Clinical and tear cytokine profiles after advanced surface ablation refractive surgery: a six-month follow-up

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Abstract

Neuropathic dry eye is one of the most frequently seen complications after corneal refractive surgery, however, its incidence decreases in a significant manner along the first six months postoperative, reaching between 10-45% incidence. However, little is known on the inflammatory status of the ocular surface during this recovery process. We aim to analyze the clinical and tear molecule concentration changes along six months after advanced surface ablation for myopia correction, in a prospective study including 18 eyes of 18 subjects who bilaterally underwent advanced surface ablation corneal refractive surgery. Clinical variables (uncorrected distance visual acuity, symptoms, conjunctival hyperemia, tear osmolarity, tear stability, corneal fluorescein staining, conjunctival lissamine staining, Schirmer test, and corneal esthesiometry) and a panel of 23 pro and anti-inflammatory cytokines/chemokines concentration in tears preoperatively and at 1, 3 and 6 months postoperatively were evaluated. We found that uncorrected distance visual acuity improved significantly from baseline at 1-month visit, symptoms improved and tear osmolarity decreased significantly from baseline at 3-month visit and there was a decrease in mechanical corneal threshold between 1-month and 3- and 6-month visits. Regarding tear molecules, IL-4, IL-5, IL-6, IL-13, IL-17A, and IFN-γ tear levels were significantly increased at all the three visits , compared to preoperative levels at V0; IL-2 and VEGF were also significantly increased at 1-month and 6-month visits, but not at 3-month visit, whereas IL-9 IL-10 and IL-12 were only significantly increased at 6-month visit. Although we found that there is a recovery in clinical variables at 6 months postoperatively (i.e. neuropathic dry eye was not developed in the sample), ocular surface homeostasis is not completely restored, as it

45 can be seen by the changes in concentration of some pro and anti-inflammatory
46 molecules measured in tears.

47 **Keywords:** advanced surface ablation; refractive surgery; ocular surface inflammation;
48 cytokines; dry eye disease.

Abbreviations

ASA: advanced surface ablation

PRK: photorefractive keratectomy

LASIK: laser in situ keratomileusis

DE: Dry eye

NGF: nerve growth factor

MMP-9: matrix metalloproteinase-9

EGF: epidermal growth factor

CGRP: calcitonin gene-related peptide

V0: baseline visit

Vm1: 1-month postoperative visit

Vm3: 3-month postoperative visit

Vm6: 6-month postoperative visit

OSDI: Ocular Surface Disease Index

ETDRS: Early Treatment Diabetes Retinopathy Study

TBUT: Tear breakup time

IFN-γ: interferon gamma

IL: interleukin

IL-1RA: IL-1 receptor antagonist

IP-10: CXCL10/interferon gamma-induced protein 10

RANTES: CCL5/regulated on activation, normal T-cell expressed and secreted

TNF-α: tumor necrosis factor

VEGF: vascular endothelial growth factor

IR: interquartile range

74 NADA: nondetects and data analysis

75 HGF: hepatocyte growth factor

76 KGF: keratinocyte growth factor

77 PDGF: platelet-derived growth factor

78 TFG: transforming growth factor

79 mOsm/L: milliosmoles/liter

80 mL/min: milliliters/minute

81 Pg/ml: picograms/milliliter

82 mm: millimeters

83 ˚C: Celsius degrees

84 CI: confidence interval

85 SD: Standard deviation;

86 NC: not calculated

87 NA: Not applicable

1. Introduction

Photorefractive keratectomy (PRK) and laser in situ keratomileusis (LASIK) are the most common surgical techniques to correct refractive errors (Shortt et al., 2013). Advanced surface ablation (ASA) is an evolution of PRK that reduce the incidence of some of the most common complications resulting from this technique, such as haze, postoperative pain or decrease in quality of vision (Pallikaris et al., 2003; Trattler and Barnes, 2008).

Neuropathic dry eye (DE) is one of the most frequently seen complications after corneal refractive surgery (Jabbur et al., 2004; Murakami and Manche, 2012), with a peak between 1 week to 3 months (Chao et al., 2014), an incidence of 10-45% at least 6 months postoperatively depending on the studies (Denoyer et al., 2015; Hovanesian et al., 2001; De Paiva et al., 2006; Shoja and Besharati, 2007), and persisting one year after surgery in 5% and in 0.8% of patients that have undergone PRK and LASIK, respectively (Bower et al. 2015). Damage to the nerve endings of the sub-basal corneal plexus in the intraoperative procedure and a neurotrophic component have been suggested as the causative factor of this type of DE (Ambrosio et al., 2008; Chao et al., 2014).

DE has been recognized as an inflammatory disorder of the lacrimal functional unit (Pflugfelder et al., 2004). An altered balance of inflammatory molecules in the tear film has been described in DE by our group (López-Miguel et al., 2014, 2016; Pinto-Fraga et al., 2018; Teson et al., 2013) and others (see refs. for a review) (Hagan et al., 2016; Tamhane et al., 2019). Regarding neuropathic DE, it has been postulated that the increase in inflammatory cytokines after corneal refractive surgery may be the cause of DE symptoms (Wilson et al., 2001b; Wilson and Ambrosio., 2001c; Leonardi et al., 2009; Alio and Javaloy, 2013; Chao et al., 2014;).

The short term (up to 7 days) production and release to the tear film of inflammatory molecules produced after the surgical wound in refractive surgery and the subsequent healing process has been widely described (Alio and Javaloy, 2013; Lee et al., 2002; Leonardi et al., 2009; Long et al., 2006; Nakamura et al. 2002; Resan et al., 2015, 2016; Suzuki et al., 2003; Tervo et al., 1997; Tomás-Juan et al., 2015; Urgancioglu et al., 2009; Wilson et al., 1999a, 1999b, 2001a). However, there are few studies evaluating the presence of some of these inflammatory mediators in tears in a longer term period after corneal refractive surgery procedures (PRK or LASIK), where it has been found a significant increase of nerve growth factor (NGF) at 1, 3 and 6 months postoperatively (Gao et al., 2014; Lee et al. 2005; Zhang et al., 2016), Interleukin 6 (IL-6) after 1 month (Gao et al. 2014), and matrix metalloproteinase-9 (MMP-9), epidermal growth factor (EGF) (González-Pérez et al., 2005), and calcitonin gene-related peptide (CGRP) (Chao et al., 2016) after 1 year of follow-up.

As incidence of neuropathic DE after refractive surgery decreases in a significant manner along the first six months postoperative, the aim of this study was to analyze the clinical and tear molecular profile up to 6 months after ASA surgery, to check if ocular surface homeostasis recovers in a similar way than clinical parameters.

2. Materials and Methods

This was a prospective, longitudinal study approved by the University of Valladolid Ethics Committee (Valladolid, Spain). The study followed the tenets of the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practices.

2.1. Patients

Subjects were subsequently included from those that presented to the refractive surgery unit at IOBA (Institute of Applied Ophthalmobiology), University of Valladolid,

Spain, that were candidates to bilateral ASA corneal refractive surgery, aged between 20 and 45 years, no history of active ocular disease, no use of topical drugs prior to surgery except artificial tears, and no systemic treatment with potential secondary effects on the ocular surface for at least 3 months before surgery. Patients with any ocular surface disease, including severe DE (levels 3 and 4 according to the DE severity grading scheme, International DE Workshop) (Lemp et al., 2007) or previous ocular surgery, were excluded. Contact lens wearers ceased contact lens use 15 days before the preoperative visit.

2.2.Surgery protocol

All surgeries were performed by the same surgeon (co-author MJM) with an Allegreto Wave® Eye-Q Excimer Laser (Alcon, Forth Worth, TX, USA). Both eyes of the same subject were operated at the same time, although only one eye was randomly included in the study. The patient's eyelids and skin surrounding the eye was prepped with 10% povidone iodine (Betadine, Meda Manufacturing, Bordeaux, France). A sterile drape was applied over the eyelids and lashes. Topical lidocaine 2% anesthetic drops (B/Braun, Barcelona, Spain) were instilled in the eye. A lid speculum was placed in the operative eye and a patch was placed over the fellow eye to avoid cross-fixation. A solution of 20% diluted absolute alcohol was applied to the central cornea for 30 seconds. The alcohol exposure was restricted to the central cornea using a 9-millimeter optical zone marker pressed onto the corneal surface. The alcohol was then removed from the optical zone marker well by absorption into a microsurgical spear sponge. After the alcohol was fully absorbed, the ocular surface was copiously irrigated with balanced salt solution to minimize toxicity to limbal germinal epithelium. Central epithelium was removed from the underlying Bowman's layer using a spatula. Excimer laser corneal ablation with an

159 optical zone of 6.5 mm and a transition zone to 9 mm was performed over the exposed
160 Bowman's layer, discarding the epithelium.
161 All patients followed the same postoperative protocol: a bandage contact lens (Acuvue®
162 Oasys®, Johnson & Johnson Vision Care Inc., Jacksonville, FL, USA) for 6 days, and topical
163 0.3% ofloxacin (Exocin®, Allergan Inc., Irvine, CA, USA), 0.1% dexamethasone (Dexafree
164 unidose®, Laboratories Théa, Clermont-Ferrand, France) and 0.18% sodium hyaluronate
165 eyedrops (Vismed, BRUDYLAB S.L., Barcelona, Spain) 4 times a day for 2 weeks. From
166 day 14 post-surgery, medications changed to topical 0.1% fluorometholone (FML®,
167 Allergan Inc.) 3 times a day for 2 weeks, twice daily for 2 weeks, and once daily for 2
168 more weeks; 0.15% sodium hyaluronate eyedrops (Hyabak®, Laboratories Théa) was
169 used 4 times a day throughout the study.
170 2.3.Clinical tests
171 Subjects were evaluated before (baseline, V0) and after surgery (at 1 month [Visit-
172 month 1; Vm1], 3 [Vm3], and 6 months [Vm6]). The following tests were performed in
173 all visits in the following order: 1) The Ocular Surface Disease Index (OSDI) questionnaire
174 was used to evaluate DE symptoms, questions being scored on a 0-100 scale, values
175 above 12 points were considered as abnormal (Schiffman et al., 2000); 2) Uncorrected
176 distance visual acuity was measured using the standard Early Treatment Diabetes
177 Retinopathy Study (ETDRS) chart; 3) The TearLab Osmolarity System (TearLab
178 Cooperation, San Diego, CA, USA) was used to measure tear osmolarity, values above
179 308 mOsm/L were considered as abnormal (Lemp et al., 2011); 4) Conjunctival
180 hyperemia was evaluated with a slit lamp using the Efron scale (0-4 range) (Efron, 1998);
181 5) Tear breakup time (TBUT) was evaluated after applying a fluorescein strip (Fluorets,
182 Chauvin, Aubenas, France), previously wetted with a preservative-free saline solution

(NaCl 0,9% 10ml, B/Braun), and subjects were observed with the cobalt blue filter over the slit lamp biomicroscope light source and a Wratten #12 yellow filter, a cut-off value of 7 seconds was considered for this test (Sullivan et al., 2010). The procedure was repeated 3 times and the mean value was recorded; 6) Fluorescein corneal staining was evaluated 2 min after that with the same filters as above 7) Conjunctival staining was evaluated using lissamine green strips (GreenGlo; HUB Pharmaceuticals LLC, Rancho Cucamonga, CA, USA) wetted with a preservative-free saline solution and applied gently into the inferior fornix. Corneal and conjunctival staining were evaluated according to the Oxford scheme (range, 0-5) (Bron et al., 2003), staining values higher than 1 were considered abnormal (Whitcher et al., 2010); 8) Schirmer test was performed placing one Schirmer sterile strip (Schirmer Tear Test Strips; Alcon Laboratories, Inc., Fort Worth, Texas, USA) in the lateral canthus of the inferior lid margin after topical anesthetic eye drops (1mg tetracaine hydrochloride and 4mg oxybuprocaine hydrochloride; Alcon Cusi, S.A., Barcelona, Spain), values of less than 5 mm in 5 minutes were considered abnormal (Lemp et al., 2007); 9) Corneal sensitivity was measured with a Belmonte's noncontact gas esthesiometer; the corneal thresholds for mechanical and thermal (hot and cold) sensitivities were determined in the central cornea, mean normal values for mechanical threshold were considered as 116.05 ± 40.37, for heat threshold were 1.78 ± 1.07, and for cold threshold were -2.42 ± 0.84 (López-de la Rosa et al., 2015).

2.4.Collection of tear samples and analysis of tear cytokine/chemokine concentration

In all visits, unstimulated basal tear samples were non-traumatically collected by capillarity from the external canthus of the eye, avoiding additional tear reflex as much as possible (Pinto-Fraga et al., 2018), 10 minutes after conjunctival hyperemia

207 evaluation and before performing any procedure that could stimulate tear secretion.
208 Samples were taken from the eye randomly included in the study. Glass capillary
209 micropipettes (Drummond, Broomall, PA, USA) were used to collect 1 µl of tears. Each
210 sample was then diluted 1:10 (up to a final volume of 10 µl) in a sterile collection tube
211 containing 9 µl of ice-cold Cytokine Assay Buffer (Milliplex, Merck-KGaA, Darmstad,
212 Germany). Tubes with tear samples were kept cold (4°C) during collection, and then
213 stored at −80°C until assayed.
214 Tear molecule concentrations were measured simultaneously with a customized 23-plex
215 assay (SPR591, HCYTO-60K, 23X-Milliplex; EMD Millipore, Burlington, Massachusetts,
216 USA) with Luminex IS-100 equipment (Luminex Corporation, Austin, Texas, USA). These
217 molecules were: EGF, CCL11/eotaxin-1, CX3CL1/fractalkine, interferon gamma (IFN)-γ,
218 interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, CXCL8/IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-
219 1 receptor antagonist (IL-1RA), CXCL10/interferon gamma-induced protein 10 (IP-10),
220 CCL2/MCP-1, CCL3/MIP-1α, CCL5/regulated on activation, normal T-cell expressed and
221 secreted (RANTES), tumor necrosis factor (TNF)-α, MMP-9, and vascular endothelial
222 growth factor (VEGF).
223 The samples were analyzed following the manufacturer's low volume sample protocol,
224 (in which only a volume of 10 ul of samples and standards are used for the assay, instead
225 of the 25 ul used in regular protocol) as previously described (Pinto-Fraga et al., 2018).
226 Data were stored and analyzed with ''Bead View Software'' (Upstate-Millipore
227 Corporation, Watford, UK).

228 2.5.Statistical Analysis

229 Statistical analysis was performed by a PhD-licensed statistician (co-author IF) using the
230 R statistical package version 3.1.1 (R Core Team; Foundation for Statistical Computing,

231 Vienna, Austria; URL: https://www.R-project.org/). Statistical significance was set at
232 $P \leq .05$.

233 Mean values and 95% confidence intervals (95% CI) were used to describe quantitative
234 variables (age, refractive error, OSDI, tear osmolarity, TBUT, Schirmer test, corneal
235 esthesiometry, and tear molecules concentration), while median values and
236 interquartile range (IR) were used for ordinal ones (UDVA, conjunctival hyperemia, and
237 corneal and conjunctival staining).

238 For cytokine analysis, to impute cytokine values below the assay detection limit, robust
239 regression on order statistics was used: this method performs a regression to impute
240 low values assuming log-normal quantiles for samples with a detection rate of at least
241 30%, after checking that the data follows a log-normal distribution. To accomplish this,
242 the nondetects and data analysis (NADA) R package was used (Lopaka, 2017). Molecules
243 detected in less than 30% of the samples were not statistically analyzed any further.
244 Cytokine expression data were transformed using the logarithmic base 2 scale. Pro-
245 inflammatory/anti-inflammatory cytokine ratios (IFNγ/IL-4, IFN-γ/IL-10, IL-6/IL-4, and IL-
246 6/IL-10) were also calculated.

247 A linear mixed effects model was used to evaluate the effect of time on clinical tests and
248 tear cytokine concentration and ratios, and Least Squares Means and their differences
249 were estimated for effect quantification, using the lme4 R package (Bates et al., 2015)
250 and R lmerTest (Kuznetsova et al., 2018). Tukey´s method was used to compute the
251 adjusted *P*-values for multiple comparisons with the lsmeans R package (Lenth, 2016).
252 Ordinal scale variables, such as conjunctival hyperemia and corneal and conjunctival
253 staining, were modeled using cumulative logit ordinal models with a mixed-effect for
254 evaluating changes over time.

To evaluate the relation between symptoms and cytokine concentration, a Pearson's correlation coefficient was performed. P-values for the no association hypothesis were adjusted for multiple comparisons using Benjamini and Hochberg method (Benjamini and Hochberg, 1995).

3. Results

Eighteen eyes of 7 males and 11 females were included in the study, 13 of them had been contact lens wearers before refractive surgery and none of them were using artificial tears habitually before entering the study. Their mean age was 34.6 (95% CI: 31.9, 37.3), range: 27-46 years, with no significant difference between males and females ($P>0.05$). Mean refractive error in the preoperative visit (V0) was -4.03 (95% CI: -3.25, -4.81), range: -1.5 to -8.00 diopters.

3.1. Clinical tests

The results of the clinical tests in all the visits can be seen in **Table 1**. Also, outcomes of DE symptoms and clinical tests throughout the study are shown in **Table 2**. As for changes over time, UDVA significantly improved between V0 and the rest of the visits ($P<.0001$), but not between postoperative visits. Symptoms (OSDI questionnaire) decreased over time, with significant differences between V0 and Vm3, V0 and Vm6, and between Vm1 and Vm6 as seen in **Figure 1A**, with symptoms under normal range at Vm3 and Vm6. Tear osmolarity also decreased significantly between V0 and Vm3 from where values were under normal limits (**Figure 1B**). There was a decrease in corneal mechanical sensitivity threshold between Vm1 and Vm3, and Vm1 and Vm6 (**Figure 1C**). The rest of the clinical tests did not show any significant changes throughout the 6 months of the study.

3.2. Tear molecules

279 The percentage of detection and the concentration of the 23 cytokines and chemokines
280 were analyzed in each tear sample (**Table 3**). Sixteen molecules had a percentage of
281 detection >30% in all visits, four were detected <30% only in Vm1 (over 30% in the rest
282 of the visits), and three molecules (CCL11/eotaxin-1, CCL3/MIP-1α, and TNF-α) had a
283 percentage of detection <30% in all visits; therefore, these three molecules were not
284 considered for further statistical comparisons.
285 The effect of time on cytokine/chemokine tear levels, as determined by the linear effect
286 model, revealed that IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17A, CXCL10/IP-10,
287 IFN-γ, and VEGF tear levels significantly varied with time (**Table 4**). Particularly, IL-2, IL-
288 4, IL-5, IL-6, IL-13, IL-17A, IFN-γ, and VEGF tear levels significantly increased at Vm1
289 compared to V0; their increase continued to be significant up to Vm6, except for IL-2
290 and VEGF which were not significantly increased at Vm3. Additionally, IL-9, IL-10, and IL-
291 12 levels were significantly increased at Vm6 (**Figure 2**). Finally, IL-4, IL-10, and IFN-γ tear
292 levels were significantly higher at Vm6 than at Vm1, and those of IL-10 were significantly
293 higher at Vm6 than at Vm3 (**Figure 2**).
294 Individual molecule concentrations of IFN-γ/IL-4, IFN-γ/IL-10, IL-6/IL-4, and IL-6/IL-10,
295 level ratios and their variation over time were also calculated as indexes of balance
296 between pro- and anti-inflammatory cytokines during the entire follow-up period
297 (**Figure 3**). Effect of time on IFN-γ/IL-10 and IL-6/IL-4 ratios was significant (*P*=0.0246,
298 *P*=0.0027, respectively). IFN-γ/IL-10 mean ratio was significantly increased at Vm3,
299 whereas IL-6/IL-4 mean ratio significantly decreased from V0 at Vm3 and Vm6.

300 3.3. Correlation between tear molecules and symptoms

301 The correlation between symptoms (OSDI questionnaire) and cytokines concentration
302 and the ratio of pro- and anti-inflammatory cytokines was analyzed (Table 5), showing

303 no significant correlation after adjustment between cytokines or cytokines ratio and
304 symptoms at any time points.

305 **4. Discussion**

306 This study demonstrated that although clinical tests reached normal values 6 months
307 after ASA surgery, 9 out of 20 tear molecules detected were still significantly increased
308 compared to preoperative values, indicating that the homeostasis of the ocular surface
309 had not yet recovered.

310 As it can be observed in Table 2, most of our subjects had symptoms and or signs of DE
311 in the baseline visit (following the cut-off values described in the methods section). Our
312 sample is composed of subjects who had decided to have refractive surgery and in
313 whom an experienced and extremely cautious ophthalmologist of our Refractive Unit
314 had prescribed such surgery as adequate. We did not intend to exclude mild DE from
315 the study while they were considered candidates to refractive surgery (Bower et al.,
316 2015; Chao et al., 2014; Yu et al., 2000; Zhang et al., 2016). It is estimated that between
317 10-50% of the candidates for refractive surgery have symptoms or clinical signs of DE
318 (Maychuk, 2016; Yu et al., 2000), however, severe DE subjects were excluded as
319 candidates for refractive surgery (Cohen and Spierer, 2018; Toda, 2018).

320 In this study, the clinical tests that changed significantly from baseline (V0) along the
321 study were symptoms (OSDI questionnaire), tear osmolarity and corneal esthesiometry
322 (mechanical threshold). Preoperative values (V0) of symptoms and tear osmolarity were
323 above what is considered as cut-off values of these tests in the majority of the subjects,
324 probably due to the fact that most of our subjects were contact lens wearers (13 out of
325 18). DE symptoms are frequent in patients prior to refractive surgery (between 38-75%)
326 (McGhee et al., 1996; de Paiva et al., 2006), often being contact lens wearers that suffer

from contact lens intolerance and thus are seeking for an alternative method for correcting their refractive errors (Cohen and Spierer, 2018; Naroo et al., 1999; Shtein, 2011). Also, an increase of tear osmolarity has been described in contact lens wearers (Golebiowski et al., 2017; López-de la Rosa et al., 2019; Nieto-Bona et al., 2018), although this is not the case in our study, as the mean of tear osmolarity in the CL wearers was 314.15 ± 20.63 and in the non CL wearers was 316 ± 20.63. In any case, all the subjects were in the range of normal to mild-moderate DE concerning the results of this test (Wolffsohn et al., 2017).

These facts can explain the lack of significant changes in clinical tests between V0 and Vm1 in our sample, while increased DE disease-related signs and symptoms in the first months postoperatively (both in LASIK and PRK/ASA techniques) has been described in the literature (Bower et al., 2015; Chao et al., 2014; Denoyer et al., 2015; Hovanesian et al., 2001; Jabbur et al., 2004; Murakami and Manche, 2012; De Paiva et al., 2006; Shoja and Besharati, 2007).

However, OSDI and tear osmolarity values decrease significantly at Vm3, reaching normal values (below the cut-off point) at 6 months (Vm6) for symptoms and at 3 months (Vm3) for tear osmolarity.

Even more, an improvement in the DE status of our subjects was found along the study (see **Table 2**). While 55.6% of subjects started the study with symptoms and signs altered, only one (5.6%) finished the study in this group. Most of these subjects finished the study with only symptoms or signs altered (72.2% at 6 month-visit). It was also found a slight increase in the number of subjects without symptoms and signs (from 11.1 at baseline to 22.2% at 6-month visit). These results could be explained by the fact that these patients were treated topically with anti-inflammatories and with artificial tears

four times a day throughout the study, as part of the post-surgery protocol, a protocol similar to the one proposed for mild DE disease treatment (Jones et al., 2017). Important to mention that no subjects in this study developed a neuropathic DE after refractive surgery, although it has been described that DE subjects are more prone to develop this complication (Toda et al., 2002; Yu et al., 2000).

Corneal esthesiometry values at V0 were similar to those described for a healthy population (López-de la Rosa et al., 2015); between V0 and Vm1 mechanical threshold increased (which means a decrease in corneal sensitivity) although without reaching statistical significance. After that, threshold values were significantly lower at Vm3 and Vm6 compared to Vm1, reaching values close to those obtained at V0. These changes have also been described in LASIK and ASA procedures by other authors (Darwish et al., 2007; Lee et al., 2006).

Corneal refractive surgery techniques provoke a logical ocular surface traumatism, which involves a complex cascade of cellular responses and interactions mediated by inflammatory molecules and growth factors triggered by the epithelial injury. An increase of EGF, eotaxin, hepatocyte growth factor (HGF), IL-1, IL-6, IL-8, IL-12, keratinocyte growth factor (KGF), NGF, platelet-derived growth factor (PDGF), transforming growth factor (TFG)-β, and TNF-α have been described in the immediate postoperative period (up to one week); all these molecules participate in the wound healing process, leading ultimately to tissue structure and function renewal (Alio and Javaloy, 2013; Lee et al., 2002; Leonardi et al., 2009; Long et al., 2006; Nakamura et al. 2002; Resan et al., 2015, 2016; Suzuki et al., 2003; Tervo et al., 1997; Tomás-Juan et al., 2015; Urgancioglu et al., 2009; Wilson et al., 1999a, 1999b, 2001a). Once this acute process is resolved, inflammatory reaction decreases progressively, but to our

knowledge there is no much information about the variation of these molecules in tears in a longer period.

In this study tear levels of a panel of 23 cytokine/chemokines were determined preoperatively and at 1, 3, and 6 months after ASA surgery in order to establish the time course of cytokine/chemokine tear levels. Our results showed that IL-2, IL-4, IL-5, IL-6, IL-13, IL-17A, IFN-γ, and VEGF tear levels were significantly increased at one month postoperatively. Interestingly, although clinical data from these patients improved significantly by this time point, all of these molecules were still significantly increased at six months after surgery; additionally, some molecules such as IL-9, IL-10 and IL-12 were only increased at Vm6. This indicates that the ocular surface inflammation process is not completely resolved by 6 months after ASA surgery. In fact, some of those molecules (IL-6, IL-17A and IFN-γ) have been found increased in DE disease (Hagan et al., 2016; Tamhane et al., 2019).

NGF, IL-6, MMP-9, EGF and CGRP (Chao et al., 2016; Gao et al., 2014; González-Pérez et al., 2012; Lee et al., 2005; Zhang et al., 2016) tear concentrations have been found increased at different time points (between 1 and 12 months follow-up) with different corneal refractive surgery techniques. We found that IL-6 was increased along the study (up to 6 months) from baseline; however Gao et al. (2014) found a significant increase at 1 month but not at 3 month evaluation, and Hessert et al. (2013) didn't find any change in a 3 month study. These differences can be attributed to different surgery techniques (LASIK, PRK or ASA) or differences in the laboratory technique used to analyze tear molecules, such as Luminex or ELISA. Regarding MMP-9 we did not find any significant change in our study, in accordance with results from Hessert et al. (2013).

TNF-α (Resan et al., 2016) and eotaxin-1 (Leonardi et al., 2009) tear levels were reported significantly increased 24h after PRK surgery. However, in our study the percentage of detection of TNF-α and eotaxin-1 was very low (<30% in all visits). That might be explained by the fact that our first postoperative time point was 1 month, when the wound healing reaction would have resolved. Our results are in accordance with other studies where TNF-α levels were undetectable or not significantly increased at 1 month from baseline (Gao et al., 2014; Hessert et al., 2013; Zhang et al., 2016).

Additionally, most cytokines followed the same tendency, increasing at V1m, decreasing at V3m, and increasing again at V6m. However, cytokine levels at Vm3, were not significantly different compared to V1m in any case. Besides, although it is not significant, values at 3 months were higher than at baseline as it can be seen in Table 3. We think that this altered pattern could be related with the corticosteroid anti-inflammatory treatment received by all patients during a 2 month-period after the surgery, which is the standard post-surgical management. This agrees with a delayed spike in tear substance P concentration at months 1 and 3 after LASIK observed by Chao et al, which has been suggested to be due to the use of topical FML for the first month after surgery (Chao et al., 2015).

While individual cytokines are usually considered either pro- or anti-inflammatory molecules, cytokines work synergistically to restore homeostasis; the balance between pro- and anti-inflammatory cytokines also seems to be more important than individual cytokine/chemokine concentration values in clinical outcomes in several human diseases (Biswas et al., 2010; Chao et al., 2017; Dodoo et al., 2001; Khan et al., 2011; Kilic et al., 2006; Liang et al., 2015). Consequently, besides the individual tear levels, we studied the ratios between pro-inflammatory (IFN-γ, IL-6) and anti-inflammatory

422 cytokines (IL-4, IL-10) throughout the entire follow-up period. We found that the IL-6/IL-
423 4 ratio (<1 in all visits) decreased over time, being significant at Vm3 compared to
424 baseline value (V0). This coincides with significant improvement in some clinical
425 parameters, including a decrease in tear osmolarity, and a decrease in the mechanical
426 threshold in corneal esthesiometry. In contrast, the IFN-γ/IL-10 ratio increased to values
427 >1 in Vm1 and, significantly at Vm3; returning to values <1 value at Vm6.
428 The correlation between symptoms (OSDI questionnaire) and cytokine concentration
429 and the ratio of pro- and anti-inflammatory cytokines was analyzed (Table 5), showing
430 no significant correlation after adjustment between cytokines and symptoms at any
431 time points. Some studies have reported an increase of pro-inflammatory cytokines
432 concentration on tears with an increase of symptoms in DE patients (Lam et al., 2009;
433 Liu et al., 2017; Tan et al., 2014; Tong et al., 2018). On the other side, a correlation
434 between symptoms and cytokine concentration in contact lens wearers was not found
435 by some authors (Martin-Montañez et al., 2016; Willcox et al., 2015), although one study
436 found a positive association between symptoms and ratios of pro-inflammatory to anti-
437 inflammatory cytokines (Chao et al., 2017). Differences in the questionnaire used to
438 evaluate symptoms, or in the study group (DE subjects, CL wearers, post-refractive
439 surgery subjects) might help to explain these differences. Studies evaluating this
440 potential association are then warranted.
441 A limitation of this study is a relatively small sample of patients. Further studies in larger
442 cohorts of patients and with longer follow-up period are warranted. Of special interest
443 would be to study the implications of our findings into developing neuropathic DE
444 and/or chronic pain after refractive surgery. Another limitation of the study is the low
445 basal tear volume that can be collected by microcapillary from patients in a reasonable

time (up to 5 minutes) without provoking a reflex secretion of tears. This fact has provoked first, that it was not possible to assay samples in duplicates and second, that the percentage of detection of some cytokines in some visits was low. We have tried to minimize this effect by using a published low volume protocol and using kits based in XMAP technology that provide a very high cytokine sensitivity values, in order to avoid high sample dilution factors which will greatly decrease assay sensitivity. Also, to analyze the results when the percentage of detection was low, we have used a ROS approach that permits imputing data with detecting percentages up to 20%, although we have increased this detection level to 30%.

In conclusion, these results indicate that ocular surface homeostasis recovery could be considered incomplete at 6 months after ASA surgery, as some tear pro-inflammatory cytokines/chemokines do not recover their basal values.

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- **Figures Legends**

Figure 1.

Changes with time in (A) symptoms, (B) tear osmolarity, and (C) corneal esthesiometry (mechanical threshold). Data is expressed as mean and 95% confidence interval. V0: baseline visit; Vm1: 1-month postoperative visit; Vm3: 3-month postoperative visit; Vm6: 6-month postoperative visit; mOsm/L: milliosmoles/liter; mL/min: milliliters/minute. Exact *P*-value of difference between visits is indicated above the brackets.

Figure 2.

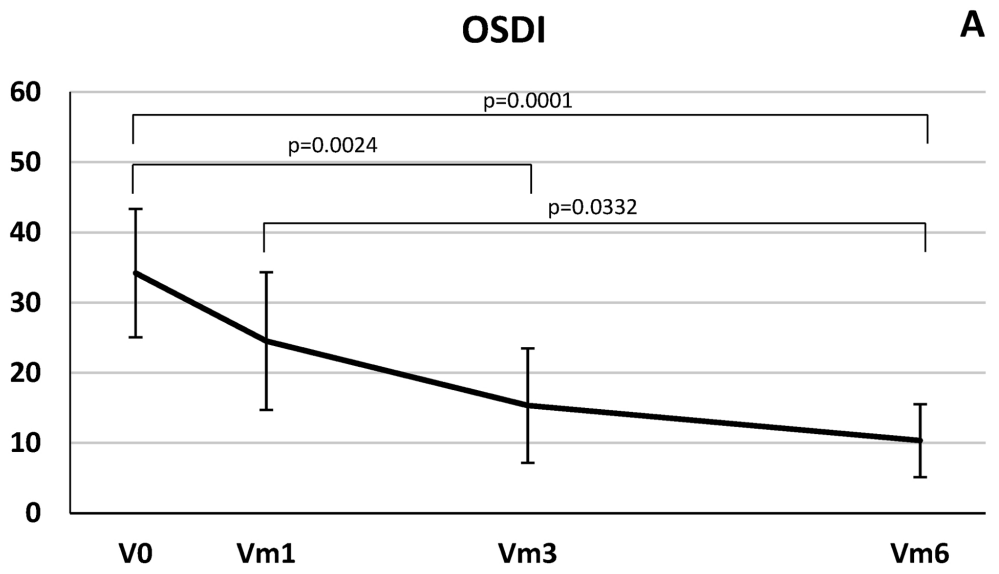
Changes with time in tear molecules: (A) IL-2, (B) IL-4, (C) IL-5, (D) IL-6, (E) IL-9, (F) IL-10, (G) IL-12, (H) IL-13, (I) IL-17A, (J) CXCL10/IP-10, (K) IFN-γ, and (L) VEGF. Data is expressed as mean and 95% confidence interval. V0: baseline visit; Vm1: 1-month postoperative visit; Vm3: 3-month postoperative visit; Vm6: 6-month postoperative visit; Pg/ml: picograms/milliliter. Exact *P*-value of difference between visits is indicated above the brackets.

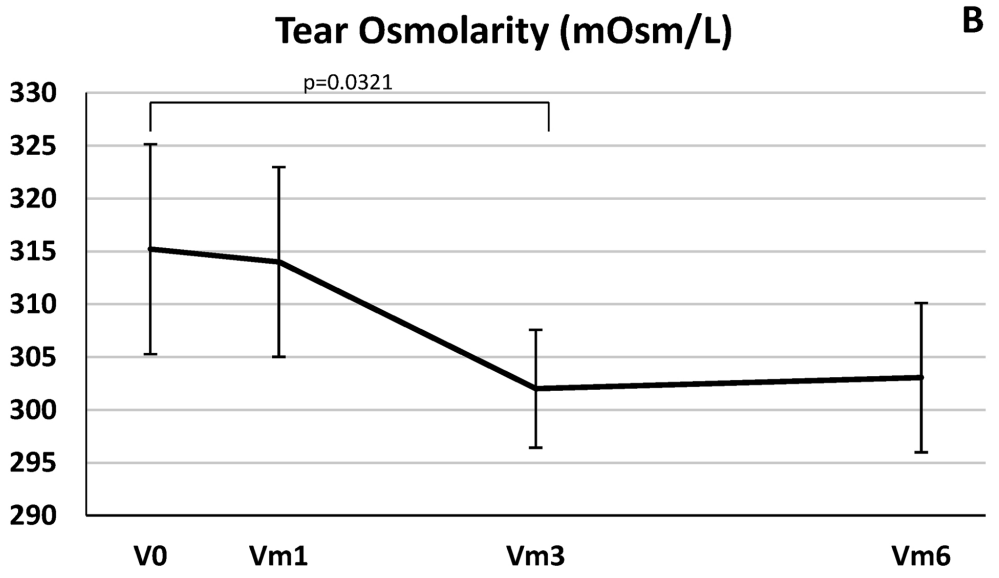
Figure 3.

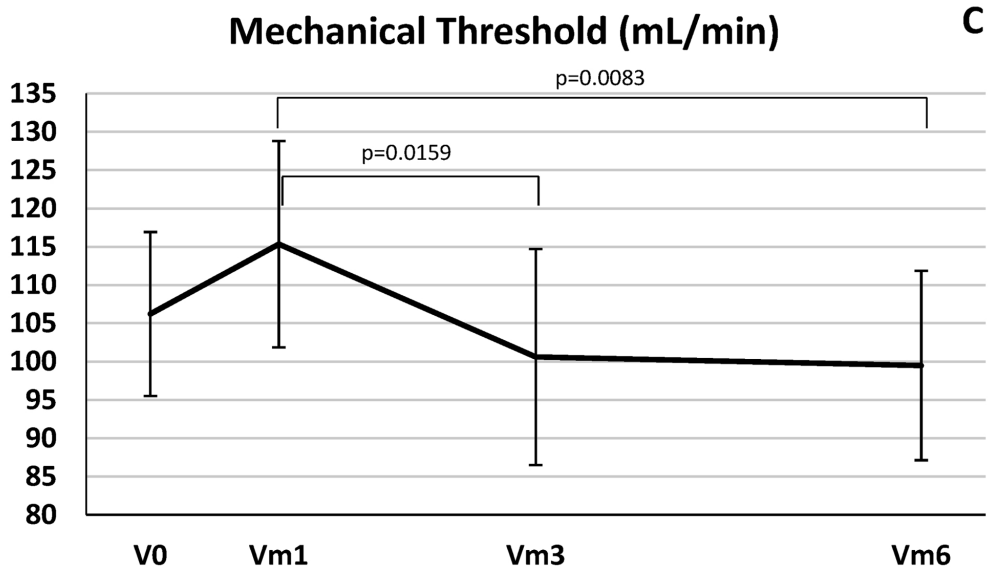
Pro-inflammatory/anti-inflammatory tear cytokine level ratios. (A) IFN-γ/IL-4, (B) IFN-γ/IL-10, (C) IL-6/IL-4, and (D) IL-6/IL-10. Data is expressed as mean and 95% confidence interval. Exact *P*-value of difference between visits is indicated above the brackets.

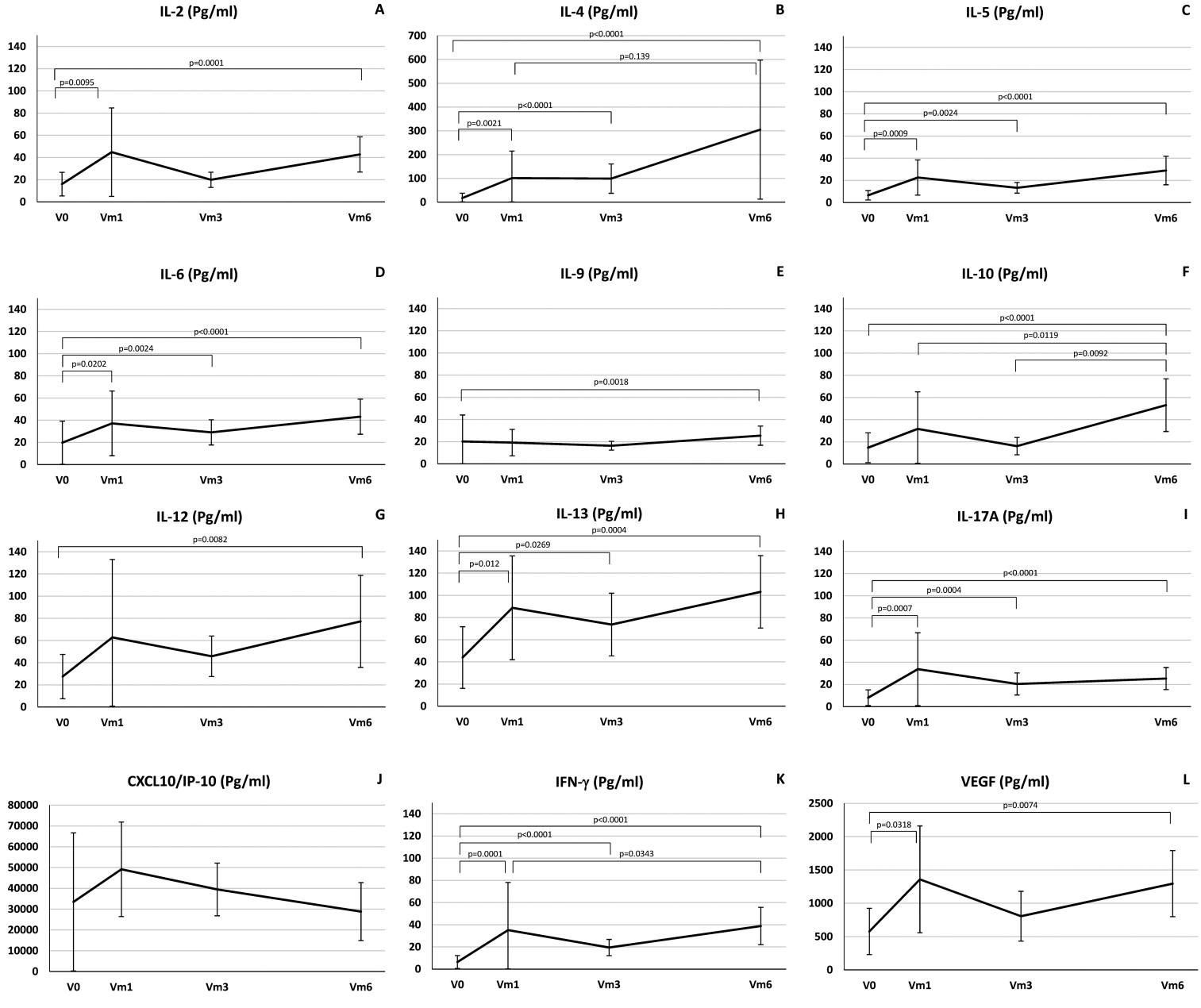
Highlights

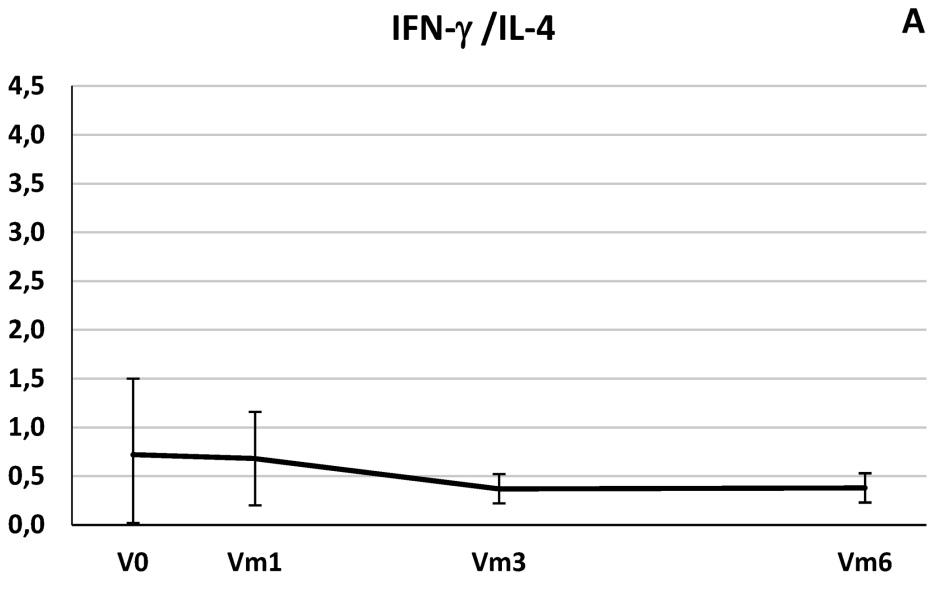
- Clinical variables completely restore after 6 months of ASA refractive surgery
- Some tear pro-inflammatory cytokines/chemokines do not recover their basal values
- Ocular surface homeostasis is not completely restored after 6 months of ASA surgery

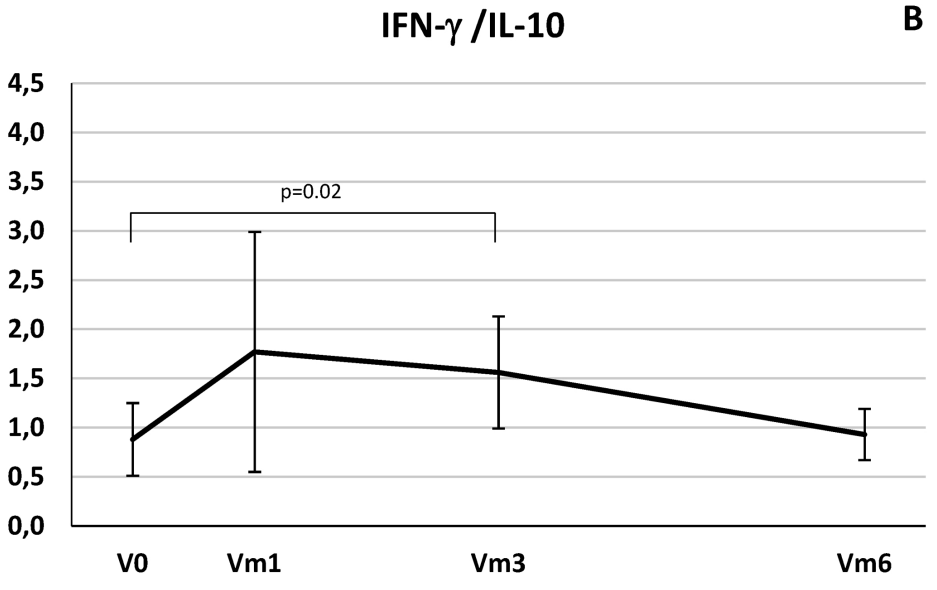

OSDI
A
60
50
40
30
20
10
0
p=0.0001
p=0.0024
p=0.0332
V0
Vm1
Vm3
Vm6

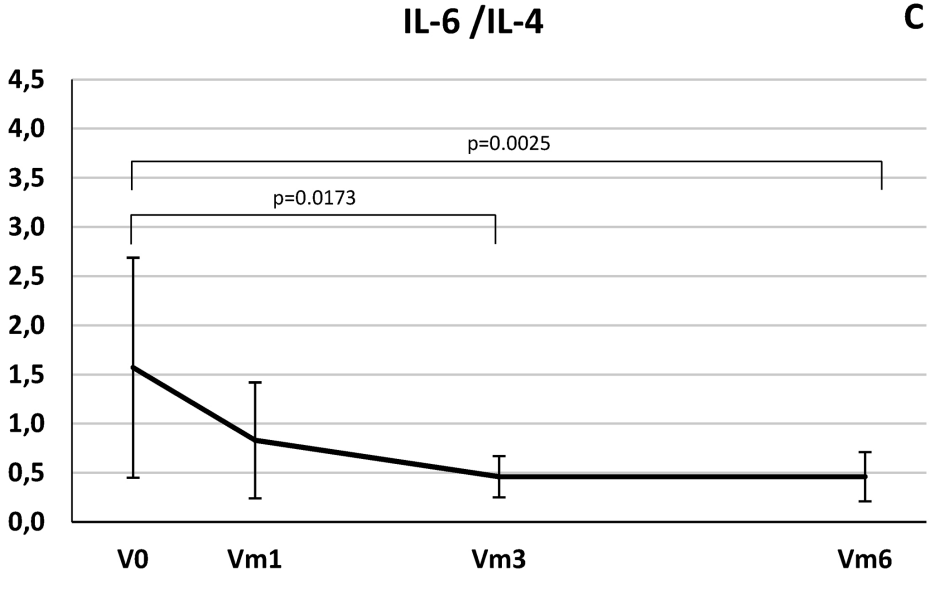

Tear Osmolarity (mOsm/L)
B
330
325
320
315
310
305
300
295
290
p=0.0321
V0
Vm1
Vm3
Vm6


Mechanical Threshold (mL/min)
C
135
130
125
120
115
110
105
100
95
90
85
80
p=0.0083
p=0.0159
V0
Vm1
Vm3
Vm6


IL-2 (Pg/ml)
A
p=0.0095
p=0.0001
IL-4 (Pg/ml)
B
p<0.0001
p=0.139
p<0.0001
p=0.0021
IL-5 (Pg/ml)
C
p<0.0001
p=0.0024
p=0.0009
IL-6 (Pg/ml)
D
p<0.0001
p=0.0024
p=0.0202
IL-9 (Pg/ml)
E
p=0.0018
IL-10 (Pg/ml)
F
p<0.0001
p=0.0119
p=0.0092
IL-12 (Pg/ml)
G
p=0.0082
IL-13 (Pg/ml)
H
p=0.0004
p=0.0269
p=0.012
IL-17A (Pg/ml)
I
p<0.0001
p=0.0004
p=0.0007
CXCL10/IP-10 (Pg/ml)
J
IFN-γ (Pg/ml)
K
p<0.0001
p<0.0001
p=0.0001
p=0.0343
VEGF (Pg/ml)
L
p=0.0074
p=0.0318
V0
Vm1
Vm3
Vm6


IFN-γ /IL-4
A
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
V0
Vm1
Vm3
Vm6


IFN-γ /IL-10
B
p=0.02
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
V0
Vm1
Vm3
Vm6


IL-6 /IL-4
C
p=0.0025
p=0.0173
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
V0
Vm1
Vm3
Vm6

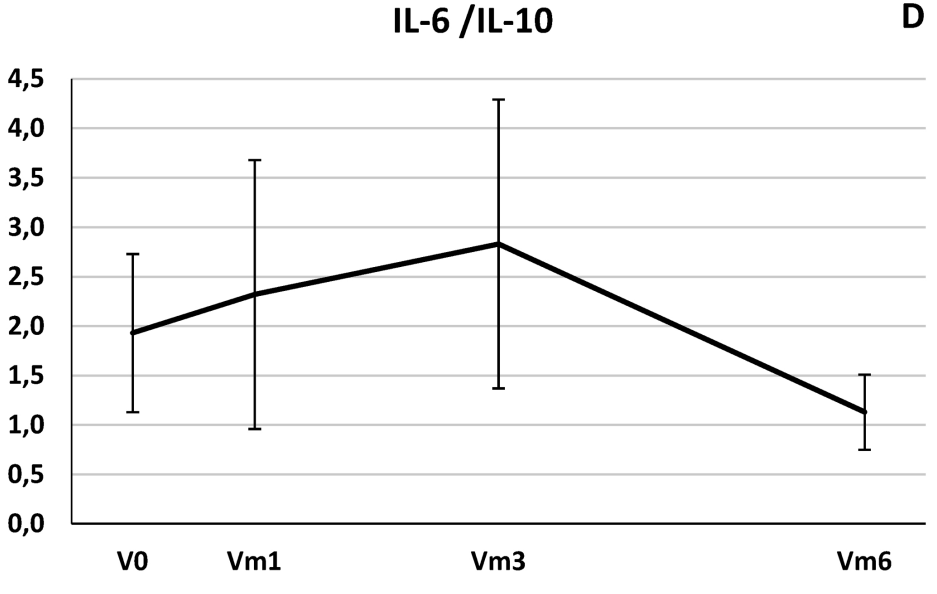

IL-6 /IL-10
D
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
V0
Vm1
Vm3
Vm6

Table 1: Clinical tests in the preoperative visit (n=18)

Clinical test (units)	Mean (95% CI) or median (IR)	Range
UDVA (Snellen feet)	20/200 (<20/200, 20/100)	<20/200 – 20/63
Refractive error (diopters)	-4.03 (-3.25, -4.81)	(-1.5) - (-8.00)
Ocular Surface Disease Index	34.22 (25.09, 43.36)	0 - 77
Conjunctival hyperemia	1 (1, 1)	1 - 2
Tear osmolarity (mOsm/L)	315.22 (305.3, 325.15)	286 - 365
Tear Break Up Time (seconds)	8.06 (6.23, 9.88)	3 - 15
Corneal fluorescein staining	0.5 (0, 1)	0 - 1
Conjunctival lissamine staining	0 (0, 1)	0 - 1
Schirmer test (mm)	14.78 (11.42, 18.14)	4 - 30
Corneal esthesiometry		
Mechanical threshold (mL/min)	106.22 (95.5, 116.94)	58 - 148
Heat threshold (˚C)	1.55 (1.31, 1.79)	1.03 - 3.06
Cold threshold (˚C)	-2.46 (-2.78, -2.15)	(-3.26) - (-0.94)

UDVA: uncorrected distance visual acuity; mOsm/L: milliosmoles/liter; mm: millimeters; mL/min: milliliters/minute; ˚C: Celsius degrees; CI: confidence interval; IR: interquartile range

Table 2: Clinical outcomes in terms of Dry Eye symptoms and tests altered throughout the study.

	V0 (Baseline)			Vm1 (at 1 month)			Vm3 (at 3 months)			Vm6 (at 6 months)		
	n	%	95% CI	n	%	95% CI	n	%	95% CI	n	%	95% CI
No symptoms, no signs	2	11.1	0 ; 25.63	0	0.0	0 ; 0	5	27.8	7.09 ; 48.47	4	22.2	3.02 ; 41.43
Symptoms OR signs	6	33.3	11.56 ; 55.11	13	72.2	51.53 ; 92.91	9	50.0	26.9 ; 73.1	13	72.2	51.53 ; 92.91
Symptoms AND signs	10	55.6	32.6 ; 78.51	5	27.8	7.09 ; 48.47	4	22.2	3.02 ; 41.43	1	5.6	0 ; 16.14

CI: confidence interval

Table 3: Tear cytokine/chemokine percentage of detection and concentration at each visit

Molecule	Visit 0 (Baseline)		Visit Vm1 (at 1 month)		Visit Vm3 (at 3 months)		Visit Vm6 (at 6 months)	
	% detection (95% CI)	**Concentration*** Pg/ml; Mean (95% CI)	**% detection (95% CI)**	**Concentration*** Pg/ml; Mean (95% CI)	**% detection** (95% CI)	**Concentration*** Pg/ml; Mean (95% CI)	**% detection** (95% CI)	**Concentration*** Pg/ml; Mean (95% CI)
IL-1β	60 (32.89, 82.54)	40.79 (6.76, 74.82)	80 (51.37, 94.69)	58.18 (23.3, 93.05)	66.7 (38.69,87.01)	27.17 (13.42, 40.91)	60 (32.89, 82.54)	22.06 (13.85, 30.27)
IL-1RA	100 (74.65, 100)	8213.33 (1055.26, 15371.41)	100 (74.65, 100)	12800.68 (3052.58, 22548.78)	93.3 (66.03,99.65)	7689.71 (241.97, 15137.45)	100 (74.65, 100)	6252.95 (-330.82, 12836.71)
IL-2	40 (17.46, 67.11)	16.03 (5.37, 26.69)	60 (32.89, 82.54)	44.84 (4.96, 84.71)	40 (17.46,67.11)	19.94 (13.06, 26.82)	80 (51.37, 94.69)	42.82 (26.95, 58.7)
IL-4	6.7 (0.35, 33.97)	17.31 (-3.14, 37.77)	46.7 (22.28, 72.58)	101.32 (-12.29, 214.93)	33.3 (12.99,61.31)	99.06 (37.51, 160.6)	60 (32.89, 82.54)	305.17 (13, 597.33)
IL-5	33.3 (12.99, 61.31)	6.55 (2.37, 10.74)	53.3 (27.42, 77.72)	22.61 (6.74, 38.48)	46.7 (22.28,72.58)	13.35 (8.54, 18.16)	66.7 (38.69, 87.01)	28.95 (16.06, 41.83)
IL-6	26.7 (8.91, 55.17)	19.74 (0.31, 39.17)	40 (17.46, 67.11)	37.15 (8.03, 66.27)	46.7 (22.28,72.58)	29.07 (17.71, 40.44)	73.3 (44.83, 91.09)	43.17 (27.34, 58.99)
CXCL8/IL-8	100 (74.65, 100)	158.13 (49.69, 266.56)	100 (74.65, 100)	199.62 (55.27, 343.97)	100 (74.65, 100)	197.55 (-33.63, 428.72)	100 (74.65, 100)	137.85 (16.29, 259.41)
IL-9	33.3 (12.99, 61.31)	20.18 (-3.73, 44.08)	53.3 (27.42, 77.72)	19.18 (7.26, 31.11)	53.3 (27.42,77.72)	16.39 (12.41, 20.38)	66.7 (38.69, 87.01)	25.46 (16.81, 34.11)
IL-10	53.3 (27.42, 77.72)	14.71 (1.28, 28.13)	53.3 (27.42, 77.72)	31.69 (-1.7, 65.09)	53.3 (27.42,77.72)	16.15 (8.36, 23.94)	80 (51.37, 94.69)	53.09 (29.32, 76.85)
IL-12	60 (32.89, 82.54)	27.51 (7.5, 47.52)	46.7 (22.28, 72.58)	62.82 (-7.43, 133.07)	73.3 (44.83,91.09)	45.79 (27.54, 64.04)	66.7 (38.69, 87.01)	77.26 (35.79, 118.73)
IL-13	40 (17.46, 67.11)	43.94 (16.18, 71.7)	73.3 (44.83, 91.09)	88.76 (41.95, 135.56)	86.7 (58.39,97.669	73.65 (45.38, 101.92)	80 (51.37, 94.69)	103.14 (70.51, 135.77)
IL-17A	26.7 (8.91, 55.17)	8.04 (0.96, 15.12)	53.3 (27.42, 77.72)	33.82 (1.03, 66.62)	46.7 (22.28,72.58)	20.43 (10.14, 30,72)	53.3 (27.42, 77.72)	25.36 (15.43, 35.29)
CCL2/MCP-1	80 (51.37, 94.69)	480. 4 (171.05, 789.75)	86.7 (58.39, 97.66)	466.47 (305.21, 627.73)	80 (51.37,94.69)	351.85 (206.77, 496.92)	93.3 (66.03, 99.65)	344.39 (255.77, 463.01)
CCL3/MIP-1A	6.7 (0.35, 33.97	NC	6.7 (0.35, 33.97)	NC	6.7 (0.35, 33.97)	NC	0 (0, 25.35)	NA
CCL5/RANTES	73.3 (44.83, 91.09	254.22 (130.86, 377.58)	73.3 (44.83, 91.09)	338.68 (139.86, 537.51)	66.7 (38.69,87.01)	203.53 (99.45, 307.61)	66.7 (38.69, 87.01)	339.38 (187.78, 490.99)
CCL11/EOTAXIN	6.7 (0.35, 33.97)	NC	6.7 (0.35, 33.97)	NC	6.7 (0.35, 33.97)	NC	0 (0, 25.35)	NA
CXCL10/IP-10	86.7 (58.39, 97.66)	33481.29 (268.4, 66694.18)	66.7 (38.69, 87.01)	49172.51 (26437.31, 71907.71)	53.3 (27.42,77.72)	39475.08 (26814.63, 52135.52)	80 (51.37, 94.69)	28813.11(14881.87,42744.34)
CX3CL1/ Fractalkine	80 (51.37, 94.69)	1318.58 (874.01, 1763.14)	80 (51.37, 94.69)	2246.9 (1187.8, 3306.01)	80 (51.37,94.69)	1825.4 (1387.66, 2263.15)	73.3 (44.83, 91.09)	2090.39 (1511.9, 2668.88)
MMP-9	93.3 (66.03, 99.65)	3603.35 (-2060.79, 9267.5)	100 (74.65, 100)	2966.37 (-1276.87, 7209.6)	93.3 (66.03,99.65)	3195.6 (426.95, 5964.24)	100 (74.65, 100)	2058.2 (81.25, 4035.15)
TNF-α	6.7 (0.35, 33.97)	NC	20 (5.31, 48.63)	NC	13.3 (2.34, 41.61)	NC	20 (5.31, 48.63)	NC
EGF	100 (74.65, 100)	1609.14 (711.99, 2506.29)	100 (74.65, 100)	2188.67 (1343.03, 3034.3)	100 (74.65, 100)	1869.73 (1212.67, 2526.79)	100 (74.65, 100)	1605.4 (1087.94, 2122.86)
IFN-γ	13.3 (2.34, 41.61)	6.36 (0.52, 12.21)	40 (17.46, 67.11)	35.17 (-7.75, 78.09)	40 (17.46,67.11)	19.44 (12.1, 26.78)	66.7 (38.69, 87.01)	38.82 (21.98, 55.66)
VEGF	73.3 (44.83, 91.09)	577.34 (229.58, 925.09)	86.7 (58.39, 97.66)	1358.96 (557.09, 2160.83)	86.7 (58.39,97.66)	805.59 (431.88, 1179.3)	86.7 (58.39, 97.66)	1294.91 (799.41, 1790.41)

*Cytokine/chemokine concentration was calculated imputing non-detected values by the robust regression on order statistic method in molecules with >30% of detection. IL-: Interleukin; MMP: metalloproteinase; CI: Confidence interval; SD: Standard deviation; Pg/ml: picograms/milliliter. NC: not calculated. NA: Not applicable.

Table 4: Effect of time on tear cytokine/chemokine concentration

Molecule	***P***	**Molecule**	***P***
IL-1β	.1994	IL-13	**.0006**
IL-1RA	.3253	IL-17A	**<.0001**
IL-2	**.0002**	CCL2/MCP-1	.5127
IL-4	**<.0001**	CCL5/RANTES	0.4432
IL-5	**<.0001**	CXCL10/IP-10	**.0419**
IL-6	**<.0001**	CX3CL1/Fractalkine	*.0687*
CXCL8/IL-8	.528	MMP-9	.7979
IL-9	**.004**	EGF	*.0648*
IL-10	**<.0001**	INF-γ	**<.0001**
IL-12	**.0079**	VEGF	**.0071**

Bold font indicates significant effect of time on molecule concentration; *italics* indicate borderline significance.

Table 5: Pearson's correlation coefficient between symptoms analyzed with the ocular surface disease index (OSDI) score and each tear cytokine level (on log2 scale).

	V0			V1			V2			V3		
	Pearson's r (95%CI)	p-value	Adjusted p-value	Pearson's r (95%CI)	p-value	Adjusted p-value	Pearson's r (95%CI)	p-value	Adjusted p-value	Pearson's r (95%CI)	p-value	Adjusted p-value
EGF	0.0385 (-0.4833;0.5401)	0.8917	0.9376	0.0129 (-0.5027;0.5217)	0.9637	0.9637	-0.0625 (-0.5569;0.4646)	0.8248	0.867	-0.1296 (-0.6019;0.4099)	0.6452	0.815
FRACTALKINE	0.1187 (-0.419;0.5948)	0.6735	0.9376	0.5619 (0.0698;0.8341)	***0.0292***	0.1454	0.2442 (-0.3064;0.6723)	0.3805	0.6549	-0.1484 (-0.614;0.3938)	0.5976	0.7968
IFNg	-0.2308 (-0.6645;0.3192)	0.408	0.9376	0.7148 (0.3195;0.8982)	***0.0027***	*0.054*	0.2897 (-0.2614;0.6983)	0.295	0.6549	-0.2039 (-0.6484;0.3443)	0.4661	0.7968
IL10	0.096 (-0.4378;0.5798)	0.7335	0.9376	0.3096 (-0.2408;0.7094)	0.2614	0.4481	0.4798 (-0.0431;0.7963)	*0.0703*	0.5576	-0.3518 (-0.7321;0.1957)	0.1984	0.7968
IL12	0.1834 (-0.363;0.6359)	0.5129	0.9376	0.599 (0.1252;0.8504)	***0.0183***	0.1454	-0.4459 (-0.78;0.0861)	*0.0958*	0.5576	-0.2189 (-0.6575;0.3304)	0.4331	0.7968
IL13	0.0364 (-0.4849;0.5386)	0.8974	0.9376	0.4671 (-0.0594;0.7902)	*0.0792*	0.2376	0.0954 (-0.4383;0.5793)	0.7353	0.8403	-0.3352 (-0.7233;0.2137)	0.2219	0.7968
IL17A	-0.2207 (-0.6585;0.3287)	0.4292	0.9376	0.5589 (0.0653;0.8327)	***0.0303***	0.1454	-0.0603 (-0.5554;0.4664)	0.8309	0.867	-0.1889 (-0.6393;0.358)	0.5002	0.7968
IL1B	-0.0153 (-0.5235;0.5009)	0.9569	0.9569	0.2322 (-0.3178;0.6654)	0.4049	0.5223	-0.1057 (-0.5862;0.4298)	0.7076	0.8403	-0.2345 (-0.6667;0.3157)	0.4003	0.7968
IL1RA	-0.1359 (-0.606;0.4045)	0.629	0.9376	-0.412 (-0.7632;0.127)	0.127	0.2771	0.2718 (-0.2794;0.6882)	0.3272	0.6549	0.6069 (0.1373;0.8537)	***0.0164***	0.3936
IL2	-0.2098 (-0.652;0.3389)	0.453	0.9376	0.4216 (-0.1156;0.768)	0.1175	0.2771	-0.2483 (-0.6748;0.3024)	0.3721	0.6549	-0.2028 (-0.6478;0.3453)	0.4685	0.7968
IL4	-0.57 (-0.8377;-0.0816)	***0.0265***	0.636	0.2281 (-0.3218;0.6629)	0.4135	0.5223	-0.2434 (-0.6719;0.3071)	0.382	0.6549	-0.0567 (-0.5529;0.4692)	0.8411	0.8411
IL5	0.0819 (-0.4492;0.5702)	0.7717	0.9376	0.4712 (-0.0541;0.7922)	*0.0762*	0.2376	0.1788 (-0.3671;0.6331)	0.5238	0.7857	-0.1859 (-0.6374;0.3607)	0.5072	0.7968
IL6	-0.3046 (-0.7066;0.246)	0.2696	0.9243	0.0577 (-0.4684;0.5536)	0.8383	0.9145	-0.3052 (-0.7069;0.2455)	0.2687	0.6549	-0.2421 (-0.6711;0.3084)	0.3846	0.7968
IL8	0.1438 (-0.3978;0.611)	0.6093	0.9376	-0.297 (-0.7024;0.2539)	0.2824	0.4518	-0.0975 (-0.5808;0.4365)	0.7295	0.8403	0.1148 (-0.4223;0.5923)	0.6836	0.8203
IL9	-0.377 (-0.7453;0.1676)	0.166	0.9243	0.3478 (-0.2001;0.73)	0.2039	0.3927	-0.2183 (-0.6571;0.331)	0.4345	0.6952	-0.1582 (-0.6202;0.3853)	0.5735	0.7968
IP10	0.154 (-0.3889;0.6175)	0.5837	0.9376	0.0649 (-0.4628;0.5586)	0.8183	0.9145	0.4183 (-0.1196;0.7663)	0.1208	0.5576	-0.0902 (-0.5758;0.4425)	0.7493	0.8411
MCP1	0.1825 (-0.3638;0.6354)	0.515	0.9376	0.0367 (-0.4847;0.5388)	0.8967	0.9357	0.1065 (-0.4292;0.5868)	0.7056	0.8403	-0.4518 (-0.7829;0.0786)	*0.0909*	0.7272

	V0			V1			V2			V3		
	Pearson's r (95%CI)	p-value	Adjusted p-value	Pearson's r (95%CI)	p-value	Adjusted p-value	Pearson's r (95%CI)	p-value	Adjusted p-value	Pearson's r (95%CI)	p-value	Adjusted p-value
MMP9	-0.3476 (-0.7299;0.2004)	0.2043	0.9243	-0.2467 (-0.6738;0.304)	0.3754	0.5223	0.0997 (-0.4348;0.5822)	0.7237	0.8403	0.1674 (-0.3772;0.626)	0.551	0.7968
RANTES	-0.0413 (-0.5421;0.4811)	0.8837	0.9376	0.2125 (-0.3364;0.6536)	0.447	0.5364	0.4964 (-0.0212;0.8042)	*0.0598*	0.5576	-0.1835 (-0.636;0.3628)	0.5126	0.7968
VEGF	0.0361 (-0.4852;0.5384)	0.8985	0.9376	0.6888 (0.2729;0.8878)	***0.0045***	*0.054*	0.4002 (-0.1409;0.7572)	0.1394	0.5576	-0.4742 (-0.7937;0.0503)	*0.0741*	0.7272
IFNg/IL4	0.4508 (-0.0799;0.7824)	*0.0917*	0.7336	0.4699 (-0.0558;0.7916)	*0.0772*	0.2376	0.3693 (-0.1763;0.7413)	0.1755	0.6017	-0.0624 (-0.5569;0.4647)	0.8251	0.8411
IFNg/IL10	-0.3249 (-0.7177;0.2248)	0.2374	0.9243	0.4198 (-0.1178;0.7671)	0.1193	0.2771	-0.2979 (-0.7029;0.2529)	0.2808	0.6549	0.359 (-0.1878;0.7359)	0.1888	0.7968
IL6/IL4	0.221 (-0.3285;0.6587)	0.4287	0.9376	-0.2365 (-0.6679;0.3138)	0.3961	0.5223	0.0149 (-0.5012;0.5232)	0.9579	0.9579	-0.0706 (-0.5625;0.4582)	0.8025	0.8411
IL6/IL10	-0.4789 (-0.7959;0.0441)	*0.0709*	0.7336	-0.3416 (-0.7267;0.2069)	0.2127	0.3927	-0.5915 (-0.8471;-0.1137)	***0.0202***	0.4848	0.3255 (-0.2241;0.718)	0.2365	0.7968

IL-: Interleukin; MMP: metalloproteinase; CI: Confidence interval.