

# Relation between Corneal Innervation with Confocal Microscopy and Corneal Sensitivity with Noncontact Esthesiometry in Patients with Dry Eye

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**PURPOSE.** An alteration in corneal innervation has been described in dry eye associated with diabetes mellitus, contact lens use, and LASIK. The purpose of this study was to evaluate whether dry eye not related to Sjögren's syndrome (NSDE) and dry eye related to primary Sjögren's syndrome (PSDE) are associated with an alteration of the corneal nerves and sensation.

**METHODS.** Twenty-one patients with dry eye (10 NSDE and 11 PSDE) and 20 healthy volunteers were studied. Healthy volunteers were divided into two groups: one younger than 60 years ( $N < 60$ ) and the other 60 years of age or older ( $N \geq 60$ ). The study of the epithelium, stroma, and subbasal corneal nerves was performed with a confocal microscope. Mechanical, chemical, and thermal sensation was evaluated using the Belmonte noncontact esthesiometer.

**RESULTS.** A statistically significant decrease in the number and density of subbasal nerves ( $P < 0.0001$ ) and the density of superficial epithelial cells ( $P < 0.0001$ ) was observed in dry eyes. The number and density of subbasal nerves was higher in the  $N < 60$  group. A significant decrease was found with respect to mechanical, chemical, and thermal sensitivity ( $P < 0.0001$ ). Sensibility was better in the healthy eyes. A strong correlation was found between the density of superficial epithelial cells and the nerves and between the number and density of subbasal nerves and sensation ( $P < 0.001$ ).

**CONCLUSIONS.** The use of confocal microscopy and noncontact esthesiometry allow the detection of the presence of corneal neuropathy in patients with dry eye. (*Invest Ophthalmol Vis Sci.* 2007;48:173–181) DOI:10.1167/iovs.06-0127

Dry eye is the most frequent cause for which patients seek ophthalmic consultation. The prevalence of dry eye is approximately 33% of the adult population older than 50 years.<sup>1</sup>

Dry eye has several diverse causes. It has been traditionally classified into hyposecretory and evaporative, although recent classifications approach more complete etiological and physiopathological aspects.<sup>2,3</sup> Though not as common as dry eye

related with age, dry eye associated with Sjögren's syndrome is considered to be the prototype because of its severity. Recently, dry eye associated with diabetes mellitus,<sup>4</sup> use of contact lenses,<sup>5</sup> and LASIK<sup>6,7</sup> have been associated with corneal innervation disturbance.

The cornea is the tissue most densely innervated in the body and receives sensory and autonomic (sympathetic and parasympathetic) nerve fibers. It has a nerve density between 20 and 40 times as much as that of the dental pulp and between 300 and 600 times as much as that of the skin.<sup>8</sup> The nerve bundles penetrate the corneal periphery in a radial manner, parallel to the superficial corneal surface at the level of the anterior stroma, losing their myelin sheath approximately 1 mm from the limbus. These bundles subdivide into small ones and turn 90° (perpendicular to the corneal surface), perforating Bowman's layer. They then turn another 90° and become situated parallel to the superficial corneal surface, between Bowman's layer and the basal layer of the corneal epithelium, where they divide again. From there, the individual nerve fibers emerge toward the most superficial layers of the corneal epithelium.<sup>9</sup> The initial studies of the arrangement of corneal nerves were based on light and electron microscopy. The main problem with these studies is that corneal nerves degenerate after 13 hours after death.<sup>8</sup> The availability of an instrument, the confocal microscope, to obtain images of the human cornea in vivo at a microstructural level, is therefore very useful for studying normal and diseased corneas.

Until recently, however, corneal sensitivity has been studied using the Cochet-Bonnet esthesiometer. This instrument has a limited use and only stimulates the mechanosensitive nerve fibers. However, it is now known that the cornea possesses mechanoreceptors, chemical receptors, and receptors for cold. In the last few years, a gas or noncontact esthesiometer has been developed that permits the application of controlled mechanical pulses, irritant chemical stimuli, and pulses of cold and hot air to determined areas of the ocular surface. In this way, we can measure in a more refined way the psychophysical characteristics of the sensation provoked by each type of stimulus.<sup>10</sup>

The objective of this study was to know whether dry eye associated with Sjögren's syndrome and dry eye not associated with Sjögren's syndrome are also related to an alteration of corneal innervation and sensitivity. In a previous study in which we used the Cochet-Bonnet esthesiometer, we did not find a correlation between corneal sensitivity and innervation.<sup>11</sup> Because of the rudimentary nature of the Cochet-Bonnet esthesiometer, we used a noncontact esthesiometer in the present study.

## MATERIALS AND METHODS

### Population and Clinical Study

Twenty healthy volunteers were studied. These were divided into two groups: one younger than 60 years ( $N < 60$  group; 10 persons; 9

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women and 1 man) whose average age was  $30.4 \pm 6.2$  years (range, 21–41) and another group 60 years or age or older ( $N \geq 60$ ; 10 persons: 8 women and 2 men) whose average age was  $65.4 \pm 3.2$  years (range, 61–70). The normal subjects were recruited from the companions of patients who attended the General Consultations in the Ophthalmology Department at San Carlos Clinical Hospital. The exclusion criteria included the use of contact lenses, the presence of ocular or systemic disease, and drug allergy.

Twenty-one dry eyes were studied. These were divided into two groups: 10 patients (8 women and 2 men) with dry eye not associated with Sjögren's syndrome (NSDE) whose average age was  $58.3 \pm 12.8$  years (range, 33–73) and 11 patients (10 women and 1 man) with dry eye associated with primary Sjögren's syndrome (PSDE) whose average age was  $61.3 \pm 11.3$  years (range, 42–77). The first group was recruited from the Unit of Ocular Surface and Inflammation in the Ophthalmology Department at San Carlos Clinical Hospital and the second group from the Rheumatology Department at San Carlos Clinical Hospital. The diagnosis of PSDE was made according to the diagnostic criteria defined by the American-European consensus group criteria (including a focus score  $\geq 1$  on labial salivary gland, or the presence of anti-SSA or anti-SSB antibodies).<sup>12</sup> None of the patients with PSDE has a diagnosis of sensory or motor neuropathy. The patients with NSDE had a Schirmer's test result with anesthesia  $< 10$  mm and symptoms of dry eye (foreign body sensation and/or dryness of the eye). The exclusion criteria were the use of contact lenses, the presence of systemic or ocular disease except dry eye, and drug allergy. All the patients with dry eye used preservative-free artificial tears. None of them or the normal subjects used topical or systemic NSAIDs (nonsteroidal anti-inflammatory drugs) at the time of examination (suspended at least 2 weeks before examination).

For the statistical analysis, the eye with the lower Schirmer's test result with anesthesia was chosen in all cases. In cases with equal Schirmer's results in both eyes, the selection criteria were, in this order, more staining with rose bengal and higher score on the symptom questionnaire.

The study was approved by the Ethics Committee of Clinical Investigations at San Carlos Clinical Hospital and was performed according to the guidelines of the Declaration of Helsinki. An informed consent of the patients and normal subjects was obtained. A detailed biomicroscopic examination of the anterior segment was performed. The ocular symptoms were determined through a questionnaire published by us.<sup>13</sup> The blink rate per minute was noted (the following definition was used for blink: a bilateral paroxysmal closure of the eyelids (duration  $< 1$  second) in the absence of a provoking external stimulus. The number of blinks was recorded for 3 minutes while the subjects were before the slit lamp. Blink rates were summarized per 1-minute period. Staining of the ocular surface was performed using rose bengal, and the staining was classified according to the method of van Bijsterveld.<sup>14</sup> Tear production was determined by Schirmer's test with anesthesia and tear clearance by our colorimetric technique. For this, 1 drop of fluorescein eye drops 0.5% and oxybuprocaine 0.4% was instilled, and after a 5-minute wait, a strip of Schirmer's paper was placed and left for 5 minutes. On reading the strip, we obtain the result of the test from the length of the wetness on the strip and the tear clearance by comparing the color of the wet part with the colors shown on a chart.<sup>15</sup>

## Confocal Microscopy

Confocal microscopy was performed in the center of the cornea. For this examination, the patient sat with the chin and forehead supported to stabilize the head. The contralateral eye fixed a flickering light source to stabilize the patient's gaze. Before the examination, a drop of anesthetic eye drops was instilled (Anestésico doble colirio; Alcon Cusí, El Masnou, Barcelona, Spain) in the inferior conjunctival fornix. During the examination, a drop of gel (Healon; Pharmacia Upjohn, Sant Cugat del Valles, Barcelona, Spain) was deposited on the objective lens, thus avoiding direct contact with the cornea. A scanning confocal microscope with slit was used (Confoscan model P4; Tomey AG, Erlangen-Tennenlohe, Germany). The technical characteristics of the instrument have been published.<sup>11,16</sup> The objective of the microscope is an immersion lens. Direct contact does not exist between the lens and the cornea, because of the immersion of the lens in a drop of gel. At the end of the examination, the cornea is examined by slit lamp, to verify its integrity. The  $x$ - $y$  position of the image and the depth of the section are controlled through the manual movement of the microscope while the position of the objective is observed in relation to the cornea and the images in real time which appear in the video monitor. During each examination, the microscope is focused various times from the tear film to the anterior chamber and vice versa. The total duration of the examination is from 2 to 2.5 minutes. The thickness of the slit remains constant during all the examinations. The lateral optical resolution of the system is 1 to 2  $\mu\text{m}$ , the amplitude of the field ( $x$ ,  $y$ ) is  $315 \times 236 \mu\text{m}$  ( $74,340 \mu\text{m}^2$ ), and the depth ( $z$ ) of the resolution of the optical section is 10  $\mu\text{m}$ .<sup>16</sup> The images in real time are recorded by videocassette recorder (model SVO-9620 PAL system,  $1768 \times 576$  pixels); Sony Corp., Tokyo, Japan) on super-VHS tapes.

The images were evaluated in a masked manner, in which the investigator (JBC) did not know to which group the images belonged. The corneal layers, which we sought to examine, were the epithelium (superficial layer and basal layer), Bowman's layer, or subbasal layer and the stroma (anterior and posterior).<sup>11</sup>

**Study of the Corneal Nerves.** The following parameters were analyzed:

Number of nerves: defined as the sum of the nerve branches present in one image.

Density: defined as the total length of the nerve fibers existent in one image, expressed in micrometers of nerve fiber within an area of  $74,340 \mu\text{m}^2$ .

Number of beadings: defined as the number of beadings existent in 100  $\mu\text{m}$  of nerve fiber.

Presence of branching pattern in one image: evaluated as positive if at least one branching pattern was present within an image or negative if not.

Grade of nerve tortuosity: classified in four grades according to a scale.<sup>11</sup>

**Study of the Corneal Epithelium and Stroma.** The cellular density was evaluated at the level of the superficial epithelium, the basal epithelial layer, the anterior stroma and the posterior stroma, through the counting of the cells existent in one image ( $74,340 \mu\text{m}^2$ ), the results are expressed in cells per square millimeter.

TABLE 1. Demographic Data

|                          | N < 60                    | N ≥ 60                    | NSDE                       | PSDE                       |
|--------------------------|---------------------------|---------------------------|----------------------------|----------------------------|
| Number of patients       | 10                        | 10                        | 10                         | 11                         |
| Gender (female:male)     | 9:1                       | 8:2                       | 8:2                        | 10:1                       |
| Age (y)                  | $30.4 \pm 5.9$<br>(21–41) | $65.4 \pm 3.2$<br>(61–70) | $58.3 \pm 12.9$<br>(33–73) | $61.3 \pm 11.3$<br>(42–77) |
| Time after diagnosis (y) |                           |                           | $5.3 \pm 2.8$<br>(1–10)    | $8.6 \pm 3.2$<br>(3–13)    |

Data are the mean  $\pm$  SD (range).

TABLE 2. Clinical Data

|                            | N<60               | N≥60               | NSDE                | PSDE                | P       |
|----------------------------|--------------------|--------------------|---------------------|---------------------|---------|
| Schirmer's with anesthesia | 18.5 ± 5.9 (12-31) | 13.6 ± 3.1 (11-21) | 5.9 ± 1.1 (4-7)     | 4.6 ± 2.0 (2-8)     | <0.001* |
| Questionnaire score        | 1.0 ± 2.2 (0-7)    | 3.2 ± 3.5 (0-10)   | 22.1 ± 10.5 (9-43)  | 28.6 ± 14.4 (11-62) | <0.001‡ |
| Blink rate                 | 16.5 ± 2.9 (12-21) | 15.5 ± 2.1 (12-18) | 15.6 ± 2.5 (13-21)  | 16.7 ± 3.8 (12-23)  | = 0.672 |
| RB staining                | 0                  | 0                  | 1.7 ± 1.0 (0.5-3.1) | 2.5 ± 0.9 (2.0-3.8) | <0.001  |

Data in parentheses are 95% confidence intervals. *P* is by ANOVA. RB, rose bengal.

\* PSDE vs. NSDE, PSDE vs. N≥60, PSDE vs. N<60, NSDE vs. N<60, NSDE vs. N≥60; *P* < 0.001, Bonferroni.

† N<60 vs. N≥60; *P* < 0.020, Bonferroni.

‡ PSDE vs. NSDE, PSDE vs. N≥60, PSDE vs. N<60, NSDE vs. N<60, NSDE vs. N≥60 and N<60 vs. N≥60; *P* < 0.001, Bonferroni.

Finally, a qualitative description was made of the abnormal findings observed in the images

## Esthesiometry

The corneal sensitivity was studied using a noncontact esthesiometer.<sup>10</sup> This instrument allows the application of pulses of air with 3 seconds' duration to the center of the cornea. The force, composition, and temperature of the air can be controlled. The mechanical stimulation consisted of a series of pulses of air with a variable flow (0-200 mL/min). The air was heated up at the tip of the esthesiometer to 50°C, so that it reached the ocular surface at 34°C and so prevented changes in the corneal temperature that can be caused by the flow of air. Chemical stimulation was performed with pulses of air, at a subthreshold flow and a neutral temperature, containing variable concentrations of CO<sub>2</sub> (0%-50%). Thermal cold stimulation was performed with pulses of air previously cooled. To prevent mechanical stimulation, the stimulus was applied with a flow 10 mL/min less than the mechanical threshold. The tip of the esthesiometer was situated 5 mm away from the corneal apex (transparent ruler). A noise (a click produced by opening the gas valve) indicated the start of the pulse. Immediately after each stimulation pulse, the subject was asked to report the presence or absence of sensation. Mechanical, thermal, and chemical thresholds were determined using the method of levels.<sup>10</sup> All the tests were performed between 10 AM and 2 PM. The temperature and the humidity were maintained constant (20°C/35%). The esthesiometry study was performed in an open manner; nevertheless, the analysis of the esthesiometric, morphologic, and clinical results was masked.

## Statistical Analysis

The results were collected in a calculation sheet (Excel 2000; Microsoft Corp., Redmond, WA) and statistical analysis performed (SPSS for Windows ver. 9.0; SPSS Sciences, Chicago, IL). The sample size was calculated to detect a significant difference in the average of the number of nerves of 1.80 (comparing the averages) with a corrected probability for multiple contrasts ( $\alpha = 0.01$ ) and a  $\beta$  error of 80%. The norm was determined with the Kolmogorov-Smirnov test. The quanti-

tative variables are expressed through the average, the standard deviation, and its confidence interval; and the qualitative variables through their frequency. The analysis of variance of one factor was performed (ANOVA) for the quantitative variables using the Bonferroni post hoc test. To study the association between quantitative variables, the Spearman correlation test was used. For the analysis of the qualitative variables, the Pearson  $\chi^2$  test was applied. *P* < 0.05 was considered statistically significant. A logistic regression analysis was performed with the purpose of finding the model that would best classify dry eyes. The reproducibility of the measurements was analyzed repeating the examination (confocal microscopy and esthesiometry) in three normal subjects 2 days after the first measurement. The number of nerves and beadlike formations and the three sensitivities were re-evaluated. The interobserver variation was calculated, comparing the results obtained by a second investigator using the same criteria. The reproducibility and the interobserver variation were analyzed by studying the variation coefficients of the different groups of data.

## RESULTS

### Clinical Data

The demographic data are summarized in Table 1. Logically, there was a significant difference in the ages (*P* < 0.001, ANOVA) between the group N<60 and the other groups (*P* < 0.001, Bonferroni) but not between each of the other groups. The duration of symptoms in the NSDE was  $5.3 \pm 2.8$  years (range, 1-10) and in the PSDE was  $8.6 \pm 3.2$  years (range, 3-13). The clinical data are presented in Table 2. The results of Schirmer's test with anesthesia were significantly different between the groups (*P* < 0.001, ANOVA). Comparing the different groups with one another, we observed a significant difference between normal eyes and dry eyes (*P* < 0.001, Bonferroni) as well as between the N<60 and the N≥60 (*P* < 0.020, Bonferroni). With respect to the responses to the questionnaire regarding the ocular surface, the difference was significant (*P* < 0.0001, ANOVA) with a difference between the normal subjects and the patients with dry eye (*P* < 0.001, Bonferroni). We did not find a statistically significant difference between the different groups in the rate of blinking. In relation to the time after diagnosis, this was significantly higher in the group of PSDE (*P* = 0.021, Mann-Whitney). The corneas of the normal subjects did not stain with rose bengal, with a significant difference between the different groups (*P* < 0.0001,  $\chi^2$ ). All patients had a normal pupillary reaction. Table 3 shows the results of the clearance, with a significant difference between the different groups (*P* = 0.004,  $\chi^2$ ).

### Confocal Microscopy

A total of 343 images were analyzed, perpendicular to the z-axis (software program, Confo-Commander ver. 2.7.1; Tomey). The reproducibility of the number of subbasal nerves was 93%; of the number of beadlike formations, 91%; of the

TABLE 3. Tear Clearance

|       | N<60   | N≥60   | NSDE   | PSDE     |
|-------|--------|--------|--------|----------|
| 1/4   | 0 (0)  | 0 (0)  | 0 (0)  | 1 (9.1)  |
| 1/8   | 0 (0)  | 0 (0)  | 4 (40) | 5 (45.5) |
| 1/16  | 2 (20) | 4 (40) | 2 (20) | 2 (18.2) |
| 1/32  | 0 (0)  | 1 (10) | 2 (20) | 3 (27.3) |
| 1/64  | 4 (0)  | 5 (50) | 2 (20) | 0 (0)    |
| 1/128 | 4 (0)  | 0 (0)  | 0 (0)  | 0 (0)    |

Values are number (%). Tear clearance was determined through our colorimetric technique. For this, 1 drop of fluorescein eye drops 0.5% and oxybuprocaine 0.4% were instilled and after the Schirmer test was completed, tear clearance was determined by comparing the color of the wet part with a table of decreasing dilutions of fluorescein.<sup>15</sup> *P* = 0.004 ( $\chi^2$ ).

TABLE 4. Confocal Data

|                                      | N<60                      | N≥60                      | NSDE                      | PSDE                      | P                    |
|--------------------------------------|---------------------------|---------------------------|---------------------------|---------------------------|----------------------|
| Number subbasal nerves               | 4.6 ± 0.8 (3–6)           | 3.2 ± 0.9 (2–5)           | 3.0 ± 0.6 (2–4)           | 2.7 ± 0.8 (2–4)           | <0.001*<br>= 0.003†  |
| Density subbasal nerves              | 787 ± 105<br>(598–902)    | 620 ± 92<br>(455–744)     | 591 ± 90<br>(487–725)     | 511 ± 106<br>(365–706)    | = 0.004‡<br><0.001§  |
| Number of beadings                   | 192 ± 61<br>(92–274)      | 197 ± 50<br>(95–289)      | 307 ± 73<br>(198–425)     | 364 ± 64<br>(273–437)     | <0.001  <br><0.002¶  |
| Density superficial epithelial cells | 1431 ± 283<br>(1003–1856) | 1339 ± 302<br>(865–1765)  | 944 ± 212<br>(655–1342)   | 971 ± 262<br>(637–1433)   | <0.002#<br><0.020**  |
| Density basal epithelial cells       | 5858 ± 702<br>(4782–7030) | 5692 ± 663<br>(4177–6452) | 5691 ± 478<br>(4992–6522) | 5744 ± 627<br>(4822–6578) | = 0.273              |
| Density anterior stromal cells       | 1062 ± 183<br>(856–1404)  | 1107 ± 194<br>(895–1456)  | 1178 ± 166<br>(938–1405)  | 1322 ± 156<br>(927–1487)  | <0.010††<br><0.050‡‡ |
| Density posterior stromal cells      | 722 ± 99<br>(564–856)     | 758 ± 116<br>(538–856)    | 724 ± 119<br>(582–938)    | 815 ± 131<br>(563–977)    | = 0.921              |

Data in parentheses are 95% confidence interval. *P* is by ANOVA.

\* PSDE vs. N<60, NSDE vs. N<60; *P* < 0.001, Bonferroni.

† N<60 vs. N≥60; *P* = 0.003, Bonferroni.

‡ N<60 vs. N≥60; *P* = 0.004, Bonferroni.

§ PSDE vs. N<60, NSDE vs. N<60; *P* < 0.001, Bonferroni.

|| PSDE vs. N<60, NSDE vs. N<60; *P* < 0.001, Bonferroni.

¶ PSDE vs. N≥60, NSDE vs. N≥60; *P* < 0.002, Bonferroni.

# PSDE vs. N<60, NSDE vs. N<60; *P* < 0.001, Bonferroni.

\*\* PSDE vs. N≥60, NSDE vs. N≥60; *P* < 0.002, Bonferroni.

†† PSDE vs. N<60; *P* < 0.010, Bonferroni.

‡‡ PSDE vs. N≥60; *P* < 0.050, Bonferroni.

mechanical sensitivity, 90%; of the chemical sensitivity, 88%; and of the thermal sensitivity, 85%. The interobserver variation was 10% for the number of subbasal nerves, 14% for the number of beadlike formations, 8% for the mechanical sensitivity, 15% for the chemical sensitivity, and 13% for the thermal sensitivity.

Table 4 shows the results of the confocal data. In relation to the number and density of subbasal nerves, a significant difference was observed (*P* < 0.001, ANOVA). Comparing the number of subbasal nerves in the different groups between one another revealed a significant difference between the two control groups (*P* = 0.003, Bonferroni) and between the N<60 group and the two dry eye groups (*P* < 0.001, Bonferroni). As regards the density of the subbasal nerves, a statistically significant difference was observed between the N<60 group and the other groups (*P* = 0.004, N<60 compared with N≥60, *P* < 0.001; N<60 compared with PSDE and NSDE, Bonferroni). A

difference in the number of beadlike formations was found (*P* < 0.001, ANOVA), with a significant difference observed between the N<60 group and the dry eye groups (*P* < 0.001, Bonferroni) as well as between the N≥60 group and the two dry eye groups (*P* < 0.002, Bonferroni). A statistically significant difference was found as regards the tortuosity of the nerve fibers (*P* < 0.001,  $\chi^2$ ) but not as regards the presence or absence of branching.

With respect to the superficial epithelial cells we found a statistically significant difference (*P* < 0.001, ANOVA). We observed a statistically significant difference between the N<60 group and the two dry eye groups (*P* < 0.002, Bonferroni) and between the N≥60 and the two dry eye groups (*P* < 0.020, Bonferroni). There was a significant difference in the density of anterior keratocytes (*P* = 0.009, ANOVA), with significant differences between the N<60 group and the PSDE group (*P* < 0.010, Bonferroni) as well as between the N≥60

TABLE 5. Mechanical, Chemical, and Cold Sensitivity Thresholds

|                                        | N<60                          | N≥60                          | NSDE                          | PSDE                          | P                                          |
|----------------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|--------------------------------------------|
| Mechanical threshold (mL/min)          | 78 ± 12<br>(58–97)            | 106 ± 21<br>(70–138)          | 134 ± 24<br>(97–170)          | 147 ± 21<br>(110–189)         | <0.001*<br>= 0.024†<br>= 0.021‡<br><0.001§ |
| Chemical threshold (%CO <sub>2</sub> ) | 15.2 ± 2.2<br>(13–19)         | 18.8 ± 1.7<br>(15–21)         | 24.2 ± 5.0<br>(20–33)         | 25.2 ± 5.4<br>(20–33)         | <0.001  <br>= 0.030¶<br><0.005#            |
| Cold threshold (°C)                    | −0.24 ± 0.09<br>(−0.38/−0.14) | −0.36 ± 0.14<br>(−0.54/−0.14) | −0.98 ± 0.23<br>(−1.34/−0.46) | −1.05 ± 0.28<br>(−1.50/−0.62) | <0.001**                                   |

Data in parentheses are 95% confidence interval. *P* is by ANOVA.

\* PSDE vs. N<60, NSDE vs. N<60; *P* < 0.001, Bonferroni.

† N<60 vs. N≥60; *P* = 0.024, Bonferroni.

‡ N≥60 vs. NSDE; *P* = 0.021, Bonferroni.

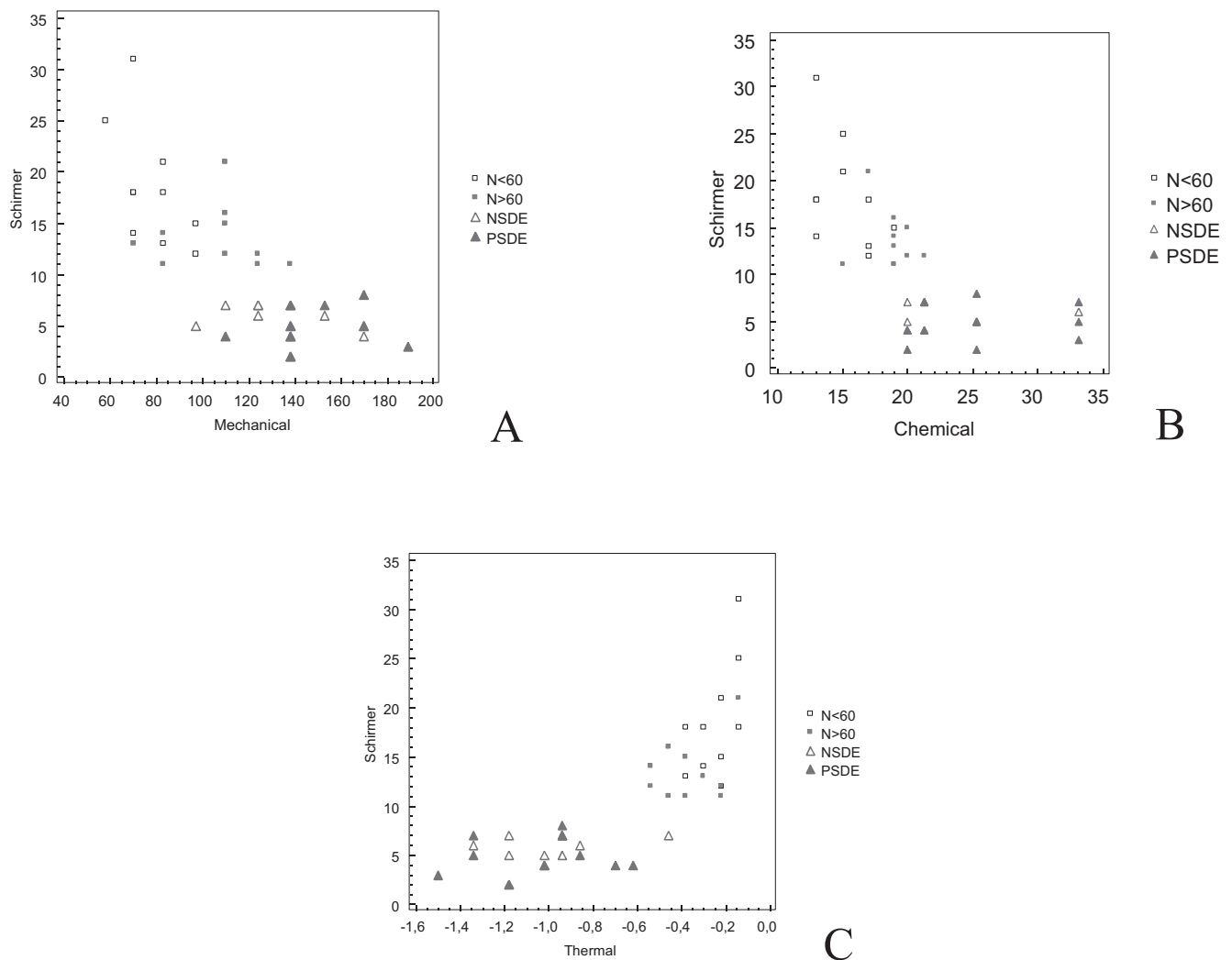
§ N≥60 vs. PSDE; *P* < 0.001, Bonferroni.

|| PSDE vs. N<60, NSDE vs. N<60; *P* < 0.001, Bonferroni.

¶ N≥60 vs. NSDE; *P* = 0.030, Bonferroni.

# N≥60 vs. PSDE; *P* < 0.005, Bonferroni.

\*\* N<60 vs. PSDE, N<60 vs. NSDE, N≥60 vs. PSDE, N≥60 vs. NSDE; *P* < 0.001, Bonferroni.



**FIGURE 1.** Correlation between Schirmer's test results and (A) mechanical thresholds ( $r = -0.747$ ;  $P < 0.001$ ), (B) chemical thresholds ( $r = -0.660$ ;  $P < 0.001$ ), and (C) thermal cold thresholds ( $r = 0.799$ ;  $P < 0.001$ ).

group and the PSDE group ( $P < 0.050$ , Bonferroni). We did not find differences in the densities of basal epithelial cells and posterior keratocytes.

No qualitative anomalies were found by confocal microscopy in neither of both groups of healthy eyes. However, we observed basal epithelial reflectivity in 36.5% of the PSDE group and in 23% of the NSDE group, nerve sprouts in 24.5% of the PSDE group and in 21% of the NSDE group, and activation of keratocytes in 35.4% of the PSDE group and in 13% of the NSDE group.

### Esthesiometry

A significant difference was found with respect to mechanical thresholds ( $P < 0.001$ , ANOVA). Comparing the different groups with one another showed the following results: N<60 compared with dry eyes ( $P < 0.001$ , Bonferroni), N<60 compared with N≥60 ( $P = 0.024$ , Bonferroni), N≥60 compared with NSDE ( $P = 0.021$ , Bonferroni), and N≥60 compared with PSDE ( $P < 0.001$ , Bonferroni). With respect to chemical thresholds, the results were also significant as follows: N<60 compared with dry eyes ( $P < 0.001$ , Bonferroni), N≥60 compared with NSDE ( $P = 0.031$ , Bonferroni), and N≥60 compared with PSDE ( $P < 0.005$ , Bonferroni). The difference was also statistically significant with respect to thermal cold thresh-

olds, when we compared healthy eyes with dry eyes ( $P < 0.001$ , Bonferroni; Table 5).

### Correlations

Within the statistically significant correlations among the studied variables, the following are of special interest. Because the density of subbasal nerves is related to its number and there is a very tight relation between those two parameters, only density of subbasal nerve correlations are shown:

Schirmer's test and mechanical, chemical, and thermal cold thresholds ( $r = -0.747$ ,  $r = -0.660$ ,  $r = 0.799$ ;  $P < 0.001$ ; Fig. 1).

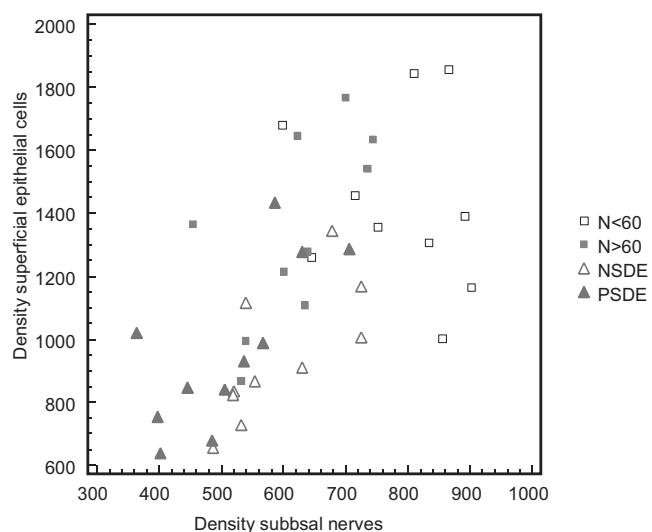
Density of subbasal nerves and superficial epithelial density ( $r = 0.624$ ;  $P < 0.001$ ; Fig. 2).

Density of subbasal nerves and the different thresholds ( $r = -0.791$ ,  $r = -0.798$ ,  $r = 0.631$ ;  $P < 0.001$ ; Fig. 3).

Questionnaire and the different thresholds ( $r = 0.555$ ,  $r = 0.498$ ,  $r = -0.678$ ;  $P < 0.001$ ; Fig. 4).

Rose bengal staining and thresholds ( $P < 0.001$ , ANOVA; Figs. 5).

In relation to the logistic regression, the parameters that best matched the dry eye model were density of superficial

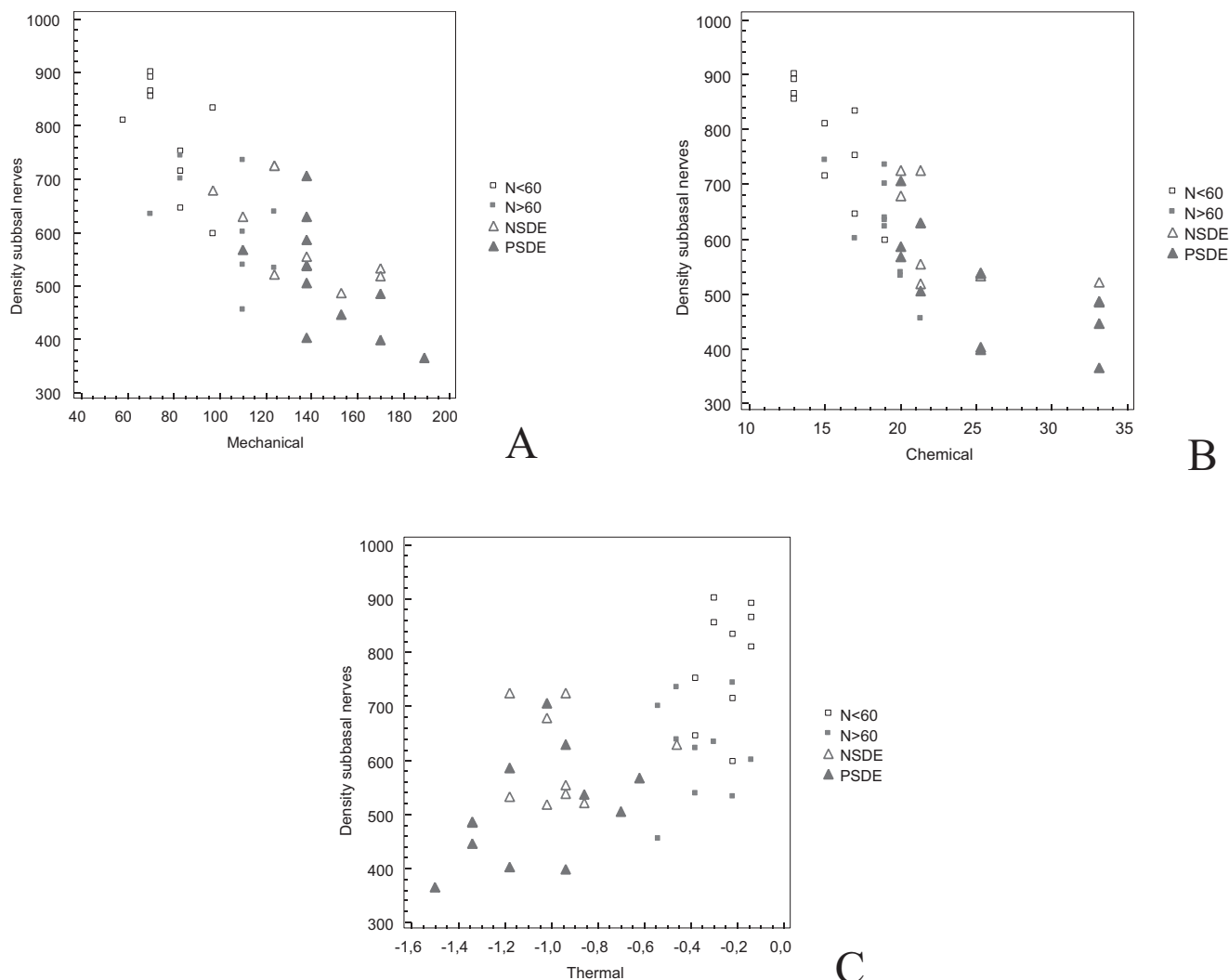


**FIGURE 2.** Correlation between density of subbasal nerves and density of superficial epithelial cells ( $r = 0.624$ ;  $P < 0.001$ ).

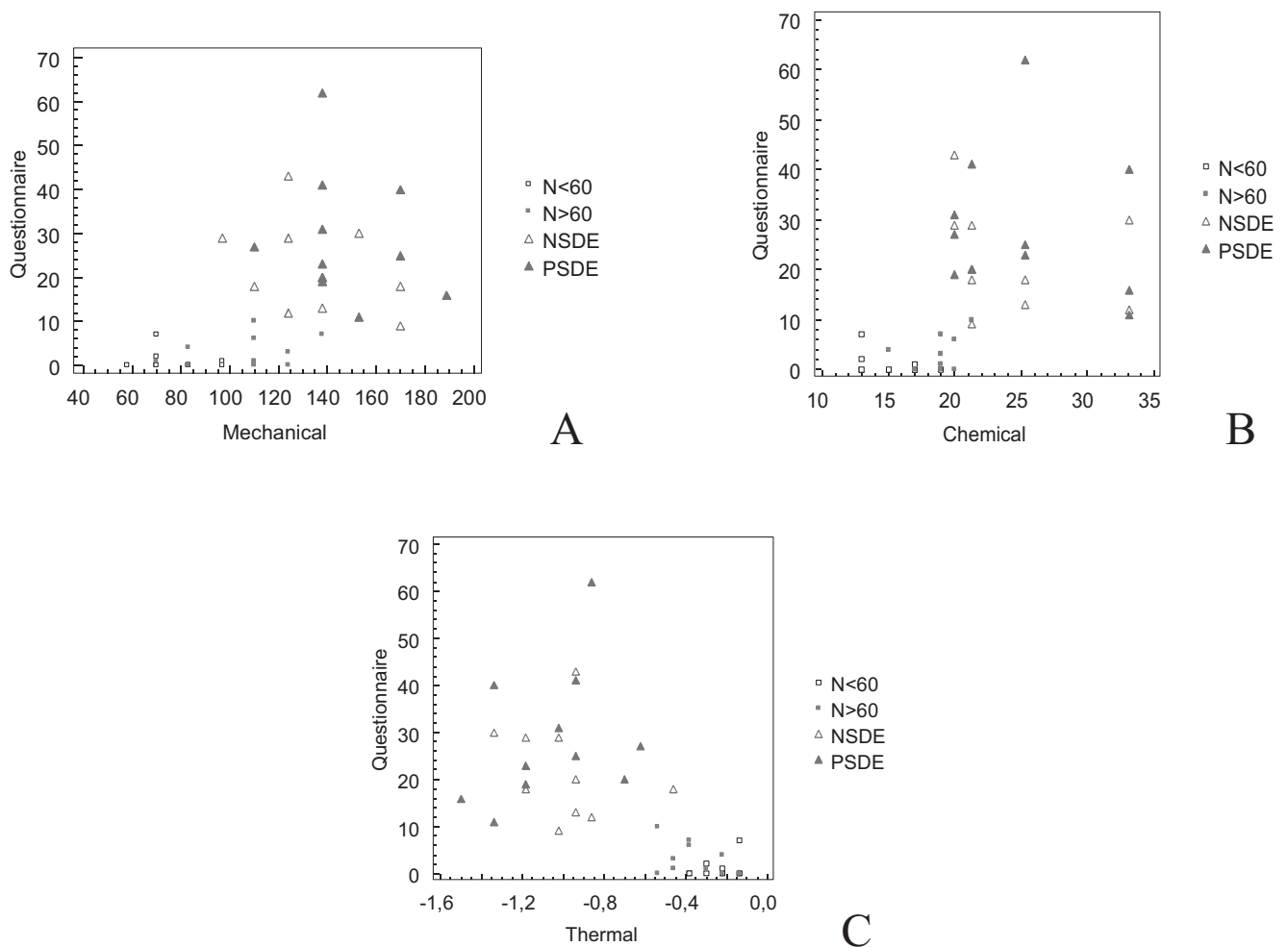
epithelial cells ( $P = 0.022$ ) and beadlike formations ( $P = 0.027$ ).

## DISCUSSION

The confocal microscope has recently been used for the microstructural clinical investigation of the human cornea. Most of the studies were qualitative and were directed toward the observation of the corneal structure after local or systemic diseases and after refractive surgery.<sup>17–19</sup> In the present study a quantitative analysis was performed. The use of the confocal microscope as an optical dissector has the advantage of being a noninvasive technique *in vivo* and makes dynamic study possible in real time. A potential limitation of this technique is that the measurements are made in the center of the cornea, and thus the results cannot be applied to the corneal periphery. It is also important in evaluating the results to note that, for the majority of the imaging tests, reproducibility and interobserver variation in our study were lower than 95% and higher than 5%, respectively. Nevertheless, comparing our results, as regards the density of the subepithelial nerves and the number of beadings in normal eyes with those of Grupcheva et al.<sup>20</sup> and Oliveira-Soto and Efron,<sup>21</sup> we found very similar results.



**FIGURE 3.** Correlation between density of subbasal nerves and (A) mechanical thresholds ( $r = -0.791$ ;  $P < 0.001$ ), (B) chemical thresholds ( $r = -0.798$ ;  $P < 0.001$ ), and (C) thermal cold thresholds ( $r = 0.631$ ;  $P < 0.001$ ).



**FIGURE 4.** Correlation between questionnaire score and (A) mechanical thresholds ( $r = 0.555$ ;  $P < 0.001$ ), (B) chemical thresholds ( $r = 0.498$ ;  $P < 0.001$ ), and (C) thermal cold thresholds ( $r = -0.678$ ;  $P < 0.001$ ).

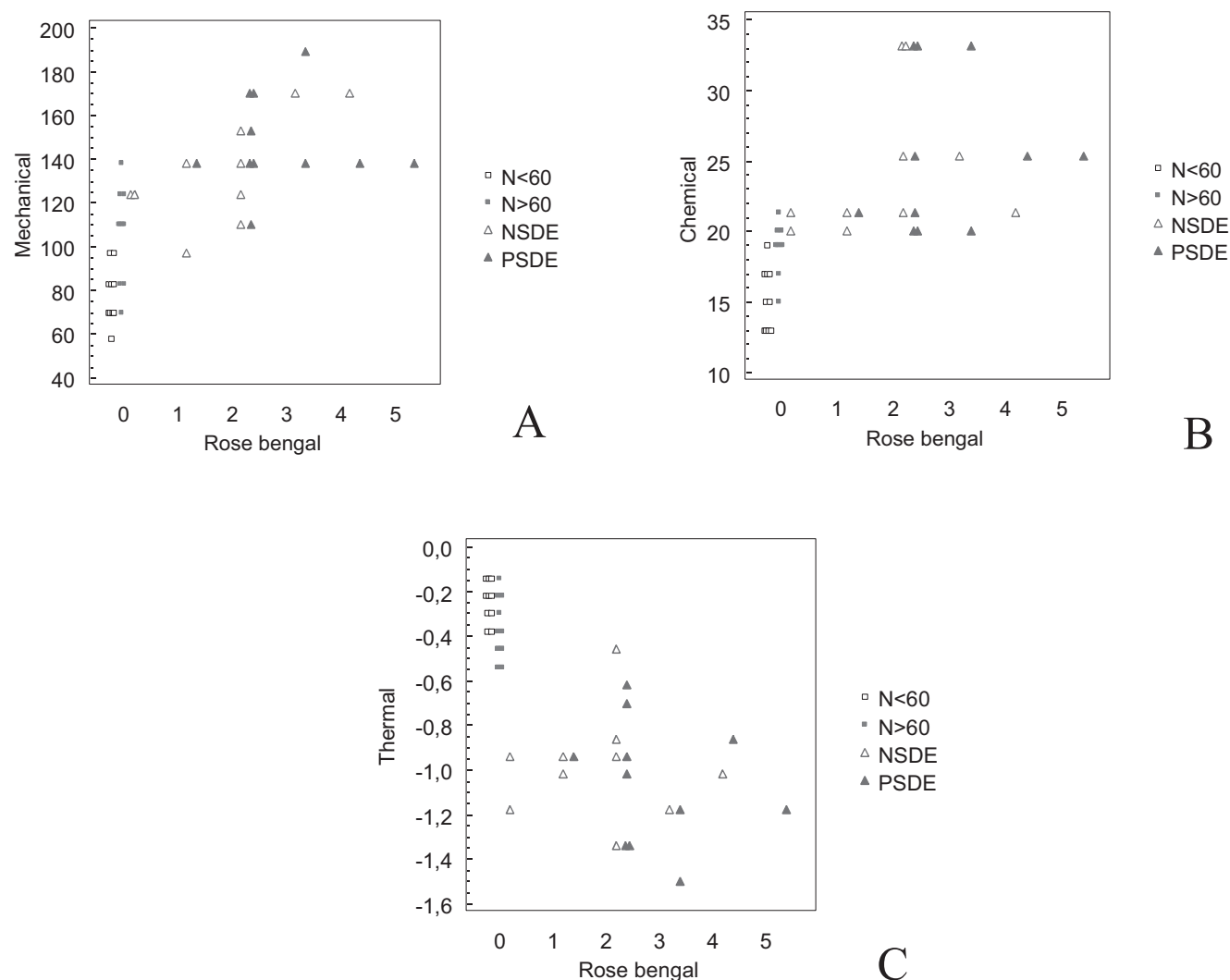
Moreover, our results coincide with those of a previous study of dry eye that we have published.<sup>11</sup>

The corneal nerves are derived fundamentally from the ophthalmic branch of the trigeminal nerve. The cornea is the peripheral human tissue most densely innervated. The nerve fibers end as free nerve endings between the epithelial cells. The epithelial cells as well as the keratocytes are innervated. The nerve fibers have an important influence in the corneal trophism and contribute to the maintenance of a healthy corneal surface. The alteration in corneal innervation produces the corneal disease neurotrophic keratitis. The most frequent causes of this disease are herpetic infection; injury to the trigeminal nerve associated with cranial, orbital, or retinal surgery; and laser therapy.

The beadings are characteristic of metabolically active transmitter-containing nerve fibers. Until now, 17 different neuropeptides and neurotransmitters have been described in the corneal nerves.<sup>9,22,23</sup> Peptidergic nerves containing neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (SP) have been demonstrated in the human cornea. The neurotrophic influence of these neuropeptides on corneal epithelial cells has been demonstrated in many experimental studies. In this way, Garcia-Hirschfeld et al.<sup>24</sup> have demonstrated that the mitotic activity in cultures containing corneal cells, together with trigeminal neurons, is higher than in those containing epithelial cells alone. The initial proliferative peak was attributed to the neuropeptide SP and the later differenti-

ation to the CGRP. The nerve fibers liberate diffusible factors, which stimulate the epithelial growth, proliferation, and differentiation and the production of collagen type VII.<sup>25,26</sup> The epithelial cells, in their turn, produce soluble factors neuronal growth factor (NGF) and glial cell-derived neurotrophic factor (GDNF) with a neurotrophic effect.<sup>9</sup>

The lower number and density of nerves at the subbasal level justify the lower corneal sensation observed in the two dry eye groups. The higher number of beadings, the presence of nerve sprouts, and the higher tortuosity are indices of a high metabolic activity, possibly directed to repair the alterations observed at the epithelial level.<sup>26</sup> Also, the activated keratocytes express NGF, and it has been observed that the overexpression of NGF induces hypertrophy of the peripheral nervous system. This effect explains why in corneas of patients with dry eye in which we observed keratocyte activation, we also found beadings and nerve sprouts. The chronic inflammation and the diminished volume and clearance of tears enriched with proinflammatory cytokines, such as interleukin (IL)-1 and -6, lead to the activation of keratocytes, which synthesize NGF and other factors of nerve growth.<sup>27</sup> We have observed that eyes with lower corneal sensitivity have a smaller number of subbasal nerves. Moreover, we have found that corneal sensitivity correlates with certain clinical parameters such as tear production (Schirmer's test) and the state of the ocular surface (staining with rose bengal). When air at corneal temperature ( $34^{\circ}\text{C}$ ) is applied to the ocular surface, the



**FIGURE 5.** Correlation between rose bengal staining and (A) mechanical thresholds ( $P < 0.001$ , ANOVA), (B) chemical thresholds ( $P < 0.001$ , ANOVA), (C) thermal cold thresholds ( $P < 0.001$ , ANOVA).

polymodal and mechano-nociceptors are stimulated.<sup>28</sup> The CO<sub>2</sub> selectively stimulates the polymodal receptors while the cooling stimulates the cold receptors.<sup>28</sup> The decrease in the three modalities of sensitivity and their correlation suggests that the lesion of the nerve terminals is nonspecific. Xu et al.<sup>29</sup> have demonstrated that corneal sensitivity in dry eyes, whether due to Sjögren's or no, is less than in normal subjects. They discovered the same correlation that we found between corneal sensitivity and Schirmer's test as well as between sensitivity and staining with rose bengal. Millodot<sup>30</sup> observed, as we did, that corneal sensitivity diminishes with age, which can justify the nervous alterations demonstrated in older patients and in age-related dry eye.

Tuominen et al.<sup>27</sup> have observed alterations in the superficial epithelium in patients with Sjögren's syndrome; however, they did not quantify these alterations. In their study, the eyes of patients with Sjögren's syndrome showed nerve sprouts, nerve tortuosity, and activation of keratocytes. We were the first to publish such changes in non-Sjögren's dry eyes.<sup>11</sup> In a recent study, Zhang et al.<sup>31</sup> found abnormal morphologic changes (tortuosity and branching) in patients dry eye that were more severe in those with Sjögren's syndrome. A strong correlation existed between the changes in nerve morphology and the degree of dry eye. Their findings are very similar to

ours; however, the greater number of nerves observed by these authors in patients with dry eye might be the result of the different resolution of different instruments and/or the small sample size in the two studies. In another study, Hosai et al.<sup>32</sup> found a decrease in corneal sensitivity in patients with dry eye that was not associated with morphologic changes. They used the Cochet-Bonnet esthesiometer, which is less sensitive than the noncontact esthesiometer. Bourcier et al.<sup>33</sup> have demonstrated that patients with dry eye exhibit corneal hypoesthesia after mechanical, chemical, and thermal stimulation. They suggest that these changes are related to the damage of corneal sensory innervation. In our study, we have shown morphologic alterations related to these sensitivity changes.

How can we relate patients' complaints with hypoesthesia? When corneal nerve endings are injured, as seems to occur in dry eye, they lose their transducing properties. Several damaged axons regenerate forming microneuromas (beadings and nerve sprouts). It is likely that this altered excitability is the origin of the dyesthesia and subjective symptoms reported by the patients.

The demonstration of the existence of nervous alterations in patients with dry eye can lead to the use of neuroprotective and/or neurotrophic eye drops for the treatment of this frequently occurring disease. In this way, Murphy et al.<sup>26</sup> demon-

strated the cure of chronic epithelial defects in dogs with topical treatment with substance P (SP); Joo et al.<sup>34</sup> showed more rapid reinnervation with NGF in an experimental model of LASIK; and most recently, we demonstrated the treatment of an epithelial defect, with documented nervous alteration, with SP and insulin-like growth factor (IGF)-1.<sup>35</sup>

In conclusion, the use of confocal microscopy and esthesiometry allow the detection of the presence of corneal neuropathy in patients with dry eye. The demonstration of such alteration in corneal innervation in patients with dry eye opens the way for possible new lines of treatment for this disease.

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