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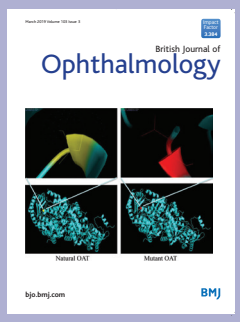

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In vivo confocal microscopy indicates an inverse relationship between the sub-basal corneal plexus and the conjunctivalisation in patients with limbal stem cell deficiency

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ABSTRACT

Background/aims Limbal stem cell deficiency (LSCD) is characterised by a marked decrease in limbal stem cells. It is classified primarily using subjective slit-lamp observations. In vivo confocal microscopy (IVCM) can non-invasively provide objective information on the condition of the limbal niche, the corneal epithelial basal cell density and the corneal sub-basal nerve plexus density (SND). We here used IVCM to evaluate changes in SND to improve LSCD classification.

Methods We evaluated and classified 38 patients (76 eyes, 44 with LSC and 32 control eyes) using the Rama, López-García and Deng (clinical and confocal) classifications and evaluated the concordance of the confocal and clinical classifications. We constructed a logistic regression model using multivariate analysis to correlate different degrees of conjunctivalisation with IVCM parameters and used receiver operating characteristic (ROC) curve analysis to establish the SND cut-off value with maximum diagnostic sensitivity and specificity.

Results The classification systems correlated moderately at best (kappa, 0.449). The corneal SND of cases (6469±6295 µm/mm^2) was less ($p<0.001$) than in controls (20911±4142 µm/mm^2). The SND, but not basal cell density, played a protective role against conjunctivalisation (OR, 0.069; 95% CI 0.008–0.619; $p=0.01$). An SND cut-off value of 17 215 µm/mm^2 yielded a sensitivity and specificity of 95.5% and 90.6%, respectively, for LSCD diagnosis.

Conclusion The density of the corneal sub-basal nerve plexus was inversely related to conjunctivalisation in LSCD. Further studies are needed to verify this and to elucidate the directionality between these factors.

INTRODUCTION

For survival, replication and engendering a healthy corneal epithelium, the different parts of the limbus (limbal niche, microenvironment and stem cells) must be well harmonised and fully functional. Limbal stem cell deficiency (LSCD) is a disease characterised by a critical reduction in the number of limbal stem cells.

Currently, LSCD is classified based primarily on symptoms and signs that are mainly observed with a slit-lamp. These classifications are therefore subjective; moreover, they do not provide the necessary information for treatment and prognosis. In vivo confocal microscopy (IVCM) is a non-invasive method that provides objective information on the condition of the limbal niche, the basal cell density of the corneal epithelium and the corneal sub-basal nerve plexus density (SND), which is reduced in the early stages of LSCD.[1]

We conducted this study to obtain better LSCD classification by evaluating changes in the sub-basal corneal nerve plexus using IVCM.

METHODS

Study design

This study investigated patients who were diagnosed with LSCD at the Virgen Macarena and Virgen del Rocio University Hospitals in Seville between 20 November 2016 and 30 May 2017. The inclusion criteria were an age over 18 years, an aetiology compatible with LSCD, in addition to signs and symptoms based on the Dua clinical criteria, such as presenting corneal conjunctivalisation, which involves the proliferation of the conjunctival epithelium and goblet cells over the corneal surface.[2] Patients who could not complete the case history review and/or basic clinical examination were excluded.

During the first visit, we collected data on medical history and clinical examinations (table 1). LSCD was classified as shown in online supplementary e-table 1e.

During the second visit, we conducted IVCM, Heidelberg retinal tomography II using the Rostock Cornea Module (HRT II-RCM; Heidelberg Engineering GmbH, Heidelberg, Germany). We mainly used the section mode (individual two-dimensional images), except in the central, inferior and superior area; we obtained two volumes of each of these areas, which involved 40 two-dimensional images taken at an 80 μm depth range (one image every 2 µm). We used this standard protocol in order to locate the sub-basal nerve plexus more accurately. After the IVCM analysis, we conducted image analysis, which entailed a cell analysis according to the parameters reported by Deng *et al*.[1] The numbers of wing and superficial basal cells were counted using the HRT II confocal microscope. The SND, given in

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Table 1 Data recorded during the first visit

Case history review	► Aetiology of limbal stem cell deficiency, according to the classification by Gris and Perez-Santonja *et al*.[18] ► Assessment of symptoms according to the López-García scale[3]: corneal erosion or recurrent ulcers in the past 6 months, presence and grade (mild, moderate or severe) of photophobia of each eye, separately, and presence and grade (mild, moderate or severe) of epiphora in each eye, separately.
Clinical examination	► Visual acuity (ETDRS optotype at 4 m). ► Intraocular pressure (Perkins tonometer). ► Slit-lamp examination to grade each patient based on the López-García,[3] Rama[4] and Deng symptom scales[1]: a. Corneal conjunctivalisation: 1. Presence or absence of conjunctivalisation (yes/no). 2. Affected corneal clock-hours sectors. 3. Degree of corneal invasion by conjunctivalisation towards the corneal apex, in millimetres, classified as follows: – <1 mm. – >1 mm and impairment <180°. – >1 mm and impairment >180°. 4. Dull corneal reflex (yes/no). 5. Central cornea involvement (yes/no). b. Corneal staining with fluorescein eye drops: – Corneal epithelial defect. – Late and persistent staining. ► 20 mg/mL fluorescein staining and Oxford scale. ► 1% lissamine green staining and Van Bijsterveld scale. ► Tear break-up time. ► Schirmer's I test, without anaesthesia.

the nerve length (µm) per area (mm^2) was measured using Image J and Neuron J software, and the average of three images of the central subbasal nerve plexus per eye was used for analysis.

The controls did not present any current or previous ocular pathology; these individuals were selected from friends or 'married-in' family members, without a close genetic relationship with the patient.

Statistical analysis

The sample size was based on the various objectives of the study as follows. First, to compare the means of two independent samples (subcategories determined according to conjunctivalisation >180° limbal and corneal advance of conjunctivalisation >1 mm), we employed the Satterthwaite test for unequal variances and determined sample size by using the sample size calculation programme nQuery Advisor Release 7.0 (Statistical Solutions, Broadway, Saugus, Massachusetts, USA) and the information provided by a pilot study. Second, in the case−control analysis, to detect a difference between them of 5000 µm/mm^2 in the variable 'SND', SD of 9000 µm/mm^2 and 5400 µm/mm^2 in two groups (obtained from a pilot study), an error α of 5% and an error β of 20% (power of 80%), we required 32 eyes in each group.

We determined the intraclass correlation coefficient to assess the reliability of measurements performed by different methods in the same participants. To this end, we calculated a sample size for which we assumed a lowest intraclass correlation coefficient of 0.6 (based on a pilot study), a 95% CI, an interval precision or amplitude of 0.15 and the use of two measurement methods.

Descriptive statistics (percentages, means and medians) were computed for the demographic and clinical variables. To compare two qualitative variables, we created contingency tables and performed the χ^2 test. To compare numerical variables, we applied the parametric Student's t-test for independent samples and the Mann-Whitney U test or the Wilcoxon test for non-normally distributed data.

We also compared the Deng,[1] López-García[3] and Rama[4] clinical classifications with Deng's confocal classification by calculating the kappa index to assess the degree of concordance with the staging scales (Landis-Koch).

To analyse the relationship between the presence of conjunctivalisation and other variables, we divided the cohort into three different types, based on the above classifications: the presence or absence of conjunctivalisation, conjunctivalisation in fewer than three or in three or more clock-hour sectors and conjunctivalisation of equal or less than 1 mm of corneal invasion and impairment of less than 180° of the limbal circumference or conjunctivalisation of more than 1 mm of corneal invasion and impairment of equal or more than 180° of the limbal circumference (table 1). This last criterion was chosen as it was considered more appropriate for performing multivariate analysis with a

Table 2 General description of the study cohort

Variables		Cases n=44 (eyes)	Controls n=32 (eyes)	P values
Age, years		52.0 (19.0)	54.7 (14.5)	0.291
Male sex		50% (participants)	50% (participants)	0.082
Laterality (right eye)		50%	50%	0.610
Case history	**Ulcers <6 months***	0 (0–4.7)	0 (0–0)	0.009
Examination	**VA ETDRS 4** m	50.8 (31.8)	81.8 (11.7)	0.001
	Schirmer I	9 (2.2–15)	15 (4.5–18)	0.153
	TBUT, s	2 (3.5–1)	7.5 (4.75–10)	<0.001
	IOP, mm Hg	12 (10–14)	12.5 (11.75–14)	0.190
	Oxford Scale	4 (2–6)	0 (0–1)	<0.001
	Van Bijsterveld Scale	4 (2–5.5)	0 (0–0.25)	<0.001
IVCM	Superficial cell density	416 (234)	499 (370)	1
	Wings cell density	3047 (1327)	4578 (950)	0.024
	Central basal cell density	4404 (2512)	6107 (628)	0.322
	Subbasal nerve plexus density, µm/mm^2	6469 (6295)	20 911 (4142)	<0.001

Continuous data are given as means (SD); qualitative variables are given as percentages; or median (25th percentile; 75th percentile).
IOP, intraocular pressure; IVCM, in vivo confocal microscopy; TBUT, tear break-up time; VA, visual acuity.

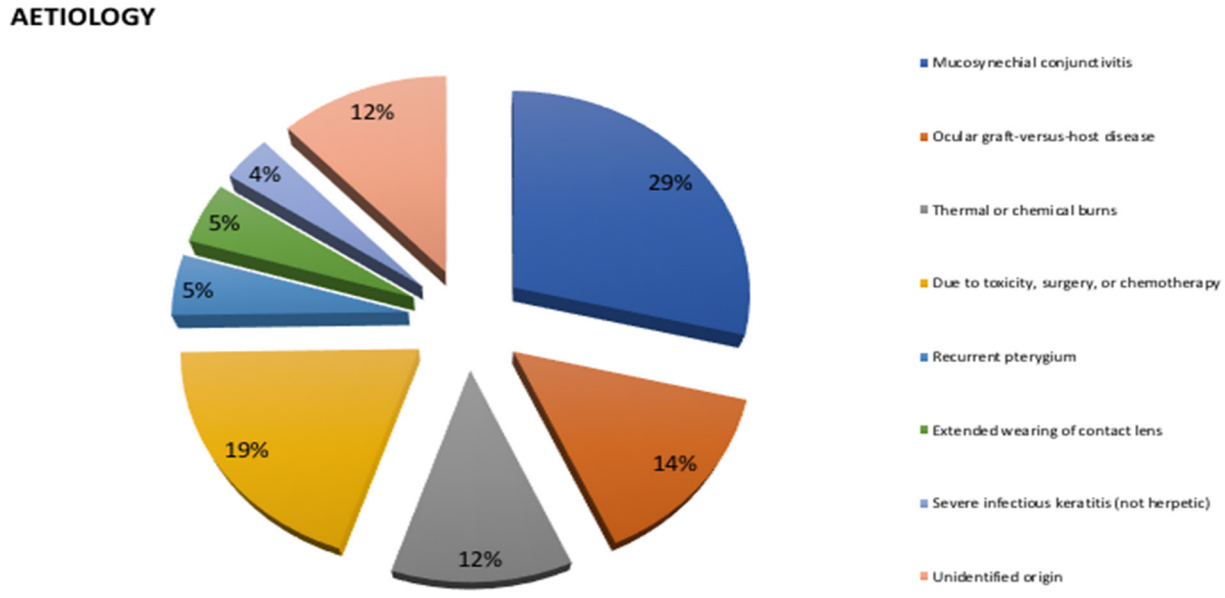

Figure 1 Aetiologies of limbal stem cell deficiency in the study cohort.

Table 3 Variables showing statistically significant differences among groups divided according to conjunctivalisation progression

	Conjunctivalisation yes/no	<3 clock-hours/ ≥3 clock-hours conjunctivalisation	<180°/ ≥180°
Variables	**P values**	**P values**	**P values**
VA ETDRS*	0.001	<0.001	0.001
Schirmer I*	0.04	0.05	0.04
TBUT*	<0.001	<0.001	<0.001
Oxford Scale*	<0.001	<0.001	<0.001
Van Bijsterveld Scale*	<0.001	<0.001	<0.001
Late persistent staining*	<0.001	<0.001	<0.001
Dull corneal reflex*	<0.001	<0.001	0.02
Central corneal involvement*	<0.001	<0.001	<0.001
Corneal epithelial defect*	0.001	0.04	0.002
Visualisation of crypts in confocal microscopy	0.005	0.016	NSSD
Symblepharon	0.01	NSSD	NSSD
Epiphora (scale)	<0.001	NSSD	NSSD
Photophobia (scale)	0.01	NSSD	NSSD
Corneal ulcers in the last 6 months	0.005	NSSD	0.004
Age	0.04	NSSD	0.04
Corneal subbasal nerve plexus density	NSSD	NSSD	0.03
Central area wings cell density	NSSD	NSSD	0.006

The variables not listed in this table demonstrated no statistically significant differences. The variables that, regardless of the criterion for dividing the groups according to progression of conjunctivalisation, always had statistically significant differences ($p < 0.05$) in the various groups are marked with '*'.
NSSD, no statistically significant differences ($p \geq 0.05$); TBUT, tear break-up time; VA, visual acuity.

non-automated method of including variables. We calculated the individual ORs and respective 95% CIs for the variables included in the model. The reliability of the model was determined using the Hosmer-Lemeshow index.

For the SND, the area under the receiver operating characteristic (ROC) curve, its 95% CI and the optimal cut-off value for distinguishing cases from controls were determined.

We used IBM SPSS V.22.0 statistical suite for Windows for all data analysis.

RESULTS

In total, we studied 76 eyes of 38 participants 32 of these eyes acted as controls (table 2).

The aetiology of the condition in the patients was determined as summarised in **figure 1.**

In the concordance analysis of the staging scales, we compared Deng's confocal classification with the Deng,[1] López-García[3] and Rama[4] clinical classifications. The respective kappa indices were 0.403 (moderate agreement), 0.449 (moderate agreement) and 0.215 (poor agreement).

The relationship between the presence of conjunctivalisation and the remaining variables is detailed in table 3. Variables not listed in table 3 demonstrated no statistically significant differences.

Table 2 summarises the general description of the main variables in the cases and controls.

As control values, we used the SND data obtained from our controls, which were similar to the well-established normal values referenced in the literature. However, the SND of cases (online supplementary e-figure 1) measured with IVCM was significantly lower 6469 (6295) $\mu m/mm^2$ ($p<0.001$) than in our controls 20 911 (4142) $\mu m/mm^2$ and than those referenced in the literature: Parissi *et al*[5]: 19 000 (4500) $\mu m/mm^2$ ($p<0.001$), Niederer *et al*[6]: 20 000 (6000) $\mu m/mm^2$ ($p<0.001$) and Lagali *et al*[7]: 18 300 (3700) $\mu m/mm^2$ ($p<0.001$) (online supplementary e-table 2).

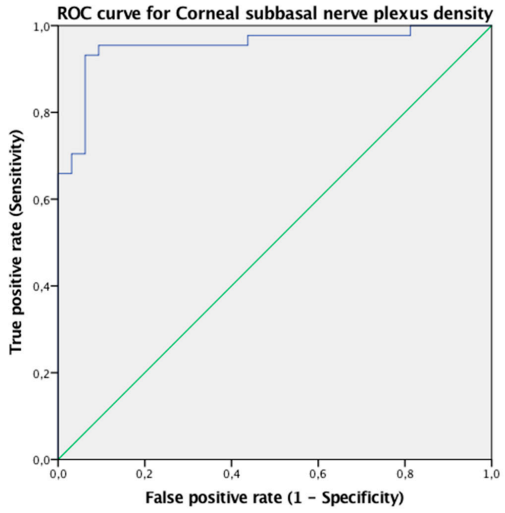

Figure 2 ROC for corneal subbasal nerve plexus density. ROC, receiver operating characteristic.

In the multivariate analysis, and through the construction of a binary logistic regression model, we found that SND acted as an independent protective factor for corneal conjunctivalisation in LSCD (OR, 0.069; 95% CI 0.008 to 0.619, p=0.01). Well-fitting models showed non-significance on the Hosmer-Lemeshow χ^2 test (p=0.623), indicating that modelled and observed predictions were not significantly different. The central basal cell density achieved an exposure value of 0.408 (0.078–2.131) and could therefore not be considered to be associated with conjunctivalisation.

ROC curve analysis of the SND in the control subjects and in patients with LSCD revealed a cut-off value of 17 215 µm/mm^2, and the resulting sensitivity and specificity were 95.5% and 90.6%, respectively (online supplementary e-figure 2). The area under the curve was 95.4%, with CI of 90.6%−100% (figure 2).

DISCUSSION

LSCD is associated with numerous conditions. To ensure a sample with sufficient statistical power for this study, we included patients with LSCD of various aetiologies. A considerable number of diseases affect limbus of the eye. Given that our centre is a reference hospital for haematopoietic stem cell transplantation in the community, 15% of our patients had ocular graft-versus-host disease.

In our analysis of the correlation of the diagnostic scales, the best correlation indices (although only moderate) were those of the Deng confocal classification[1] with the López-García classification of signs and symptoms,[3] as well as with the Deng clinical classification.[1] However, the Deng confocal classification correlated poorly with the Rama classification.[4]

SND has been widely described as the most reproducible and reliable measure of the state of neuropathic damage in other diseases, such as diabetic polyneuropathy and small fibre neuropathy.[8 9] Thus, we chose to use SND as the measure of reference. The basal cells of the corneal epithelium and the corneal sub-basal nerves stimulate the growth, proliferation, regeneration, differentiation and possibly cell migration through trophic factors, such as neuropeptides and neurotransmitters.[10] Moreover, Niederer *et al*[6] have reported that, although SND decreases with age, along with corneal sensitivity, central basal cell density does not.

The reduction in the SND with age could explain the delay in corneal injury healing.[11] Rosenberg *et al* demonstrated the relationship between the reduction in the SND and the increase in recurrent erosions, and the delay in healing of the corneal epithelium in patients with diabetes.[12] Moreover, the relationship between corneal neovascularisation and innervation has been widely studied, although it remains unclear which of these occurs first. According to Ferrari *et al*, neovessels and the nerve plexus in the cornea inhibit each other. Animal models have shown that an induction of corneal neovascularisation caused the disappearance of nerves in the area, while denervation led to neovascularisation within 7 days.[13] Experimental models have demonstrated a delay in corneal healing and the onset of persistent epithelial defects after corneal denervation.[14 15]

Based on these findings, we studied the state of the corneal nerve plexus and corneal cell densities in patients with LSCD. To assess the nerve plexus, we determined nerve density according to Misra *et al*,[8] Tavakoli *et al*[9] and Hertz *et al*.[16] We used the HRT-II confocal microscope with the Rostock cornea module, given its laser scanning ability, to assess nerve density. These microscopes are reported to yield the most reproducible, reliable and comprehensive results, particularly in terms of nerve counts, as they provide better contrast and do not lose image quality in the peripheral regions, as do other confocal microscopes.[17] We paid particular attention to the central cornea because, as other authors have stated, the peripheral basal cells are typically located in the deep anterior area of the palisades of Vogt, which is frequently damaged in these patients and is difficult to locate by IVCM.[1] The SND values obtained from our controls (20911±4142 µm/mm^2) were similar to those reported in the literature. The SND in our LSCD cohort was markedly lower (6469±6295 µm/mm^2) than that reported by Parissi *et al*[5] in healthy patients (19000±4500 µm/mm^2; p<0.001). Our values were also lower than those reported by Niederer[6] (20000±6000.50 µm/mm^2; p<0.001) and Lagali (18300±3700 µm/mm^2; p<0.001).[7] These results are consistent with the concept that SND reduction can be used as a diagnostic and severity criterion in LSCD, and is related to corneal neovascularisation.[1 13]

Using IVCM, Deng *et al* studied corneal changes that occur in LSCD (27 eyes of 20 patients) and concluded that some of these changes are associated with the early stages of LSCD. Conjunctivalisation was referred to as a late phenomenon in LSCD, given that the corneal epithelium cells did not show the conjunctival phenotype in the early disease stages.[1] Those authors proposed that the central basal cell density and SND are useful parameters in the early stages of the disease and established cut-off values for the diagnosis of LSCD at 7930 central basal cells/mm^2 and 53 sub-basal plexus nerves/mm^2, with high sensitivities and specificities. For basal cells, this cut-off showed a sensitivity of 95.5% and a specificity of 100%. For SND, the cut-off showed a sensitivity of 87% and a specificity of 91.7%, indicating that a reduction of 15.4% and 47.7%, respectively, could be associated with the early stages of LSCD. Those authors also studied and compared the central basal cell density and SND in patients with LSCD in the early stages with those of healthy controls (12 eyes of 10 patients) and found a reduction of 38% and 58%, respectively, in these parameters in patients.[1] They did not assign greater weight to either of these parameters in diagnosing LSCD.

We therefore set out to establish which parameter was more strongly related to corneal conjunctivalisation and which might be a confounding factor in LSCD. To this end, we performed a multivariate analysis and established a logistic regression model. SND was an independent protective factor for conjunctivalisation

(OR, 0.069; 95% CI 0.008 to 0.619, p=0.01), while basal cell density achieved an exposure value of 0.408, with an interval that included 1 (0.078–2.131), and was therefore not associated with conjunctivalisation.

Similarly, other IVCM variables in our study, such as the density of superficial, wing, central basal, and peripheral basal cells, the presence of limbal crypts and the presence of the palisades of Vogt did not show a significant effect. Deng *et al* confirmed the importance of corneal innervation in maintaining the corneal epithelium through the stimulation of 'proliferation, regeneration, differentiation, and possibly migration'. This is likely to be mediated by neuropeptides, neurotransmitters, glial cell-derived neurotrophic factors and neural growth factors.[1] Neither the study by Deng *et al* nor our own clarified whether neovascularisation or neural deprivation caused the other phenomenon, although both studies agreed that the reduction in the SND precedes neovascularisation. Although it is unclear whether the degeneration and reduction of the corneal sub-basal nerve plexus leads to depletion of the basal epithelial cells, or the converse, our study suggested that the reduction in the SND is a key factor and might be more important than the reduction in the central basal cell density of the cornea (online supplementary e-figure 2). This probably involves factors, secreted by the corneal nerve, which regulate the maintenance of a healthy corneal epithelium. Thus, measurement of the SND through IVCM, non-invasively provides objective data that can assist in the early diagnosis and classification of patients. The ROC curve, constructed with a very high accuracy level (area under the curve of 95.4% with a 95% CI of 90.6% to 100%), indicated an SND cut-off value that could distinguish between cases and controls with good sensitivity and specificity (95.5% and 90.6%, respectively). However, studies with larger sample sizes are needed to help explain the limits between intermediate and advanced stages of the condition and to clarify the directionality of the relationship between the SND, epithelial basal cells and conjunctivalisation.

Our findings show that the corneal nerve density of the corneal subbasal nerve plexus is inversely related to conjunctivalisation in LSCD. However, with a small and diverse sample size, the results should be interpreted cautiously, as the findings might not be transferable to all patients with LSCD, since this pathology has a varied aetiopathogenesis and is associated with different diseases. Further studies with larger, more homogeneous sample sizes will be necessary to assess the relationship and direction of causality between corneal SND and conjunctivalisation.

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