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ORIGINAL ARTICLE

IgE Ratio in Tears: A Predictive Tool of Ocular Allergic Inflammation

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ABSTRACT

Purpose: To demonstrate the tear IgE (measured/exuded) ratio (R) as a useful biological marker of ocular allergy in order to distinguish severe from less severe inflammatory status.

Methods: Tear samples and sera from 78 ocular allergy patients and 19 control subjects were analyzed. Total IgE and albumin were measured for calculating the tear IgE-R defining two subgroups (SG) of samples: R $\geq$ 4-SG and R < 4-SG. Eosinophil cationic protein, Th1 and Th2 cytokines (IFN- γ , IL-4, -5, -6, -8 and -10) and protein electrophoretic profiles were also investigated in tears.

Results: The R < 4-SG compared to the R $\geq$ 4-SG shows higher levels of tear albumin, eosinophil cationic protein, and Th1 and Th2 cytokines. Moreover, each subgroup presents a specific protein profile.

Conclusion: This study showed that an IgE-R lower than four must be carefully interpreted as a warning sign of a severe inflammatory context and should be also associated with an exploration of immunological profile.

Keywords: IgE ratio, inflammation, ocular allergy, tear protein profile, tears

In 2001 the European Academy of Allergy and Clinical Immunology (EAACI) proposed an ocular hypersensitivity (OHS) nomenclature based on the distinction between allergic and nonallergic OHS.^{1,2} OHS was further divided into IgE- and non-IgE-mediated and approved by the World Allergy Organization (WAO) in 2004, for any allergic event and independently of the target organ.^{3,4} In 2012, on the basis of this nomenclature, Leonardi et al. proposed a new classification of ocular atopies⁵ summarized in Figure 1. Seasonal allergic conjunctivitis (SAC) and perennial allergic conjunctivitis (PAC) are similar forms of mild severity and are not sight-threatening because neither SAC nor PAC has corneal involvement.⁵ They are enhanced by the

successive reintroduction or the permanent exposure to a similar allergen generating a *de novo* IgE synthesis and the release of inflammatory mediators such as histamine, tryptase, leukotriene, and cytokines.⁶ Vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC) are severe forms and are IgE-mediated in 50% of cases.⁵ These persistent and chronic forms of ocular allergy (OA) could induce corneal damage and be vision-threatening. The diagnosis of OA is proposed on the basis of clinical history with signs and symptoms. According to the diagnostic algorithm, a set of laboratory tests is proposed.⁷ Among them, tear fluid protein analysis could provide a battery of potential targets for monitoring the pathology and providing a better

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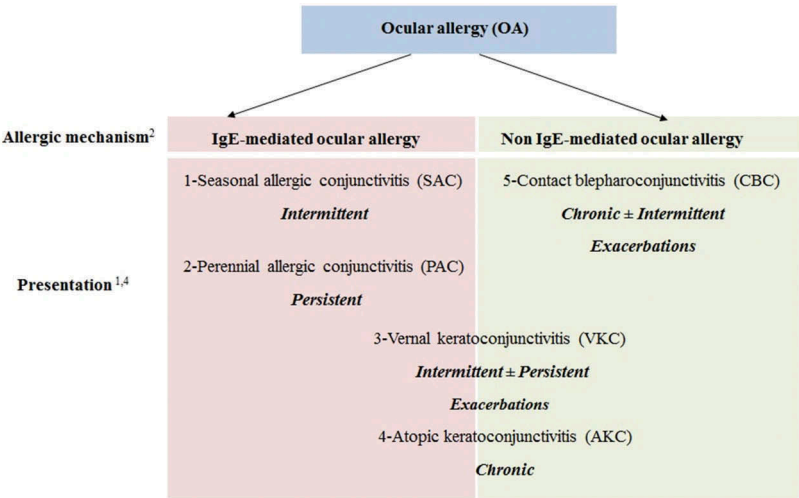


FIGURE 1. Classification and nomenclature of ocular allergy syndromes. Five major ocular allergy syndromes: seasonal allergic conjunctivitis (SAC); perennial allergic conjunctivitis (PAC); vernal keratoconjunctivitis (VKC); atopic keratoconjunctivitis (AKC); contact blepharoconjunctivitis CBC.

pathophysiological understanding. Using ELISA, multiplex bead arrays and proteomic techniques, several mediators, such as total and specific IgE, protease enzymes, chemokines, and cytokines have been identified.⁶ Increased levels of tear IgE as well as eosinophil cationic protein (ECP) have been considered as a disease marker of OA^{6,8–11} and tear ECP was described as a useful marker of severity in AKC¹².

To assess a local IgE production in tears, an indirect evaluation using a tear IgE ratio (R) described by Liotet¹³, can be used. This ratio is calculated as the relation of the measured IgE level (m-IgE) divided by the exuded IgE level (e-IgE) into the tears according to the following formula: $R = m\text{-IgE}/e\text{-IgE}$. A ratio higher or equal to 4 is indicative of a local synthesis of IgE. To assess the robustness and usefulness of this ratio determination in the diagnosis of OA, three technical approaches: fluoroenzyme immunoassay, multiplex bead-based immunoassays, and capillary electrophoresis – were used to quantitate ECP and cytokines, and to identify the tear protein profile, respectively. The aim of this study was to consolidate the measured/exuded tear IgE ratio, with the ECP level, the Th1/Th2 cytokines and the main tear proteins in order to improve the comprehension of the tear inflammatory mediator context.

MATERIAL AND METHODS

Collection of Tears and Serum

This study was performed on 78 patients with OA referred to the medical laboratory of the Centre Hospitalier National d’Ophtalmologie des Quinze-Vingts, who exhibited a significant level of IgE in tears (>0.15 kU/L). Serum was collected from all patients and tears were collected from these patients in both eyes

except for one patient, corresponding to 78 sera and 155 tear samples. As emphasized by Leonardi⁶, in many cases of allergic conjunctivitis there is a unilateral predominance. Furthermore, each eye may be considered as a unique immunological entity.¹⁴ For these reasons, tears were collected from both eyes. The patients had not been using any topical drug or antiallergic treatment for at least 2 weeks before sampling. Serum and tear samples were collected with the patient’s agreement. Nineteen healthy subjects constituted the control (CTL) group. Demographic data are shown in Table 1. Healthy subjects did not have any history, sign or symptom of allergic or ocular surface disease. Tears were gently collected from the external canthus using a sterile microcapillary plastic pipette (CML, Labelians, France), avoiding reflex tearing as much as possible. For each subject, tears were collected from both eyes as far as possible. The mean volume of tears collected was between 70 and 80 µl. Tears were then immediately expelled from the pipettes into polypropylene vials and centrifuged at 4000 g for 15 min at room temperature (RT). Supernatants were stored at –20°C until further analysis. Blood samples were collected using humeral venipuncture using a 21-gauge butterfly needle into a serum vacutainer tube, then centrifuged at 4000 g for 10 min at RT, to collect serum.

TABLE 1. Demographic table of the population included in the study. Age of subjects is represented as mean and standard deviation.

	Allergic group	Control group
No. of subjects	78	19
Sex (females:males)	25:53	9:10
Age (years)	45 ± 18	24 ± 6
No. of serum samples	78	-
No. of tear samples	155	19

Quantitation of IgE and Albumin in Serum and Tear Samples

Total IgE in serum and tear samples were measured using an ultrasensitive enzyme-linked immunosorbent assay (ELISA) with a final reading of the fluorescence using Unicap 100® (ThermoFisher, France). Briefly, the anti-IgE, covalently coupled to the ImmunoCap reaction vessel, reacts with the total IgE in the sample. After washing, enzyme-labeled antibodies against IgE were added to form a complex. After incubation, unbound enzyme-anti-IgE antibodies were washed away and the bound complex was then incubated with a developing agent. After stopping the reaction, fluorescence was measured, which is directly proportional to the concentration of IgE in the sample. Tear- and serum-albumin were measured using nephelometric immunodetection (Image 800®, Beckmann, Miami, FL, USA) according to the manufacturer's recommendations.

Tear IgE Ratio (R) Calculation and Subgroup Determination

This ratio is based on the calculation of the analytical measurement of IgE in tears (*m-IgE*) and the calculated rate of exuded IgE (*e-IgE*) from serum as follow $R = m\text{-IgE}/e\text{-IgE}$.

The *e-IgE* values are calculated according to the following formula:

$\text{Serum-IgE} [(S\text{-IgE}) \times (\text{Tear-Albumin (T-Alb)}/\text{Serum-Albumin (S-Alb)})] \times 0.65$ where '0.65' corresponds to the hydrodynamic immunoglobulin radius. According to a cut-off of '4' two subgroups (SG) were established.

Quantitation of Eosinophil Cationic Protein (ECP) in Tears

Tear ECP levels were determined using the fluoroenzyme immunoassay (ImmunoCAP®; ThermoFisher, France) in a volume of 40 µL of undiluted tear sample. The lower limit of detection (LOD) according to the manufacturer was 0.5 µg/L.

Quantitation of Cytokines in Tears Using Multiplex Bead Analysis

IL-4, IL-5, IL-6, CXCL-8, IL-10, and IFN-γ were measured, using microspheres as the solid support for immunoassay and according to the manufacturer's recommendations (MILLIPLEX® Map Kit High Sensitivity; Millipore, Germany). Briefly, tear samples were diluted twice in each well and incubated with the premixed antibody-coated beads overnight at 4°C.

Washed beads were further incubated with biotin-labeled anti-human cytokine antibody for 1 h at RT followed by streptavidin-phycoerythrin for 30 min at RT. Samples were analyzed using a Bioplex 200 system® (Bio-Rad, Marnes-la-Coquette, France). The LOD of these cytokines were: 1.83 pg/mL for IL-4, 0.49 pg/mL for IL-5, 0.18 pg/mL for IL-6, 0.31 pg/mL for CXCL-8, 1.46 pg/mL for IL-10 and 0.61 pg/mL for IFN-γ.

Proteomic Analyses of Tear Proteins and Immunoglobulins

Capillary Electrophoresis

Chip-based analyses were performed using the Agilent 2100 Bioanalyzer system (Agilent, Waldbronn, Germany) in combination with the Protein 230 LabChit kit, for sizing and quantifying protein samples from 14 to 230 kDa. All chips were prepared according to the manufacturer's recommendations and as previously described.¹⁵ Briefly, 4 µL of tears were diluted in sample buffer with 1 M dithiothreitol (DTT). The samples were denatured at 95°C for 5 min, and then loaded onto chips prefilled with the gel/dye mixture. Separated proteins were detected by laser-induced fluorescence. The software of the Agilent 2100 Bioanalyzer expresses each protein peak as a percentage versus total protein and for each sample gives a tear profile electropherogram associated with a gel-like image. The sample buffer contains an upper and lower marker of known molecular weight, which can be used for semi-quantitative analysis.

Western Blot Analysis: Detection of IgG, IgA, and Kappa (K) – Lambda (λ) Light Chains (LCs)

Twenty micrograms of tear proteins from samples were subjected to SDS-PAGE analysis on a 12% SDS polyacrylamide precast gel (Mini-Protean® TGX, Bio-Rad, France). Briefly, proteins were transferred onto a nitrocellulose membrane using the Turbo-Blotting® Transfer System (Bio-Rad) according to the manufacturer's recommendations. Membrane was successively incubated and detected using, with an anti-human IgG, anti-human IgA, and anti-human (K-λ) LCs horseradish peroxidase-conjugated antibodies (Sigma, St. Louis, MO, USA).

Data Analysis

Data are presented as mean ± standard error of the mean (SEM). Differences between groups were identified using the nonparametric Mann-Whitney U test. Spearman's rho correlation was used to examine the relationships between the targets detected in tears. *P*-value of less 0.05 was considered to be statistically significant. Statistical analysis was performed using GraphPad prism7 software (La Jolla, CA, USA).

RESULTS

IgE Ratio Determination: $R \geq 4$ -SG and $R < 4$ -SG

As expected by the formula of IgE Ratio ($R = m\text{-IgE}/e\text{-IgE}$), the $R < 4$ -SG displayed higher levels of serum IgE ($618 \text{ ku/L} \pm 138$ vs $374 \text{ ku/L} \pm 130$), but lower levels of measured tear IgE ($m\text{-IgE}$) ($5.85 \text{ ku/L} \pm 2.10$ vs $31.38 \text{ ku/L} \pm 20.08$) and slightly higher levels of exuded IgE ($e\text{-IgE}$) ($2.79 \text{ ku/L} \pm 0.74$ vs $2.00 \text{ ku/L} \pm 0.98$). Healthy subjects do not express $m\text{-IgE}$ in tears ($<0.15 \text{ ku/L}$). Likewise, $e\text{-IgE}$ calculation includes, among others, the levels of tear albumin (T-Alb), the results show that the $R < 4$ -SG displayed higher levels of T-Alb than the $R \geq 4$ -SG ($0.41 \text{ g/L} \pm 0.05$ vs $0.14 \text{ g/L} \pm 0.03$; $p < .001$, Figure 2). Healthy subjects displaying tear albumin values under 0.02 g/L . No difference was found for serum-albumin levels between the two subgroups.

Tear Cytokines and ECP Levels according to the IgE Ratio

Tears from healthy subjects contained detectable levels of cytokines IL-4, CXCL-8, IL-10, IFN- γ and ECP, and levels

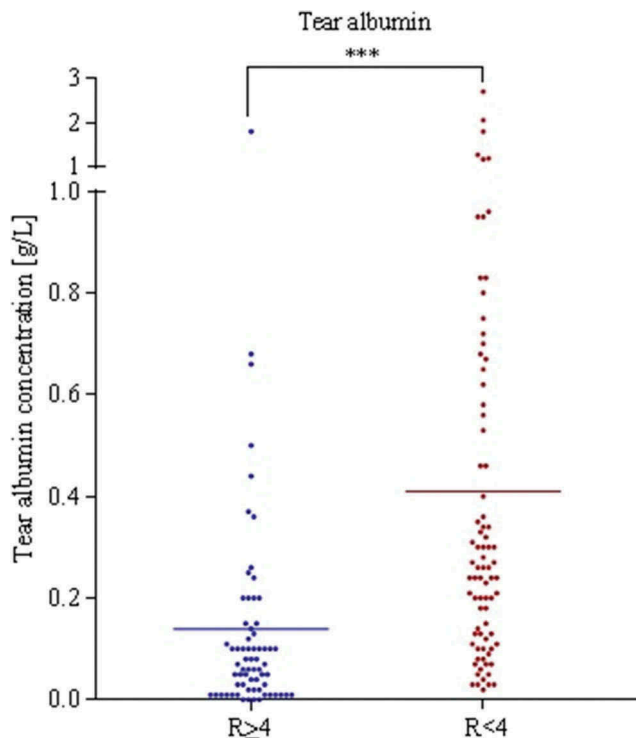


FIGURE 2. Tear albumin measurement according to the IgE ratio (R). Tear albumin levels in patients with $R < 4$ ($n = 87$; $0.40 \text{ g/L} \pm 0.04$) are higher than in patients with $R \geq 4$ ($n = 70$; $0.13 \text{ g/L} \pm 0.03$). Data are presented as mean $\pm$ SEM; nonparametric Mann-Whitney test was performed.

under the LOD for IL-5 and IL-6. All cytokines and ECP, except CXCL-8 (Figure 3(a,b)) were significantly up-regulated in the $R < 4$ -SG compared to the $R \geq 4$ -SG and CTL (Tables 2–3). In both subgroups, IL-4 was correlated with IFN- γ and IL-10 (Figure 4(a)). Specific correlations between levels of IL-10 vs IFN- γ and levels of IL-6 vs IL-4 were found only in the $R < 4$ -SG (Figure 4(b)). ECP and CXCL-8 showed considerable variations giving high standard deviations related to a substantial discrepancy between the minimum and maximum values: ECP ($0\text{--}2000 \text{ pg/mL}$) – CXCL-8 ($0\text{--}1400 \text{ pg/mL}$) for the $R < 4$ -SG. Interestingly, a significant correlation was found between ECP and CXCL-8 ($R = 0.63$; $p < .001$) for the $R < 4$ -SG.

Tear Proteins

Immunoglobulin light chains (Ig-LCs, Figure 5(a)), zinc- α 2-glycoprotein (ZAG-2, Figure 5(b)) and lysozyme-C (Lyso-C, Figure 5(c)) were significantly modulated in the $R < 4$ -SG. In this group, Ig-LCs increased compared to the $R \geq 4$ -SG ($4.16\% \pm 0.39$ vs $2.6\% \pm 0.29$), ZAG-2 decreased vs the CTL ($2.55\% \pm 0.26$ vs $3.20\% \pm 0.36$), and Lyso-C decreased compared to the $R \geq 4$ -SG as well as to the CTL with, respectively ($33.47\% \pm 0.73$ vs $37.45\% \pm 0.79$ vs $37.63\% \pm 2.62$). Lactoferrin was only modulated in the $R \geq 4$ -SG compared to the CTL ($24.35\% \pm 0.50$ vs $21.65\% \pm 0.70$, Figure 5(d)). Lipocalin-1 (Figure 5(e)) and IgA heavy chains (IgA-HCs, Figure 5(f)) did not show a significant difference between these groups. The values in percent versus total protein content, found in each group are presented for the main tear proteins in Table 4. Figure 6(a) shows an overlay of the electropherograms from two representative patients from the $R < 4$ -SG (red line) and the $R \geq 4$ -SG (blue line). The patient from the $R < 4$ -SG exhibits an increase peak with a shoulder between 59 and 62 kDa (peak no. 5) corresponding to albumin and the IgG-HCs presumably coeluted (10). Moreover, the peak at 26 kDa (peak no. 3) should correspond to LCs from IgG and IgA. Furthermore, the no significant correlation ($R = 0.12$) between the LCs and the IgA-HCs could be explained by the presence of second origin of LCs coming from IgG. Nevertheless, a significant correlation was found between the LCs and the IgA-HCs, for the $R \geq 4$ -SG ($R = 0.58$; $p < .0001$). To improve this corresponding coelution of IgG and IgA an immunodetection by western blot analysis was achieved. IgG was detected in 62.5% of tear samples in the $R < 4$ -SG tears versus 22.2% for the $R \geq 4$ -SG (Figure 6(b)). These results were significantly correlated to the increased rate of the LCs in the $R < 4$ -SG ($4.16\% \pm 0.39$) versus ($2.48\% \pm 0.28$) for the $R \geq 4$ -SG (Table 4). These findings are consistent with the electrophoretic profiles obtained with the bioanalyzer.

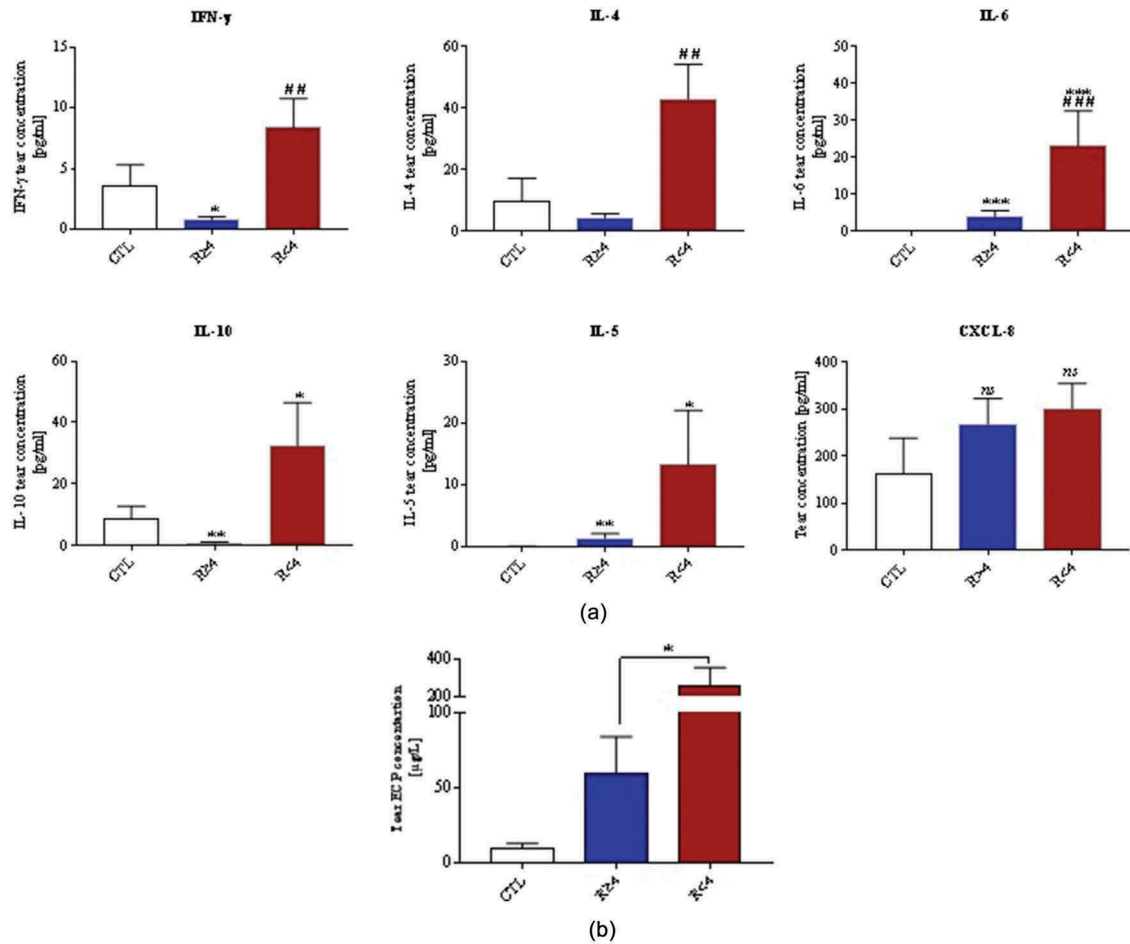


FIGURE 3. Cytokine and ECP quantitation in tears. A. Tear cytokine levels of IFN- γ , IL-4, IL-6, IL-10, IL-5, and CXCL8. $p^* < 0.05$, $p^{**} < 0.01$ and $p^{***} < 0.001$, between R-SG and CTL. $p^{\#} < 0.05$, $p^{\#\#} < 0.01$ and $p < 0.001$, between R < 4-SG and R $\geq$ 4-SG. Data are presented as mean and SEM; nonparametric Mann-Whitney test was performed. B. Eosinophil cationic protein (ECP) quantitation in tears. $p^* < 0.05$ between R < 4-SG and R $\geq$ 4-SG. Data are presented as mean $\pm$ SEM (CTL: $n = 13$; 9.36 ± 3.15) (R $\geq$ 4-SG: $n = 57$; 59.59 ± 24.39) (R < 4-SG: $n = 35$; 254.3 ± 101.1).

TABLE 2. Cytokines levels in tears according to subgroups. Tear cytokine detected by multiplexed bead assay. IFN γ , interferon γ ; IL, interleukin; CXCL8, C-X-C motif chemokine ligand 8; LOD, limit of detection.

Groups	<i>n</i>	IFN- γ	IL-4	IL-6	IL-10	IL-5	CXCL8
(pg/mL for all values)							
Control	19	3.62 ± 1.68	9.60 ± 7.55	< LOD	8.69 ± 4.21	< LOD	84.11 ± 15.35
R $\geq$ 4	52	0.77 ± 0.23	4.31 ± 1.41	3.92 ± 1.63	0.96 ± 0.34	1.37 ± 0.73	244.4 ± 51.28
R < 4	58	8.38 ± 2.41	43 ± 11.29	23.16 ± 9.39	32.44 ± 13.96	13.28 ± 8.803	211.7 ± 39.44

DISCUSSION

The eye is a complex organ with susceptibilities to allergic reactions involving its own immunological features, which can evolve in a unilateral or bilateral way in the same individual. These functions can be modified in case of even mild impairment of the blood-tear barrier and the passage of low-molecular-weight molecules from the vascular to the ocular compartment. Within an allergic population with ocular manifestations and

independently of the clinical patterns, this study defined two subgroups according to the IgE ratio ($R = m\text{-IgE}/e\text{-IgE}$): a R < 4-SG and a R $\geq$ 4-SG.

In both groups the presence of albumin in tears compared to healthy subjects, is a hallmark of the blood-tear barrier permeability, nevertheless, patients from R < 4-SG display mainly higher albumin levels than patients from R $\geq$ 4-SG. This finding is corroborated through a stronger inflammatory response according to the ECP and cytokine levels in tears of this group. The presence of albumin in tears may have various origins and it remains

TABLE 3. ECP levels in tears according to subgroups. Tear ECP detected by fluoroenzyme immunoassay. ECP, eosinophil cationic protein. Values are expressed as mean $\pm$ SEM in pg/mL for cytokines and μ g/mL for ECP.

Groups	<i>n</i>	ECP (μ g/mL)
Control	13	9.36 $\pm$ 3.15
R $\geq$ 4	57	59.59 $\pm$ 24.39
R < 4	35	254.3 $\pm$ 101.1

unclear whether it is a cause or a consequence of a local inflammatory process.¹⁶ The distribution of patients in R < 4-SG according to tear albumin levels could be stratified into two groups (Figure 2): first, the R < 4-SG1 with tear albumin levels lower than 0.17 g/L (*n* = 27; 31.1%) corresponding to the mean value of tear albumin found in R $\geq$ 4-SG and the R < 4-SG2 with tear albumin levels $\geq$ 0.17 g/L (*n* = 60; 68.9%). The R < 4-SG1 displays tear albumin concentrations similar to the R $\geq$ 4-SG, but associated with an inflammatory immunological response according to our results. This distribution reveals the variability of albumin levels in tears. Tear albumin concentrations were already reported in patients with OA ranging from 0.170 $\pm$ 0.021 g/L¹⁷ to 1.430 $\pm$ 0.820 g/L.¹⁸ Therefore, the albumin levels could be influenced by the sampling method¹⁹, the circadian cycle²⁰ and the different analysis methods.^{21,22,23} Furthermore, the relationships between the clinical OA phenotypes and the specific inflammatory immunologic markers in tears have been established^{6,9,23} which provided a better understanding of OA pathogenesis. Among them, ECP, which is secreted by activated eosinophils, with a toxic action, remains a specific inflammatory marker for the IgE-mediated allergy and is correlated with the severity of the disease²⁴ and the late phase of the allergic reaction.^{25,26} Additionally, CCL11 (C-C motif chemokine ligand 11) was also described in tears of SAC, VKC, and AKC.^{26,27} Likewise, CCL11 and IgE seem to be the major contributors in SAC pathogenesis²⁶, but, as emphasized by Leonardi *et al.*,⁵ the level of ECP, increased in all forms of allergic conjunctivitis, may be the only abnormal laboratory parameter observed in VKC.²⁸

Interestingly, ECP was correlated with CXCL-8 in the R < 4-SG, supporting the inflammatory status of this subgroup. This observation is substantiated by previous studies that demonstrated an enhancement

of the activity of eosinophils and an increased amount of ECP released, associated with an increased concentration of CXCL-8.²⁷ According to Kawashima *et al.*, this phenomenon would be related to the stimulation of neutrophils by the chemokine CXCL-8, which increased the recruitment and the trans-basement membrane migration of eosinophils.²⁸

In the present study, Th1 and Th2 responses were evaluated by measuring a Th1-type cytokine such as IFN- γ , and Th2-type cytokines such as IL-4, IL-5, IL-6 and IL-10. These cytokines, as well as chemokines such as CXCL-8, are known to be increased in chronic OA inflammation.^{9,29} The results of this study show that the R < 4-SG is characterized by combined Th1 and Th2 responses. As it was previously described,³⁰ increased expression of IFN- γ , the main Th1 cytokine, correlates with disease severity, particularly in AKC, and could play an important role in inflammatory cell recruitment and activation of mast cells. Likewise, Th2 cells play an important role in the development of allergic disorders involving secretion of IL-4 and IL-5,³¹ and this Th2 response is generally associated with chronic allergic diseases such as VKC and AKC.⁶

The severity status attributed to the R < 4-SG population is strengthened by the Th1 and Th2 responses mediated by IFN- γ , IL-4, and IL-6, likewise through the robust correlation between IL-4 and IFN- γ . In agreement with Leonardi *et al.*,⁹ these data show a strong correlation between these cytokines, with no specific Th1 or Th2 profile for any subgroup.

IL-4 was detected in tears in the early phase following allergen exposure,³² IFN- γ was linked to more severe and chronic forms of OA such as VKC or AKC.³³

This correlation between IL-4 and IFN- γ is not in accordance with the immunological paradigm that IL-4 cross-regulates IFN- γ .

In the present study, the effector cytokines, *i.e.*, IL-4, IL-10, and IFN- γ , have a particular profile in the R < 4-SG: IL-4 is strongly correlated with both IFN- γ and IL-10. The concomitant expression of these cytokines would include type 1 T regulatory cells (Tr1) expressing IL-10 and IFN- γ as well as Th cells coexpressing IL-10 and IL-4.³⁴ These correlations reflect the balance between both the Th and Treg systems presumably linked to chronic exposure to the allergen and the repetitive costimulations of the Th cells for this group. These profiles were previously

TABLE 4. Percentages of tear proteins and immunoglobulins measured with microcapillary electrophoresis in controls and subgroups. Five major proteins were quantified from electropherograms: lysozyme-C; lipocalin-1; ZAG, Zn- α -glycoprotein; lactoferrin; Ig, Immunoglobulin; LCs, light chains; HCs, heavy chains. Data are expressed as relative percentage of each protein versus total protein content.

Groups	<i>n</i>	Lysozyme-C	Lipocalin-1	ZAG	Lactoferrin	Ig-LCs	IgA-HCs
Percentages (%)							
Control	19	37.63 $\pm$ 2.62	21.97 $\pm$ 0.85	4.38 $\pm$ 0.48	21.65 $\pm$ 0.70	3.1 $\pm$ 0.37	2.30 $\pm$ 0.49
R $\geq$ 4	52	37.45 $\pm$ 0.79	20.03 $\pm$ 0.75	3.20 $\pm$ 0.36	24.35 $\pm$ 0.50	2.6 $\pm$ 0.29	1.65 $\pm$ 0.21
R < 4	64	33.47 $\pm$ 0.73	20.2 $\pm$ 0.55	2.55 $\pm$ 0.26	23.13 $\pm$ 0.91	4.16 $\pm$ 0.39	1.70 $\pm$ 0.20

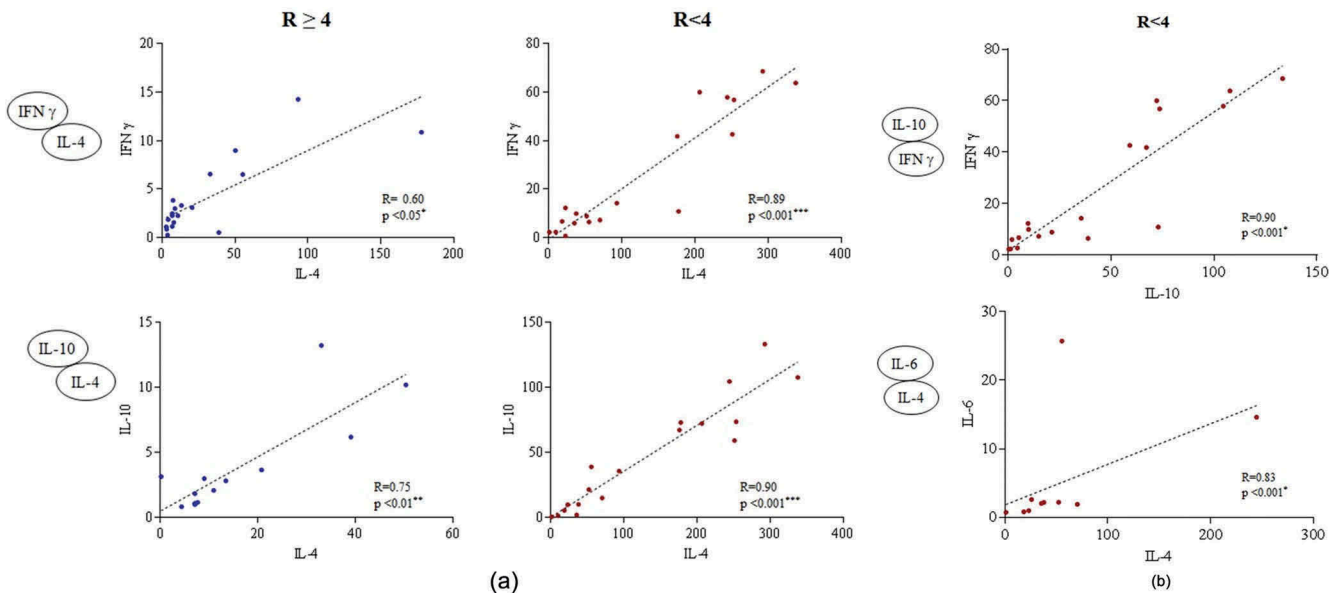


FIGURE 4. Correlation between pairs of cytokines in each subgroup. A. In $R < 4$ -SG, IL-4 shows a robust correlation with IFN- γ and IL-10, compared with $R \geq 4$ -SG. B. In the $R < 4$ -SG, a strong correlation is found between IL-10/IFN- γ and IL-6/IL-4. Pearson correlation was applied, correlation coefficient $p < 0.05$, $p^{**} < 0.01$ and $p^{***} < 0.001$.

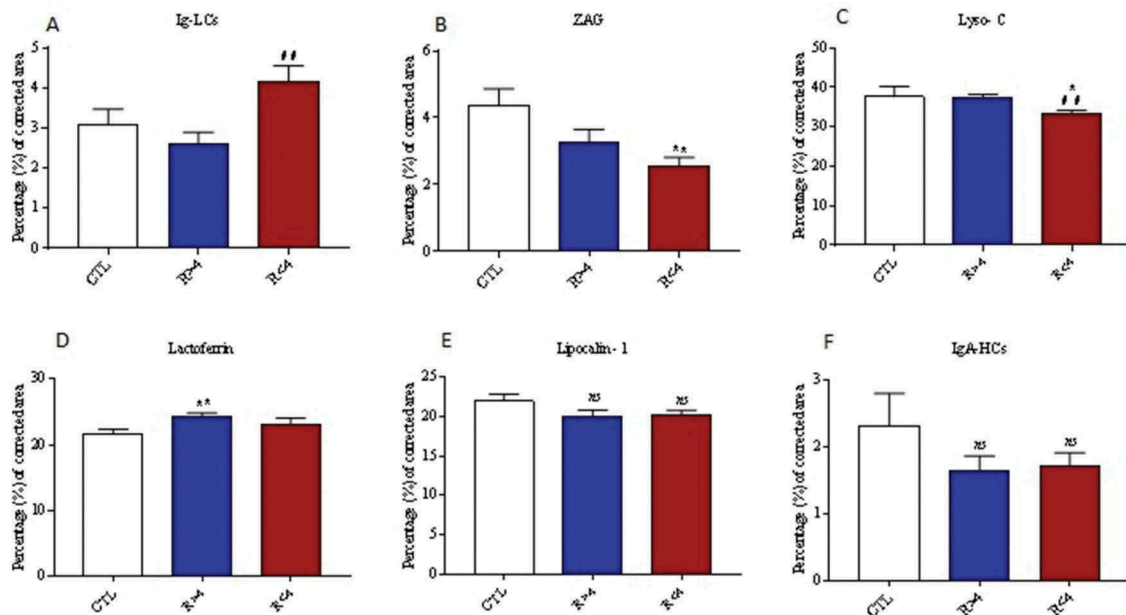


FIGURE 5. Capillary electrophoresis quantitation as a percentage of tear proteins according to the IgE ratio (R). A. Immunoglobulin light chains (Ig-LCs), B. Zinc- α glycoprotein (ZAG-2), C. lysozyme-C (Lyso-C), D. lactoferrin, E. lipocalin-1, F. immunoglobulin A-heavy chains (IgA-HCs). Data are presented as means and SEM. $p^* < 0.05$ and $p^{**} < 0.01$, respectively, between $R < 4$ -SG and CTL. $p^{***} < 0.01$ between $R < 4$ -SG and $R \geq 4$ -SG.

described in patients' tears with ocular allergy as SAC, PAC, AKC or VKC.^{9,24,33} Indeed, the presence of albumin in tears is accompanied by the presence of other proteins with similar or lower molecular weight. Among these proteins, cytokines are of particular interest because their concentration in blood can increase up to 1,000-fold during infection, trauma or systemic allergy.^{35,36}

Nevertheless, the $R \geq 4$ -SG which exhibited a high rate IgE in tears compared to $R < 4$ -SG (data not shown), displays lower IL-4 tear level. However, the dosage of these immunoglobulins relates only to free proteins that are not complexed with mast cells and granulocytes. This paradoxical result can, therefore, be explained by the absence of allergen in the ocular compartment. As

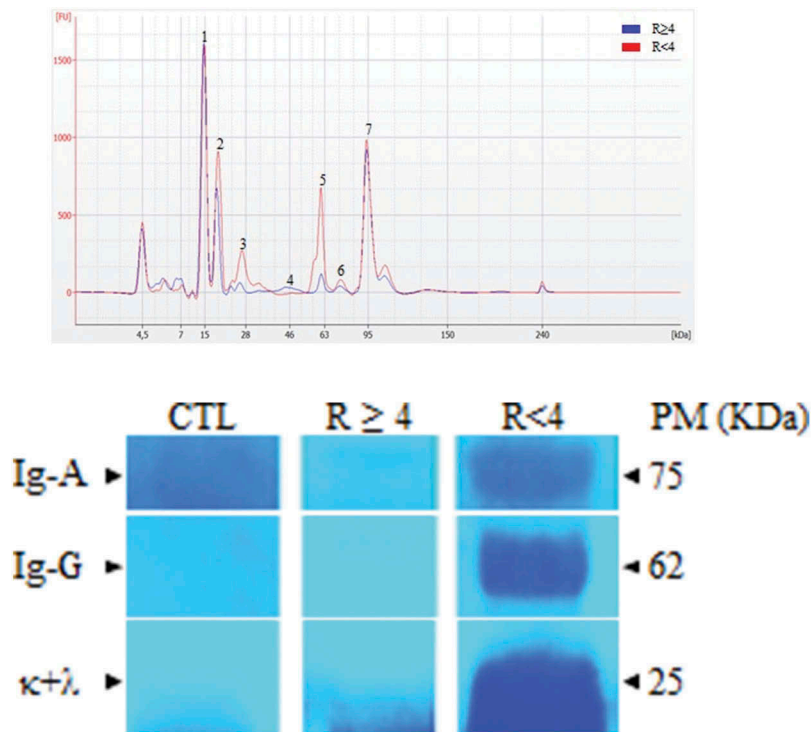


FIGURE 6. Tear protein electrophoresis and immunostaining. A. Comparison between subgroups $R \geq 4$ -SG (red line) and $R < 4$ -SG (blue line). The two electropherograms overlap. The number under each peak corresponds to a specific protein: (1) lysozyme-C, (2) lipocalin-1, (3) immunoglobulin (Ig) light chains, (4) Zn- α 2-glycoprotein, (5) albumin, (6) IgA (α) heavy chains, (7) lactoferrin. B. Detection of immunoglobulins: each lane of Western blot corresponds to CTL (control group), $R \geq 4$ -SG and $R < 4$ -SG with immunostaining versus IgA (α) and IgG (γ) heavy chains, and kappa (K) + lambda (λ) light chains.

described by Kaleshnikov *et al.*,³⁷ the IgE receptor (Fc ϵ RI) activation occurs only when the receptor-bound IgE are cross-linked via antigens and this is necessary for the induction of IgE-mediated allergic reactions. The absence of allergen prevents degranulation or leukotriene release and does not lead to an acute inflammatory reaction. In fact, several chemokines and cytokines have been found overexpressed in OA and these present findings highlight the usefulness of the IgE ratio to discriminate a severe ocular context according to the Th1, Th2, and ECP responses but none of them seems to be ideal for use as a diagnostic or prognostic factor. Nevertheless, the pathophysiological orientation provided by the ratio does not exclude studying each patient on a case-by-case basis.

The second part of this study evaluated a particular tear protein profile according to the ratio using more widespread analytical techniques such as capillary electrophoresis and immunodetection by Western blot. Unexpectedly, the IgA-HC levels were similar in both subgroups. Although their protective role in the inflammatory allergic reaction has been already reported,^{38–40} they did not appear to be a discriminant criterion in this study. IgA is the first line of antibodies produced in the early stage of an allergic immunization, and IgG is the second defensive line of antibodies and are persistent antibodies. Immunodetection shows that more than 62%

of the samples with $R < 4$ displayed IgG in tears versus 22% for samples with $R \geq 4$. Thus, the secretion of IgG is compatible with a late sensitization humoral response corresponding to a persistent or chronic presentation of an allergen for the $R < 4$ -SG.

To our knowledge, the tear protein profile (i.e., Lipoc-1, Lyso-C, Lacto, and ZAG-2) has been especially studied in a context of dry eye disease (DED) but rarely in OA.⁴⁰ Nevertheless, the present study demonstrated a particular protein profile in the $R < 4$ -SG with a significant decrease of the Lyso-C and ZAG-2 concentrations. Lyso-C has primarily a bacteriolytic function through an enzymatic action that damages bacterial cell walls. This action is driven in a synergic way with lactoferrin.⁴¹ It is also present in cytoplasmic granules of the macrophages and the polymorphonuclear neutrophils. Thus, this decrease exposes the eye to an increased susceptibility to infections. ZAG-2 is involved in the lipid breakdown in adipocytes and plays a possible role in immunity⁴² but its specific role in tears remains unknown. This decrease affecting only two proteins is not linked to an evaporation mechanism⁴³ and does not seem to have been described in cases of DED. Moreover, the treatment of allergic conjunctivitis including oral or topical antihistamines may generate iatrogenic DED through undesirable anticholinergic side effects^{44,45} or a toxic reaction, especially due to extensive and

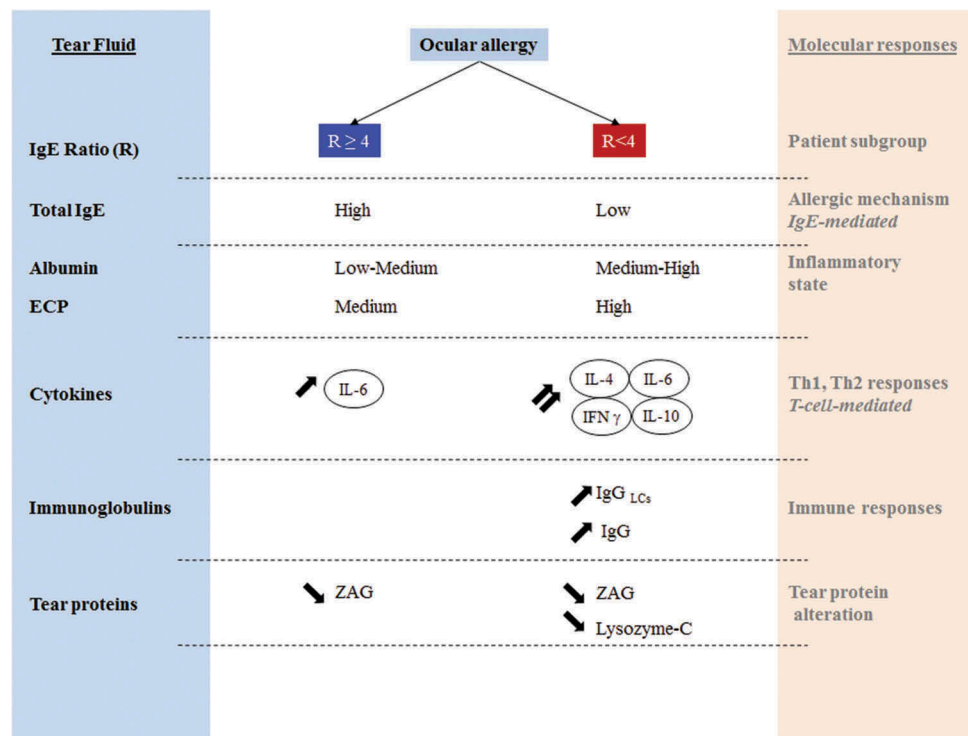


FIGURE 7. Tear mediators and molecular responses in ocular allergy: summary of molecular findings.

prolonged use of preservative-containing eye drops. It is therefore useful to appreciate the tear protein profile in order to associate or rule out possible DED diagnosis.⁴⁶ Nevertheless, considering this profile as characteristic of severe inflammatory context, further investigations with a larger cohort of patients should be performed. Furthermore, it would be interesting to pay particular attention to atopic and non-atopic forms of OA to allow a better understanding of possible discrepancies of systemic allergic reactions and local ocular allergies. As highlighted by Manzouri et al.,⁴⁷ the diagnosis of these different forms remains difficult in particular if it is not associated with other manifestations of atopy such as rhinitis, asthma or eczema. Likewise, according to the EAACI criteria,⁴ the non-atopic forms IgE-mediated of OA are related to different etiologies: Hymenoptera venom, helminthiasis, drugs or other. It, therefore, seems obvious that the etiological and symptomatic management of ocular allergies will be based on a diagnosis, sometimes difficult to make.

In Figure 7, we have summarized our major findings according to the various biological measurements to distinguish both subgroups. Although the application of the multiplexed bead-based assays allows assessing the tear cytokine profile, it remains difficult to adapt in a routine laboratory practice. Therefore, we suggest paying particular attention to the IgE ratio when it is lower than 4 and using an additional set of laboratory tests such as the tear ECP assay and immunofixation technique, which can easily replace Western blot, in order to better assess the immunological and inflammatory status of this fluid.

CONCLUSION

This study concerning the practical interest of the tear IgE ratio confirms its usefulness in estimating the local production of IgE in tears when it is high, and points out the importance of attentively interpreting cases with a low ratio in order to take into account the inflammation level and its mechanisms. The measurement of the IgE ratio in tears is a useful and discriminant tool to confirm the diagnosis of OA, particularly in chronic or persistent forms when the etiology is difficult to determine based on clinical criteria and allergic anamnesis. In front of a ratio lower than 4, we propose to systematically include achievement of the ECP and the immunofixation assays in the laboratory methods routinely used for tear exploration.

ABBREVIATIONS

AKC	atopic keratoconjunctivitis
CTL	control group
CXCL8	C-X-C Motif Chemokine Ligand 8
DTT	dithiothreitol
EAACI	European Academy of Allergy and Clinical Immunology
ECP	eosinophil cationic protein
e-IgE	exuded IgE
IgA-HCs	immunoglobulin A-heavy chains-LCs:
	immunoglobulin light chains

Lacto	lactoferrin
LOD	limits of detection
Lyso-C	lysozyme-C
m-IgE	measured IgE
OA	ocular allergy
OHS	ocular hypersensitivity
PAC	perennial allergic conjunctivitis
SAC	seasonal allergic conjunctivitis
SDS-PAGE	sodium dodecylsulfate –polyacrilamide gel electrophoresis
SEM	standard error of the mean
SG	subgroup
S-Alb	serum albumin
S-IgE	serum IgE
T-Alb	tear albumin
VKC	vernal keratoconjunctivitis
WAO	World Allergy Organization
ZAG-2	zinc- α 2 glycoprotein

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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