



In-vivo confocal microscopy of the ocular surface: ocular allergy and dry eye

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Purpose of review

To summarize recent studies on in-vivo confocal microscopy (IVCM) findings in ocular allergy and dry eye disease (DED), highlighting the role of IVCM in the advancement of knowledge of these diseases.

Recent findings

IVCM provided new data on ocular surface changes in both ocular allergy and DED. Corneal and conjunctival epithelial and inflammatory cells, corneal nerves, and Meibomian glands showed peculiar patterns of abnormalities, not easily discernable with current clinical exams in these two diseases and their subtypes. At present, small sample size of researches, and poor standardization and evidence of image analysis and interpretation are the most challenging issues.

Summary

Ocular allergy and DED are common and increasing healthcare problems, and need better understanding of pathogenesis and natural history, more reliable endpoints, and more tailored diagnostic and therapeutic approaches. IVCM allows quick, noninvasive, steady-state respectful examination of the ocular surface at cellular level to be performed and has potential to be used in the future as a biomarker and to contribute to optimize the tailored management of these diseases.

Keywords

conjunctiva, cornea, dry eye disease, in-vivo confocal microscopy, ocular allergy

INTRODUCTION

Ocular allergy and dry eye disease (DED) are common conditions, with more than 20% of prevalence, which increase healthcare problems, adversely affect the quality of life, and increase work-related issues and economic burdens [1^a,2,3]. They include a wide spectrum of clinical subtypes with several partially overlapping symptoms of ocular surface disease. The need for a better understanding of pathogenesis and natural history, for more reliable endpoints, and for more tailored diagnostic and therapeutic approaches is, at present, a major concern [1^a].

Although ocular allergy and DED have different pathogenic mechanisms, clinical differences may not always be as clear-cut as expected, because of a common inflammatory involvement of the same ocular surface structures, including corneal and conjunctival epithelia. Therefore, high-tech diagnostic instruments are rapidly becoming important additional tools for correct management of these diseases.

In-vivo confocal microscopy (IVCM) is an emerging noninvasive technology that allows us to see

ocular surface structures at the cellular level, providing images comparable to histological methods without significantly affecting the steady state of the target tissues [4]. The application of this technology to the cornea provided us with new important chances to analyze this thin, transparent, avascular, and multilayered tissue. The ability of laser IVCM technology to go beyond the cornea and to examine the different components of the ocular surface has opened new doors for studying the physiology and pathology of this complex morpho-functional unit. Changes in inflammation, innervation, epithelial cells, and Meibomian glands are the most suitable ocular surface issues to be studied by IVCM [5^a].

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KEY POINTS

- IVCN provides new data on changes in the ocular surface morpho-functional unit in both DED and ocular allergy.
- Corneal and conjunctival epithelial and inflammatory cells, corneal nerves, and Meibomian glands show peculiar patterns of abnormalities revealed by IVCN that are not easily discernible with current clinical exams for ocular allergy and DED and associated subtypes.
- The evolution of IVCN will allow us to identify biomarkers in ocular allergy and DED, and the optimization of tailored management of these diseases.

The aim of this review is to summarize recent key findings in IVCN of the ocular surface in ocular allergy and DED, focusing on the promising role of this modality in clinical research and practice.

OCULAR ALLERGY

Allergic eye diseases present several different manifestations, ranging from acute, self-limited, mild forms of seasonal/perennial allergic conjunctivitis to chronic, severe, sight-threatening atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC) [6]. VKC and AKC represent major therapeutic challenges, and for this reason, several recent research efforts have focused on the pathogenesis, biomarkers, and clinical management of these two allergic diseases. In this review, we will use the term 'ocular allergy' referring to these chronic allergic syndromes (VKC and AKC), excluding seasonal or perennial allergic conjunctivitis.

Epithelial and stromal changes

There does not appear to be any datum regarding the conjunctival cell density in ocular allergy. The central corneal confocal features of AKC patients compared with controls by Hu *et al.* [7] in 2008 showed a significant AKC-related decrease in basal epithelial cell density (6563.6 vs. 8916.7 cells/mm²) and in the ratio of basal epithelial to superficial epithelial cell density (7.5 vs. 8.9) [7].

Recent data published by Leonardi *et al.* [8^{***}] on VKC showed decreased corneal basal epithelial cell density (5704 vs. 6770 cells/mm²). The same study described several VKC-related abnormalities of the superficial epithelium, such as increased cell diameter (27.8 vs. 20.5 μm), reflectivity (54.48 vs. 37.3), presence of activated cell hyper-reflective nuclei (59 vs. 10%), and an abnormal nucleocytoplasmic ratio (26.4 vs. 7.5%). There was also a

significant decrease in keratocyte density in the anterior corneal stroma (690 vs. 844 cells/mm²), showing that the disease involved more than the superficial cornea [8^{***}].

A recent study [9] described the IVCN appearance of limbal morphology in VKC. The Vogt palisades were characterized by atrophy or absence of stromal papillae and the disappearance of bright basal cells. These changes were accompanied by infiltration of numerous Langerhans cells and vessel dilation. The presence of normal Vogt palisades at superior limbus was significantly higher in the tarsal form of VKC, followed by the mixed form, both of which were significantly higher than that in the bulbar form (85.7 vs. 28.6 vs. 20%, respectively) [9].

Inflammation

Reports on IVCN detection of AKC inflammatory cells described the presence of diffuse, round, highly reflective infiltrates in the ulcerated cornea [7]. Hu *et al.* [10] reported elevated densities of tarsal conjunctival inflammatory cells in AKC patients, and that cyclosporine A (CsA) treatment reduced the densities, but not to levels found in normal control eyes (1448 vs. 696 vs. 394 cells/mm², respectively). In these patients, conjunctival inflammatory cell density was negatively correlated with tear stability and corneal sensitivity, and positively correlated with the vital staining scores (Fig. 1). IVCN revealed papillary lesions that showed remarkable fibrosis in patients using CsA [10]. Recent confocal data on inflammation in VKC patients compared with healthy controls showed an increased presence of activated keratocytes (59 vs. 10%) and stromal dendritic cells (54 vs. 0%) [8^{***}]. Bulbar and tarsal conjunctival dendritic cells were also increased relative to controls (365.6 vs. 42.1 cells/mm² and 232.2 vs. 0 cells/mm², respectively), as were roundish hyper-reflective inflammatory cells in the bulbar and tarsal conjunctivas (724.8 vs. 108.4 cells/mm² and 903.6 vs. 385.2 cells/mm², respectively) [9] (Fig. 2).

In-vivo confocal microscopy comparisons among clinical subtypes of VKC (tarsal, bulbar, and mixed form) highlighted the increased prevalence of activated keratocytes in the mixed form (50 vs. 48 vs. 83.3%, respectively) [8^{***}]. There was also an increased density of tarsal dendritic cells in the tarsal form (257.5 vs. 81.6 vs. 291.3 cells/mm², respectively) [9].

Corneal nerves

In 2008, Hu *et al.* [7] first reported IVCN data on changes in the sub-basal nerve plexus (SNP) in ocular allergy. In this study, AKC patients compared

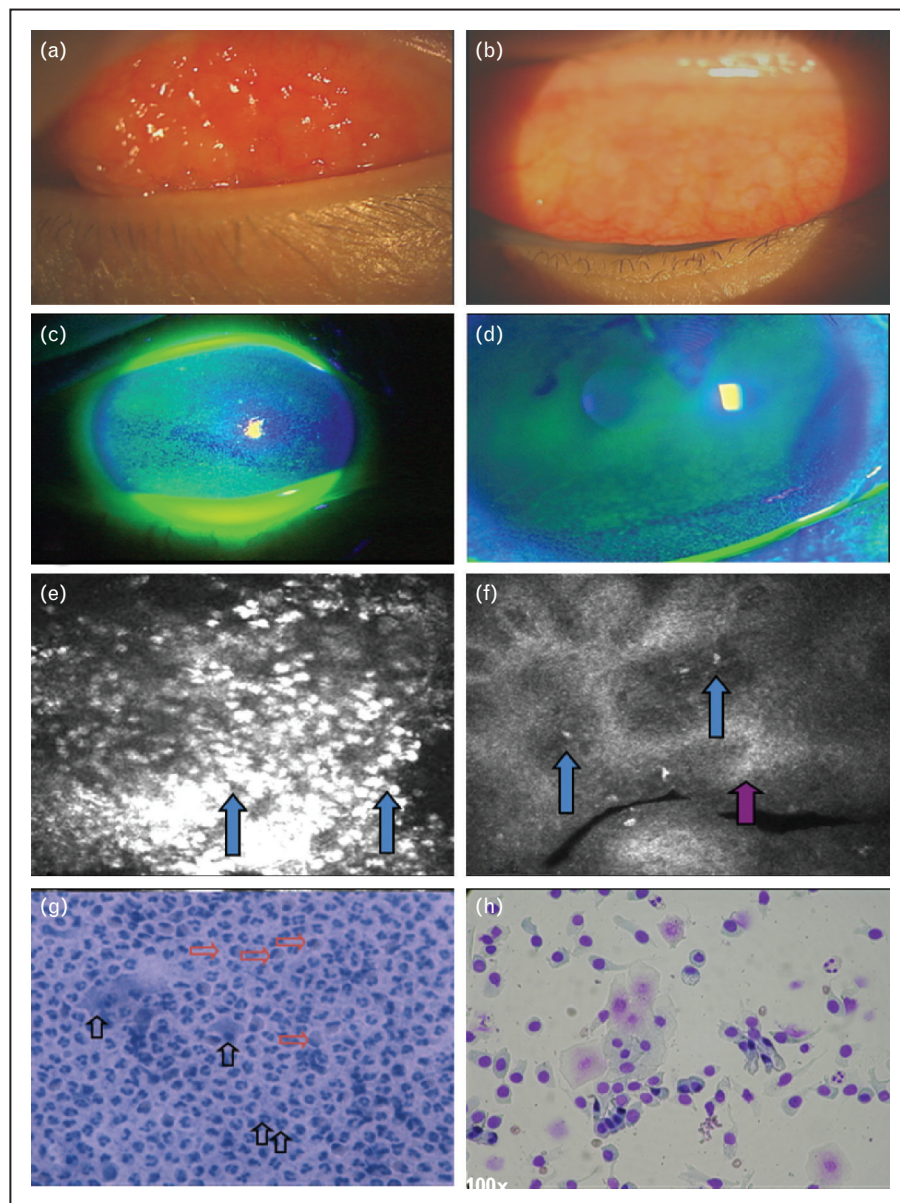


FIGURE 1. Ocular surface changes after topical cyclosporine treatment in a patient with AKC. Both tarsal papillae (b vs. a) and corneal fluorescein staining (d vs. c) greatly improved. Clear reduction of conjunctival inflammatory cell (arrows) density is highlighted by both IVCM (f vs. e) and impression cytology (h vs. g). AKC, atopic keratoconjunctivitis; IVCM, in-vivo confocal microscopy (unpublished – courtesy of Murat Dogru, Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan).

with healthy individuals, showed significantly decreased sub-basal long nerve fibers and nerve branch densities (11.8 vs. 32.6 and 32.6 vs. 67.7 nerves/mm²). These confocal parameters were positively correlated with corneal sensitivity, tear break-up time, and basal epithelial cell density, and negatively correlated with corneal staining. Moreover, thick stromal nerves with obscure borders, sharp deflection, and bifurcation abnormalities were observed in eyes with AKC [7].

Leonardi *et al.* [8^{••}] extensively explored corneal innervation in VKC, describing both SNP and stromal nerve changes (Fig. 3). SNPs showed VKC-related reductions of nerve fiber density (9930 vs. 13670 μm/mm²), number of nerve fibers (4.9 vs. 6.4 n/image), and nerve bead-like formations (9.8 vs. 12.5 n/100 μm), and increased nerve tortuosity (25 vs. 0%, presence grade 3–4). Stromal nerves showed VKC-related increased rates of thickness (60.7 vs. 11%), tortuosity (34.4 vs. 13.9%),

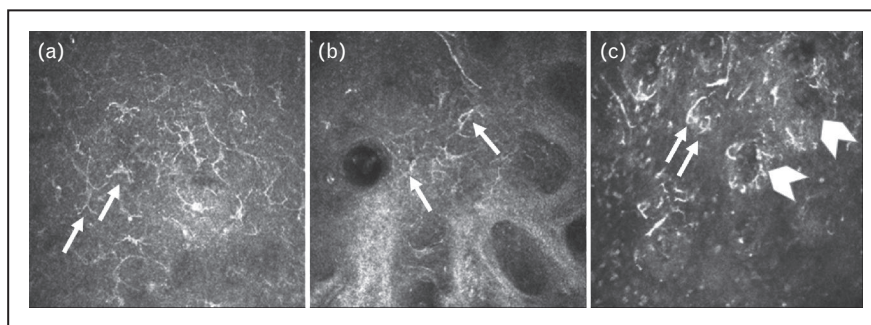


FIGURE 2. IVCM images of ocular surface in VKC showing severe dendritic and inflammatory cells infiltration (arrows) in bulbar (a) and tarsal (b) conjunctiva, and in Meibomian gland interstice (c). Meibomian gland acinar units (arrowheads) appear rare and atrophic. IVCM, in-vivo confocal microscopy; VKC, vernal keratoconjunctivitis (unpublished – courtesy of Jianjiang Xu, Department of Ophthalmology, Eye & ENT Hospital of Fudan University, Shanghai, China).

branching (47.5 vs. 13.9%), and deflection (54.1 vs. 0%) [8^{***}]. Among the clinical subtypes of AKC, only the rate of abnormal stromal nerve tortuosity was significant (35.3, 20, and 52.2% for tarsal, limbal, and mixed forms, respectively) [8^{***}].

Meibomian glands

In 2011, Le *et al.* [9], in a study of conjunctival changes in VKC patients, also reported IVCM images of severe Langerhans cell infiltration around the Meibomian glands (Fig. 2). Recently, Ibrahim *et al.* [11^{***}] highlighted severe Meibomian gland changes in AKC patients. IVCM revealed extensive fibrotic changes in Meibomian gland acinar units, more extensive than that observed in obstructive Meibomian gland dysfunction (MGD). AKC patients, compared with obstructive MGD and healthy controls, had higher periglandular inflammatory cell density (1192 vs. 712.9 vs. 60.6 cells/mm²), decreased Meibomian gland acinar unit longest diameters (43.8 vs. 98.9 vs. 59.9 μm) and areas (646.0 vs. 2119.5 vs. 716.5 μm²), and decreased acinar unit

density (40.1 vs. 70.3 vs. 120.1 n/mm²) [11^{***}]. Confocal Meibomian gland changes in AKC showed potential differences from those in patients with obstructive MGD. Contrary to the enlarged Meibomian gland acinar units found in obstructive MGD patients, AKC patients had shrunken Meibomian glands, with extensive periglandular fibrosis.

DRY EYE

Dry eye disease is a multifactorial disease of the ocular surface morpho-functional unit, an integrated system comprising the tear film, lacrimal glands, cornea, conjunctiva, Meibomian glands, lids, and the sensory and motor nerves that connect them. DED is accompanied by increased tear film osmolarity and inflammation and has the potential to damage the ocular surface [12]. IVCM may not only lead to improved understanding of the pathophysiological mechanisms of DED, but aid in the assessment of prognosis and treatment of this complex disease as well [4]. Recently, IVCM provided us with important information on

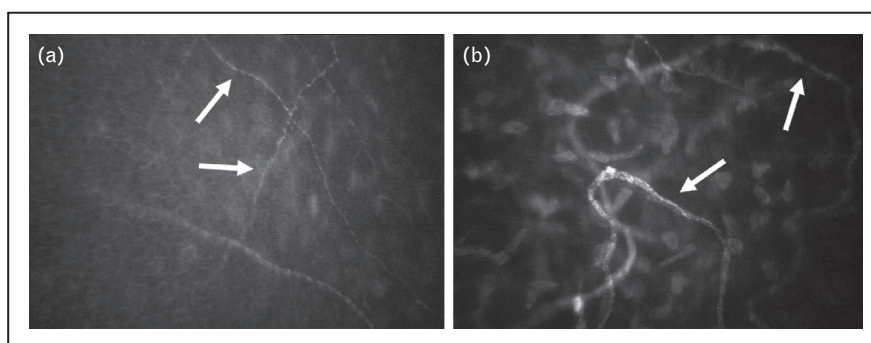


FIGURE 3. IVCM images of corneal nerves (arrows) in VKC. Sub-basal nerves (a) show decreased density and abnormal course. Stromal nerves (b) appear abnormally thick and convoluted. IVCM, in-vivo confocal microscopy; VKC, vernal keratoconjunctivitis (unpublished – courtesy of Andrea Leonardi, Ophthalmology Unit, Department of Neuroscience, University of Padua, Padua, Italy).

cellular, nervous, and glandular changes in different types of dry eye.

Epithelial and stromal changes

Recently, comparison of IVCN data for matched groups of patients with primary Sjogren's syndrome, hypo-secretive non-Sjogren dry eye (non-SSDE), MGD, and control individuals showed significant DED-related changes of ocular surface epithelial densities [5[•]]. All cornea (941–1506 vs. 1902 cells/mm²; range of DED values vs. controls), bulbar (984–1403 vs. 2136 cells/mm²), and tarsal (990–1433 vs. 2381 cells/mm²) conjunctiva and eyelid margin (982–1213 vs. 1623 cells/mm²) presented a decreased superficial epithelial cell density. Basal epithelial densities were also reduced in each examined tissue (3616–4875 vs. 5164 cells/mm², 3062–3177 vs. 4044 cells/mm², 4082–4287 vs. 4626 cells/mm² for bulbar conjunctiva, tarsal conjunctiva, and eyelid margin, respectively) except for the cornea, where it was increased (6498–6933 vs. 6304 cells/mm²). These data partly confirmed previous confocal findings [13[•]], and led us to hypothesize that changes in epithelial cell densities are meaningful indicators of tissue damage arising from mechanical, trophic, and inflammatory phenomena.

Literature reports evidence of decreased corneal stromal keratocyte density in dry eye [5[•],13[•]], as net outcome of synchronous increase of apoptotic phenomena, proteolytic activity, and proliferative stimuli.

In-vivo confocal microscopy presents obvious advantages over other modes of assessing ocular surface cell organization and density. First, as a minimally invasive procedure, it is more respectful of the ocular surface steady state compared with relatively invasive methods such as impression or brush cytology. This led us to apply this technology to evaluate conjunctival squamous metaplasia based on the mean individual epithelial cell area (MIECA), nucleocytoplasmic ratio, and goblet cell density [13[•],14]. Analysis of MIECA and nucleocytoplasmic ratio revealed significantly worse scores in dry eye patients compared with controls ($P < 0.0001$). The IVCN scores were not significantly different from cytological results, and the correlation between the two techniques was very strong (MIECA: r^2 0.557; nucleocytoplasmic ratio: r^2 0.765). Thus, MIECA and nucleocytoplasmic ratio appear to be two promising quantitative parameters of IVCN cytology [14].

For evaluation of goblet cell density, IVCN has produced some conflicting data [13[•]] and major concerns [15]. Specifically, a recent report studying

the tarsal conjunctivas in young, older, and patients with Sjogren's syndrome showed poor inter-observer agreement. Further, some IVCN results seemed unreasonable from a biological point of view. For instance, goblet cell density in patients with Sjogren's syndrome (24 eyes) was reported to be 688 ± 318 cells/mm², whereas that for young healthy individuals (24 eyes) was 36 ± 41 cells/mm² [15].

Inflammation

In-vivo confocal microscopy studies [5[•],13[•]] provided us with several partially validated parameters to be used in the evaluation of ocular surface inflammation (Fig. 4). The most used parameters included dendritic cells, presumed leukocytes, and activated keratocytes. Dendritic cells, interpreted as antigen-presenting cells, are more commonly visualized at the level of the corneal SNP, and more rarely in the conjunctiva and eyelid margin. Comparing different subtypes of DED and healthy controls, Sjogren's syndrome and MGD patients showed significant increases in the density of corneal dendritic cells (82–169 vs. 53 cells/mm²) [5[•]]. Moreover, recent studies on rheumatoid arthritis showed that the prevalence of these cells is associated with ocular surface disease [16,17[•]], and the density is correlated with the cytokine concentration in tear fluid [17[•]]. Further, the density of dendritic cells promptly responds to changes in ocular surface inflammatory activity [17[•]].

In-vivo confocal microscopy assessment of presumed leukocytes has recently been partially validated in tarsal and bulbar conjunctivas [13[•],15]. Compared with healthy individuals, all Sjogren's syndrome, non-SSDE, and MGD patients have increased conjunctival densities of these cells (177–498 vs. 87 cells/mm²) [5[•]]. More challenging is the assessment of inflammatory cells in the interstices of Meibomian glands. Using IVCN, Ibrahim *et al.* [18] quantified presumed Meibomian gland leukocyte density. They reported that as a MGD diagnostic tool, the density of leukocytes was 100% for both sensitivity and specificity with a cut-off at 300 cells/mm² [18]. However, we and other authors considered it too difficult to identify and count single periglandular cells. These groups performed semiquantitative analyses (1–4 grading) of the punctiform elements viewable in the interstices. The presence of the punctiform elements was defined as the 'inhomogeneous appearance of Meibomian gland interstice' and showed increased scores in patients with Sjogren's syndrome [5[•],19], MGD [5[•]], contact lens wearers [20], and glaucoma [21].

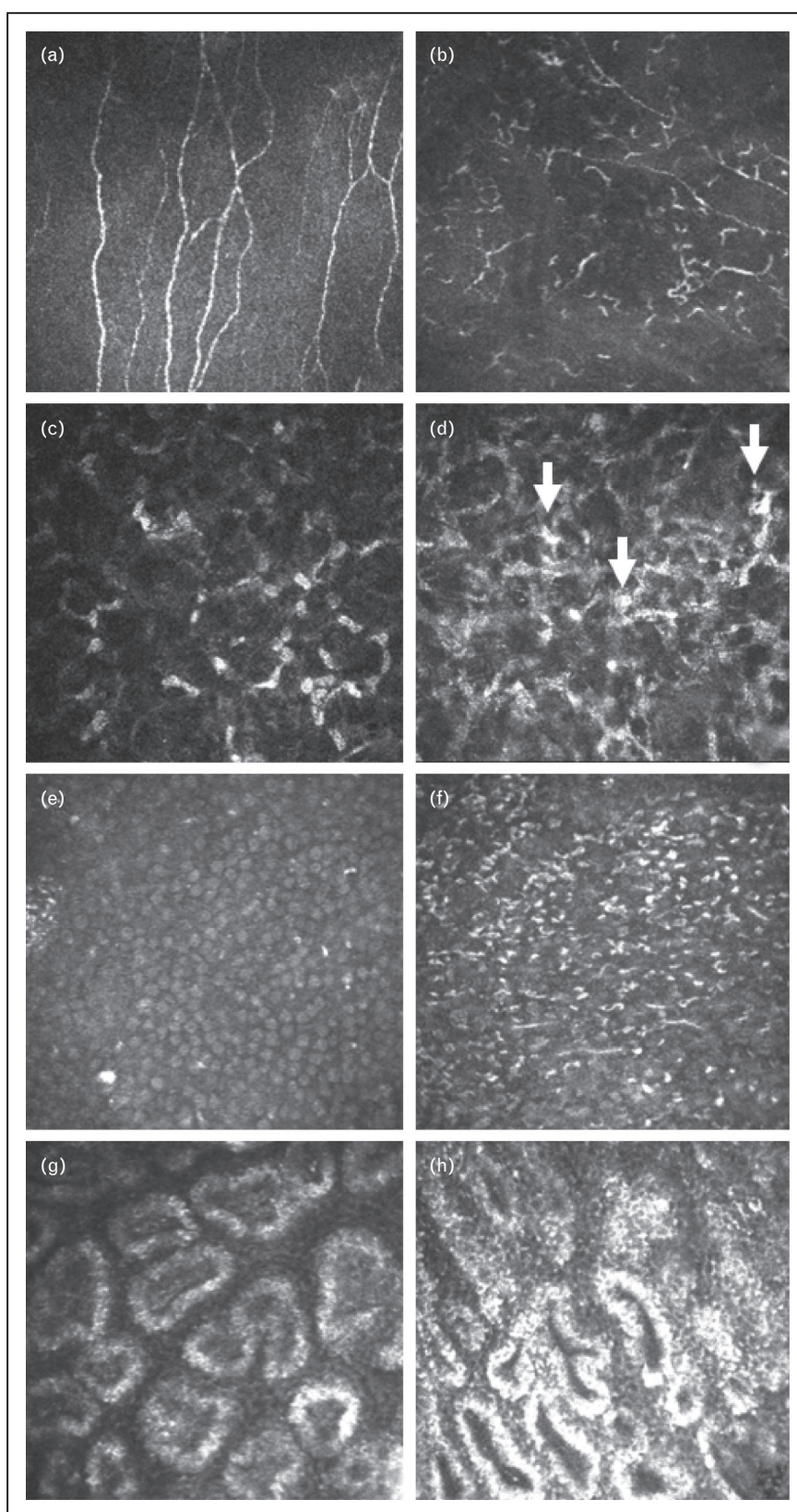


FIGURE 4. IVCM signs of inflammation: DED patient (SSI) compared to healthy individual. DED patient shows increased density of corneal dendritic cells at the level of a damaged sub-basal nerve plexus (b vs. a), hyper-reflective activated keratocytes (d, arrows vs. c), presumed conjunctival inflammatory and dendritic cells (f vs. e), inhomogeneous appearance of Meibomian gland interstice (h vs. g) (previously published [5]). DED, dry eye disease; IVCM, in-vivo confocal microscopy.

Activated keratocytes are hyper-reflective stromal cells, identified in confocal reports, but not histologically described, and could be interpreted in more than one way [5[■],13[■],22]. They could be normal stromal cells in which the confocal light ray strikes at an unusual angle. However, these findings are associated to corneal inflammation, as reported in patients with DED [5[■],13[■]], or Graves' orbitopathy, where this was the only confocal sign that was significantly different between active and inactive orbitopathy [22]. It is likely that these cells are keratocytes in a particular state of metabolic activation induced by pro-inflammatory cytokines. Alternatively, they could be stromal bone marrow-derived cells.

In the future, IVCN dynamic assessment of ocular surface inflammation may be of help in evaluating dry eye severity. Additionally, it can likely guide clinical treatment and aid in the evaluation of the efficacy of anti-inflammatory drugs in clinical trials [4,13[■],17[■]].

Corneal sub-basal nerve plexus

The cornea is the most densely innervated tissue in the body, and it has a key role in DED. Corneal nerves are involved in maintenance of ocular surface homeostasis by regulating epithelial cell trophisms, and the secretion and distribution of tears. IVCN allows the description of the morphology, density, and disease-induced alterations of corneal nerves, particularly the SNP [4].

Recently, several small studies quantitated and analyzed morphologic DED-related SNP changes and found partially conflicting data [13[■]]. The most frequent findings were decreased density or number of fibers and increased tortuosity and bead-like formations [13[■]]. More recently, Labbé *et al.* [23[■]] reported DED-related decreased nerve density (9.426 vs. 15.956 mm/mm²) and number (24.73 vs. 39.06 n/mm²), but with no significant changes in nerve tortuosity and beading. Villani *et al.* [5[■]], comparing Sjogren's syndrome, non-SSDE, MGD, and healthy individuals, found that DED patients had decreased numbers of nerves (2.9–4.3 vs. 5.8 n/frame), and increased tortuosity (1.5–2.5 vs. 1.2, grading 1–4) and bead-like formations (218–304 vs. 116 n/100 μm).

The inconsistencies of these data are probably because of the small sample sizes and differences in the clinical features of the patients enrolled. However, IVCN is playing a key role in providing us with information on DED-related neuropathy and neuro-inflammation.

Meibomian glands

Ibrahim *et al.* [18] first validated the confocal parameters of Meibomian gland acinar longest diameter, acinar shortest diameter, and acinar unit density for the diagnosis of MGD. The sensitivities of their findings were between 81 and 90%, and the specificities were between 81 and 96%. More recent studies [5[■],13[■]] confirmed increased acinar diameters (94–106 vs. 49–53 μm) and orifice diameters (48–50 vs. 32–35 μm), increased secretion reflectivity, and decreased acinar unit density (57–64 vs. 110–121 n/mm²) in MGD. For contact lens wearers, acinar diameter is decreased (36 vs. 45 μm) [20]. In graft-vs.-host disease, the Meibomian gland acini are decreased in both the longest diameter (37.3 vs. 60.4 μm) and the shortest diameter (17.7 vs. 26.6 μm), and acinar unit density is also decreased (57.8 vs. 88.8 n/mm²) [24]. Results recently reported for Primary Sjogren's syndrome patients have less obvious interpretation, but showed predominance of confocal signs of inflammation, without dilatative morphologic changes, leading to hypothesize the occurrence of an inflammatory-atrophic nonobstructive MGD [5[■],19]. Recent research described different forms of age-related Meibomian gland changes, reporting that asymptomatic older individuals mainly showed signs of atrophic, nonobstructive, age-related MGD [25[■]]. In all cases, IVCN provides new information regarding the patterns of Meibomian gland changes associated with different subtypes of DED.

CONCLUSION

Recently, IVCN has been used increasingly to examine several components of the ocular surface morpho-functional unit. The uniqueness of this technology allows performance of quick, noninvasive, steady-state respectful examinations at the cellular level. It enables the acquisition of new information on specific pathogenic patterns and responses to treatments. At present, the small sample size of research studies, and poor standardization and evidence of exam analysis and interpretation are the most challenging issues. In the future, the evolution of IVCN will allow us identify biomarkers of ocular allergies and DED, and contribute to the optimization of tailored management of these diseases.

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Conflicts of interest

There are no conflicts of interest.

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