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Research article

# Inflammation-related molecules in tears of patients with chronic ocular pain and dry eye disease

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ABSTRACT

The purpose of this study was to analyze inflammation- and pain-related molecules in tears of patients suffering from chronic ocular pain associated with dry eye (DE) and/or a previous corneal refractive surgery (RS). Based on history, symptomatology, and clinical signs, the subjects (n = 180, 51.0 ± 14.7 years, 118 females, 62 males) in this cross-sectional study were assigned to one of five groups: DE and chronic ocular pain after RS (P/DE-RS, n = 52); asymptomatic subjects, i.e., without DE and chronic ocular pain, after RS (A-RS, n = 30); DE and chronic ocular pain without previous RS (P/DE-nonRS, n = 31); DE, no pain, and no previous RS (DE-nonRS, n = 35); and asymptomatic subjects with no previous RS (controls, n = 32). The tear concentrations of 20 cytokines and substance P (SP) were analyzed by immunobead-based assay and enzyme-linked immunosorbent assay, respectively. We found that tear levels of interleukin (IL)-10 and SP were increased in the RS groups. There were significant differences in IL-8/CXCL8 among the five groups. Nerve growth factor (NGF) tear levels were significantly higher in P/DE-RS than in DE-nonRS and controls. IL-9 had the highest percentage of detection in the P/DE-RS and P/DE-nonRS groups, while macrophage inflammatory protein (MIP)-1α, IL-2, and interferon (IFN)-γ were higher in the P/DE-RS, A-RS, and P/DE-nonRS groups. IL-17A was detected only in the A-RS group. Moderate correlations were observed in the A-RS, P/DE-nonRS, DE-nonRS and controls groups. A positive correlation was obtained between growth related oncogene concentration and tear break-up time (rho = 0.550; p = 0.012), while negative correlation was found between monocyte chemoattractant protein-3/CCL7 and conjunctival staining (rho = −0.560; p = 0.001), both in the A-RS group. IL-10 correlated positively with ocular pain intensity (rho = 0.513; p = 0.003) in the P/DE-nonRS group. Regulated on Activation Normal T Cell Expressed and Secreted/CCL5 correlated negatively with conjunctival staining (rho = −0.545; p = 0.001) in the DE-nonRS group. SP correlated negatively with corneal staining (rho = −0.559; p = 0.001) in the controls. In conclusion, chronic ocular pain was associated with higher IL-9 tear levels. IL-10, SP, MIP-1α/CCL3, IL-2, and IFN-γ were associated with previous RS. Higher levels of IL-8/CXCL8, MIP-1α/CCL3, IL-2, and IFN-γ were associated with DE-related inflammation, while NGF levels were related to chronic ocular pain and DE in RS patients. These findings suggest that improved knowledge of tear cytokines and neuromodulators will lead to a more nuanced understanding of how these molecules can serve as biomarkers of chronic ocular pain, leading to better therapeutic and disease management decisions.

## 1. Introduction

The corneal nociceptive systems provide several protective functions, including the preservation and restoration of the tear film. The consequences of damage to the nociceptive pathways include conditions ranging from dry eye (DE) disease to centralized oculofacial neuropathic pain syndrome. This pain is characterized by a huge disparity between the high intensity of symptoms and the lack of external clinical signs (Rosenthal and Borsook, 2016). Around 89% of DE patients experience

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**Abbreviations**

| | |
|---|---|
| ANOVA | analysis of variance |
| A-RS | asymptomatic subjects who had refractive surgery |
| B/B0 | Bound/Maximum Bound |
| CELab | Controlled Environment Laboratory |
| CI | confidence interval |
| Controls | subjects with no ocular symptoms and no previous ocular surgeries |
| DE | dry eye |
| DE-nonRS | patients with dry eye but no pain and with no previous refractive surgery |
| EGF | epidermal growth factor |
| ELISA | enzyme-linked immunosorbent assay |
| GRO | growth related oncogene |
| IFN | interferon |
| IL | interleukin |
| IL-1Ra | interleukin-1 receptor antagonist |
| LASIK | laser in situ keratomileusis |
| MCP | monocyte chemoattractant protein |
| MIP | macrophage inflammatory protein |
| MMP | matrix metallopeptidase |
| NGF | nerve growth factor |
| NRS | Numerical Rating Scale |
| OSDI | Ocular Surface Disease Index |
| P/DE-nonRS | patients with dry eye and chronic ocular pain who did not have refractive surgery |
| P/DE-RS | patients with dry eye and chronic ocular pain after refractive surgery |
| RANTES | Regulated on Activation Normal T Cell Expressed and Secreted |
| RS | refractive surgery |
| SP | Substance P |
| TBUT | tear break-up time |
| TNF | tumor necrosis factor |

some degree of ocular pain (Satitpitakul et al., 2017). This pain can be neuropathic, but it can also be nociceptive and triggered by inflammation (Galor et al., 2015).

The onset of chronic pain associated with post-operative corneal procedures, such as corneal refractive surgery (RS), has also been described (Nettune and Pflugfelder, 2010). One hypothesis to explain chronic post-surgical corneal pain is that damage occurs to the corneal nerve plexus during surgery and results in neuropathic pain that progresses to chronicity (Theophanous et al., 2015). This alteration of the corneal nerves has also been suggested as the causative factor for laser in situ keratomileusis (LASIK)-associated DE, which affects around 50% of patients undergoing RS (Chao et al., 2014).

The neuropathic pain associated with RS and DE has not been fully characterized; therefore, it is difficult to diagnose. Consequently, the treatment remains extremely challenging. The response to medical therapies is often poor, which forces a multi-step approach (Dieckmann et al., 2017; Goyal and Hamrah, 2016). In addition, both ocular pain and DE greatly affect the quality of life, and it represents a significant economic burden (Stapleton et al., 2017). Therefore, there is a growing interest in objectively identifying molecules of inflammation and/or pain that can serve as tools for diagnosis, monitoring, and treatment (Roy et al., 2017).

A wide variety of molecules are currently associated with neuropathic and/or inflammatory pain. These include cytokines such as interleukin (IL)-1β, IL-4, IL-6, IL-8/CXCL8, IL-10, IL-17, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1/CCL2, fractalkine/CX3CL1, and regulated on activation normal T cell expressed and secreted (RANTES)/CCL5 (Ferrari et al., 2014; Hung et al., 2017; Khan et al., 2017; Liou et al., 2013; Üçeyler et al., 2006; Verri et al., 2006; White et al., 2005; Zhang and An, 2007). Some tear cytokines, such as IL-1, IL-6, IL-8/CXCL8, TNF-α, matrix metallopeptidase (MMP)-9, interferon (IFN)-γ, and RANTES/CCL5, are also involved in DE (Enríquez-de-Salamanca et al., 2010; Hagan et al., 2016; López-Miguel et al., 2016; Pinto-Fraga et al., 2018; Taketani et al., 2020; Yoon et al., 2007). In addition to cytokines, the neuromodulators substance P (SP) and nerve growth factor (NGF) have been associated with pain and DE (Ferrari et al., 2014; Hagan et al., 2016; Kuruvilla et al., 2019; Sacchetti et al., 2020).

However, most of the above studies that analyzed the role of cytokines involved in pain were conducted in animal models and addressed other pathologies such as fibromyalgia, spinal cord injury, and spinal degeneration (Khan et al., 2017; Üçeyler et al., 2006; White et al., 2005), with only a few studies focusing on ocular pain (Ferrari et al., 2014; Kuruvilla et al., 2019). To our knowledge, the tear molecular profile of subjects with chronic ocular pain after RS has not been studied in depth, nor has it been compared with other ocular surface pathologies such as DE. Therefore, the aim of this study was to develop a molecular profile of tear cytokines and related molecules that are associated with chronic ocular pain and DE and/or RS.

## 2. Materials and methods

This was a single-center, cross-sectional study approved by the Ethics Committee of the Valladolid University Clinical Hospital. This study complied with the Tenets of the Declaration of Helsinki and the Good Clinical Practices. The nature of the research was explained to the participants and informed consent was obtained prior to their participation in this study.

### 2.1. Patients sample

Five groups of participants were recruited: 1) patients with DE and chronic ocular pain after RS (P/DE-RS); 2) asymptomatic subjects, i.e., without DE and chronic ocular pain, after RS (A-RS); 3) patients with DE and chronic ocular pain who did not have RS (P/DE-nonRS); 4) patients with DE but no pain and with no previous RS (DE-nonRS); and 5) subjects with no ocular symptoms and no previous ocular surgeries (controls).

The criteria for determining the presence of DE were having DE-related symptoms (Ocular Surface Disease Index [OSDI] score $\geq$13) and at least two of the following conditions in both eyes: fluorescein tear break-up time (TBUT) $\leq$ 7 s, corneal fluorescein staining $\geq$ grade 1 (Oxford scale), conjunctival lissamine green staining $\geq$ grade 1 (Oxford scale), and Schirmer test with topical anesthesia $\leq$5 mm in 5 min. The presence of chronic ocular pain was indicated when the Numerical Rating Scale (NRS) score was $\geq$2 (Satitpitakul et al., 2017), and the pain lasted at least 3 months (Nicholas et al., 2019).

Inclusion criteria were the following: 1) age $\geq$18 years; 2) diagnosis of DE and/or ocular pain in the pertinent groups as defined above; 3) LASIK at least 3 months before in the two groups who had previous RS. Exclusion criteria were the following: 1) any ocular surface disease in the last 3 months or any ocular surgery in the last 6 months (except DE, pain, and RS in the pertinent groups); 2) diagnosis of any systemic disease that could have an ocular component in the last 3 months; 3) initiation of any systemic medication that could affect the ocular surface health in the previous 3 months; 4) initiation of lacrimal punctum occlusion in the previous 3 months; 5) contact lens wear in the 7 days prior to the study; or 6) any topical medication and lubricants in the 24 and

12 h, respectively, before the study.

Control subjects had to meet the same inclusion and exclusion criteria, but they did not meet the inclusion diagnostic criteria for DE, ocular pain, and RS.

### 2.2. Clinical examination and tear collection

All participants were evaluated after 30 min under a normal controlled environment conditions (23°C temperature, 50% relative humidity and no localized air flow) in the Controlled Environment Laboratory (CELab) (www.visionrd.com/celab/) (Calonge et al., 2017). The purpose of this was to normalize the environmental conditions in which clinical evaluations were conducted to eliminate the effects of a changing external environment (López-Miguel et al., 2014; Tesón et al., 2013). During the 30 min of adaptation, a medical history was taken. After that, clinical examination and tear sample collection were performed (also under normal controlled conditions) in the following sequence, always between 9.30 a.m. and 2.00 p.m.

Symptomatology: Ocular surface symptoms were evaluated with the self-administered OSDI and NRS questionnaires. The OSDI assesses DE-related symptoms through 12 questions, and the overall score (0–100) classifies patients into severity groups: 0–12: absence of symptoms; 13–22: mild symptoms; 23–32: moderate symptoms; and 33–100: severe symptoms (Miller et al., 2010; Schiffman et al., 2000). The NRS was used to evaluate ocular pain and consists of a numbered line from 0 to 10 that measures pain intensity: 0–1: no pain; 2–4: mild pain; 5–7: moderate pain; and 8–10: severe pain (Satitpitakul et al., 2017).

Tear sample collection: Basal tear samples were collected non-traumatically from the external canthus of the eye using glass capillary micropipettes (Drummond Scientific Co., Broomall, PA, USA) as previously described (López-Miguel et al., 2016). Reflex tearing was avoided as much as possible. Basal tears (1 μl) were collected for cytokine analysis from a randomly selected eye. Tear samples for cytokine analysis were then diluted (1:10) in a cryotube containing 9 μl of ice-cold Milliplex Cytokine Assay Buffer (Millipore Merck, Madrid, Spain). A 2-μl tear sample was also collected from the contralateral eye for SP analysis. These samples were diluted (1:25) in the corresponding SP assay buffer. All samples were kept cold (4 °C) during the study visit and then immediately frozen (−80 °C) until assayed.

Tear stability: Tear stability was evaluated by measuring TBUT with a SL-D7 slit lamp (Topcon Corporation, Tokyo, Japan) emitting a cobalt blue light that passed through a Wratten No. 12 yellow filter (Eastman Kodak, Rochester, NY, USA). Sodium fluorescein strips (I-DEW FLO, Entod ResearchCell UK Ltd, London, UK) were wetted with sodium chloride and applied into the inferior fornix. After 2 min, three measurements of TBUT were performed in both eyes, and the mean was recorded. TBUT below 7 s was considered to be abnormal (Craig et al., 2017).

Corneal and conjunctival integrity: Fluorescein staining was used to grade corneal integrity after evaluating TBUT. The extent of corneal staining was recorded in both eyes according to the Oxford grading scale (scale, 0–5) (Bron et al., 2003). The integrity of the nasal and temporal conjunctiva was assessed by staining the conjunctiva with lissamine green strips (I-DEW green, Entod Research Cell UK Ltd, London, UK) according to the Oxford grading scale (scale, 0–5) (Bron et al., 2003).

Tear production: Tear production was assessed in both eyes using the Schirmer test with anesthesia. Five minutes after instilling a drop of topical anesthesia (tetracaine 0.1% and oxybuprocaine 0.4%; Alcon Cusí, Barcelona, Spain), a sterile Schirmer strip (I-DEW tear strips, Entod Research Cell UK Ltd, London, UK) was placed in the external inferior fornix of both eyes. After 5 min with eyes closed, the wet length of the strips was recorded. Values $\leq$ 5 mm were considered to be abnormal (Lemp et al., 2007).

### 2.3. Tear cytokine and SP concentration analyses

The concentration of 20 cytokines was measured simultaneously in tear samples by X-MAP technology with a customized 20-plex immunobead-based assay (SPR 1549 Custom 20-plex Magnetic Human Cytokine MilliplexMAP panel; Millipore, Merck, MA, USA) and a Luminex IS-100 (Luminex Corporation, Austin, TX, USA) following the manufacturer's low volume protocol which uses 10 μl of sample/standards per assay, as previously described (Enríquez-de-Salamanca et al., 2010). The molecules analyzed were epidermal growth factor (EGF), fractalkine/CX3CL1, IL-1β, IL-1 receptor antagonist (Ra), IL-2, IL-4, IL-6, IL-8/CXCL8, IL-9, IL-10, IL-17A, MCP-1/CCL2, MCP-3/CCL7, TNF-α, IFN-γ, growth related oncogene (GRO), macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, NGF, and RANTES/CCL5. Standard curves were used to convert fluorescence units to cytokine concentration (pg/ml). Minimum detectable concentrations (in pg/ml) according to the manufacturer specifications were as follows: 3.2 pg/ml for EGF, fractalkine/CX3CL1, IL-1Ra, IL-2, IL-4, IL-8/CXCL8, IL-9, IL-10, IL-17A, MCP-3/CCL7, IFN-γ, GRO, MIP-1α/CCL3, MIP-1β/CCL4, NGF, and RANTES/CCL5; 9.6 pg/ml for IL-1β and MCP-1/CCL2; 8 pg/ml for IL-6; and 6.4 pg/ml for TNF-α. Data were stored and analyzed with the "BeadView Software'' (Upstate-Millipore Corporation, Watford, UK).

Tear levels of SP were measured using a competitive enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's protocol. The optical density at 420 nm was read in a SpectraMAx MS spectrophotometer (Molecular Devices, San Jose, CA, USA). Subsequently, absorbance values were used to calculate the % Bound/Maximum Bound (B/B0) for each sample, and the values were converted into concentration (pg/mL) using a standard curve (concentration range from 3.9 to 500 pg/ml and sensitivity [80% B/B0] of approximately 8 pg/ml) with the SoftMax Pro software v4.8 (Molecular Devices, CA, USA).

### 2.4. Statistical analyses

Statistical analyses were performed using the R software (version 3.6.1) (The R Foundation, 2019). The sample size was calculated to detect clinical differences by an analysis of variance (ANOVA) with a factor of 5 levels (statistical power of 80% and 1% of level of significance to maintain reasonable experiment-wise type I error rates when adjusting for multiple comparisons). A minimum of 23 subjects per group was estimated to detect as significant an effect size set at a Cohen's Coefficient, f, of 0.4.

Quantitative data were expressed as mean values and 95% confidence interval (CI), while percentages were used for qualitative data. Comparison of sex distribution in the study groups was assessed using the Chi-square test; pairwise comparisons were examined by applying the Benjamini & Hochberg correction (Benjamini and Hochberg, 1995). Comparison of age among the study groups was evaluated by one-way ANOVA; pairwise comparisons were analyzed by independent samples t-tests and applying the Benjamini & Hochberg correction. P-values $\leq$ 0.05 were considered statistically significant. For DE-related signs (TBUT, corneal and conjunctival staining, and Schirmer test), the values for both eyes were averaged, but no statistical comparisons were performed because these were only used to correctly assign subjects in each study group.

For tear molecules, regression on order statistics was used to impute the tear molecule concentrations below the assay detection limit (Lee, 2017). Molecules with percentage of detection >50% were considered as quantitative molecules. The concentrations of these were log-transformed (log 2) to normalize the distribution. Then, to compare concentrations among the 5 study groups, the propensity score (Rosenbaum and Rubin, 1983) and the least absolute shrinkage and selection operator (LASSO) methods were applied using the "gImnet" R package to balance the groups and compensate for sex and age differences (Friedman et al., 2010). Subsequently the survey package was used to fit

a linear model with weights (Lumley, 2004). The molecules with percentage of detection <50% (IL-2, IL-9, IL-17A, IFN-γ, and MIP-1α/CCL3) were considered qualitatively with two possible values: detected or undetected. Contingency tables and the propensity score method were used to compare the qualitative molecule levels among the 5 study groups. Pairwise comparisons were evaluated by applying the Benjamini & Hochberg correction.

Correlations between quantitative tear molecules and clinical characteristics were analyzed in each study group. Pearson correlation coefficient, r, was used for normally distributed variables, while Spearman correlation coefficient, rho, was used for non-normally distributed variables. P-values ≤ 0.05 were considered statistically significant.

## 3. Results

The study included 180 subjects who were distributed among the 5 groups as follows: P/DE-RS, 28.9%; A-RS, 16.7%; P/DE-nonRS, 17.2%; DE-nonRS, 19.4%; and controls, 17.8%. Of the subjects, 34.4% were males and 65.6% were females, and the study population age range was 23–89 years. There were significant differences in the gender distribution, with females composing the lowest percent in the A-RS group, 53.3%, and the highest in the P/DE-nonRS group, 87.1% (Table 1). There were also significant differences in age among the groups, with the P/DE-RS group being the youngest, 37.1 (34.8–39.4) years and the DE-nonRS group being the oldest, 62.9 (58.9–66.9) years. While there were significant differences in both the gender and age distributions, there were no significant differences in the gender pairwise comparisons.

### 3.1. Clinical characteristics

The intensity of ocular pain was moderate in the P/DE-RS and P/DE-nonRS groups (Table 2). DE-related symptoms were moderate in the DE-nonRS group and severe in the two groups with ocular pain, i.e., P/DE-RS and P/DE-nonRS. Mild DE-related clinical signs were present in the P/DE-nonRS and DE-nonRS groups.

### 3.2. Tear levels of neuropathic and inflammatory pain-related molecules

EGF, IL-1Ra, IL-8/CXCL8, MCP-1/CCL2, GRO, and SP were detected in at least 93.5% of the subjects in all groups (Table 3). Fractalkine/CX3CL1, IL-1β, IL-4, IL-6, IL-10, MCP-3/CCL7, TNF-α, MIP-1β/CCL4, NGF, and RANTES/CCL5 were detected in variable ranges from 30% to 98.1% of the subjects depending on the study group. IL-2, IL-9, IL-17A, IFN-γ, and MIP-1α/CCL3 were below the detection cutoff level of 50%, and they were statistically analyzed as qualitative variables (detected/undetected).

The tear concentration of quantitatively analyzed molecules and the percentage of detection of qualitatively analyzed molecules in each study group were adjusted by sex and age (Table 4). There were significant differences in the tear concentrations of IL-8/CXCL8, IL-10, and SP, as well as the percentage of detection of IL-2, IL-9, IL-17A, IFN-γ, and MIP-1α/CCL3. There were no significant differences in the pairwise comparisons of IL-8/CXCL8, IL-2 and IFN-γ, although the highest concentration of IL-8/CXCL8 was present in the P/DE-nonRS group followed by the DE-nonRS group. The highest percentage of detection of IL-2 and IFN-γ was in the two RS groups, P/DE-RS and A-RS, and the P/DE-nonRS group. There were significant differences in the post-hoc pairwise comparisons of IL-10 and SP tear concentrations and the percentages of detection of IL-9, IL-17A, and MIP-1α/CCL3. Both IL-10 and SP were significantly increased in the RS groups, P/DE-RS and A-RS (Fig. 1).

For IL-9, the highest percentage of detection occurred in both groups with pain symptomatology, P/DE-RS and P/DE-nonRS (Fig. 2). IL-17A was detected only in the A-RS group, 26.7%, so there were significant differences with the other study groups. For MIP-1α/CCL3, the highest percentage of detection was in both RS groups, P/DE-RS and A-RS, followed by the P/DE-nonRS (Fig. 2).

Additionally, tear levels of IL-4, TNF-α, and NGF were on the borderline of statistical significance (Table 4). There were no significant differences among the groups in the post-hoc pairwise comparison for tear IL-4 and TNF-α. Whereas the post-hoc pairwise comparisons for NGF showed that the concentration was significantly higher in the P/DE-RS group than in DE-nonRS and controls.

### 3.3. Correlations between tear molecules and clinical characteristics

Significant correlations were observed in all study groups (see Appendix A in supplementary data). Most of these correlations were weak (i.e., correlation coefficient <0.5). However, some moderate correlations (correlation coefficient >0.5) were found in the A-RS, P/DE-nonRS, DE-nonRS and controls groups. Particularly, in the A-RS a positive moderate correlation was obtained between tear GRO concentration and TBUT, while negative correlation was found between MCP-3/CCL7 and conjunctival staining (Fig. 3A). In the P/DE-nonRS group IL-10 levels correlated positively with NRS ocular pain (Fig. 3B) and RANTES/CCL5 correlated negatively with conjunctival staining in the DE-nonRS group (Fig. 3C). Finally, SP tear levels correlated negatively with corneal staining in the controls (Fig. 3D).

## 4. Discussion

Chronic ocular pain is very disabling and difficult to treat because the pathophysiological mechanisms are still poorly understood, and there are no standard criteria for diagnosis (Galor et al., 2018; Réaux-Le Goazigo et al., 2017). Understanding the molecular mechanisms involved in chronic ocular pain is crucial to develop an effective therapeutic and management strategy (Réaux-Le Goazigo et al., 2017). Previous studies have reported the role of some cytokines and neuromodulators as markers of inflammation and pain (Ferrari et al., 2014; Hagan et al., 2016; Hung et al., 2017; Khan et al., 2017; Liou et al., 2013; Sacchetti et al., 2020; Üçeyler et al., 2006; Verri et al., 2006; White et al., 2005; Zhang and An, 2007). However, the literature is scarce regarding molecular mediators in patients with chronic ocular pain associated with DE or RS.

**Table 1**
Demographic characteristics of the study groups.

| Demographic characteristics | P/DE-RS (n: 52) | A-RS (n: 30) | P/DE-nonRS (n: 31) | DE-nonRS (n: 35) | Controls (n: 32) | P-value* |
|---|---|---|---|---|---|---|
| **Sex (% females)** | 63.5 | 53.3 | 87.1 | 68.6 | 56.2 | **0.0435** |
| **Age (years)** | 37.1 (34.8–39.4) | 42.2 (39.5–44.8)[a] | 60.8 (56.6–65.0)[b] | 62.9 (58.9–66.9)[b] | 59.5 (56.3–62.8)[b] | **<0.0001** |
| **Years with ocular symptoms** | 5.3 (4.05–6.55) | NA | 9.2 (5.60–12.84) | 10.0 (6.95–13.05) | NA | – |
| **Years since RS** | 7.8 (6.33–9.27) | 11.5 (10.19–12.81) | NA | NA | NA | - |

Variables are presented as means and 95% confidence intervals in parentheses except for sex which is presented as female percentage. *P-values for the comparison of the 5 study groups. P-values ≤ 0.05 were considered statistically significant and are shown in bold. [a]) P = 0.032 compared to P/DE-RS group. [b]) P < 0.0001 compared to P/DE-RS group. P/DE-RS: patients with DE and chronic ocular pain after RS; A-RS: asymptomatic subjects who had RS; P/DE-nonRS: patients with DE and chronic ocular pain who did not have RS; DE-nonRS: patients with DE but no pain and with no previous RS; Controls: subjects with no ocular symptoms and no previous ocular surgeries; DE: dry eye; RS: refractive surgery; NA: not applicable.

**Table 2**
Study group clinical test descriptive data.

| Questionnaire/Test | P/DE-RS | A-RS | P/DE-nonRS | DE-nonRS | Controls |
|---|---|---|---|---|---|
| **NRS ocular pain (score, 0–10)** | 5.23 (4.45–6.01) | 0.28 (−0.03–0.59) | 6.48 (5.80–7.16) | 0.31 (0.07–0.55) | 0.20 (−0.03–0.43) |
| **OSDI questionnaire (score, 0–100)** | 58.49 (52.38–64.60) | 8.06 (6.95–9.17) | 44.43 (37.48–51.38) | 30.56 (24.50–36.62) | 3.60 (2.42–4.78) |
| **TBUT (seconds)** | 4.51 (3.87–5.15) | 5.51 (4.20–6.82) | 3.65 (3.24–4.06) | 3.30 (2.92–3.68) | 5.15 (4.45–5.85) |
| **Corneal staining (Oxford scale, 0–5)** | 1.12 (0.90–1.34) | 0.93 (0.63–1.23) | 1.26 (1.01–1.51) | 1.04 (0.75–1.33) | 0.20 (0.10–0.30) |
| **Conjunctival staining (Oxford scale, 0–5)** | 0.71 (0.51–0.91) | 0.18 (0.05–0.31) | 1.10 (0.88–1.32) | 1.15 (0.92–1.38) | 0.80 (0.59–1.01) |
| **Schirmer test with anesthesia (0–35 mm)** | 8.09 (5.87–10.31) | 13.88 (11.70–16.06) | 9.31 (6.69–11.93) | 9.27 (6.53–12.01) | 9.42 (7.28–11.56) |

Variables are presented as questionnaire or test result means with 95% confidence interval in parentheses. P/DE-RS: patients with DE and chronic ocular pain after RS; A-RS: asymptomatic subjects who had RS; P/DE-nonRS: patients with DE and chronic ocular pain who did not have RS; DE-nonRS: patients with DE but no pain and with no previous RS; Controls: subjects with no ocular symptoms and no previous ocular surgeries; DE: dry eye; RS: refractive surgery; OSDI: Ocular Surface Disease Index; NRS: Numerical Rating Scale; TBUT: tear break-up time.

**Table 3**
Percentage of detection of the 20 cytokines and SP analyzed in tears in each study group.

| Molecules | P/DE-RS % (95 CI) | A-RS % (95 CI) | P/DE-nonRS % (95 CI) | DE-nonRS % (95 CI) | Controls % (95 CI) |
|---|---|---|---|---|---|
| **EGF** | 100.0 (91.43–100) | 100.0 (85.87–100) | 100.0 (86.27–100) | 100.0 (87.68–100) | 100.0 (86.66–100) |
| **Fractalkine/CX3CL1** | 82.7 (69.18–91.31) | 86.7 (68.36–95.64) | 58.1 (39.26–74.93) | 51.4 (34.28–68.28) | 50.0 (33.63–66.37) |
| **IL-1β** | 32.7 (20.72–47.22) | 46.7 (28.8–65.36) | 61.3 (42.29–77.58) | 45.7 (29.22–63.13) | 46.9 (29.51–64.97) |
| **IL-1Ra** | 100.0 (91.43–100) | 100.0 (85.87–100) | 93.5 (77.16–98.87) | 94.3 (79.48–99) | 93.7 (77.78–98.91) |
| ***IL-2*** | *25.0 (14.48–39.23)* | *33.3 (17.94–52.86)* | *12.9 (4.22–30.76)* | *5.7 (1–20.52)* | *3.1 (0.16–18)* |
| **IL-4** | 78.8 (64.91–88.48) | 83.3 (64.55–93.7) | 83.9 (65.53–93.91) | 71.4 (53.48–84.76) | 71.9 (53.02–85.6) |
| **IL-6** | 48.1 (34.22–62.22) | 66.7 (47.14–82.06) | 64.5 (45.38–80.17) | 80.0 (62.54–90.94) | 59.4 (40.79–75.78) |
| **IL-8/CXCL8** | 100.0 (91.43–100) | 100.0 (85.87–100) | 100.0 (86.27–100) | 100.0 (87.68–100) | 100.0 (86.66–100) |
| ***IL-9*** | *28.8 (17.55–43.27)* | *10.0 (1.75–33.13)* | *25.8 (12.54–44.93)* | *2.9 (0.15–16.62)* | *12.5 (4.08–29.93)* |
| **IL-10** | 57.7 (43.26–70.99) | 73.3 (53.83–87.02) | 71.0 (51.76–85.11) | 57.1 (39.52–73.24) | 56.2 (37.88–73.16) |
| ***IL-17A*** | *0.0 (0–8.57)* | *16.7 (6.3–35.45)* | *0.0 (0–13.73)* | *0.0 (0–12.32)* | *0.0 (0–13.34)* |
| **MCP-1/CCL2** | 100.0 (91.43–100) | 100.0 (85.87–100) | 100.0 (86.27–100) | 100.0 (87.68–100) | 96.9 (82–99.84) |
| **MCP-3/CCL7** | 51.9 (37.78–65.78) | 73.3 (53.83–87.02) | 93.5 (77.16–98.87) | 91.4 (75.81–97.76) | 81.2 (62.96–92.14) |
| **TNF-α** | 30.8 (19.12–45.26) | 30.0 (15.41–49.56) | 61.3 (42.29–77.58) | 57.1 (39.52–73.24) | 59.4 (40.79–75.78) |
| ***IFN-γ*** | *42.3 (29.01–56.74)* | *56.7 (37.66–74.02)* | *35.5 (19.83–54.62)* | *28.6 (15.24–46.52)* | *28.1 (14.4–46.98)* |
| **GRO** | 100.0 (91.43–100) | 100.0 (79.95–100) | 100.0 (86.27–100) | 100.0 (87.68–100) | 100.0 (86.66–100) |
| ***MIP-1α/CCL3*** | *36.6 (23.97–51.09)* | *43.3 (25.98–62.34)* | *16.1 (6.09–34.47)* | *8.6 (2.24–24.19)* | *9.4 (2.45–26.17)* |
| **MIP-1β/CCL4** | 48.1 (34.22–62.22) | 56.7 (37.66–74.02) | 74.2 (55.07–87.46) | 62.9 (44.95–78.01) | 68.7 (49.86–83.25) |
| **NGF** | 98.1 (88.42–99.9) | 86.7 (68.36–95.64) | 77.4 (58.46–89.72) | 71.4 (53.48–84.76) | 75.0 (56.25–87.87) |
| **RANTES/CCL5** | 51.9 (37.78–65.78) | 63.3 (43.91–79.46) | 87.1 (69.24–95.78) | 74.3 (56.43–86.89) | 71.9 (53.02–85.6) |
| **Substance P** | 100.0 (91.27–100) | 100.0 (85.44–100) | 100.0 (85.44–100) | 100.0 (87.36–100) | 100.0 (86.66–100) |

Data are presented as percentages with 95% confidence intervals in parentheses. Molecules with a percentage of detection lower than 50% are in italics. P/DE-RS: patients with DE and chronic ocular pain after RS; A-RS: asymptomatic subjects who had RS; P/DE-nonRS: patients with DE and chronic ocular pain who did not have RS; DE-nonRS: patients with DE but no pain and with no previous RS; Controls: subjects with no ocular symptoms and no previous ocular surgeries; DE: dry eye; RS: refractive surgery; CI: confidence interval; EGF: epidermal growth factor; IL: interleukin; IL-1Ra: interleukin-1 receptor antagonist; MCP: monocyte chemoattractant protein; TNF: tumor necrosis factor; IFN: interferon; GRO: growth related oncogene; MIP: macrophage inflammatory protein; NGF: nerve growth factor; RANTES: Regulated on Activation Normal T Cell Expressed and Secreted.

Our results showed differences among the study groups in the tear concentration of IL-10, SP, IL-8/CXCL8, and NGF. Particularly, we found the highest values of IL-10 tear concentration in both the symptomatic and asymptomatic RS groups. IL-10 is an anti-inflammatory molecule that is associated with decreased hyperalgesia after sciatic nerve chronic constriction injury (Wagner et al., 1998). Higher *IL-10* gene expression occurs in patients after peripheral nerve injury but without neuropathic pain compared to patients with neuropathic pain (Held et al., 2019). Higher microRNA levels of IL-10 have also been found in patients with painless neuropathy than in patients with painful neuropathy and healthy controls (Üçeyler et al., 2007). Some authors point out that IL-10 can be considered a prototypical analgesic cytokine (Üçeyler and Sommer, 2007). Our results suggest that increased tear secretion of IL-10 is related to RS itself, not to DE or chronic ocular pain. This agrees with previous results from our research group who found that IL-10 concentration increased significantly at 6 months after advanced surface ablation RS (González-García et al., 2020). The high IL-10 tear concentration in the A-RS group might play a protective role and explain why these patients were asymptomatic.

SP is a neuropeptide involved in neurogenic inflammation and in modulation of pain transmission (Ferrari et al., 2014; Taketani et al., 2020). It promotes the migration and proliferation of corneal epithelial cells (Garcia-Hirschfeld et al., 1994; Nishida et al., 1996). Our results indicated that SP tear levels were significantly increased in the RS groups, but there were no significant differences between symptomatic (P/DE-RS) and asymptomatic (A-RS) individuals. Thus, the high SP tear concentration seems to be associated with RS, but not with the presence of ocular pain. This could be because damage to the corneal nerves releases SP, which abolishes the immune privilege and promotes a protective immune response to infectious agents (Paunicka et al., 2015). Chao et al. (2015) have shown that the SP tear level increase in LASIK patients is related to decreased nerve density, and it may facilitate post-LASIK corneal reinnervation, although it was only evaluated up to 3 months post-LASIK. Patients included in our study had undergone RS 9.20 ± 5.09 years ago. Some authors suggested that complete regeneration of corneal nerve plexus occurs 2–5 years after RS, although that is not yet completely established (Chao et al., 2014). However, based on the concentration of SP found in our RS groups, we hypothesize that reinnervation was altered or not completed in these individuals. Further studies are needed to critically assess the process of reinnervation after RS and the relationship of it to the presence of inflammation and pain-related molecules in tears.

Our results also revealed significant differences in IL-8/CXCL8 tear levels among the study groups, with the P/DE-nonRS patients having the

**Table 4**
Concentrations (pg/ml) or percentages of detection of tear molecules in each study group (adjusted by sex and age).

| Molecules | P/DE-RS | A-RS | P/DE-nonRS | DE-nonRS | Controls | P-value[a] |
|---|---|---|---|---|---|---|
| Quantitatively analyzed molecules: mean concentration (95% confidence interval) pg/ml | | | | | | |
| **EGF** | 2060.52 (1711.41–2480.52) | 1713.98 (1420.35–2068.32) | 1930.68 (1472.40–2531.60) | 1599.85 (1256.41–2037.16) | 1601.75 (1245.65–2059.63) | 0.62 |
| **Fractalkine/ CX3CL1** | 1006.35 (721.15–1404.35) | 1657.48 (557.54–3095.33) | 908.9 (421.67–1959.87) | 609.59 (338.16–1098.87) | 857.31 (520.24–1412.76) | 0.30 |
| **IL-1β** | 8.61 (6.70–11.07) | 10 (4.73–21.13) | 14.98 (8.00–29.03) | 6.05 (4.12–8.90) | 7.78 (6.17–9.81) | 0.27 |
| **IL-1Ra** | 5858.85 (3851.30–8912.86) | 4887.66 (2953.82–8087.57) | 5821.77 (3091.04–10964.94) | 3098.18 (1523.05–6302.31) | 2530.71 (1398.84–4578.45) | 0.42 |
| **IL-4** | 239.29 (144.95–395.01) | 248.82 (116.12–533.20) | 151.89 (68.93–334.72) | 77.21 (37.64–158.40) | 78.26 (40.72–150.39) | *0.059* |
| **IL-6** | 43.5 (30.55–61.94) | 26.41 (14.52–48.03) | 48.78 (22.39–106.27) | 26.6 (15.11–46.83) | 16.66 (9.42–29.50) | 0.16 |
| **IL-8/CXCL8** | 171.61 (126.30–233.17) | 161.97 (117.34–223.57) | 479.71 (240.57–956.57) | 205.5 (122.32–344.90) | 89.07 (53.91–147.17) | **0.04** |
| **IL-10** | 21.44 (14.62–31.44) | 32.75 (15.60–68.77) | 15.5 (6.74–35.63) | 5.82 (2.98–11.37) | 7.44 (4.29–12.90) | **0.02** |
| **MCP-1/CCL2** | 404.31 (304.93–536.08) | 392.87 (301.72–511.55) | 456.57 (313.71–628.42) | 460.14 (347.60–609.14) | 390.47 (272.38–559.75) | 0.92 |
| **MCP-3/CCL7** | 161.48 (127.75–204.10) | 227.64 (140.84–367.94) | 220.39 (154.64–314.08) | 190.03 (141.91–254.46) | 187.64 (132.40–265.91) | 0.68 |
| **TNF-α** | 5.22 (3.91–6.96) | 2.41 (1.03–5.68) | 10.1 (5.08–20.08) | 5.27 (3.59–7.74) | 6.75 (4.9–9.30) | *0.07* |
| **GRO** | 3644.14 (3075.28–4318.24) | 2908.3 (2407.63–3513.08) | 5736.46 (3703.26–8885.95) | 4068.65 (3095.12–5348.40) | 3432.93 (2633.95–4474.25) | 0.26 |
| **MIP-1β/CCL4** | 13.89 (10.01–19.23) | 17.08 (7.13–40.89) | 26.84 (9.70–74.21) | 5.8 (2.78–12.13) | 9.00 (5.29–15.30) | 0.18 |
| **NGF** | 18.82 (15.07–23.51) | 11.27 (6.95–18.25) | 8.19 (4.64–9.57) | 6.82 (4.86–9.57) | 6.31 (4.57–8.71) | *0.07* |
| **RANTES/CCL5** | 56.75 (41.05–78.45) | 94.24 (47.61–186.54) | 69.3 (41.92–114.48) | 42.2 (24.52–72.61) | 44.9 (27.76–72.60) | 0.25 |
| **Substance P** | 2296.66 (1721.19–3064.53) | 2590.46 (1861.46–3604.95) | 1062.94 (817.23–1382.54) | 1088.44 (821.03–1442.94) | 1519.04 (1382.08–1792.80) | **0.002** |
| **Qualitatively analyzed molecules: percentage of detection (95% confidence interval)** | | | | | | |
| **IL-2** | 25.4 (13.08–42.83) | 30 (15.83–48.9) | 22.9 (12.01–38.56) | 4.1 (0.42–18.65) | 5.2 (0.79–20) | **0.009** |
| **IL-9** | 28.2 (15.19–45.75) | 9.4 (1.33–34.2) | 44.3 (29.51–60.07) | 1.8 (0.01–15.45) | 13.6 (4.91–30.39) | **0.0001** |
| **IL-17A** | 0 (0–11.87) | 26.7 (13.33–45.46) | 0 (0–10.19) | 0 (0–12.67) | 0 (0–12.49) | **<0.0001** |
| **IFN-γ** | 57.4 (40.15–73.18) | 61.5 (42.84–77.54) | 57.3 (41.4–71.85) | 29.8 (15.95–48.05) | 30 (16.24–48.13) | **0.0069** |
| **MIP-1α/CCL3** | 47.8 (31.4–64.72) | 37.2 (21.41–55.92) | 33.9 (20.64–49.97) | 6.2 (1.16–21.56) | 12.3 (4.16–28.85) | **0.0003** |

[a] P-values for the comparison of the 5 study groups. P-values ≤ 0.05 were considered statistically significant and are shown in bold. P-values > 0.05 but ≤0.07 were considered on the borderline of statistical significance and are shown in italics. P/DE-RS: patients with DE and chronic ocular pain after RS; A-RS: asymptomatic subjects who had RS; P/DE-nonRS: patients with DE and chronic ocular pain who did not have RS; DE-nonRS: patients with DE but no pain and with no previous RS; Controls: subjects with no ocular symptoms and no previous ocular surgeries; DE: dry eye; RS: refractive surgery; EGF: epidermal growth factor; IL: interleukin; IL-1Ra: interleukin-1 receptor antagonist; MCP: monocyte chemoattractant protein; TNF: tumor necrosis factor; GRO: growth related oncogene; MIP: macrophage inflammatory protein; NGF: nerve growth factor; RANTES: Regulated on Activation Normal T Cell Expressed and Secreted; IFN: interferon.

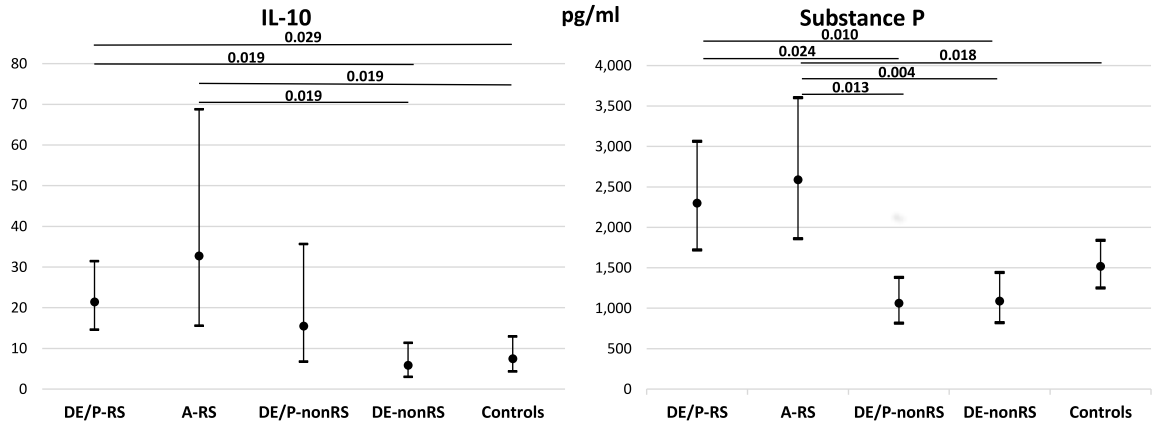


**Fig. 1. Post-hoc pairwise comparisons of the IL-10 and SP tear concentrations.** Data are presented as mean concentrations (pg/ml) and 95% confidence intervals. P/DE-RS: patients with DE and chronic ocular pain after RS; A-RS: asymptomatic subjects who had RS; P/DE-nonRS: patients with DE and chronic ocular pain who did not have RS; DE-nonRS: patients with DE but no pain and with no previous RS; Controls: subjects with no ocular symptoms and no previous ocular surgeries; DE: dry eye; RS: refractive surgery; IL: interleukin.

highest concentration followed by the DE-nonRS patients. IL-8/CXCL8 is a pain-associated molecule that induces persistent mechanical nociceptor hypersensitivity together with TNF-α and IL-1β (Enríquez-de--Salamanca et al., 2010; Sachs et al., 2002). Some authors suggest that IL-8/CXCL8 plays an important role in neuropathic pain following nerve injury or disc herniation (Khan et al., 2017; Kim et al., 2011). However, other authors have not found such relationships with neuropathic pain (Bäckryd et al., 2016). IL-8/CXCL8 is released in inflammation or prolonged desiccation stress and several studies found that this cytokine, along with others, reflect the severity of DE (Lam et al., 2009; López-Miguel et al., 2016). Our results could also be explained by the fact that TNF-α, a cytokine that stimulates the production of other cytokines such as IL-8/CXCL8, is increased in the P/DE-nonRS group (Carrasco Otero, 2011).

Likewise, tear levels of NGF were significantly higher in P/DE-RS than in DE-nonRS and controls. NGF is a neurotrophic factor

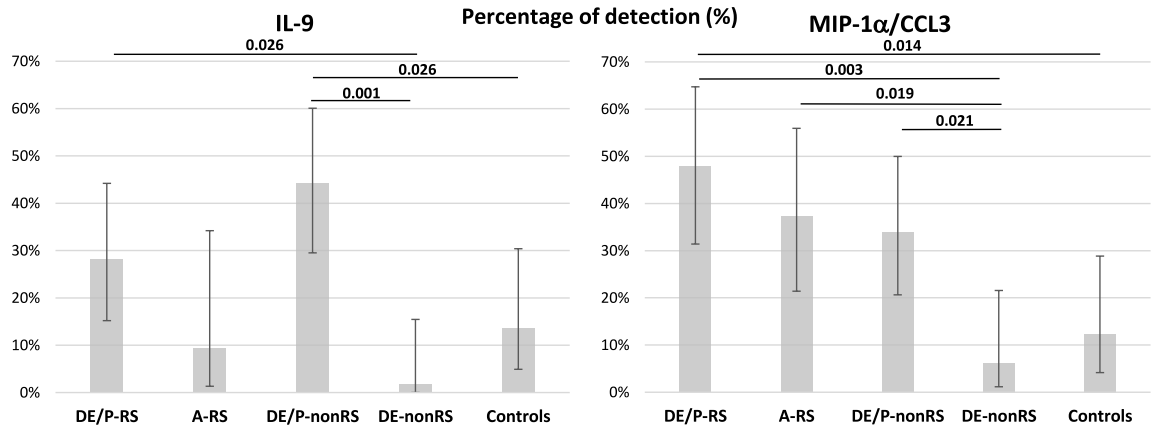


**Fig. 2. Post-hoc pairwise comparisons of the IL-9 and MIP-1α/CCL3 percentage of detection in tears.** Data are presented as percentage of detection and 95% confidence interval. P/DE-RS: patients with DE and chronic ocular pain after RS; A-RS: asymptomatic subjects who had RS; P/DE-nonRS: patients with DE and chronic ocular pain who did not have RS; DE-nonRS: patients with DE but no pain and with no previous RS; Controls: subjects with no ocular symptoms and no previous ocular surgeries; DE: dry eye; RS: refractive surgery; IL: interleukin; MIP: macrophage inflammatory protein.

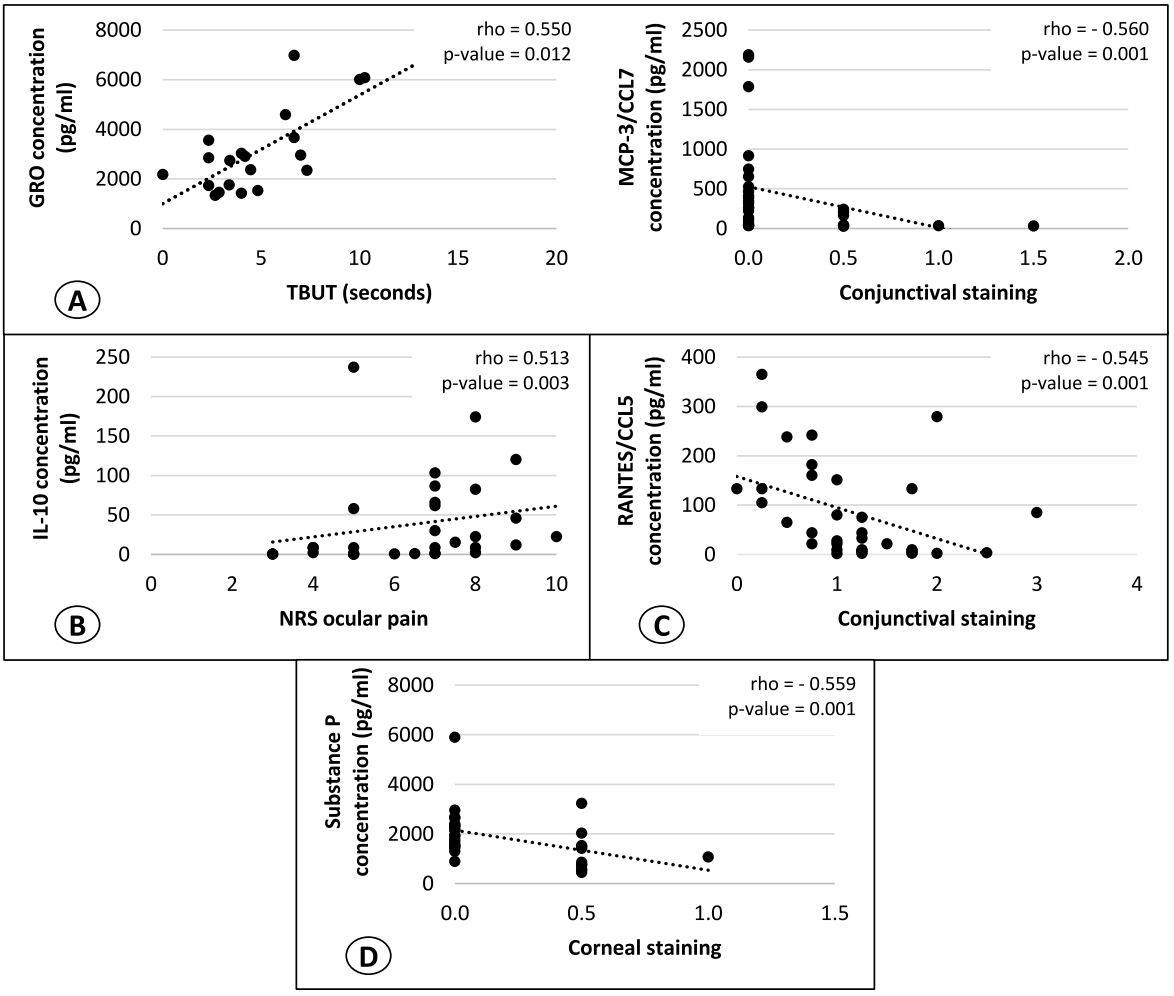


**Fig. 3. Significant and moderate correlations between tear molecules and clinical characteristics in the A-RS (A), P/DE-nonRS (B), DE-nonRS (C) and controls (D) groups.** A-RS: asymptomatic subjects who had refractive surgery; P/DE-nonRS: patients with dry eye and chronic ocular pain who did not have refractive surgery; DE-nonRS: patients with dry eye but no pain and with no previous refractive surgery; controls: subjects with no ocular symptoms and no previous ocular surgeries; GRO: growth related oncogene; TBUT: tear break-up time; MCP: monocyte chemoattractant protein; IL: interleukin; NRS: Numerical Rating Scale; RANTES: Regulated on Activation Normal T Cell Expressed and Secreted.

considered to be an endogenous mediator in some persistent pain states (Skaper, 2018). There is increasing evidence that points to this crucial role in the generation of pain and hyperalgesia (Lewin et al., 2014; Skaper, 2018). Additionally, nerve regeneration after LASIK induces the release of many growth factors such as NGF (Hyung et al., 2005). This could explain why we found the highest concentration of NGF in the P/DE-RS group. Topical exogenous application of NGF has been suggested as a potential treatment for restoring corneal integrity and improving corneal sensitivity (Gong et al., 2021). Further, blocking antibodies directed against NGF have remarkable analgesic potency in human clinical trials for other painful conditions such as osteoarthritis, lower back pain, and interstitial cystitis. Thus, anti-NGF medication has the potential to make a major impact on day-to-day chronic pain treatment (Lewin et al., 2014).

In the present study, there were significant differences in the percentages of detection of IL-9, IL-17A, MIP-1α/CCL3, IL-2, and IFN-γ. Because of the low levels of detection, they were analyzed only qualitatively as either detected or non-detected. IL-9, a pro-inflammatory cytokine, is thought to be involved in the pathogenesis of immune and inflammatory disorders. However, despite its pro-inflammatory function in chronic inflammatory diseases, one study suggested that IL-9 serves as a master regulator in the resolution of chronic inflammation in arthritis (Rauber et al., 2017). Our research group found that the *IL-9* gene is related to DE as it is one of the 4 genes (along with *IL-6*, *EGFR,* and *NAMPT*) that produces the best predictive model for the occurrence of graft versus host disease–associated DE (Cocho et al., 2015). Also, our group reported that the tear concentration of IL-9 was elevated at 6 months after advanced surface ablation corneal RS (González-García et al., 2020). The results of the present study showed an increased percentage of detection of IL-9 in both groups of patients with pain, i.e., P/DE-RS and P/DE-nonRS. To our knowledge, there are no studies that support an association between IL-9 and ocular pain. However, increased IL-9 plasma levels were found in patients with unstable angina pectoris and chest pain compared to controls (Lin et al., 2013). In addition, upregulation of IL-9 was detected in the lumbar region of the spinal cord in a mouse model for streptozotocin-induced diabetic neuropathic pain (Zychowska et al., 2013). The association already found between IL-9 and these systemic painful pathologies supports our results and, therefore, the relationship with ocular pain. Further studies regarding the role of this molecule in chronic ocular pain are warranted.

Regarding IL-17A, some authors have proposed it as a biomarker and a therapeutic target for DE (Tarn et al., 2019). Furthermore, upon nerve injury such as corneal epithelial abrasion, IL-17A promotes neuroinflammatory responses like the proliferation of astrocytes and the secretion of pro-inflammatory cytokines towards the injury site, contributing to the onset of neuropathic pain and hypersensitivity (Sun et al., 2017). Our research group found that IL-17A levels were significantly increased at 6 months after advanced surface ablation RS (González-García et al., 2020). In the present study, IL-17A was detected only in the A-RS group (26.7%). This might be because DE patients included in this study, based on their clinical characteristics, had mild DE, whereas increased IL-17A levels have been related more to severe DE cases.

MIP-1α/CCL3 is produced by cells during infection or inflammation by promoting the recruitment of leukocytes to the site of inflammation (Bhavsar et al., 2015). It is also involved in wound healing by supporting migration of macrophages to tissue lesions. The recruitment of inflammatory lineages could contribute to the development of allodynia, hyperalgesia, and chronic pain by altering nociceptive transduction through the activation of chemokine receptors in the dorsal root ganglia (Liou et al., 2013; Sun et al., 2016). This agrees with our results for MIP-1α/CCL3 that was detected in higher concentrations for RS patients and in P/DE-nonRS patients. This suggests that MIP-1α/CCL3 has a relationship with RS (ocular tissue damage), chronic ocular pain, and DE (inflammation).

IL-2 was detected at low rates in all study groups, which may be due to its short half-life (Lin et al., 2000). However, the detection rate of IL-2, as well as that of IFN-γ, was higher in both RS groups and in the P/DE-nonRS group. The similarity of detection rate for both cytokines could be because IL-2, among other molecules, promotes IFN-γ production by natural killer cells, and therefore higher levels of IL-2 would be associated with higher levels of IFN-γ, and vice versa. IFN-γ and IL-2 trigger the cellular immune response when a potentially harmful agent is detected (Carrasco Otero, 2011). Also, some authors have found higher levels of IL-2 in patients with moderate DE and exacerbated symptoms than in subjects with moderate DE but no exacerbated symptoms and controls (Li et al., 2020). Our research group found higher levels of IL-2 and IFN-γ in tears at 6 months after advanced surface ablation RS compared to preoperative levels (González-García et al., 2020). These could explain the relationship of IL-2 and IFN-γ with RS and high symptomatic DE.

Regarding the molecular profile of our RS groups, their comparison with other types of ocular surgery would be of great interest to study the similarities or differences between them. In the present section we have discussed the results of our LASIK RS groups with those previously obtained by our research group in advanced surface ablation-operated subjects, and found that in both populations IL-10, IL-9, IL-17A, IL-2 and IFN-γ molecules were increased. However, González-Garcia et al. (2020) also found significantly increased levels of IL-4, IL-5, IL-6, IL-12, IL-13 and vascular endothelial growth factor, whereas we found increased levels of SP, NGF and MIP-1α/CCL3. These discrepancies are probably due to the different molecules analyzed (they did not evaluate SP and NGF levels, while we did not assess IL-5, IL-12, IL-13 and vascular endothelial growth factor levels), the type of surgery (advanced surface ablation versus LASIK) and the time elapsed since surgery in each population (6 months versus 9.20 ± 5.09 years). In addition, the aim and methodology of both studies were very different. González-Garcia et al. (2020) performed a longitudinal study in which they evaluated tear molecules before and after surgery, while we conducted a cross-sectional study to analyze the molecular profile of patients who underwent RS many years ago and compare this profile with DE patients and controls.

In addition, correlations between tear molecules and clinical parameters were also evaluated in the present study. Moderate correlations have been found between GRO and tear stability, MCP-3/CCL7 and RANTES/CCL5 and conjunctival epithelial integrity, IL-10 and ocular pain intensity, and SP and corneal epithelial integrity. The relationship between ocular symptoms and some cytokines, such as IL-10, has also been reported in the literature (Cocho et al., 2016; Jung et al., 2015; Massingale et al., 2009; Willems et al., 2021). Lambiase et al. (2011) found a significant correlation between SP and tear stability, but they did not evaluate the relationship of SP with conjunctival staining. Other studies did not obtain a significant correlation between GRO and tear stability (Agrawal et al., 2016) and RANTES/CCL5 and conjunctival epithelial integrity (Cocho et al., 2016), which may be because the pathologies addressed (DE with human immunodeficiency virus infection and ocular chronic graft versus host disease) have different pathophysiologic bases than those of the present study.

In summary, our results show that some molecules differ in patients with or without chronic ocular pain associated with DE and/or a previous RS. IL-10 and SP were particularly associated with the RS procedure. IL-8/CXCL8 concentration was increased in the P/DE-nonRS group, which corroborates its association with DE inflammation. NGF appears to be related with the development of chronic ocular pain and DE in RS patients. The detection percentage of IL-9 was elevated in the two groups of patients with chronic ocular pain, which indicates that this cytokine may play a role in the process of producing the pain. On the other hand, the percentages of detection of MIP-1α/CCL3, IL-2, and IFN-γ were raised in both RS groups and in the P/DE-nonRS group, which indicates an association with RS, DE inflammation, and pain symptomatology. Also, correlations between tear molecules and clinical parameters were found in each study group. These specific correlations

and molecular profiles found in the different study groups may help explain the differences in symptomatology and clinical parameters, and corroborate that a condition is not defined by the level of a single molecule but by the balance of a group of molecules (Enríquez-de-Salamanca et al., 2010). Understanding these molecular bases underlying ocular pain will help in the search for effective, selective and personalized therapies (Hagan et al., 2016).

However, this study has some limitations. One limitation is that only patients with LASIK RS were included. In addition, the DE patients without previous RS had mainly mild clinical signs of DE. It would be interesting to analyze and compare the tear molecular profiles in patients with severe DE and other types of ocular surgery (refractive and others) and also in patients with different types of ocular pain (nociceptive/neuropathic/mixed). Another limitation is that only a single timepoint was tested. A comparison with different timepoints would be of interest. Also, a second assay using a different method was not performed for independent confirmation of the results; nevertheless, the methods used in this study, Luminex and ELISA, are well-established, show consistent results, and are widely used for molecule determination in tears (de Jager et al., 2003; dupont et al., 2005; Hagan and Tomlinson, 2013; Zhao et al., 2018). Finally, analysis of the corneal nerve plexus morphology by in vivo confocal microscopy and the correlation of the plexus changes with molecular changes in tears would help in the understanding of chronic ocular pain. Further studies addressing this are warranted.

In conclusion, our study outcomes show that development of chronic ocular pain is related with IL-9 tear levels, whereas IL-10, SP, MIP-1α/CCL3, IL-2, and IFN-γ levels are related to RS itself. Also, IL-8/CXCL8, MIP-1α/CCL3, IL-2, and IFN-γ appear to be associated with DE inflammation, and NGF is associated with the development of chronic ocular pain and DE in RS patients. Specific correlations between molecules related to neuropathic and inflammatory pain and clinical parameters according to the presence of ocular pain, RS or DE corroborate the different molecular profiles in these groups of patients. This information may help to better understand their different pathophysiological basis and symptomatology. These findings suggest that improved knowledge of tear cytokines and neuromodulators will lead to a more nuanced understanding of how these molecules can serve as biomarkers of chronic ocular pain, leading to better therapeutic and disease management decisions.

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## Declarations of interest

None.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2022.109057.

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