In vivo confocal microscopy of the corneal sub-basal nerve plexus in medically controlled glaucoma

Running head: sub-basal nerve plexus in glaucoma

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Abstract

The present study investigated the corneal sub-basal nerve plexus (SNP) modifications in glaucoma. Ninety-five glaucomatous patients were enrolled and divided into: Group 1 and 2, preserved and preservative-free mono-therapy (30 and 28 patients), and Group 3, multi-therapy (37). Thirty patients with dry-eye disease (DED) and 32 healthy subjects (HC) served as controls. *In vivo* confocal microscopy evaluated the nerve fibers density (CNFD), length (CNFL), thickness (CNFT), branching density (CNBD), and dendritic cell density (DCD).

CNFD, CNFL, and CNBD were reduced in Group 3 and DED compared to HC ($p<0.05$). CNFL was reduced in Group 3 compared to Group 2 ($p<0.05$), and in Group 1 compared to HC ($p<0.001$). CNFD, CNBD, and CNFT did not differ between glaucomatous groups. DCD was higher in Group 3 and DED compared to HC and Group 2 ($p<0.01$). Group 3 showed worse ocular surface disease index (OSDI) scores compared to Group 1, 2, and HC ($p<0.05$). CNFL and DCD correlated with OSDI score in Group 3 ($r=-0.658$, $p<0.001$; $r=0.699$, $p=0.002$).

Medical therapy for glaucoma harms the corneal nerves, especially in multi-therapy regimens. Given the relations with the OSDI score, SNP changes seem features of glaucoma-therapy related OSD, and negatively affect the patient's quality of life.

Introduction

Corneal nerve fibers are devoted to several crucial functions such as corneal sensitivity and trophism, epithelial integrity, proliferation and promotion of wound healing, and protection and homeostasis of the entire ocular surface (Patel & McGhee, 2005). The corneal sub-basal nerve plexus (SNP) originates from the ophthalmic branch of the trigeminal nerve and from sympathetic and parasympathetic nerve fibers. Once reaching the corneo-scleral limbus, corneal nerve fibers centripetally distribute branches to the intermediate corneal stroma, forming a moderately dense network, and to the sub-epithelium, forming a dense plexus. After penetration of the Bowman's membrane, nerves form a dense SNP, branches of which terminate in the corneal epithelium.

In vivo confocal microscopy (*IV*CM) can non-invasively characterize the SNP morphology, providing highly magnified images and opening a window into live histology. In infectious keratitis, *IV*CM revealed a significant reduction of nerve fiber length, number, and density, and documented inflammatory cells dispersed in the peri-fibers interstice (Patel & McGhee, 2005; Cruzat et al. 2017; Kokot et al. 2018). In diabetes, *IV*CM observed a reduction of nerve fiber length, density, and branching, especially in patients with clinical signs of peripheral diabetic neuropathy (Tavakoli et al. 2010; Kim et al., 2013; Dell'Omo et al., 2018; Roszkowska Licitra et al., 2021). A reduction of fiber density and an increase of tortuosity, significantly correlating with the corneal sensitivity, was reported in dry eye (Labbè et al., 2012; Steger et al., 2015; Giannaccare et al., 2019). In corneal refractive surgery and keratoplasty, *IV*CM findings correlated with the recovery of visual acuity and corneal sensitivity over time (Stachs et al., 2010).

Numerous studies demonstrated that medical therapy for glaucoma disrupts all ocular surface structures, with the cornea being one of the most affected (Baratz et al., 2006; Martone et al., 2009; Labbè et al., 2012; Mastropasqua et al., 2014; Mastropasqua et al. 2016 and 2017; Agnifili et al., 2013 and 2018). Because of the key functions exerted by corneal nerves on the ocular surface homeostasis, several studies investigated the SNP features in patients with medically controlled glaucoma (Baratz et al., 2006; Martone et al., 2009; Labbè et al., 2012; Villani et al., 2016; Agnifili

et al., 2019; Baghdasaryan et al., 2019). Overall, the majority of these studies documented a decrease in density, number, and length, and an increase in reflectivity and tortuosity of nerve fibers; the presence of punctate reflective elements (assumed to be inflammation-related). As suggested by Labbè et al. (2012), these aspects were potentially relevant for the health of the entire ocular surface, since anti-glaucoma medications, especially those containing preservatives (benzalkonium chloride, BAK), produced an anesthetic effect and a neurotrophic keratopathy with secondary dry eye. To date, no previous study was designed to elucidate whether SNP alterations are related to the complexity of therapy, whether they may represent additional features of the glaucoma therapy-related ocular surface disease (OSD), or whether there are relationships between medical therapy, corneal nerve modifications and patients' quality of life (QoL).

Therefore, we designed the present confocal study to characterize the SNP morphology in different therapeutic regimens of anti-glaucoma therapy, and to investigate whether SNP features correlate with the OSD index (OSDI) questionnaire and the National Eye Institute Visual Function Questionnaire-25 (NEI VFQ-25) scores, to establish their impact on the OSD and QoL.

Material and Methods

Patient selection

This observational single-center study was conducted between April 2020 and May 2021 at the Ophthalmology Clinic of the "G. d'Annunzio" University of Chieti-Pescara (Chieti, Italy). The research was approved by our internal review board (Department of Medicine and Aging Science of the "G. d'Annunzio" University of Chieti-Pescara, Italy) and adhered to the tenets of the Declaration of Helsinki.

For the purpose of the study, we considered patients with medically controlled primary open angle glaucoma (POAG), and two control groups represented by patients with dry eye disease (DED) and healthy subjects (healthy controls: HC). Written informed consent was obtained from all subjects prior to enrolment, after explanation of the nature and possible consequences of the study.

Patients with glaucoma were divided into three therapeutic regimens according to the number of intra-ocular pressure (IOP) lowering medications, and the presence of preservative: preserved mono-therapy (Group 1: one BAK containing medication); preservative-free (PF) mono-therapy (Group 2: one BAK-free medication); multi-therapy (Group 3; more than two medications). Ninety-five Caucasian glaucomatous patients were enrolled, according to the given features for each study group in the protocol; for controls, thirty DED patients and thirty-two healthy subjects were consecutively enrolled.

Glaucomatous patients had to have the following inclusion criteria: a diagnosis of POAG (open angle at gonioscopy, IOP > 22 mmHg at diagnosis), visual field (VF) test (30-2 test, full-threshold, Humphrey field analyzer II 750; Carl Zeiss Meditec, Inc., Dublin, CA, USA) with at least three contiguous points on the total deviation probability plot at the less than 2% level, Glaucoma Hemifield Test (GHT) outside normal limits, and glaucomatous features of the optic disc consistent with the VF alterations. The disease had to be medically controlled at the moment of enrolment (IOP<18 mmHg), with IOP lowering therapy unmodified during the 12 months prior to enrolment. Unpreserved artificial tears treatments were allowed.

Exclusion criteria were a recent history (< 6 months) of systemic, intra-ocular or ocular surface inflammatory diseases, a diagnosis of DED or the presence of symptoms indicating DED before starting anti-glaucoma medications, topical or systemic therapies potentially inducing corneal toxicity, secondary glaucoma, corneal dystrophies, previous ocular surgery including cataract and refractive surgery, ocular trauma, chemical burn, contact lens wear, diabetes, pregnancy, and breastfeeding.

Inclusion criteria for DED were based on the TFOS DEWS II Diagnostic Methodology Report: OSDI>12 and (BUT<10 seconds or CFS>2, according to Oxford grading scale) (Wolffsohn et al., 2017). Exclusion criteria were diabetes mellitus or other neurodegenerative diseases; any other ocular surface diseases other than DED; concomitant treatments with drugs potentially modifying the ocular surface status in the last six months; eyelid malposition or lid movement disorders; previous corneal refractive surgery or cataract, glaucoma, contact lens wear, pregnancy, and breastfeeding. Previous or ongoing eyelid hygiene and/or unpreserved artificial tears treatments were allowed.

HC had to show a completely normal ophthalmological assessment, with normal clinical ocular surface tests. Exclusion criteria were history of systemic or intra-ocular inflammatory diseases or any ocular surface disease, systemic or topical therapies in the last six months that could have modified the ocular surface, previous ocular surgery or refractive surgery, contact lens wearing, pregnancy, and breastfeeding.

Patient examination

1. OSDI and NEI VFQ-25 questionnaires, and ocular surface clinical tests

The OSDI and NEI VFQ-25 questionnaires were administered immediately after enrollment, to stage the OSD and assess patient's QoL. Afterwards, patients underwent break-up time (BUT), corneal fluoresceine staining (CFS), a careful slit-lamp examination of both the anterior and posterior segment of the eye, and (30 minutes after BUT and CFS measurements) Schirmer test I (STI) without topical anesthesia, in the order suggested by the DEWS guidelines (Wolffsohn et al.,

2017). BUT was recorded as the average of three consecutive measurements. STI result was expressed as the length of the strip that was wet after 5 minutes. CFS was evaluated with 1% sodium fluorescein and scored 0 to 3 according to the Oxford grading scale (Bron et al., 2003).

2. Corneal sensitivity

Thirty minutes after clinical tests, corneal sensitivity was measured using the Cochet-Bonnet aesthesiometer (Luneau, France); ambient conditions (humidity, temperature, and light) of the dedicated room were carefully controlled to standardize measurements. Corneal aesthesiometry (CA) was measured three times in each eye, and the mean value was considered for the analysis.

3. IVCM of SNP

*IV*CM was performed after questionnaires and clinical tests to analyze the SNP (between Bowman's layer and the basal epithelium; 50-80 μm of depth), using the Heidelberg Retinal Tomography III coupled with a Rostock Cornea Module (Heidelberg Engineering, GmbH, Heidelberg, Germany). After topical anesthesia with 0.4% oxybuprocaine and the application of a drop of 0.2% polyacrylic gel as coupling medium between the contact cap of the objective lens and cornea, the confocal examination was conducted over a central to mid-peripheral area of the cornea, of about 5 mm in diameter, as previously reported (Patel et al., 2005; Kokot et al., 2018). *IV*CM examinations were performed using both automatic scans for sequential images and manual frame acquisition, with the automatic brightness mode to maintain the same illumination intensity. For each subject forty sequential images were collected, whereas ten randomly chosen high-quality images without artefacts and not overlapping by more than 20% between them, were selected for the analysis.

Confocal parameters

To describe the SNP features, we considered the corneal nerve fiber length (CNFL), thickness (CNFT), density (CNFD), branch density (CNBD), and the dendritic cells (DCs) density (DCD). The ImageJ software (version 1.52n, National Institute of Health, USA) with NeuronJ plug-in (version 1.4.3), freely available online at http://imagej.nih.gov/ij/ and provided in the public domain by the National Institutes of Health (Bethesda, MD, USA), were used to analyze these parameters.

CNFL: the nerve tracing function, embedded in the ImageJ plugin, was used to trace the total extension of all nerves in each frame; the density of the nerve fibers was calculated in mm/mm^2. *CNFT* (μm): NeuronJ was used to measure the nerve fiber thickness, defined as the mean of three measures of the thickness of long nerve fibers within a frame, without artifacts or motion blur. *CNFD* (n/frame): this parameter was calculated dividing numbers of all identifiable fibers (manually identified) by the standardized area of the confocal image (400 x 400 μm). To evaluate the *CNBD* (n/frame), the total number of visible fiber branches was manually calculated and divided by the standardized area of the confocal image (400 x 400 μm). *DCD* (cells/mm^2): this parameter was calculated using the analysis software in the confocal microscope, by averaging numbers of DCs from ten randomly selected images, counted manually within the region of interest, as previously described (Mastropasqua et al., 2016). DCs may present an immature or mature aspect: immature DCs show a large body with fewer and shorter processes, if any, whereas mature (or activated) DCs show a slender cell body from which a network of long membrane processes extends resembling dendrites of nerve cells.

*IV*CM measurements were performed by two different operators (LB and GDO). Both operators were masked to the subject's history and grouping. Both eyes per patient were evaluated, but one eye per subject was randomly chosen for data analysis. All data were analyzed by a masked investigator (FO), and results were averaged.

The main outcomes of the study were CNFL, CNFT, CNFD, CNBD, and DCD; the secondary outcomes were the OSDI and NEI VFQ-25 scores. Interobserver agreement was investigated for all confocal parameters.

Statistical Analysis

Statistical analysis was performed with SPSS Advanced Statistical TM 25.0 Software (2017; SPSS, Inc., Chicago, IL, USA). The sample size was calculated considering difference between groups of almost 10%, a power of 80% and type I error rate (a) of 5%. A Shapiro-Wilk test was used to test the normality of distribution. Parametric tests were used for the analysis of variables with normal

distribution, and non-parametric tests for variables not normally distributed (CNFL, CNBD, OSDI, BUT, STI, and CA). Student's t-test and χ^2 test were used to evaluate age and gender differences between groups. Mean and standard deviation are presented for variables and number for categorical variables. A one-way ANOVA with post hoc Tukey for multi-comparison was used to assess differences among groups for normally distributed variables, and the Kruskal-Wallis test with post hoc Wilcoxon for non-normally distributed variables. A P value <0.05 was considered statistically significant. Correlations between *IV*CM parameters, ocular clinical tests and OSDI and NEI VFQ-25 scores were determined using a non-parametric measure by the Spearman's index.

Results

Demographic and clinical data

Demographic and clinical data of glaucomatous patients and controls are reported in Table 1, whereas the therapeutic regimens in glaucoma Groups are reported in Table 2. No significant differences were found in gender, age, and IOP between Groups. The VF mean defect (MD) was significantly worse in glaucoma groups compared to healthy subjects and patients with DED ($p<0.05$), and between Group 3 and Groups 1 and 2 ($p<0.05$). Patients with glaucoma were in an early to moderate perimetric stage (Hodapp et al., 1993) and more than 70% of patients with DED were at a level 2 in severity (TFOS DEWS II criteria) (Wolffsohn et al., 2017).

Questionnaires and ocular surface clinical tests

Table 3 reports data concerning questionnaires and ocular surface tests. In detail, Group 3 showed significantly worst OSDI and NEI VFQ-25 scores compared to Groups 1 and 2, and HC ($p<0.05$ and $p<0.001$, respectively). Patients controlled with a preserved mono-therapy presented a slightly increased OSDI score compared to patients controlled with a PF mono-therapy ($p<0.05$). The OSDI score was similar between Group 3 and DED, whereas the NEI VFQ-25 score was reduced in Group 3 compared to Groups 1 and 2, and HC. The NEI VFQ-25 score did not differ between mono-therapy groups, HC and DED.

BUT, STI, and CFS were significantly worse in Group 3 and DED compared to HC ($p<0.05$), and between Group 3 and Groups 1 and 2 ($p<0.05$). CA was reduced by about 50% in Group 3 and DED (without differences between them) compared to HC and glaucoma mono-therapy groups ($p<0.001$) but was similar between HC and Groups 1 and 2.

In vivo confocal microscopy data

*IV*CM data are reported in Table 4. SNP and DCs were clearly recognized in all patients, with features similar to those described in previous confocal studies (Baratz et al., 2006; Martone et al., 2009; Ranno et al., 2011; Labbè et al., 2012; Mastropasqua et al., 2014; Mastropasqua et al., 2016; Villani et al., 2016; Dell'Omo et al., 2018).

In a comprehensive view, confocal parameters were worse in Group 3 and DED compared to HC (Figures 1-3), with no significant differences between Group 3 and DED. In a more detailed analysis, CNFD and CNBD were significantly reduced in Group 3 and DED compared to HC ($p<0.05$ and $p<0.001$, respectively). CNFL showed significantly worse values in Group 3 compared to Group 2 ($p<0.05$), and in Group 1 compared to HC ($p<0.001$); CNFD, CNFT, and CNBD did not significantly differ between glaucoma groups. CNFT was reduced only in DED in comparison with HC ($p<0.001$). DCD were significantly higher in Group 3 and DED compared to HC ($p<0.001$) and Group 2 ($p<0.01$), with Group 3 and DED patients presenting a higher incidence of activated (mature) DCs (Figure 3).

SNP parameters estimated by Investigator 1 were not significantly different from that estimated by the Investigator 2, with a high interobserver agreement and a moderate agreement according to Cohen's k coefficient (0.41-0.60; agreement percentage of 72.3%). (Table 5).

Correlations

Spearman's correlation analysis indicated that CNFL negatively correlated with CFS and OSDI score in Group 3 ($r=-0.576$, $p=0.003$ and $r=-0.658$, $p<0.001$, respectively), and DED ($r=-0.672$, $p=0.002$ and $r=-0.701$; $p<0.001$, respectively). DCD significantly correlated with CFS and OSDI score in Group 3 ($r=0.723$, $p<0.002$ and $r=0.699$, $p= 0.002$, respectively).

Discussion

In line with literature, the present study found that medical therapy for glaucoma induces significant alterations to the corneal SNP (Martone et al., 2009; Ranno et al., 2011; Labbè et al., 2012; Villani et al., 2016; Agnifili et al., 2019; Baghdasaryan et al., 2019; Rossi et al., 2019). ~~As additional new aspects~~Moreover, our results lead to hypothesize that SNP alterations may represent one of the features characterizing the glaucoma therapy-related OSD and could impact on patient QoL. ~~In particular,~~ IOP lowering medications harm the SNP especially in therapy regimens requiring multiple eyedrops ~~administration during the day~~, producing~~when~~ nerve alterations that appear similar to those observed in DED.

When focusing on ~~therapy regimens~~glaucoma groups, the most part of confocal parameters did not significantly differ between preserved and PF mono-therapies, and between patients on mono-therapy and healthy controls. Overall, these aspects seem to indicate that the preservative does not markedly disturb corneal nerves when the therapy regimen requires a preserved medication administered one or two times per day. On the other hand, CNFL presented significant differences between patients on monotherapy and controls, probably indicating that this parameter could be ~~the most accurate and, thus, a~~an ~~potential~~ early indicator of SNP alteration, when clinical signs are still lacking.

These findings are partially in line with literature, since some studies reported that a single BAK-containing eyedrop per day does not induce major alterations to some structures, such as Meibomian glands, whereas other studies found that this regimen may damage or stimulate others structures such as epithelia or DCs (Martone et al., 2009; Agnifili et al., 2013; Shtein & Callaghan 2013; Mastropasqua et al., 2016; Agnifili et al., 2018; Bhattacharya et al., 2020). The different effects of the preservative on the ocular surface components may presumably depend on the different resistance or response of cells and tissues to stress stimuli. In addition, though IVCM clearly differentiate the effects of preservatives on the entire ocular surface, and confocal findings correlate with clinical indicators of the ocular surface status, the observed differences on SNP

aspects could also depend on a different ability of IVCM to detect changes for each single nerve component (Mastropasqua et al., 2015 and 2016; Frezzotti et al., 2014).

In a deeper analysis ~~of confocal parameters~~, multi-treated patients presented worse fiber density values compared to unpreserved mono-therapies, whereas thickness, branch density and length did not differ between groups. ~~In addition, the branch density was significantly worse in multi-treated patients compared to healthy controls.~~ A comprehensive interpretation of these findings could be that corneal nerves globally preserve their normal morphology ~~, without evident signs of iatrogenic neuropathy,~~ when medical therapy requires one medication per day. Conversely, the earlier response to therapy is probably the inflammation of the environment surrounding nerve fibers, ~~On the other side,~~as the DCD increase in ~~DCD in patients controlled with a~~ preserved mono-therapies~~edication~~ indicate~~s that the earlier response to therapy is the inflammation of the environment surrounding nerve fibers~~. This agrees with previous studies which suggested that inflammation represents the first step of changes in the glaucoma therapy-related OSD ~~and, thus, is one of the most frequently recognizable alterations~~ (Villani et al., 2016; Agnifili et al., 2018). ~~In fact, i~~In a study conducted on stable medically controlled glaucoma patients, Villani et al. (2016) suggested that ~~medical therapy stimulates~~ neuroinflammatory processes ~~, which~~ represent the earliest signs of SNP modifications, ~~that are~~ recognizable even without evident nerve loss. When the number of eyedrops administered per day increases, the most remarkable modification~~s~~ ~~is~~ are the DCD increase ~~DCD increase, which presents two-fold values compared to patients controlled with a single medication. On the other hand, when directly considering nerve fibers, the finest detectable SNP feature change is~~ and the nerve branches reduction. These~~is~~ result~~s~~ agree~~s~~ with recent evidence found in patients with diabetes, where the corneal nerve branch density reduction represented an early sign of peripheral neuropathy (Ferdousi et al. 2019).

~~The second~~Others remarkable aspects observed in multi-treated patients were~~as~~ the~~a~~ ~~r~~CNFD and CNFL reduction ~~of the nerve fiber density and length~~, which have been demonstrated to correlate with the ~~stage of the~~ OSD severity in different conditions (Cruzat et al., 2017). Therefore, in

patients under a complex therapy regimen, early and late signs of toxic corneal neuropathy seem to simultaneously coexist, probably depending on an asynchronous involvement of nerve fibers.

A surprising result of our study was that nerve fibers showed a normal thickness in the three groups of ~~therapy but~~glaucoma but was reduced in DED. This aspect appears in contrast with the results of a recent study conducted on type 2 diabetes mellitus, which found that the reduction of corneal nerve fiber thickness was one the earlier marker of the diabetic keratopathy (Dell'Omo et al., 2018). ~~Given that in glaucoma this aspect was not previously investigated, comparisons cannot be drawn. However, i~~One may~~t could be~~ hypothesize~~d~~ that the sequential order of SNP alterations between the glaucoma therapy-related OSD and other forms of OSD could be different, with thickness modifications probably appearing later in glaucoma.

As mentioned above, differences between Group 3 and DED for any of the confocal parameters were not observed. The close similarity of the ocular surface between glaucomatous patients in multi-therapy and patients with DED was in accordance with previous evidence that defined the glaucoma therapy-related OSD an iatrogenic form of dry eye (Labbè et al., 2012; Mastropasqua et al., 2015 and 2016; Dell'Omo et al., 2018).

Our results are generally in line with previous confocal studies on glaucoma (Baratz et al., 2006; Martone et al., 2009; Ranno et al., 2011; Labbè et al., 2012; Villani et al., 2016; Agnifili et al., 2019; Baghdasaryan et al., 2019). Labbè and coworkers (2012) reported a significant reduction of the sub-basal nerve fiber density and number of branching in multi-treated patients, without differences between glaucoma and DED. In an ancillary report to the ocular hypertension treatment study (OHTS), it was reported that anti-glaucoma medications reduced the number and density of SNP after six years of therapy (Baratz et al., 2006). Martone and coworkers observed a lower sub-basal nerve fiber number and an increased tortuosity in different therapy regimens, with worse values in patients controlled with a preserved mono-therapy or in multi-therapy (Martone et al., 2009). ~~However, in disagrees with our results, the best nerve preservation observed in this study was in patients treated with PF beta-blockers in mono-therapy. This aspect could depend on the fact~~

~~that our mono-therapy groups also included prostaglandin analogs, which may induce SNP alterations even without preservatives. On the other hand, Rossi et al. (2019) reported an improvement of corneal nerve parameters after switching preserved therapies to PF-tafluprost. These inconsistencies in literature indicate that the effects of preservatives on corneal nerve fibers are still unclear and matter of debate.~~

Our study found that CNFL and DCD significantly correlated with and OSDI and CFS scores. Given that higher OSDI and CFS scores indicate the presence of ~~a form of~~ OSD, these correlations suggest that the reduction of the nerve fiber density, along with inflammatory modifications interesting the SNP environment, represent potential hallmarks of the glaucoma therapy-related OSD. ~~Conversely, no correlation was observed between CA and confocal parameters. As previously hypothesized, this may depend on a potential anesthetic effect of both preservatives and the active compounds (Kozobolis et al., 2005; Labbè et al., 2012).~~

Overall, OSDI and NEI VFQ-25 scores, which differently explore the patient QoL, were worse in Group 3 (and DED) compared to mono-therapies. First, these results indicate that complex therapy regimens are strong determinants of the OSD in glaucoma. Second, the correlations between the OSDI score and CNFL and DCD~~, even in the absence of correlations between NEI VFQ-25 and SNP parameters,~~ suggest that the iatrogenic corneal neuropathy could affect the patient QoL. Finally, ~~these results further confirm that~~ the ocular surface in glaucomatous patients under multi-therapy and DED is almost similar, with a reduced QoL in both conditions (Mastropasqua et al., 2016; Agnifili et al., 2018).

The present study presents some limitations. ~~First, we did not consider all SNP confocal parameters potentially analyzable. However, given that it is not clarified what are the best indicators of the corneal nerve plexus condition, we evaluated those that were more frequently considered in literature. Second~~First, beside the OSDI questionnaire, we used the NEI VFQ-25 survey to assess the quality of life. This survey, though is well-validated for patients with glaucoma, does not represent the correct way to explore the QoL in ocular surface diseases. Therefore, direct

comparisons between glaucoma groups and DED could be biased by the nature of the survey. However, the OSDI score may in part overcome this inaccuracy.

~~Third~~Second, given that this was an observational study, we cannot state which nerve fiber alteration appears first, how the duration of treatment progressively modifies SNP, and how SNP features change as years of therapy increase. Fogagnolo and coworkers (2015) investigated these aspects in a prospective study in which the effects of prostaglandin analogs on SNP were evaluated on naïve-to-therapy glaucomatous patients. The authors found that while preserved latanoprost induced the development of nerve branching pattern and nerve beading, PF tafluprost did not. Of note, they also found that: i) both formulations activated stromal keratocytes immediately after the initiation of treatment, with a progressive increase over time; ii) keratocytes activation occurred earlier in patients using the preserved treatment; and iii) morphological changes of the nerves appear only after 9-12 months. Therefore, it seems that there is a sort of period of latency before SNP changes appear, and that the keratocyte activation is the initial change that progressively promote further nerve modifications. These results are in line with our suggestions, ~~even~~ since we hypothesized that ~~though~~ inflammatory changes can be the~~could be hypothesized as some of the~~ earlier in the cascade of events leading to~~ier~~ SNP modifications. Interestingly, during the first year of therapy, Fogagnolo et al. (2015) found that the ocular surface tests remained unchanged. These results led to the conclusion that corneal and SNP changes have a relevant clinical impact only in longer follow-up and in presence of a concomitant OSD. A related aspect to consider is that each different class of drugs and, between the same class the different active compounds, may differently harm SNP. Unfortunately, in our study we did not evaluate mono-therapy groups based on the drug class or the type of active compound, but on presence or absence of the preservative. However, previous studies demonstrated that the effects of IOP lowering medications on SNP depend, besides the treatment regimen and the presence of preservative, also by the drug class, and the subtype of active compound (Martone et al., 2009; Fogagnolo et al., 2015).

~~Forth~~Third, the nerve plexus, as in other clinical conditions, cannot be entirely visualized in confocal frames or in planar reconstructions (Lagali et al., 2018); therefore images, that are subjectively selected, can only reproduce an in vivo estimate of the morphological conditions. Fourth, we did not specifically consider the impact of PF-lubricants, common contextual therapy in glaucoma and DED, on SNP; however, the use of lubricants could have produced only a mitigation of the OSD without adjunctive detrimental effects on corneal nerves. ~~Finally, further prospective studies aimed at concomitantly investigate SNP along with other ocular surface structures, will clarify the exact role of corneal nerves alterations in the OSD development and the order as they appear.~~

Conclusions

In the present study we found that medical therapy for glaucoma induces significant morphological alterations of SNP, especially in complex therapy regimens. From a clinical point of view, these alterations may represent additional features of the glaucoma therapy-related OSD and may contribute to clarify the relationship between glaucoma therapy, OSD and QoL. In this optic, further studies investigating whether a less harmful medical therapy, the suspension of IOP lowering medications after glaucoma surgery, the use of anti-inflammatory agents, or potential forthcoming neurotrophic agents (such as the nerve growth factor), may improve the SNP conditions and favorably affect the OSD or the QoL, will clarify the real clinical significance of corneal nerve alterations in glaucoma.

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References

Agnifili, L., Fasanella, V., Costagliola, C., Ciabattoni, C., Mastropasqua, R., Frezzotti, P. & Mastropasqua, L. (2013). In vivo confocal microscopy of meibomian glands in glaucoma. *Br J Ophthalmol* **97**(3), 343-349.

Agnifili, L., Mastropasqua, R., Fasanella, V., Brescia, L., Scatena, B., Oddone, F. & Mastropasqua, L. (2018). Meibomian gland features and conjunctival goblet cell density in glaucomatous patients controlled with prostaglandin/timolol fixed combinations: a case control, cross-sectional study. *J Glaucoma* **27**(4), 364-370.

Agnifili, L., Brescia, L., Oddone, F., Sacchi, M., D'Ugo, E., Di Marzio, G., Perna, F., Costagliola, C. & Mastropasqua, R. (2019). The ocular surface after successful glaucoma filtration surgery: a clinical, in vivo confocal microscopy, and immune-cytology study. *Sci Rep* **9**(1), 11299.

Baghdasaryan, E., Tepelus, T.C., Vickers, L.A., Huang, P., Chopra, V., Sadda, S.R. & Lee, O.L. (2019). Assessment of Corneal Changes Associated with Topical Antiglaucoma Therapy Using In vivo Confocal Microscopy. *Ophthalmic Res* **61**(1), 51-59.

Baratz, K.H., Nau, C.B., Winter, E.J., McLaren, J.W., Hodge, D.O., Herman, D.C. & Bourne W.M. (2006). Effects of glaucoma medications on corneal endothelium, keratocytes, and subbasal nerves among participants in the ocular hypertension treatment study. *Cornea* **25**(9), 1046-1052.

Bhattacharya, P., Edwards, K., Harkin, D. & Schmid, K.L. (2020). Central corneal basal cell density and nerve parameters in ocular surface disease and limbal stem cell deficiency: a review and meta-analysis. *Br J Ophthalmol* **104**(12), 1633-1639.

Bron, A.J., Evans, V.E. & Smith, J.A. (2003). Grading of corneal and conjunctival staining in the context of other dry eye tests. *Cornea*. **22**(7), 640-650.

Cruzat, A., Qazi, Y. & Hamrah, P. (2017). In Vivo Confocal Microscopy of Corneal Nerves in Health and Disease. *Ocul Surf* **15**(1), 15-47.

Dell'Omo, R., Cifariello, F., De Turris, S., Romano, V., Di Renzo, F., Di Taranto, D., Coclite, G., Agnifili, L., Mastropasqua, L., & Costagliola, C. (2018). Confocal microscopy of corneal nerve plexus as an early marker of eye involvement in patients with type 2 diabetes. *Diabetes Res Clin Pract* **142**, 393-400.

Ferdousi, M., Romanchuk, K., Mah, J.K., Virtanen, H., Millar, C., Malik, R.A. & Pacaud, D. (2019). Early corneal nerve fibre damage and increased Langerhans cell density in children with type 1 diabetes mellitus. *Sci Rep* **9**(1):8758.

Fogagnolo, P., Dipinto, A., Vanzulli, E., Maggiolo, E., De Cilla', S., Autelitano, A. & Rossetti L. (2015). A 1-year randomized study of the clinical and confocal effects of tafluprost and latanoprost in newly diagnosed glaucoma patients. *Adv Ther* **32**(4):356-369.

Frezzotti, P., Fogagnolo, P., Haka, G., Motolese, I., Iester, M., Bagaglia, S.A., Mittica, P., Menicacci, C., Rossetti, L., & Motolese, E. (2014). In vivo confocal microscopy of conjunctiva in preservative-free timolol 0.1% gel formulation therapy for glaucoma. *Acta Ophthalmol* **92**(2):e133-40

Giannaccare, G., Pellegrini, M., Sebastiani, S., Moscardelli, F., Versura, P. & Campos, E.C. (2019). In vivo confocal microscopy morphometric analysis of corneal subbasal nerve plexus in dry eye disease using newly developed fully automated system. *Graefes Arch Clin Exp Ophthalmol* **257**(3), 583-589.

Hodapp, E., Parrish, R.K. II & Anderson DR. (1993). Clinical Decisions in Glaucoma. St Louis: The CV Mosby Co;52-61.

Kim, G., Singleton, J.R., Mifflin, M.D., Digre K.B., Porzio M.T. & Gordon Smith, A. (2013). Assessing the reproducibility of quantitative in vivo confocal microscopy of corneal nerves in different corneal locations. *Cornea* **32**, 1331-1338.

Kokot, J., Wylęgała, A., Wowra, B., Wójcik, Ł., Dobrowolski, D. & Wylęgała, E. (2018). Corneal confocal sub-basal nerve plexus evaluation: a review. *Acta Ophthalmol* **96**(3), 232-242.

Kozobolis, V.P., Detorakis, E.T., Maskaleris, G., Koukoula, S.C., Fountoulakis, N., Chrysochoou, F. & Konstas, A.G. (2005). Corneal sensitivity changes following the instillation of latanoprost, bimatoprost, and travoprost eyedrops. *Am J Ophthalmol* **139**(4), 742-743.

Labbé, A., Alalwani, H., Van Went, C., Brasnu, E., Georgescu, D. & Baudouin, C. (2012). The relationship between subbasal nerve morphology and corneal sensation in ocular surface disease. *Invest Ophthalmol Vis Sci* **53**(8), 4926-4931.

Lagali, N. S., Allgeier, S., Guimarães, P., Badian, R. A., Ruggeri, A., Köhler, B. & Rolandsson, O. (2017). Reduced Corneal Nerve Fiber Density in Type 2 Diabetes by Wide-Area Mosaic Analysis. *Investigative Opthalmology & Visual Science,* **58**(14), 6318.

Martone, G., Frezzotti, P., Tosi, G.M., Traversi, C., Mittica, V., Malandrini, A., Pichierri, P., Balestrazzi, A., Motolese, P.A., Motolese, I. & Motolese, E. (2009). An in vivo confocal microscopy analysis of effects of topical antiglaucoma therapy with preservative on corneal innervation and morphology. *Am J Ophthalmol* **147**(4), 725-735.

Mastropasqua, L., Agnifili, L., Mastropasqua, R., Fasanella, V., Nubile, M., Toto, L., Carpineto, P. & Ciancaglini, M. (2014). In vivo laser scanning confocal microscopy of the ocular surface in glaucoma. *Microsc Microanal* **20**(3), 879-894.

Mastropasqua, R., Agnifili, L., Fasanella, V., Curcio, C., Brescia, L., Lanzini, M., Fresina, M., Mastropasqua, L. & Marchini, G. (2015). Corneoscleral limbus in glaucoma patients: in vivo confocal microscopy and immunocytological study. *Invest Ophthalmol Vis Sci* **56**(3), 2050-2058.

Mastropasqua, R., Agnifili, L., Fasanella, V., Lappa, A., Brescia, L., Lanzini, M., Oddone, F., Perri, P. & Mastropasqua, L. (2016). In vivo distribution of corneal epithelial dendritic cells in patients with glaucoma. *Invest Ophthalmol Vis Sci* **57**(14), 5996-6002.

Mastropasqua, R., Agnifili, L., Fasanella, V., Nubile, M., Gnama, A.A., Falconio, G., Perri, P., Di Staso, S. & Mariotti, C. (2017). The Conjunctiva-Associated Lymphoid Tissue in Chronic Ocular Surface Diseases. *Microsc Microanal* **23**(4), 697-707.

Patel, D.V. & McGhee, C.N. (2005). Mapping of the normal human corneal sub-Basal nerve plexus by in vivo laser scanning confocal microscopy. *Invest Ophthalmol Vis Sci* **46**(12), 4485-4488.

Ranno, S., Fogagnolo, P., Rossetti, L., Orzalesi, N. & Nucci, P. (2011). Changes in corneal parameters at confocal microscopy in treated glaucoma patients. *Clin Ophthalmol* **5**, 1037-1042.

Rossi, G.C.M., Scudeller, L., Lumini, C., Mirabile, A.V., Picasso, E., Bettio, F., Pasinetti, G.M. & Bianchi, P.E. (2019). An in vivo confocal, prospective, masked, 36 months study on glaucoma patients medically treated with preservative-free or preserved monotherapy. *Sci Rep* **9**(1), 4282.

Roszkowska Licitra, C., Tumminello, G., Postorino, E.I., Colonna, M.R., & Aragona, P. (2021). Corneal nerves in diabetes. The role of in vivo corneal confocal microscopy in the assessment of peripheral small fiber neuropathy. *Surv Ophthalmol* **66**(3), 493-513.

Shtein, R.M. & Callaghan, B.C. (2013). Corneal confocal microscopy as a measure of diabetic neuropathy. *Diabetes* **62**(1), 25-26

Stachs, O., Zhivov, A., Kraak, R., Hovakimyan, M., Wree, A. & Guthoff, R. (2010). Structural-functional correlations of corneal innervation after LASIK and penetrating keratoplasty. *J Refract Surg* **26**(3), 159-167.

Steger, B., Speicher, L., Philipp, W. & Bechrakis, N.E. (2015). In vivo confocal microscopic characterization of the cornea in chronic graft-versus-host disease related severe dry eye disease. *Br J Ophthalmol* **99**(2), 160-165.

Tavakoli, M., Quattrini, C., Abbott, C., Kallinikos, P., Marshall, A., Finnigan, J., Morgan, P., Efron, N., Boulton, A.J.M. & Malik, R.A. (2010). Corneal confocal microscopy: a novel noninvasive test to diagnose and stratify the severity of human diabetic neuropathy. *Diabetes Care* **33**(8), 1792-1797.

Villani, E., Sacchi, M., Magnani, F., Nicodemo, A., Williams, S.E., Rossi, A., Ratiglia, R., De Cillà, S. & Nucci, P. (2016). The Ocular Surface in Medically Controlled Glaucoma: An In Vivo Confocal Study. *Invest Ophthalmol Vis Sci* **57**(3), 1003-1010.

Wolffsohn, J.S., Arita, R., Chalmers, R., Djalilian, A., Dogru, M., Dumbleton, K., Gupta, P.K., Karpecki, P., Lazreg, S., Pult, H., Sullivan, B.D., Tomlinson, A., Tong, L., Villani, E., Yoon, K.C., Jones, L. & Craig J.P. (2017). TFOS DEWS II Diagnostic Methodology report. *Ocul Surf* **15**(3), 539-574.

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Figure Legends

Figure 1. *IV*CM of SNP in healthy controls, glaucoma Groups, and DED

In healthy subjects the nerve plexus appears regularly organized with a higher fibers' density and normal branching compared to glaucoma and DED (A). Groups 1 and 2 (B and C) do not show significant differences between them; however, the presence of preservative seems to induce qualitative modifications, such as increased tortuosity, and the presence of an increased number of dendritic cells x(arrow) compared to healthy controls. Group 3 (D) and DED (E) present an evident reduction of nerve fibers density, branching and length, with a significant increase of punctate hyper-reflective elements (black arrowheads, presumably inflammatory signs) versus Groups 1 and 2 and healthy controls.

Figure 2. Planar reconstructions of SNP

To provide a wider representation of SNP, we manually created a planar reconstruction of a limited region of interest in a healthy (A), glaucomatous multi-treated (BAK-preserved prostaglandin analog and beta-blocker/CAI fixed combination) (B) and DED patient (C), by juxtaposing neighboring frames. Group 3 and DED show inflammation-related changes (punctate hyper-reflective elements and some scattered dendritic cells (arrowhead)) along with a fiber density reduction.

Figure 3. Dendritic cells distribution in healthy controls, glaucoma Groups, and DED

Compared to healthy controls (A) and monotherapy Groups (B and C), dendritic cells (arrowheads) present a higher density in Group 3 and DED (D and E), and mostly appear in their mature and activated form (E).