**Abstract:**

Purpose: To evaluate by in vivo laser scanning confocal microscopy (LSCM) the corneal findings in moderate-to-severe dry eye patients before and after treatment with topical corticosteroid and to associate the confocal findings to the clinical response. Methods: Fifty eyes of 50 patients with moderate-to-severe dry eye were included in this open label, masked study. Exclusion criteria were any systemic or ocular condition (other than dry eye) and any systemic or topical treatment (except artificial tears), ongoing or performed in the previous 3 months, with known effect on the ocular surface. All patients were treated with loteprednol etabonate ophthalmic suspension 0.5% q.i.d. for 4 weeks. Baseline and follow-up (day 30±2) visits included Ocular Surface Disease Index (OSDI) questionnaire, full eye exam and central cornea LSCM. We compared data obtained before and after treatment and looked for associations between baseline data and steroids-induced changes. Basing on the previously validated OSDI Minimal Clinically Important Difference, we re-analyzed the baseline findings comparing patients clinically improved after treatment (CIAS) to patients not clinically improved (NCIAS).

Results: OSDI score and LSCM dendritic cell density (DCD) significantly decreased after treatment. Baseline DCD correlated with both OSDI and DCD steroid-related changes (r=-0.44, P<0.05 and r=-0.70, P<0.01, respectively; Spearman) and was significantly higher in CIAS patients compared to NCIAS patients (164.1±109.2 vs 72.4±45.5 cells/mm², P<0.01; independent samples t-test).

Conclusion: LSCM examination of DCD allows detection of treatment-related inflammation changes and shows previously unknown associations between confocal finding and symptoms improvement after treatment. These promising preliminary data suggest the need for future studies testing the predictive value of DCD for clinical response to topical corticosteroids.
In this study, we evaluated by corneal LSCM the changes related to treatment with topical corticosteroids in moderate-to-severe dry eye patients. Based on the previously validated OSDI Minimal Clinically Important Difference, we divided the study populations into clinically improved after steroids (CIAS) and not clinically improved after steroids (NCIAS) and we re-analyzed the baseline findings, comparing these 2 groups. Our results contribute to open interesting prospects for the use of LSCM in clinical practice and in clinical research as a non-invasive biomarker to assess inflammation. Moreover, the baseline difference in dendritic cells density between CIAS and NCIAS patients, suggests the need for future studies, planned to test the predictive value of LSCM for clinical response to treatment with steroids. We think that this approach might contribute to bridge the current gap between the pathogenesis-driven classification and the severity-driven management of dry eye.
Detailed response to review

Editor in Chief's comments:

Edoardo, it is important that you address each of the reviewer comments in a revised submission and note, specifically by line number, where the comments have resulted in changes in the text. Please also follow the Managing Editor's instructions below for reformatting your figure. TONY

Dear Tony, Editors and Reviewers, your efforts are gratefully acknowledged in reviewing and strengthening our manuscript with your recommendations.

Topical Editor's comments:

The authors presented interesting results from a pilot study that highlights the potential clinical implications of IVCM to understand the disparity between common clinical signs and symptoms.

Both Reviewers have raised important questions for the authors to further improve the quality of the manuscript.

Please change the line numbering in your revision to "continuous", so that they do not restart on every new page.

Done. Sorry for the wrong setting in the previous submission.

Reviewer #1:

In this work, Villani et al. examine the effects of topical steroids on dendritic cell density (DCD) and corneal nerves in patients with dry eye using in vivo confocal microscopy (IVCM). This is an interesting and important paper that addresses a long overlooked component of dry eye pathogenesis - the alteration of cornea nerves. Although still in the preliminary stages of investigation, IVCM has potential to provide a powerful tool to assess the effects of inflammation on corneal structure and function, thereby providing a measure by which to monitor disease development, progression and treatment outcomes.
The current investigation is well thought out with an adequate sample size, and appropriate baseline measures to define the study groups. Studies such as this are necessary to ultimately define the usefulness of IVCM as a clinic readout for ocular surface disease.

Areas of concern:

One obvious and major concern about this study is the lack of a control group. It is impossible to know what changes in IVCM-generated parameters should be considered clinically significant without proper controls.

Thank you for your appreciation. We agree with your major concern and we discussed that in the manuscript, writing that further studies, using an appropriate more robust study design, are needed. However, we think that this research may be an important pilot study and an important step to explore potentials of IVCM as a biomarker in dry eye.

The meaning of "minimal clinically important difference" is not adequately defined.

Methods, ln81, ADDED: “According to Miller KL et al.19 “Subject Global Assessment” data, MCID thresholds were set at 6.1, 5.3, and 13.4 for 13<OSDI<22 points, 23<OSDI<32 points, and 33<OSDI<100 points, respectively.”

Results, ln102, ADDED: “Applying to our study population the OSDI MCID,19 set at 5.3 for the 7 patients with baseline OSDI >23 and <32 and set at 13.4 for the 43 patients with baseline OSDI>33,…”

Discussion, ln146, ADDED: “In order to assess therapy-related clinically significant improvement of symptoms, we used the previously validated OSDI MCID19. The MCID is defined as “the smallest difference in score in that domain of interest which subjects perceive as beneficial and which would mandate, in the absence of troublesome side effects and excessive cost, a change in the patient’s management.”34 We think that this instrument, when applied to ocular surface disease symptoms and dry eye patients, may represent an important progress in both clinical management and clinical research endpoints’ development.”

Additional details outlining the analysis software and techniques used to quantify cell types and nerves are necessary.
Ln 54, CHANGED: “Data for superficial and basal epithelial cell density, anterior, posterior, and activated keratocyte density,\textsuperscript{5} sub-basal dendritic cell density (DCD),\textsuperscript{7,10,14,15} and sub-basal nerve length and tortuosity were acquired.”

Ln 58, CHANGED: “Images selection and analysis were performed by a single masked investigator, following previously published and validated procedures.\textsuperscript{7,10}”

Ln 62, ADDED: “Epithelial (superficial and basal) and stromal (anterior and posterior) cells were counted at the first and at the last clearly visible epithelial and stromal layer. Activated kerocytes were defined as stomal cells with hyper-reflective ovoid or multilobate nuclei.\textsuperscript{8,16,17}”

Ln 65, CHANGED: “The total length of nerves in each image, defined as the sum of the length of all the nerve fibres within a frame, was calculated using the segmented line drawing tool of ImageJ software (available in the public domain at http://imagej.nih.gov/ij/).”

How well does DCD actually reflect the inflammatory state at the ocular surface? How closely does a change in DCD parallel other readouts of ocular inflammation? What is the biological significance of a change in DCD in the setting of dry eye? If using DCD as a read out for pro-inflammatory activity has been validated, then it needs to be clearly described.

If DCD provides a readout for proinflammatory activity, it is somewhat counterintuitive that an increase in DCD would be noted in patients that experience clinical significant improvements following treatment with steroid. Please discuss.

Ln 135, ADDED: “Previous reports on sub-basal dendritic cells, in particular, interpreted as Langerhans antigen-presenting cells,\textsuperscript{14} showed increased DCD in inflammatory conditions\textsuperscript{4,31,32} and correlations between DCD and both corneal immunohistochemistry analysis\textsuperscript{15} and tear fluid inflammatory cytokines concentration.\textsuperscript{10,33}”

Ln 140, ADDED: “To the best of our knowledge, this is the first research describing steroid-related prompt change of a largely studied, validated, and repeatable in vivo confocal inflammatory parameter.”

We agree with your last observation. Our dye eye patients showed a decrease in DCD after therapy with steroid.

________________________
Reviewer #2:

Villani et al. present data on a prospective study using in vivo laser scanning confocal microscopy (LSCM) to evaluate corneal changes in dry eye patients treated with topical corticosteroids over a 4-week period. They show that there are significant changes in various clinical and confocal findings when comparing baseline and 4-week follow-up visits after therapy. They further demonstrate that baseline dendritic cell density (DCD) is significantly higher at baseline in patients classified as showing clinical improvement after steroids compared to patients that were not clinically improved after therapy. The authors conclude that their findings using LSCM to evaluate DCD allows for detection of treatment-related inflammation changes and possible predictive value of DCD for clinical response to topical steroid therapy for treatment of dry eye.

The findings are interesting and have potential clinical implications in this field.

Thank you for your appreciation.

The authors did acknowledge the important study limitation of not having a control group. However, the following comments and suggestions need to be addressed:

Abstract

Results section: Please clarify the correlation between baseline DCD and OSDI and DCD steroid-related changes. How were they calculated? Were the correlations determined by using the differences between baseline and follow-up OSDI and DCD changes or the absolute end point values? Please state a positive or negative correlation for clarity.

We clarified correlation findings both in the abstract and in the manuscript.

Abstract, ADDED: “(r=−0.44, P<0.05 and r=−0.70, P<0.01, respectively; Spearman)”

Ln98, CHANGED: “The DCD baseline values were significantly correlated with both OSDI and DCD steroid-related changes, defined as V1-baseline values (r=−0.44, P<0.05 and r=−0.70, P<0.01, respectively; Spearman).”

Methods

Line 24: Did the authors intended to use the term "afferent"? Suggest replacing with "referred" to describe patients being directed to study center.

Done as suggested, thank you.
Study design and procedures

Line 9: Were there any unscheduled visits due to worsening symptoms in the study? Please indicate and discuss in results.

Ln91, ADDED: "No unscheduled visits due to worsening symptoms were performed during the study period."

Statistical Analysis

Line 24-25: THE OSDI-MCID suggests ranges of improvement depending on initial severity classification based on OSDI. It was indicated that the Delphi Panel report was used to classify disease severity in the study. However, the determination of MCID is based on classification using the OSDI score. Based on this, please expand and clarify how severity classification was determined and how MCID was determined. Were subjects reclassified based on OSDI scores? Was the upper or lower limit for MCID used to determine CIAS vs. NCIAS?

The Delphi Panel grading was used to include the patients (“We consecutively recruited 50 patients (41 women and 9 men; average ± standard deviation age 54.6±10.1 years, range 35-77 years) with moderate-to-severe dry eye…”).

In order to assess MCID, using SGA-related data, thresholds were set on the basis of the OSDI score of each patient.

Methods, ln81, ADDED: “According to Miller KL et al.19 “Subject Global Assessment” data, MCID thresholds were set at 6.1, 5.3, and 13.4 for 13<OSDI<22 points, 23<OSDI<32 points, and 33<OSDI<100 points, respectively.”

Results, ln102, ADDED: “Applying to our study population the OSDI MCID,19 set at 5.3 for the 7 patients with baseline OSDI >23 and <32 and set at 13.4 for the 43 patients with baseline OSDI>33, …”

Discussion, ln146, ADDED: “In order to assess therapy-related clinically significant improvement of symptoms, we used the previously validated OSDI MCID19. The MCID is defined as “the smallest difference in score in that domain of interest which subjects perceive as beneficial and which would mandate, in the absence of troublesome side effects and excessive cost, a change in the patient’s management.”34 We think that this instrument, when applied to ocular surface disease
symptoms and dry eye patients, may represent an important progress in both clinical management and clinical research endpoints’ development.”

Results

Comments mentioned previously for indication of follow-up visits and clarification on correlation findings.

Done as suggested (please, see above).

The study reports interesting findings highlighting the significant changes between baseline and V1 after treatment. Was further analysis performed such as a multivariate regression analysis using either OSDI or DCD as outcome measures to determine explanatory variables correlated OSDI or DCD measures? Variables include clinical data as well as patient demographics.

This kind of analysis would be an elegant approach to be included in future more robust studies. Our statistician warned us not to include that in the present analysis, because we would have several independent variables and this is a pilot study without an adequately calculated sample size.

The results indicate that baseline DCD was significantly higher in CIAS vs. NCIAS patients. Was there a difference in V1 DCD or a change in DCD from baseline to V1 between these two groups of patients that might explain for improvement in OSDI scores?

Were differences in V1 clinical and confocal data between CIAS and NCIAS evaluated?

“At V1, DCD was significantly decreased in CIAS (P<0.01, paired samples t-test) but not in NCIAS patients, with no further difference between the 2 groups (65.4±43.9 vs 61.8±47.8, not significant; independent samples t-test).”

Figure 1: suggest highlighting typical DC and nerve fiber in figure and a figure key to explain these features for readership unfamiliar with LCSM.

We reformatted the figure as suggested by the Managing Editor.
If you feel that is essential to highlight typical findings in the figure, we will do that. We respectfully prefer to avoid adding symbols that could interfere with clinical interpretation of the images. Several images of typical confocal appearance of DC and nerves have already been published by our group and by other researchers and a number of them are referenced in this paper. Moreover, this paper has been submitted to be included in a “Dry Eye” special issue; the same issue will include an invited contribution where we will show typical confocal findings in dry eye.

If you agree, we only changed the figure legend.

Figure 1 Legend, CHANGED: “LSCM images acquired approximately at corneal apex at sub-basal level. Frames show sub-basal nerve plexus fibers running roughly in parallel and dendritic bright objects (dendritic cells) with different densities.

Panels A and B, from a CIAS patient, show high DCD at baseline (A) and dramatically decreased DCD after (B) 4 weeks of treatment with loteprednol etabonate ophthalmic suspension 0.5% QID.

Panels C and D, from a NCIAS patient, show low DCD at baseline (C) and slightly decreased DCD after (D) 4 weeks of treatment with loteprednol etabonate ophthalmic suspension 0.5% QID.”

Table 2 - gender P-value indicates "n.d." with no explanation in table legend for "n.d."

Table 2, ADDED: n.d.: not determined

Discussion

The important conclusions from the study are based primarily on determination of DCD. Please discuss whether the DCD findings in this study population are comparable to previous publications of DCD in normal and/or dry eye patients. Is the DCD valid based on previous publications? Please discuss.

Ln153, ADDED: “In our dry eye patients, the mean baseline DCD was comparable with the higher range of previously published LSCM data (139 vs 56-127 cells/mm²). This is not surprising because in this study we selected moderate-to severe dry eye patients. Interestingly, baseline DCD in CIAS patients was significantly higher, similar with values previously found in Sjogren..."
Syndrome patients (164 vs 169 cells/mm$^2$), while baseline DCD in NCIAS patients was comparable with the lower range of previously published data on dry eye patients (72 vs 56-127 cells/mm$^2$). These findings suggest that CIAS and NCIAS patients, almost undistinguishable on the basis of usual clinical examinations and characterized by similar grade of disease, might be very different from a pathogenic point of view and LSCM assessment of DCD might be a biomarker able to detect this difference. If CIAS patients’ DCD and response to treatment confirm the mainly inflammatory nature of their ocular surface disease, NCIAS patients’ lack of LSCM signs of inflammation and poor response to treatment open new and interesting questions on the core pathogenic mechanism of their ocular surface disease;“

Line 24: It is mentioned several times throughout the manuscript that LSCM is "easy and quick" method for assessing inflammation biomarkers. This may hold true for experienced users. However, this may prove difficult in a typical clinical practice setting. Suggest removing "easy and quick" because capturing images with confocal via applanation of the cornea requires cooperation from patients and a skilled technician. Post-processing and interpretation of images by experienced technicians are also needed to determine DCD.

Done, as suggested.

The authors mentioned study limitation of not having a control group in the study. Patient compliance to treatment is a critical parameter in any clinical study. Please discuss whether patient compliance with treatment was evaluated in this study and how it may impacted the outcome.

We agree that this is an important parameter to be included in well-designed clinical trials, but the protocol of this pilot study did not include specific procedures to assess compliance to therapy. We simply asked to patients at V1 if they had regularly instilled the therapy and we have had positive feedbacks from all the patients. However, we have no reasons to hypothesize a lower compliance to therapy in patients with baseline lower DCD.

Ln92, ADDED: “At V1, all patients referred good tolerability and good compliance to the treatment.”
Managing Editor's instructions for reformatting figures for publication:

Expand to 7.5" final width and move the figure section letter for each figure section to the upper left of each section. The Editorial Office prefers the use of 14 pt Arial bold capital letters for the figure section letters. Submit as a grayscale TIFF file with at least 350 pixels/inch (ppi) resolution. If a color image is available, please also upload an identical RGB color file that can be used for the online version of the journal.

Feel free to contact me via email if you have any questions concerning your figures. Thanks, Kurt.

Done, as suggested. Thank you.
Corneal Confocal Microscopy in Dry Eye Treated with Corticosteroids

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Short title: Corneal Confocal Microscopy in Dry Eye Treated with Corticosteroids

2 tables; 1 figures

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ABSTRACT

Purpose. To evaluate, by in vivo laser scanning confocal microscopy (LSCM), the corneal findings in moderate-to-severe dry eye patients before and after treatment with topical corticosteroid and to associate the confocal findings to the clinical response. Methods. Fifty eyes of 50 patients with moderate-to-severe dry eye were included in this open label, masked study. Exclusion criteria were any systemic or ocular condition (other than dry eye) and any systemic or topical treatment (except artificial tears), ongoing or performed in the previous 3 months, with known effect on the ocular surface. All patients were treated with loteprednol etabonate ophthalmic suspension 0.5% q.i.d. for 4 weeks. Baseline and follow-up (day 30±2) visits included Ocular Surface Disease Index (OSDI) questionnaire, full eye exam and central cornea LSCM. We compared data obtained before and after treatment and looked for associations between baseline data and steroids-induced changes. Based on the previously validated OSDI Minimal Clinically Important Difference, we re-analyzed the baseline findings comparing those patients clinically improved after treatment (CIAS) to patients not clinically improved (NCIAS). Results. OSDI score and LSCM dendritic cell density (DCD) significantly decreased after treatment. Baseline DCD correlated with both OSDI and DCD steroid-related changes (r=-0.44, P<0.05 and r=-0.70, P<0.01, respectively; Spearman) and was significantly higher in CIAS patients compared to NCIAS patients (164.1± 109.2 vs 72.4±45.5 cells/mm², P<0.01; independent samples t-test). Conclusions. LSCM examination of DCD allows detection of treatment-related inflammation changes and shows previously unknown associations between confocal finding and symptoms improvement after treatment. These promising preliminary data suggest the need for future studies testing the predictive value of DCD for a clinical response to topical corticosteroids.
Key words: dry eye, inflammation, corticosteroids, cornea, confocal microscopy
Dry eye syndrome is a common ocular disorder, with a prevalence of 5% to 30% of the adult population.\textsuperscript{1} Inflammation of the ocular surface is a major pathogenic mechanism in the etiology of dry eye, and it is potentially an important biomarker of this multifactorial disease.\textsuperscript{2} The management of inflammation in moderate-to-severe dry eye includes the use of topical corticosteroids,\textsuperscript{3} with level I evidence of effectiveness published for a number of formulations.\textsuperscript{3}

In vivo laser scanning confocal microscopy (LSCM) has a resolution comparable to histological techniques and provides a non-invasive tool that allows the study of the living ocular surface structures at the cellular level. This technology has been applied to several ocular surface conditions,\textsuperscript{4} including dry eye.\textsuperscript{5,6} LSCM application to dry eye recently showed 2 main advances: new opportunities to analyze simultaneous information from the whole ocular surface morpho-functional unit,\textsuperscript{6,7} and promising results in the study of inflammation\textsuperscript{7-9} and in the monitoring of the response to therapy.\textsuperscript{10-12} Specifically, the effectiveness of LSCM in measuring changes related to dry eye disease and related to treatment\textsuperscript{5,6,10-12} is of interest with the potential to be able to provide new clinical applications.

The purpose of the present study was two-fold. First, we evaluated by corneal LSCM the changes related to treatment with topical corticosteroids in moderate-to-severe dry eye patients. Second, we analyzed the LSCM findings in patients clinically improved and not clinically improved with the treatment.
METHODS

Patients

This study adhered to the tenets of the Declaration of Helsinki, and all of the subjects provided written informed consent before examination.

We recruited 50 consecutive patients (41 women and 9 men; average ± standard deviation age 54.6±10.1 years, range 35-77 years) with moderate-to-severe dry eye who were referred to our general clinic. Each diagnosis was made according to the modified dry eye severity grading classification of the Delphi Panel Report. Inclusion criteria were dry eye symptoms for more than 6 months, reduced break up time (BUT < 10 sec), reduced Schirmer score (<10 mm/5 min), and positive corneal and conjunctival staining. Exclusion criteria were trauma or ocular surgery in the previous 6 months, any systemic or ocular disease (other than dry eye), and any systemic or topical treatment with a known effect on the ocular surface (except artificial tears) ongoing or performed in the previous 3 months.

Study Design and Procedures

The study protocol included a baseline visit, V0, and a follow-up visit, V1, 28 - 32 days later. Unscheduled visits were performed when due to worsening symptoms. All enrolled patients were treated with loteprednol etabonate ophthalmic suspension 0.5% (Bausch & Lomb Inc., Rochester, NY, USA) four times daily for 4 weeks after the baseline visit.

During both visits all patients underwent a full eye exam including ocular surface disease index (OSDI) questionnaire, assessment of the best corrected visual acuity, biomicroscopic
examination of the ocular surface, measurement of the tear film BUT, corneal staining with fluorescein and bulbar conjunctival staining with lissamine green scored according to the Collaborative Longitudinal Evaluation of Keratoconus (CLEK) scheme,\textsuperscript{13} IOP measurement, and Schirmer test without topical anesthesia.

LSCM study of central cornea was performed using the HRT II with Corneal Rostock Module (Heidelberg Engineering, Dossenheim, Germany) with a scanning wavelength of 670 nm. The objective lens (63X immersion; Zeiss, Oberkochen, Germany) was covered by a polymethacrylate sterile cap (Tomo-Cap, Heidelberg Engineering) and had a working distance of 0.0 to 2.0 mm. The examination area was 400 X 400 µm. Before each examination, a drop of oxybuprocaine chlorohydrate 0.4% and ophthalmic gel (polyacrylic gel 0.2%) were separately instilled into the lower conjunctival fornix. The exam was conducted approximately at the corneal apex. With the microscope in the acquisition modality “Section Mode”, we set the depth to zero at the most viewable superficial epithelial layer, and then we manually acquired images of all corneal layers until the endothelium was reached. The same procedure was repeated 3 times in each eye.

**Image Analysis**

Data for superficial and basal epithelial cell density, anterior, posterior, and activated keratocyte density,\textsuperscript{8} sub-basal dendritic cell density (DCD),\textsuperscript{7, 10, 14, 15} and sub-basal nerve length and tortuosity were acquired. Each LSCM parameter was obtained by selecting the best quality image from each of the 3 antero-posterior scans and averaging the results. Image selection and analysis were performed by a single masked investigator, following previously published and
validated procedures. Briefly, cell density was determined through the manual cell counting procedure present in the software, taking into consideration the whole area marked as available for the cell count. Cells that were partially within the area analyzed were counted only along the right and lower margins. Epithelial (superficial and basal) and stromal (anterior and posterior) cells were counted at the first and at the last clearly visible epithelial and stromal layer. Activated keratocytes were defined as stromal cells with hyper-reflective ovoid or multilobate nuclei. Results were expressed in cells per square millimeter. The total length of nerves in each image, defined as the sum of the length of all the nerve fibres within a frame, was calculated using the segmented line drawing tool of ImageJ software (available in the public domain at http://imagej.nih.gov/ij/). The tortuosity was evaluated according to grading performed by comparison with the reference images.

For each parameter showing a significant difference between V1 and V0, 15 confocal images were randomly selected and re-analyzed by a second independent masked investigator in order to assess the inter-observer agreement.

**Statistical Analysis**

Data derived from the worst eye, defined as the one with the lower BUT, were used for statistical analysis. All of the data were expressed as the average ± standard deviation. We compared baseline clinical and confocal data to V1 data using the t-test for repeated measures. K coefficient was used to test inter-observer agreement. We also tested correlations between baseline data and steroid-induced changes with Spearman's correlation coefficient.
Based on the previously validated OSDI Minimal Clinically Important Difference (MCID), we divided the study populations into clinically improved after steroids (CIAS) and not clinically improved after steroids (NCIAS). According to Miller KL et al. “Subject Global Assessment” data, MCID thresholds were set at 6.1, 5.3, and 13.4 for 13<OSDI<22 points, 23<OSDI<32 points, and 33<OSDI<100 points, respectively. We re-analyzed the baseline findings, comparing CIAS to NCIAS groups with the independent samples t-test. For each variable in which we found no significant difference between the two groups, we calculated the minimum detectable difference (MDD; β=0.80) for that variable. Statistical significance was set at P<0.01. Statistical analysis was performed with commercial software (SPSS for Windows v.19.0; SPSS Sciences, Chicago, IL, USA).

RESULTS

No adverse events, including clinically significant intra-ocular hypertension, were observed during topical steroid therapy. No unscheduled visits due to worsening symptoms were performed during the study period. At V1, all patients referred good tolerability and good compliance to the treatment.

OSDI score, fluorescein and lissamine green staining, DCD, and hyper-reflective keratocytes density all significantly decreased from baseline to V1 (Table 1). However, corticosteroid treatment did not induce significant differences in epithelial and keratocyte cell densities, sub-basal nerve length, or nerve tortuosity.
The DCD baseline values were significantly correlated with both OSDI and DCD steroid-related changes, defined as V1-baseline values \( (r=-0.44, P<0.05 \) and \( r=-0.70, P<0.01, \) respectively; Spearman). The 2 masked investigators analyses of DCD and hyper-reflective keratocytes density showed “substantial agreement” \( (k=0.76) \) and “almost perfect agreement” \( (k=0.91), \) respectively.

For our study population the OSDI MCID,\(^{19}\) set at 5.3 for the 7 patients with baseline OSDI >23 and <32 and set at 13.4 for the 43 patients with baseline OSDI>33, we identified 36 (72%) CIAS and 14 (28%) NCIAS patients. At baseline, there were no significant differences in any of the variables measured except for DCD which was significantly higher in CIAS, 164.1± 109.2, compared to NCIAS, 72.4±45.5 (independent samples t-test, \( P<0.01, \) Table 2, Fig.1). At V1, DCD was significantly decreased in CIAS (\( P<0.01, \) paired samples t-test) but not in NCIAS patients, with no further difference between the 2 groups (65.4±43.9 vs 61.8±47.8, not significant; independent samples t-test).

**DISCUSSION**

The 2007 International Dry Eye Workshop\(^2\) analyzed and classified dry eyes based on pathogenic mechanisms and highlighted the role of inflammation in this disease. In addition to this pathogenesis-driven approach, the Workshop provided clinical classification and management recommendations based on severity grading.\(^3,20\)

Based on the concept that inflammation is a key component of the pathogenesis of dry eye, several anti-inflammatory therapies, including topical corticosteroids, play a role in treatment of
moderate-to-severe dry eye.\textsuperscript{3, 21, 22} Loteprednol etabonate efficacy in this disease has already been reported in both research and clinical settings.\textsuperscript{23-25} Moreover, this is a site-active corticosteroid that undergoes a predictable and relatively rapid metabolism to an inactive metabolite. This characteristic improves the safety profile, reduces the risk of increasing intraocular pressure, and makes it a good candidate for use in inflammatory ocular surface conditions.\textsuperscript{23}

In clinical practice, the current severity-driven grading of dry eye provides poor direct information on inflammatory activity. The simultaneous evaluation of symptoms (maybe related to inflammation), tears secretion and stability (may be causes of inflammation), and superficial epithelial changes (may be caused by inflammation) seems to provide a complex of information more suitable for disease classification, i.e. in hypo-secretive and hyper-evaporative forms, than for assessment on inflammation activity. To study the role of immunity and inflammation in patients, tools are needed to visualize immune cells in vivo.\textsuperscript{26} In the last few years, LSCM has shown potentials in analyzing dry eye-related inflammation in several ocular surface components,\textsuperscript{4-7} including the corneal epithelium\textsuperscript{27} and stroma,\textsuperscript{8} conjunctiva,\textsuperscript{9, 28} and meibomian glands.\textsuperscript{29} It also has the ability to detect changes due to treatment.\textsuperscript{10, 30}

In this study, sub-basal dendritic cells and hyper-reflective activated stromal keratocytes significantly decreased after treatment with loteprednol. Both of these cell types have previously been hypothesized to be signs of inflammation in confocal images.\textsuperscript{4-8, 10, 26, 31} Previous reports on sub-basal dendritic cells, in particular, interpreted as Langerhans antigen-presenting cells,\textsuperscript{14} showed increased DCD in inflammatory conditions\textsuperscript{4, 31, 32} and correlations between DCD and
both corneal immunohistochemistry analysis\textsuperscript{15} and tear fluid inflammatory cytokines concentration.\textsuperscript{10, 33}

The inter-observer repeatability of these cells assessment, already tested in patients and healthy subjects,\textsuperscript{10, 26, 31} has been confirmed by our results. To the best of our knowledge, this is the first research describing steroid-related prompt change of a largely studied, validated, and repeatable in vivo confocal inflammatory parameter.

Our results contribute to open interesting prospects for the use of LSCM as a non-invasive biomarker to assess ocular surface inflammation. We feel that our most interesting LSCM results may be the correlation between baseline DCD and steroid-related OSDI changes and the baseline difference in DCD between CIAS and NCIAS patients. In order to assess therapy-related clinically significant improvement of symptoms, we used the previously validated OSDI MCID\textsuperscript{19}. The MCID is defined as “the smallest difference in score in that domain of interest which subjects perceive as beneficial and which would mandate, in the absence of troublesome side effects and excessive cost, a change in the patient’s management.”\textsuperscript{34} We think that this instrument, when applied to ocular surface disease symptoms and dry eye patients, may represent an important progress in both clinical management and clinical research endpoints’ development.

In our dry eye patients, the mean baseline DCD was comparable with the higher range of previously published LSCM data (139 vs 56-127 cells/mm\textsuperscript{2}).\textsuperscript{5-7} This is not surprising because in this study we selected moderate-to severe dry eye patients. Interestingly, baseline DCD in CIAS patients was significantly higher, similar with values previously found in Sjogren Syndrome.
patients (164 vs 169 cells/mm²), while baseline DCD in NCIAS patients was comparable with the lower range of previously published data on dry eye patients (72 vs 56-127 cells/mm²). These findings suggest that CIAS and NCIAS patients, almost undistinguishable on the basis of usual clinical examinations and characterized by similar grade of disease, might be very different from a pathogenic point of view and LSCM assessment of DCD might be a biomarker able to detect this difference. If CIAS patients’ DCD and response to treatment confirm the mainly inflammatory nature of their ocular surface disease, NCIAS patients’ lack of LSCM signs of inflammation and poor response to treatment open new and interesting questions on the core pathogenic mechanism of their ocular surface disease.

This preliminary research has important limitations, including the lack of a control group, but these findings suggest the need for future studies, using an appropriate more robust study design, planned to test the predictive value of DCD for clinical response to treatment with steroids. We think that this approach might have great potential to enable new clinical applications of LSCM and, above all, to help bridge the current gap between the pathogenesis-driven classification and the severity-driven management of dry eye.

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REFERENCES


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Table 1. Significant changes between baseline and V1 for clinical and confocal data.

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<th>Baseline</th>
<th>Visit 1</th>
<th>P</th>
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<tbody>
<tr>
<td>OSDI score</td>
<td>52.3±20.7</td>
<td>41.8±20.0</td>
<td>&lt;0.01</td>
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<tr>
<td>Fluorescein corneal staining</td>
<td>5.07±1.1</td>
<td>4.05±0.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lissamine green conjunctival staining</td>
<td>6.47±1.3</td>
<td>4.82±1.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dendritic cell density (cells/mm²)</td>
<td>138.4±106.7</td>
<td>64.4±45.</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hyper-reflective keratocytes (cells/mm²)</td>
<td>61.2±16.7</td>
<td>46.5±13.3</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

OSDI, ocular surface disease index; P-values determined by t-tests for repeated measures.
Table 2. Baseline clinical and confocal data: CIAS vs NCIAS.

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<tr>
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<th>CIAS (n=36)</th>
<th>NCIAS (n=14)</th>
<th>P</th>
<th>MDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (female:male)</td>
<td>30:6</td>
<td>11:3</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Age</td>
<td>52.3±9.7</td>
<td>60.5±12.8</td>
<td>n.s.</td>
<td>11.26</td>
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<tr>
<td>OSDI</td>
<td>54.5±22.5</td>
<td>46.8±17.4</td>
<td>n.s.</td>
<td>20.48</td>
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<tr>
<td>BUT (seconds)</td>
<td>4.9±2.3</td>
<td>5.4±2.1</td>
<td>n.s.</td>
<td>3.21</td>
</tr>
<tr>
<td>Fluorescein corneal staining</td>
<td>4.9±1.0</td>
<td>5.3±1.4</td>
<td>n.s.</td>
<td>1.68</td>
</tr>
<tr>
<td>Lissamine green conjunctivae staining</td>
<td>6.6±1.2</td>
<td>6.11±1.2</td>
<td>n.s.</td>
<td>1.92</td>
</tr>
<tr>
<td>Schirmer test (mm/5 minutes)</td>
<td>4.58±1.6</td>
<td>4.58±1.5</td>
<td>n.s.</td>
<td>2.36</td>
</tr>
<tr>
<td>Superficial epithelial cells density (cells/mm²)</td>
<td>1485.9±290.0</td>
<td>1469.8±284.1</td>
<td>n.s.</td>
<td>425.94</td>
</tr>
<tr>
<td>Basal epithelial cells density (cells/mm²)</td>
<td>6559.2±230.9</td>
<td>6534.36±214.3</td>
<td>n.s.</td>
<td>317.65</td>
</tr>
<tr>
<td>Anterior keratocytes density (cells/mm²)</td>
<td>1082.3±154.5</td>
<td>1070.3±151.4</td>
<td>n.s.</td>
<td>226.03</td>
</tr>
<tr>
<td>Posterior keratocytes density (cells/mm²)</td>
<td>812.3±106.6</td>
<td>835.66±102.7</td>
<td>n.s.</td>
<td>155.65</td>
</tr>
<tr>
<td>Hyper-reflective keratocytes density (cells/mm²)</td>
<td>56.1±6.3</td>
<td>48.9±5.8</td>
<td>n.s.</td>
<td>8.97</td>
</tr>
<tr>
<td>Sub-basal dendritic cells density (cells/mm²)</td>
<td>164.1±109.2</td>
<td>72.4±45.5</td>
<td>&lt;0.01</td>
<td>n.d.</td>
</tr>
<tr>
<td>Sub-basal nerves length (µm/mm²)</td>
<td>17.8±3.9</td>
<td>16.6±3.2</td>
<td>n.s.</td>
<td>5.31</td>
</tr>
<tr>
<td>Sub-basal nerves tortuosity (Grade 0-4)</td>
<td>2.4±0.6</td>
<td>2.3±0.6</td>
<td>n.s.</td>
<td>0.90</td>
</tr>
</tbody>
</table>

P, P-value by independent samples t-test; MDD, minimum detectable difference; n.d.: not determined; n.s.: not significant; OSDI, ocular surface disease index; BUT, breakup time.
FIGURE LEGENDS

Figure 1. Dendritic cells response to topical steroids. LSCM images acquired approximately at corneal apex at sub-basal level. Frames show sub-basal nerve plexus fibers running roughly in parallel and dendritic bright objects (dendritic cells) with different densities. Panels (A) and (B), from a CIAS patient, show high DCD at baseline (A) and dramatically decreased DCD after (B) 4 weeks of treatment with loteprednol etabonate ophthalmic suspension 0.5% QID. Panels C and D, from a NCIAS patient, show low DCD at baseline (C) and slightly decreased DCD after (D) 4 weeks of treatment with loteprednol etabonate ophthalmic suspension 0.5% QID.
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