

**Profiling ocular surface responses to preserved and non-preserved topical glaucoma medications: a two-year randomised evaluation study**

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## ABSTRACT

**Background:** Use of topical glaucoma medications has been reported to cause ocular surface (OS) discomfort and inflammation. This study explores the profile of inflammatory cytokines and OS symptoms induced in response to preserved and non-preserved drops.

**Methods:** Prospective, randomized evaluation on thirty-six treatment-naïve patients over 24 months of three differently preserved glaucoma drop preparations: Preservative-free (PF), Polyquad (PQ), and Benzalkonium chloride (BAK). Study participants were evaluated at baseline and then at 1, 3, 6, 12 and 24 months whilst on medication. At each visit, participants completed the Ocular Surface disease index (OSDI) questionnaire, had basal tear sampling and impression cytology (IC) of the conjunctival epithelium. Quantitative polymerase chain reaction was performed to measure the gene expression of inflammatory cytokines (Interleukin (IL)-6, IL-8, IL-10, IL-12A, IL-12B, IL-17A, IL-1 $\beta$ , and tumour necrosis factor (TNF)- $\alpha$ ) in the IC samples. Corresponding protein expression of cytokines in tear samples was assessed by the Becton-Dickinson cytometric bead arrays.

**Results:** Compared to PF and PQ groups, mRNA and protein expression of IL-6, IL-8, and IL-1 $\beta$  increased in samples from the BAK group in a time-dependent fashion, whereas all other cytokines showed a non-significant increase. In the BAK group, there was a strong correlation between OSDI and the levels of IC/IL-1 $\beta$  ( $r=0.832$ , R squared= $0.692$  and  $p=0.040$ ); IC/IL-10 ( $r=0.925$ , R squared= $0.856$  and  $p=0.008$ ) and tear/IL-1 $\beta$  ( $r=0.899$ , R squared= $0.808$  and  $p=0.014$ ).

**Conclusion:** BAK-preserved topical drops stimulate a sterile inflammatory response on the OS within 3-months which is maintained thereafter. Whereas PF-drops and PQ-preserved drops showed no significant OS inflammation.

**Keywords:** Glaucoma, Inflammation, Ocular surface, Glaucoma medical therapies, Cytokines

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## 1. INTRODUCTION

Primary open-angle glaucoma (POAG) is the leading cause of irreversible blindness worldwide.<sup>1-3</sup> It affects approximately 73.6 million people globally with an estimated increase to 111.8 million in 2040 including 3.7% of UK and 2.6% of Oceania population aged between 48-92 years.<sup>1,3-5</sup> Anti-glaucoma topical medications are widely used to control intra-ocular pressure (IOP) in POAG patients and are usually the first line treatment recommended.<sup>1,2,4</sup> Evidence suggests that patients receiving long-term topical anti-glaucoma drops may develop ocular surface (OS) disease (conjunctival hyperaemia, dry-eye like syndrome and irritability) or experience aggravation of pre-existing OS disease.<sup>6</sup>

Preservatives are used to prolong the shelf-life of glaucoma medications and some evidence suggested that their detergent properties increased the penetration of the active ingredients of the glaucoma drops.<sup>6,7</sup> Benzalkonium chloride (BAK) is a quaternary ammonium compound that is the most widely used preservative in topical ophthalmic medication.<sup>6,7</sup> It has been shown to have toxic effects on OS epithelia, inducing low-grade inflammation<sup>7-10</sup> and may pose a risk for failure of glaucoma surgery.<sup>11,12</sup> Polyquad (PQ) is polyquaternium-1 belonging to the family of polycationic polymers and is commonly used in contact-lens solution and topical ophthalmic medications.<sup>6</sup> Experimental studies have demonstrated that PQ does not induce cytotoxic effects on the OS.<sup>13,14</sup> However, the long-term effects of PQ-preserved topical glaucoma medications on OS epithelia and patient experience remains unknown.

The aim of this study was to evaluate the inflammatory effects of three different preserved glaucoma drop preparations on the OS of treatment naïve patients. This involved the profiling of inflammatory cytokines on the ocular surface over a 24-

month period and their correlation to changes in ocular surface discomfort as measured with the Ocular Surface Disease Index (OSDI) patient reporting instrument.

## **2. METHODS**

### **2.1 Study design**

The research was approved by the Nottingham Research Ethics Committee-1 (REC Ref: 13/EM/0225), the Research and Innovation unit of the Nottingham University Hospitals, National Health Service Trust (Ref: 12OY006) and the Medicine and Health Regulatory Authority (EudraCT number: 2013-000581-10), UK. All research was conducted in accordance with the tenets of the Declaration of Helsinki. Patients who met the inclusion and exclusion criteria were enrolled (Supplementary Table 1). Informed consent was obtained from each patient prior to enrolment.

A single-center, pragmatic randomized controlled study design was adopted to explore the effect of preserved (bimatoprost 0.01%, travoprost 0.04%) and non-preserved (latanoprost 0.005%, timolol 0.5%, dorzolamide 2%) topical anti-glaucoma medications on the expression of cytokines at the OS. Treatment-naïve glaucoma patients requiring topical hypotensive medication were enrolled and randomized into three groups, receiving either benzalkonium chloride (BAK), polyquad (PQ) or preservative free (PF) drops. A computer-based randomization process provided by the Clinical Trials Unit of the University of Nottingham was used to determine study group allocation. Study visits were scheduled at baseline (prior to commencement of medication), 1-, 3-, 6-, 12- and 24-months following therapy initiation. There are no published longitudinal studies that demonstrated inflammatory effects of the preserved and PF drops on the patient OS. Therefore, as a guide to estimate the sample size, we utilised the outcomes of our previous

impression cytology studies<sup>15-17</sup> that were unrelated to glaucoma drops. It was estimated that n=8 samples/group will have 85% power at alpha=0.05 to detect 2-fold difference (standard deviation = 0.5) in cytokine levels between the groups. As a precautionary step and considering the possibility of attrition of samples and patient drop out during the study period, we collected samples from total 36 patient with 12 enrolled in each treatment group.

At each visit, impression cytology and tear samples were taken, and patients completed the OS disease index (OSDI) questionnaire. Where monotherapy was insufficient to control IOP, drops with the same class of preservative were added for further IOP lowering. For PQ allocated patients only 2 PQ-preserved options exist (travoprost 0.004% monotherapy or travoprost 0.004%/timolol 0.5% combined therapy) if dual therapy was insufficient then the third medication added was PF.

## **2.2 OSDI assessment**

The standard OSDI questionnaire consisting of 12 questions for assessment of symptoms, functional limitations and discomfort related to dry eye was used.<sup>18,19</sup> The questionnaire was administered by trained ophthalmic staff at the start of study and then at each follow-up visits. The baseline questionnaire was elicited prior to study group allocation. In this questionnaire each question is graded on a scale of 0 to 4, with 0 indicating 'none of the time' and 4 indicating 'all the time'. OSDI was calculated as: (sum of scores x 25) / (total number of questions answered). An OSDI score between 0 and 100 was obtained. Scores below 12 reflected normal OS health and above 33 indicated severe dry-eye condition.

## **2.3 Sample collection**

Samples were collected using a microcapillary glass tube (5  $\mu$ L, Drummond Scientific Co., PA) from canthal and inferior fornixes<sup>20</sup> and stored at -80°C. Conjunctival

epithelium was collected using impression cytology (IC) as previously reported.<sup>15</sup> Briefly, cellulose-ester discs of pore-size 0.45µm (Millipore Corporation, MA) and diameter of 13mm were cut into halves and applied to the upper and lower bulbar conjunctiva under topical anaesthesia (proxymetacaine hydrochloride 0.5%) for 10 to 15 seconds. The discs were gently peeled off and transferred into RNA lysis buffer (buffer RLT; Qiagen, UK).

#### **2.4 Total RNA isolation and cDNA synthesis**

Total RNA was isolated from the IC samples using RNeasy mini kit (Qiagen, UK) as per manufacturer's instructions. 150ng of total RNA was then reverse transcribed into cDNA using QuantiTect RT kit (Qiagen, UK).

#### **2.5 Quantitative real-time polymerase chain reaction (qPCR)**

QPCR was performed to measure the relative fold-change of genes of interest using TaqMan probe assays (Supplementary Table 2; Applied Biosystems, UK). Each reaction was prepared to 20µL final volume containing 5µL of diluted cDNA (1 in 5 with nuclease-free (NF) water), 10µL of TaqMan gene expression master mix, 1µL of gene-of-interest, 1µL of endogenous gene (hypoxanthineguanine phosphoribosyltransferase-1 (HPRT1)) and 3µL of NF water. Data was acquired on Mx3005p real-time PCR instrument (Stratagene/Agilent Technologies, UK) and analysed using delta-delta CT method as previously described.<sup>21</sup>

#### **2.6 Cytokine measurement**

Tear cytokines were measured using a BD-cytometric bead array (CBA) human inflammatory cytokines kit (BD Biosciences, UK) as previously reported.<sup>22</sup> The tear samples were diluted 1 in 25 to a final volume of 50µL. To diluted tear samples, 50µL of each capture beads (phycoerythrin (PE) conjugated) and PE-detection reagents was added. The mixture (150µL) was incubated in the dark for 3 hours



and then washed (x2 with wash buffer) *via* centrifugation. The resulting pellet was re-suspended in 300 $\mu$ L of wash buffer for beads analysis using BD-LSR II flow cytometer (BD Biosciences, UK). The acquired data was further analysed using the FCAP array software *version 3.0* (BD Biosciences, UK) and plotted using the Prism 7.0 software (GraphPad software Inc. CA).

## 2.7 Statistical analysis

QPCR and CBA data were statistically analysed using the Prism 7.0 (GraphPad software Inc. CA). Two-way ANOVA was performed to evaluate the statistical differences between PF vs PQ, PF vs BAK, and PQ vs BAK, respectively. As our aim was to compare the effect of preservatives with preservative-free drops, we only reported the statistical data for the PF vs PQ and PF vs BAK groups.

## 2.8 Masking

Masking of patients and clinicians to treatment was not possible. However, IC and tear samples were masked to group allocation and the analysis of biomarkers was undertaken in a masked fashion.

# 3. RESULTS

## 3.1 Demographics and patient samples

A total of 36 patients were recruited based on the study criteria (table 1) and randomized in to three groups: 12 in each of the preservative-free (PF), polyquad (PQ) group and benzalkonium chloride (BAK) group. One participant from the BAK group withdrew after baseline sample collection.

**3.1.1 Impression cytology:** 11 of the 35 IC samples could not be used due to low RNA quantity and/or RNA quality. Cytokine gene expression analysis using QPCR

was performed on the following IC samples (n=7 in PF; n=8 in PQ; and n=9 in BAK).

**3.1.2 Tear samples:** It was not possible to analyse 8 of the 35 tear samples due to low collection volume (samples < 2  $\mu$ L were not used). BD-CBA assay was only performed on complete patient samples (collection at all time points) in each group (n=7 in PF; n=8 in PQ; and n=9 in BAK).

Demographic information of patients whose samples (n=24 in total had complete samples for all time-points) were analysed in this study are provided in table 1. Supplementary table 3 reports the intra-ocular pressure (IOP; mm Hg), visual acuity (VA) and visual field mean deviation (MD) of the three patients' groups at baseline and 24-month. There was a reduction in mean IOP at 24-month in all groups compared to baseline. Mean group VA and mean MD in all groups remained unchanged at 24-month. All patients were started on single drop treatment (latanoprost in PF, travoprost in PQ and bimatoprost in BAK) (Supplementary Table 3). In the PF group, all participants remained on a single drop throughout the study, one patient was started on an ocular lubricant (OL) at 12-month. In the PQ group, four of the eight patients started dual drop therapy (travoprost + timolol) beginning at 3-month. From 12-month onwards, two patients were on three drops with the third added drop (dorzolamide) being a PF formulation. In addition, four of the patients on single drop in PQ group also received an OL. In the BAK group, two patients were moved to dual drop therapy (bimatoprost + timolol) starting 12-month. None of the patients in BAK group were commenced on an OL throughout the study. Refer the Consolidated Standards of Reporting Trials (CONSORT) flow-diagram (supplementary figure 1) and the CONSORT checklist (supplementary table 4) for further information.

## 3.2 Cytokine gene expression in conjunctival impression cytology samples

All IC samples showed constitutive gene expression for *IL-1 $\beta$* , *TNF- $\alpha$* , *IL-6*, *IL-8*, *IL-10* and *IL-12a* but not *IL-12b* and *IL-17a*. Three of the 6 cytokines namely *IL-1 $\beta$* , *IL-8* and *IL-6* showed increased expression in the BAK group (figure 1).

**3.2.1 Interleukin 6 (IL-6):** In the BAK group, *IL-6* mRNA showed more than a 2-fold increase in 7/9 samples at all time-points compared to baseline, but this did not reach statistical significance compared to PF and PQ groups. On the other hand, the *IL-6* mRNA levels in the PF and PQ groups was modestly increased at all time-points with no significant difference noted compared to baseline.

**3.2.2 Interleukin 8 (IL-8):** In the BAK group, *IL-8* mRNA (9/9 samples) was increased more than 4-fold starting at 1-month with significant elevated levels noted at 3-month (4.76-fold increase,  $p=0.0380$ ) and 6-month (5.18-fold increase,  $p=0.0445$ ). Upon reaching 12-month, *IL-8* was slightly reduced, but still remained elevated (9/9 samples) compared to PF and PQ groups. Unlike the BAK group, *IL-8* mRNA levels did not change significantly in PF and PQ groups at any time-point compared to baseline.

**3.2.3 Interleukin 1 $\beta$  (IL-1 $\beta$ ):** In the BAK group, *IL-1 $\beta$*  mRNA increased in a time-dependent manner from 3-month onwards with significantly increased level noted at 6-month (6.03-fold increase,  $p=0.0023$ ; 9/9 samples), and remained at a similar high level until 24-month compared to baseline and all time-points in PF and PQ groups. In the PQ group, there was a 2.92-fold increase in *IL-1 $\beta$*  mRNA levels at 24 months. There was no change noted in the PF group at all time-points.

**3.2.4 Interleukin 10 (IL-10):** *IL-10* mRNA expression demonstrated a 1.5-fold increase in PF group starting 1-month and remained unchanged until 24-month (4/7

samples). In the PQ group, *IL-10* showed reduced expression until 12-month with slight elevation noted by 24-month (in 7/8 samples). In the BAK group, *IL-10* mRNA (in 5/9 samples) was modestly increased in a linear fashion starting at 3-months but failed to reach statistical significance compared to baseline and all time-points in PF and PQ groups.

**3.2.5 Tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) and Interleukin 12a (IL-12a):** *TNF- $\alpha$*  and *IL-12a* mRNA did not change significantly in either of the treatment groups.

### 3.3 Quantification of cytokines in tear samples

The baseline level of IL-6, IL-8, and IL-1 $\beta$  ( $83.34 \pm 55.63$  pg/mL,  $1119.40 \pm 674.68$  pg/mL and  $2.06 \pm 5.65$  pg/mL, respectively) in all patients randomized into three groups were not significantly different (figure 2). The number of patients who showed cytokines level above the detection limit of the BD-CBA assay kit has been detailed in supplementary table 5.

**3.3.1 Interleukin 6 (IL-6):** In the BAK group, IL-6 showed elevated levels starting at 6-month time-point with significantly increased levels noted at 24-month ( $161.06 \pm 73.91$  pg/mL;  $p=0.0368$ ; 9/9 samples) compared to PF group ( $41.69 \pm 36.12$  pg/mL; 5/7 samples). There was also a mild non-significant elevation in IL-6 levels in the PQ group beginning at 6 months, which remained at a similar level until 24-month time-point.

**3.3.2 Interleukin 8 (IL-8):** In the BAK group, IL-8 showed increased levels beginning at 3-month ( $2770.51 \pm 1016.05$  pg/mL;  $p=0.0244$ ; 9/9 samples), which remained elevated until 24 months ( $1827.54 \pm 1038.21$  pg/mL;  $p=0.0388$ ; 9/9 samples) compared to PF group ( $328.42 \pm 186.45$  pg/mL; 6/8 samples). In the PQ

group, IL-8 was modestly increased with no statistical significance noted at all time-points compared to PF group.

**3.3.3 Interleukin 1 $\beta$  (IL-1 $\beta$ ):** In the BAK group, IL-1 $\beta$  showed significant elevated levels starting at 3-month ( $11.79 \pm 8.18$  pg/mL;  $p=0.0243$ ; 6/9 samples) with elevated levels noted at 24-month ( $17.09 \pm 9.74$  pg/mL;  $p=0.0187$ ; 6/9 samples) compared to PF group ( $1.58 \pm 3.54$  pg/mL; 2/7 samples). A mild elevation of IL-1 $\beta$  levels was also noted at all time-points for the PQ group.

**3.3.4 Other cytokines:** IL-10, IL-12p70 (a heterodimer encoded by *IL-12a* gene), and TNF- $\alpha$  was detected at inconsistent low-levels in up to 2 of 8 patient samples (refer Supplementary table 5 for details regarding number of patients that showed cytokines above the detection limit of BD-CBA assay kit).

### **3.4 Ocular Surface Disease Index (OSDI) evaluation**

The mean OSDI score was evaluated for the patients whose samples were used for cytokine estimation. All patients randomized to the PF, PQ, and BAK groups showed similar OSDI scores at the baseline (mean OSDI = 5.22) and there was no significant difference between the groups (figure 3).

For the PF group, the mean OSDI score was less than 12 for 6/7 patients at all time-points.

For the PQ group, the mean OSDI score was more than 12 starting at 6-month in 4/8 patients, with 1 of 8 patients was scored more than 20 at 24-month.

For the BAK group, the mean OSDI score was more than 20 at 12-months (5/9 patients;  $p<0.0001$ ), with 3 of 9 patients was scored more than 30 at 24-month ( $p<0.0001$ ) compared to PF group.

As shown in table 2, the linear correlation between OSDI and cytokines [OSDI vs IC-cytokines and OSDI vs tear-cytokines] was measured using the Pearson correlation test method. We have noted a significant correlation between OSDI and IC/IL-1 $\beta$  ( $r=0.832$ ,  $R\text{ squared}=0.692$  and  $p=0.040$ ), OSDI and IC/IL-10 ( $r=0.925$ ,  $R\text{ squared}=0.856$  and  $p=0.008$ ) and OSDI and tear/IL-1 $\beta$  ( $r=0.899$ ,  $R\text{ squared}=0.808$  and  $p=0.014$ ). However, the correlation between OSDI and tear level for TNF- $\alpha$ , IL-10 and IL-12p70 was not determined since these were only detected in insignificant levels (in up to 2 of 8 samples).

#### 4. DISCUSSION

Topical drops are the primary treatment for patients with glaucoma. Evidence suggests that the presence of BAK preservative in drops may lead to ocular irritation, tear-film instability, chronic inflammation, subconjunctival fibrosis, and increase the risk for failure of glaucoma filtration surgery.<sup>8,9,23</sup> BAK-preserved drops have been implicated in the development of subclinical ocular surface inflammation through increased levels of inflammatory mediators.<sup>10,24,25</sup> Unlike previous reports, which are often cross sectional, in this study we have undertaken a three-way evaluation of different preservative effects on treatment-naïve patients and profiled the temporal effect of these on cytokines in IC and tear samples.

Cytokines and chemokines are known to be secreted from a variety of cell types in response to infectious or inflammatory stimuli. These proteins may then trigger activation of intracellular signalling, enhancing cell proliferation/differentiation or lead to programmed cell death.<sup>26</sup> IL-1 $\beta$  and TNF- $\alpha$  are known to induce other cytokines/chemokines and profibrotic growth factors leading to sterile inflammation<sup>27</sup> and tissue fibrosis.<sup>28</sup> IL-6 and IL-8 (CXCL8) are proinflammatory cytokine with the

ability to amplify autoimmune responses *via* the activation of infiltrating neutrophils and T-cells.<sup>26</sup>

Impression cytology provides an alternative to biopsy or scraping to obtain conjunctival cells for cell-surface markers analysis using flow cytometry<sup>25</sup> and gene expression studies of host defense genes.<sup>29</sup> Normal conjunctival epithelium collected using the IC technique has been shown to express constitutive mRNA levels of *IL-1*, *IL-6*, *IL-8*, *TNF- $\alpha$* .<sup>30</sup> In response to inflammatory stimuli, these have been produced both at mRNA and protein level.<sup>30</sup> Elevated expression of IL-1 $\beta$ , IL-6 and IL-8 have been reported in the conjunctival IC samples of patients with Sjogren's syndrome keratoconjunctivitis sicca<sup>31</sup> and dry-eye condition.<sup>32</sup> Conjunctival IC samples from patients on BAK-preserved anti-glaucoma drops show elevated expression of human leucocyte antigen (HLA)-DR, IgE, IL-8 and two chemokine receptors (CCR4 and CCR5)<sup>10,24,25</sup> and increased levels of T helper (Th)-1 inflammatory cytokines have been demonstrated in tear samples of patients receiving preserved anti-glaucoma medications for more than 6-months duration.<sup>33</sup> BAK also induces cytokine production from trabecular meshwork cells in laboratory studies.<sup>34</sup>

Our study demonstrates that mRNA for cytokines are constitutively expressed in conjunctival epithelium samples at baseline. An explanation for increased levels of IL-6, IL-8 and IL-1 $\beta$  in IC in the tear samples of patients randomised to the BAK group during the study could be that these cytokines are sensitive to any inflammatory stimuli and their homeostatic balance is key for maintenance of immune-privilege status of OS.<sup>35</sup> Although we have demonstrated the matching trend of both mRNA and protein expression of cytokines in samples from BAK group, it is likely that conjunctival epithelium may not be the only source for tear cytokines. Other possible sites for cytokine secretion in response to BAK are corneal

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epithelium,<sup>36</sup> tenon's fibroblast<sup>37</sup> and limbal-resident immune cells.<sup>38</sup> A previous study has demonstrated that the levels of tear IL-1 $\beta$  and TNF- $\alpha$  increased in patients on multiple BAK-containing drops compared to those on single drop therapy *i.e.*, concentrating effect of BAK on ocular inflammation.<sup>33</sup> Here, all our patients randomized to the BAK group were on a single drop and have shown time-dependent increase in IL-6, IL-8 and IL-1 $\beta$  levels.

The advantages of PF over preserved medication for glaucoma treatment has been widely reported.<sup>39-41</sup> Similarly, as evident from OSDI scoring, our patient group on PF and PQ containing drops have not complained of OS discomfort which can be correlated with reduced levels of inflammatory cytokines. On the other hand, those randomized to BAK-preserved drops had a high OSDI score, which strongly correlated to elevated levels of IL-1 $\beta$ . Recent phase III-b clinical trials have demonstrated that switching patients from BAK-preserved latanoprost to PF-tafluprost have improved OS tolerability and patient compliance within 12-weeks.<sup>41</sup>

PQ has been previously shown to be safe and did not induce cytotoxic/inflammatory effects on OS epithelium in *in-vitro* or *in-vivo* studies.<sup>13,14</sup> Samples from our patient cohort using PQ-preserved drops have shown moderately high levels of IL-1 $\beta$  and IL-6 from 12-month onwards. This is also mimicked in the trends noted in the OSDI results. Our results suggest that chronic, long-term use of PQ preserved drops could induce delayed subclinical OS inflammation.

One criticism could be that some of the effects may be related to other non-preservative components of the drops. Previous *in-vitro* studies have successfully compared the inflammatory and cytotoxic effects of widely used prostaglandin analogues: latanoprost, travoprost, and bimatoprost.<sup>42,43</sup> Notably, none of these three analogues was shown to induce inflammatory markers in cultured conjunctival



epithelial cells. Similarly, our study has also demonstrated that the addition of PF-timolol between 12- and 24-month duration as a second active drug has no significant effect on the cytokine levels; even in PF-latanoprost group. This is consistent with previous studies that demonstrated non-toxic effects of PF-timolol and PF-latanoprost on OS epithelium compared to BAK-preserved timolol and latanoprost drops, respectively.<sup>30,39</sup> Clinical studies have shown that patients on bimatoprost frequently develop ocular hyperemia.<sup>44-47</sup> This effect of bimatoprost 0.03% on OS has been confirmed to be due to vasodilation mediated *via* over-production of nitric oxide synthase, but not due to the induction of inflammatory cytokines.<sup>44,48</sup>

Our observation that BAK is responsible for the increased expression of cytokines is consistent with numerous studies that evaluated the inflammatory markers in tears of patients treated with preservative-containing and PF drops.<sup>8-10,25,33,38</sup> These published observations though cannot exclude the possibility of an unknown inflammatory effect from bimatoprost. The magnitude of effect if any exists in our BAK patient group is likely to be negligible and it does not detract from the observations or conclusions of this report. However, a future study is warranted to validate and compare the OS responses in patients on PF-bimatoprost 0.01% versus BAK-bimatoprost 0.01%, PF-latanoprost 0.005% versus BAK-latanoprost 0.005%, and PF-travoprost 0.04% versus BAK-travoprost 0.04%.

The strengths of this study are following:

1. A first of its kind, prospective evaluation of the changes in ocular surface markers of inflammation in treatment-naïve patients that were randomised to three preserved options available for glaucoma drops.
2. Baseline sampling of treatment-naïve patients for comparative analysis of cytokines over a period of 24-months have successfully reduced the effect of

confounding variables. BL samples showed no significant differences in the baseline cytokine levels across the three groups.

3. Statistical correlation between the ocular surface inflammatory changes and clinical symptoms as measured by the OSDI tool was successfully demonstrated.

The weakness is that we have recruited and analysed relatively small numbers (due to poor sample quality and/or quantity). Although even with this small sample size, we have demonstrated a clear effect on inflammatory markers in IC and tear samples. The use of different prostaglandin analogues in each of the preservative groups introduces slight uncertainty into the possibility that some of the inflammatory effects in the BAK group may be related to active ingredient, bimatoprost.

In conclusion, our results have demonstrated that patients on BAK preparation showed increased OS inflammation compared to those on PF and PQ-preserved preparations. The inflammatory effect of BAK was detectable starting 3 months and sustained for the duration of the 24-month follow-up. The strong correlation seen between OSDI scores and increased levels of cytokines in BAK group supports the conclusion that BAK induces ocular discomfort in patients.<sup>7-10</sup> There is a suggestion that PQ is also capable of producing delayed OS inflammatory response, which may lead to ocular surface discomfort.

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## TABLES

**Table 1:** Demographic of patients' whose samples were evaluated for cytokines profiling

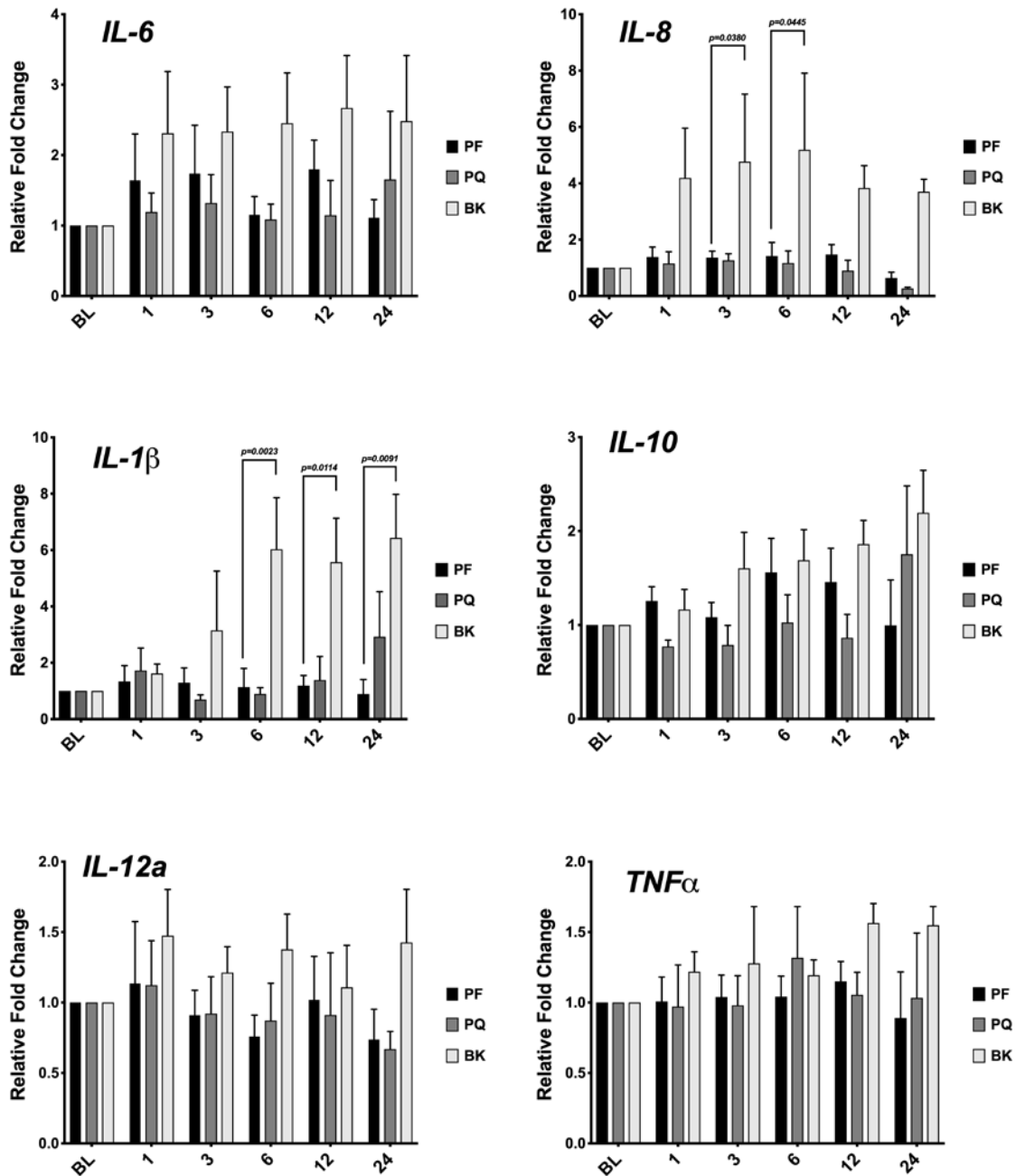
	<i>Preservative - free</i>	<i>Polyquad</i>	<i>Benzalkonium chloride</i>
<i>Gender (male/female)</i>	3/4	2/6	6/3
<i>Age in years mean (SD)</i>	67.9 (5.0)	70.3 (12.7)	63.9 (10.9)

**Table 2:** Pearson correlation of OSDI with cytokines level in conjunctival impression cytology (IC) and tear samples of patients in benzalkonium chloride (BAK) group

	IC (mRNA)	Tears (protein)
<b>IL-6</b>		
<i>r</i>	0.687	0.794
<i>95% CI</i>	-0.280 to 0.962	-0.048 to 0.976
<i>R squared</i>	0.473	0.630
<i>p value</i>	0.131	0.059
<b>IL-8</b>		
<i>r</i>	0.295	0.245
<i>95% CI</i>	-0.679 to 0.892	-0.706 to 0.881
<i>R squared</i>	0.087	0.060
<i>p value</i>	0.570	0.639
<b>IL-1<math>\beta</math></b>		
<i>r</i>	0.832	0.899
<i>95% CI</i>	0.062 to 0.981	0.324 to 0.989
<i>R squared</i>	0.692	0.808
<i>p value</i>	0.040	0.014
<b>TNF-<math>\alpha</math></b>		
<i>r</i>	0.757	
<i>95% CI</i>	0.053 to 0.995	N.D.
<i>R squared</i>	0.516	
<i>p value</i>	0.067	
<b>IL-10</b>		
<i>r</i>	0.925	
<i>95% CI</i>	0.458 to 0.992	N.D.
<i>R squared</i>	0.856	
<i>p value</i>	0.018	
<b>IL-12</b>		
<i>r</i>	0.286	
<i>95% CI</i>	-0.684 to 0.890	N.D.
<i>R squared</i>	0.081	
<i>p value</i>	0.582	

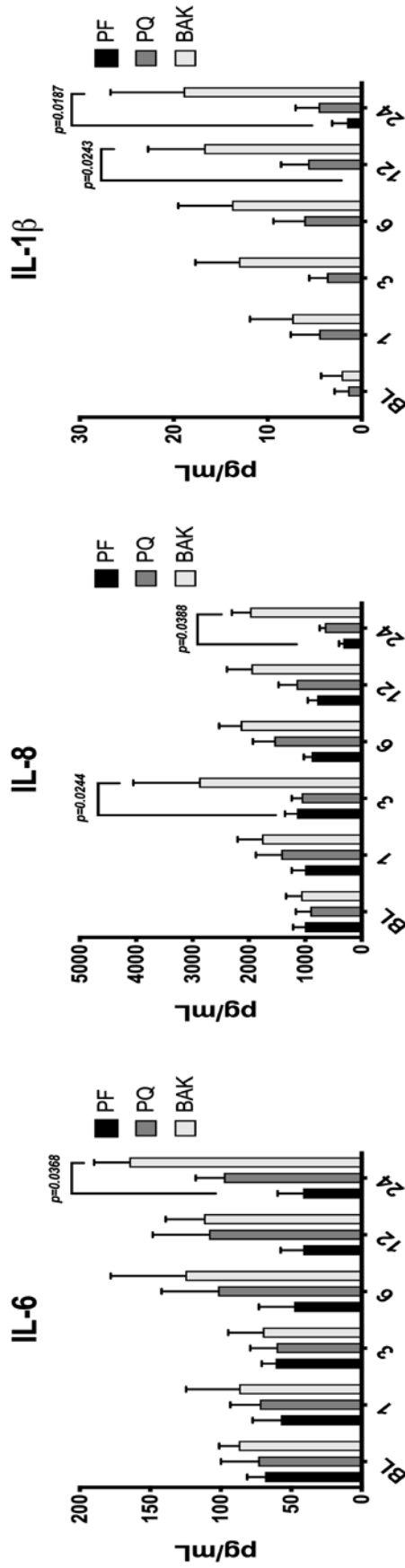
*N.D.* - Not determined; *CI* – Confidence interval

## FIGURES

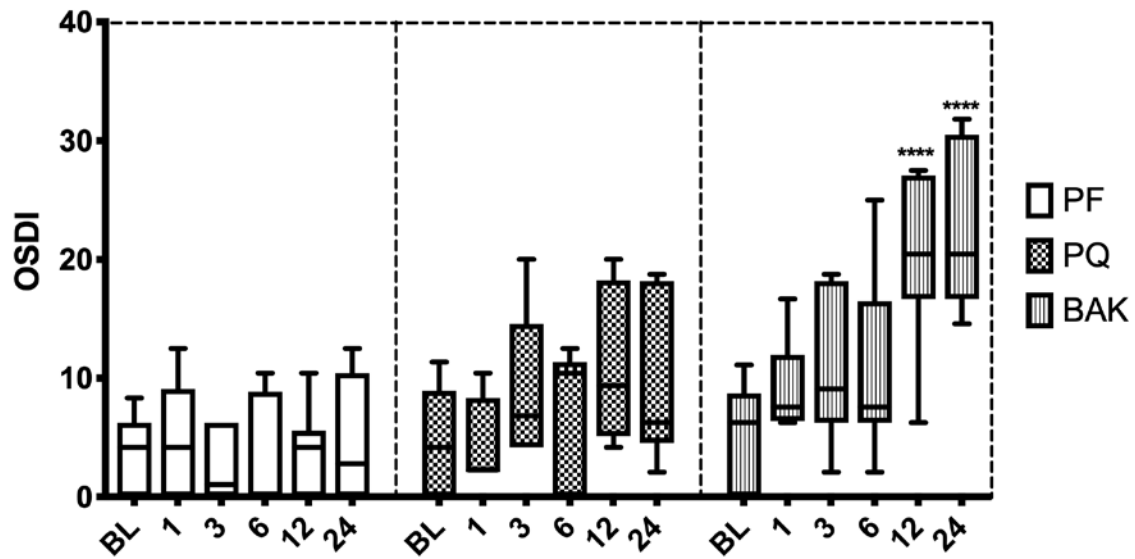


**Figure 1:** Gene expression analysis of cytokines in conjunctival impression cytology (IC) samples. Based on 24 patients with complete data collection. Relative fold change

(RFC; Y-axis) of *IL-6*, *IL-8*, *IL-1 $\beta$* , *TNF- $\alpha$* , *IL-10* and *IL-12a* in IC samples that are collected at different durations (in months; X-axis) from patients on anti-glaucoma medications that are preservative-free (PF) and preserved with polyquad (PQ) and benzalkonium chloride (BAK), respectively. RFC is represented as mean  $\pm$  standard deviation. Two-way ANOVA was performed to assess the statistical significance between PF vs PQ and PF vs BAK groups with alpha level set at  $p=0.05$ .



**Figure 2:** Quantification of inflammatory cytokines in tear samples. Based on 24 patients with complete data collection. Cytokines (IL-6, IL-8, IL-1 $\beta$ , TNF- $\alpha$ , IL-10 and IL-12p70) were measured in tear samples that are collected at different durations (in months; X-axis) from patients on anti-glaucoma medications that are preservative-free (PF) and preserved with polyquad (PQ) and benzalkonium chloride (BAK), respectively. Data for TNF- $\alpha$ , IL-10 and IL-12p70 was not shown due to insignificantly low levels. Number of patients that showed cytokines above detection limit were provided in Supplementary figure 4. Quantity of cytokines (in pg/mL) is represented as mean  $\pm$  standard deviation. Two-way ANOVA was performed to assess the statistical significance between PF vs PQ and PF vs BAK groups with alpha level set at  $p=0.05$ .



**Figure 3:** Ocular surface disease index (OSDI). Box plot represents the OSDI score for  $n=24$  patients with complete data collection. OSDI was calculated based on questionnaire completed by patients, randomised to preservative-free (PF) and preserved (polyquad (PQ) and benzalkonium chloride (BAK)) anti-glaucoma medications, at baseline (BL) and at each subsequent visit (1, 3, 6, 12 and 24 months; X-axis). Data is represented as mean (solid line in the box) with minimum and maximum OSDI values indicated as vertical bars. Two-way ANOVA was performed to assess the statistical significance between PF vs PQ and PF vs BAK groups with alpha level set at  $p=0.05$ . \* denotes  $p<0.05$  and \*\*\* denotes  $p<0.001$ .