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

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