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*Pre-Print*

Entropy of corneal nerve fibers distribution observed by laser scanning confocal microscopy : a noninvasive quantitative method to characterize the corneal innervation in Sjogren s Syndrome Patients

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**ABSTRACT**

Sjogren s Syndrome (SS) is a progressive autoimmune condition mainly affecting the salivary and lacrimal glands with an incidence of primary SS between 1/100 and 1/1000. SS implies an alteration in the epithelium and subepithelium innervation, with consequent reduction of corneal sensitivity. It is necessary to have a noninvasive quantitative methods able to characterize the status of the corneal nerve fibers of the patients in order to choose and follow the best therapy. Entropy (information dimension) of the nerve corneal fibers distribution observed by confocal microscopy was evaluated in patients with primary SS (n=30, 6 males, 24 females, 21-81 years), diagnosed by biopsy of salivary gland and blood tests and in sex- age-matched healthy subjects (n=12). Corneal nerve fiber density, Langerhans cell count and cell density in the nerve plexus images were also evaluated. In selected patients salivary gland atrophy degree was also evaluated. Nerve corneal distribution observed by confocal microscopy is fractal. Entropy of the corneal nerve distribution statistically distinguishes between Sjogren s Syndrome patients and healthy subjects: patients present a lower value of

information dimension of the corneal nerve fibers distribution than healthy individuals ( $p < 0.001$ ). Percentage of grouped cases classified by entropy according to the subjects (selected patients vs healthy) showed a 100 % sensitivity and 96 % specificity,  $p < 0.0001$  with a low value of coefficient of variation among the individuals (6-7 times lower than the other morphometric indexes).. Entropy correlated with the severity of the disease (salivary gland atrophy degree,  $p < 0.01$ ). Evaluation of entropy of the corneal nerve distribution observed by a laser confocal microscopy appears able to quantitatively and noninvasively characterize an aspect of the SS patients in relation to the recognition of an impairment of their ocular surface, giving us for the first time a method to objectively and precisely characterizes the corneal innervation status in the SS patients.

**Key words:** Sjogren s Syndrome, Laser scanning microscopy, Cornea, Corneal nerve fibers, Autoimmune diseases, Fractal analysis, Information dimension, Entropy

## INTRODUCTION

The incidence of primary Sjogren s Syndrome (SS), a progressive autoimmune condition mainly affecting the salivary and lacrimal gland (Buchholz et al., 2006; [McGinnigles et al., 2012](#)), varies between 1/100 and 1/1000 ([Moss et al., 2004](#)). In primary SS (SSI) the presence of specific antibodies and signs of the infiltration of mononuclear cells in the exocrine glands accompany reduced tear and saliva secretion, producing the manifestation of a keratoconjunctivitis sicca (KCS) . In secondary SS (SSII), the typical symptoms of the primary form are coupled by other well-defined autoimmune disorders: rheumatoid arthritis, Systemic lupus erythematosus, scleroderma (Bherens et al., 2006; [Barabino et al., 2010](#)).

The ocular surface is a morphofunctional unit comprising lacrimal film, cornea, limbus, conjunctiva, mucoepidermal junction, accessory and principal lacrimal glands, Meibomian gland, and nervous plexus. The alteration of one of the individual components of the ocular surface system is followed by pathologic events that extend to the other structures. Experimental and clinical studies hypothesized that in SS, KCS may be the primitive disease of the ocular structure, only part dependent on the deficit of lachrymal secretion and therefore susceptible to an evolution its own. Surface

morphologic changes, including the presence of inflammatory cells and the expression of histocompatibility antigens (HLA-DR), suggest the participation of epithelial cells in an important inflammatory and immunologic process in the pathogenesis of KCS associated with SS (Stern et al., 2002) . Furthermore, SS implies an alteration in the epithelium and subepithelium innervations, with consequent reduction of corneal sensitivity. It is necessary to study the modification of the corneal surface further in the depth, for better understanding of the physiopathology of KCS associated with SS, to differentiate from the degenerative form and for determining the right therapeutic treatment (Nichols et al., 2000).

Traditional methodological clinical and instrumental diagnostics study comprehend an algorithm of multiple tests including: Schirmer test, tear film break-up time (BUT), ocular surface evaluation with fluorescein and Lissamine Green staining (Nichols et al., 2000; Zhang et al., 2010). The use of *in vivo* confocal microscopy permits a completely new approach of in the study of the microscopic morphology of the cornea, offering a resolution comparable to histologic examination without invasivity and it is quickly and easily repeatable (Villani et al., 2007; Nenitez-del-castillo et al., 2007; Zhang et al., 2010; [Kojima et al., 2010](#)).

In order to quantitatively characterize the corneal nerve distribution in Sjogren's Syndrome we have evaluated the entropy (a fractal parameter) of corneal nerve fibers distribution observed by confocal microscopy in patients and in control subjects.

## **MATERIALS AND METHODS**

The patients came to our attention at the Ophthalmology Division after being sent for a visit by the Rheumatology Clinic, where a diagnosis of SS was made according to the Japanese consensus criteria (diagnosed by biopsy of salivary gland and blood tests). Informed consent was obtained from all

subjects. Examination procedures were board reviewed and the study was conducted in accordance with the tenets of the Declaration of Helsinki.

### **Patients**

Thirty patients (6 men and 24 women) with primary Sjogren's Syndrome (aged between 21 and 81 years) and 12 sex-, age-matched controls were studied. None of patients had a history of Steven Johnson syndrome, lymphoma, AIDS, corneal dystrophy and inflammation, therapy with antiglaucoma drugs, steroids, use of contact lenses. The results were compared with the same examination parameters performed on the healthy control subjects.

### **In vivo Laser Scanning Confocal Microscopy**

*In vivo* laser confocal microscopy, 670 nm red wavelength helium neon diode laser source, was performed on all subjects with a HRT II Rostock Corneal Module (Heidelberg Engineering GmbH, Dossenheim, Germany). The confocal laser scanning device uses a 60 x objective water immersion lens. The images measure 400  $\mu\text{m}$  x 400  $\mu\text{m}$ , and the manufacturer specifies an optical section thickness of 4  $\mu\text{m}$ . The module uses an entirely digital capture system. *In vivo* confocal microscopy was performed in the centre of the cornea by the same operator. A drop of anesthetic

(oxybuprocaine chloride 0,4%) was instilled in the lower conjunctival fornix before examination. During the test, the object lens of the microscope was covered with gel (hydroxypropylmetil cellulose) and came in direct contact with the corneal surface. Proper alignment and position of the head was maintained with the help of a dedicated target mobile red fixation light for the contralateral eye. A digital camera mounted on a side arm provided a lateral view of the eye and objective lens to monitor the position of the objective lens on the surface of the eye. A scan of the cornea was performed for each participant in the central area of the corneal nerve fiber plexus for each eye; the procedure lasted

2 to 5 minutes. A drop of antibiotic was instilled in the lower conjunctival fornix at the end of each examination. The cornea was then examined by slit-lamp to ensure its integrity.

### **Evaluation of image entropy**

Single-pixel contours of images of the corneal fiber plexus were extracted by a Canny edge filter (setting sigma = 2; high threshold = 20; low threshold = 10; Digital Image Magnifier software by Strikos Nikolaos: <http://www.softoxi.com/digital-image-magnifier.html>) and submitted to analysis to determine the information dimension (entropy, D1) of the images (Pitsianis et al., 1989). To evaluate the information present in the pattern (entropy), information dimension, a robust estimate from a finite amount of data that gives the probability of finding a point in the image, was calculated. The set was covered with boxes of linear size,  $d$ , keeping track of the mass,  $m_i$  (the amount of pixels) in each box (from 1 to 100 pixels), and calculated the information entropy  $I(d)$  from the summation of the number of points in the  $i$ -th box divided by the total number of points in the set multiplied for its logarithm (Falconer, 1990). The slope of the log-log plot of information entropy vs.  $1/\text{box side length}$  represented the information dimension of the distribution. Inter- and intra-observers errors of the entire procedure were  $< 3\%$

The log-log plots used to calculate the information dimension showed a straight line with high correlation coefficient, always above a value equal to 0.99, thus justifying the fractal approach (figs. 1-2, bottom). The methodology was implemented by using the Benoit 1.3 software (TruSoft Intl Inc: <http://trusoft-international.com/benoit.html>) and a personal computer. The method was validated by measuring computer generated Euclidean and fractal shapes of known information dimensions (Circumference = -0.7%; Square = + 0.4%; Triadic Koch island = -0.9%; Sierpinski's Triangle = -1.5%). Entropy values from 6 images of the same patient were averaged.

### **Corneal nerve fiber density, Langerhans cell count and cell density**

Corneal nerve fiber density, cell count and cell density were evaluated by an expert ophthalmologist manually analyzing all the scanned 400 x 400 micron images. Results were averaged,

### **Salivary gland biopsy**

The salivary gland biopsy permitted to confirm the diagnosis and to measure the degree of the salivary glands atrophy in the patients by an expert pathologist. Patients were assigned to two classes: low atrophy (#1 and #2 classes) and high atrophy (#3 and #4 classes).

### **Statistical tests**

The Kruskal-Wallis test was applied in order to verify significant differences between the groups. A linear regression analysis was applied in order to verify the significance of the log-log plot. In order to evaluate the significance of fractal dimension with respect to the subjects a ROC curve analysis was performed (SigmaPlot 11, systat software, inc, USA) .

## **RESULTS**

Figures 1-2, top left, show the corneal nerve fibers inside the cornea in the healthy individuals and in the SS patients, respectively, after in vivo laser scanning confocal microscopy. The nerve fibers inside the cornea and their single pixel contours after a Canny edge filter are shown in figures 1-2, top right. Fractal analysis, applied over the skeletonized images, reveals that the nerve corneal distribution is fractal (log log plot is linear,  $p < 0.001$ , in the healthy individuals as well in the patients (figures 1-2, bottom). Entropy evaluation of the images of the nerve corneal distribution observed by laser scanning confocal microscopy statistically distinguishes between Sjogren s syndrome patients and healthy subjects showing a reduction of the entropy of corneal nerve distribution in the patients ( $p < 0.001$ , table 1, figure 3). The percentage of grouped cases classified by entropy (D cut-off =1.22) according

to the subjects (SS patients vs healthy) shows a 96 % sensitivity and 98% specificity,  $p < 0.0001$  (table 2). Entropy of the corneal nerve fibers correlates with salivary gland atrophy degree ( $p < 0.01$ , table 3, figure 4). Coefficient of variation of entropy resulted 7 times lower than the one of cell count or cell density (table 1, table 4, respectively).

## DISCUSSION

Fractal analysis is able to study the complexity of biological structures, and perform diagnosis and prognosis in the patient, also in our hands (Losa and Nonnemacher, 1996; [Masters, 2004](#); Traversi et al., 2008; [Bianciardi et al., 2013](#); [Bianciardi et al., 2014](#)). Fractal analysis has been performed, here, to evaluate the information dimension, or entropy, of the distribution of the corneal nerve fiber observed by confocal microscopy.

Entropy of the corneal nerve fiber distribution appeared lower in the SS patients than in healthy subjects and it correlated with high significance with the salivary gland atrophy, i.e. the severity of the disease. The automatic, objective, entropy index showed high sensitivity and specificity and its coefficient of variation resulted 6-7 times lower than the one of other (manual, subjective) morphometric indexes used in the disease, like as corneal nerve fiber density, Langerhans cell count and cell density.

## CONCLUSION

Entropy evaluation of the corneal nerve fiber distribution observed by laser scanning confocal microscopy is able to give an objective method to automatically and precisely quantize an aspect of the Sjogren's patient. This noninvasive technique permits, for the first time, to objectively characterize the SS patient corneal innervation status so helping us to choose and follow the best therapy. The analysis is inexpensive and not time-consuming (1 minute on average, to obtain the

skeletonized images and to perform the fractal dimension analysis by a standard PC and cheaper ready-made software).

## CONFLICT OF INTERESTS

The Authors declare no any conflict of interests

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## TABLES

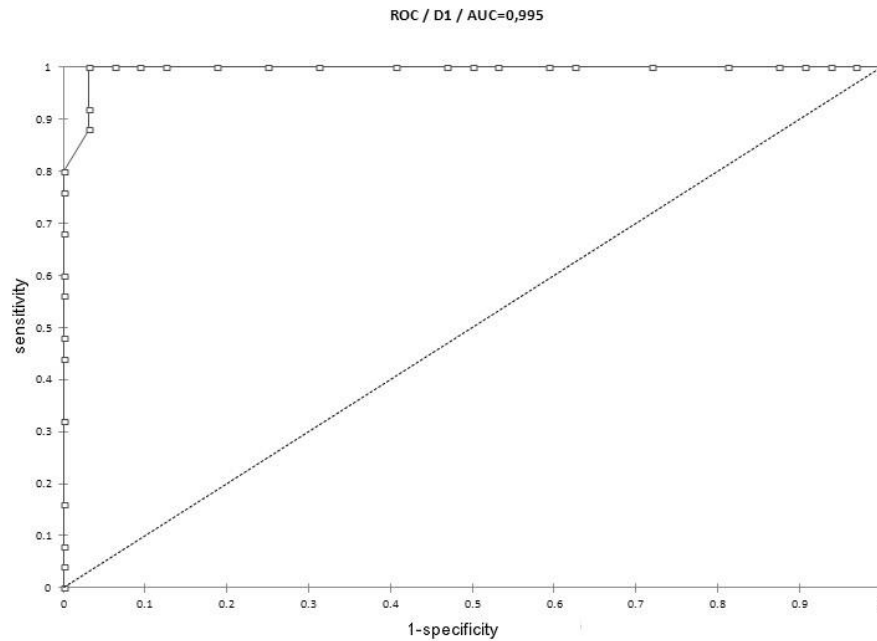
**Table 1**

	Entropy CV%
Healthy Controls	1.30 (0.04) 3%
Sjogren s Syndrome	1.14 (0.06) 5%
P	0.001

*Information dimension (entropy) of the corneal nerve fiber distribution observed by laser scanning microscopy in Sjogren s Syndrome patients is lower than in healthy subjects. Mean values (standard deviation).*

**Table 2.** ROC analysis, entropy (D1) of corneal nerve fibers distribution, Sjogren s Syndrome patients (primary SS) vs. healthy subjects

Samples	Sensitivity	Specificity	D1,Cut-off	AUC	p
Normal vs SS	1.00	0.96	1.22	0.995	0.0001



*High discriminant power of fractal dimension index (entropy)*

**Table 3**

	Entropy
High atrophy	1.10 (0.02)
Low atrophy	1.17 (0.02)
P	0.01

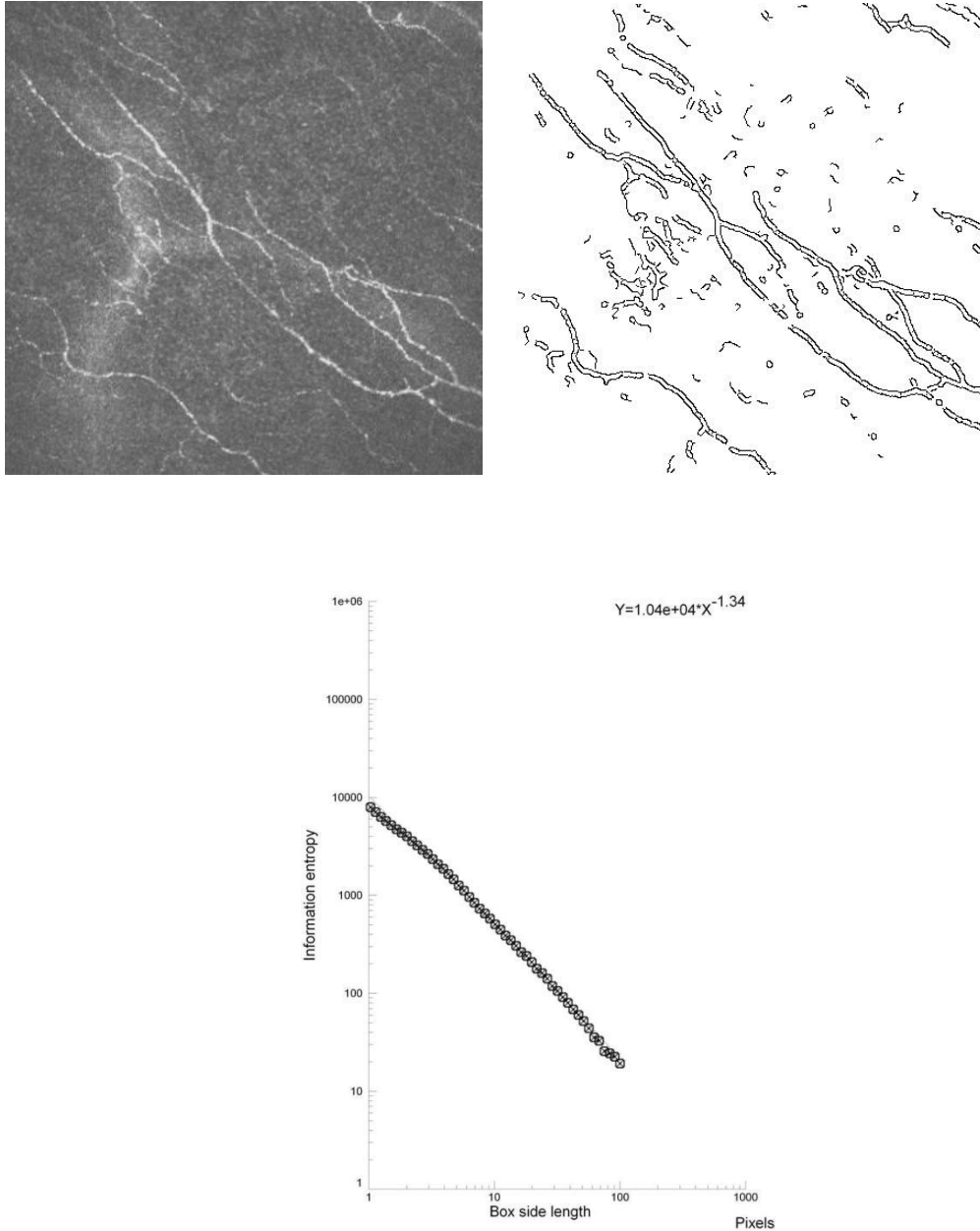
*Entropy correlates with the degree of salivary glands atrophy. High atrophy =3 - 4 classes, Low atrophy = 1 or 2. Mean values (standard deviation).*

**Table 4**

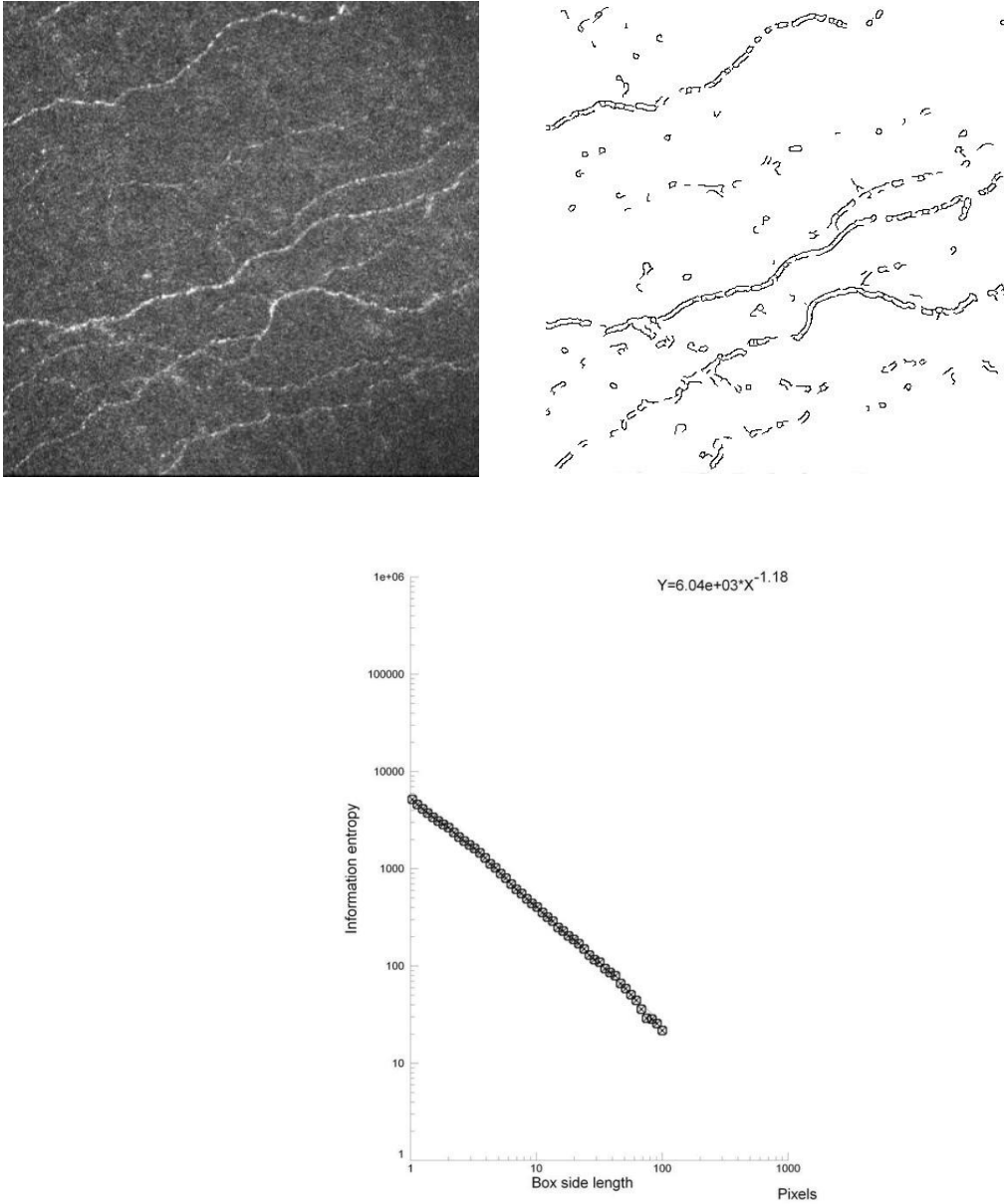
	Nerve fiber density CV%	Cell Count/field CV%	Cell Density CV%
Healthy Controls	8.1 (2.1) 26%	5 (1.4) 28%	37 (10) 27%
Sjogren s Syndrome	4.9 (1.0) 20%	3 (1.0) 33%	22 (7) 32%
P		0.001	0.001

*Corneal nerve fiber density, Langerhans cell count and cell density in the corneal fiber plexus. Mean values (standard deviation). Coefficient of variation resulted 6-7 times higher than the one of entropy (see, table 1).*

## FIGURES

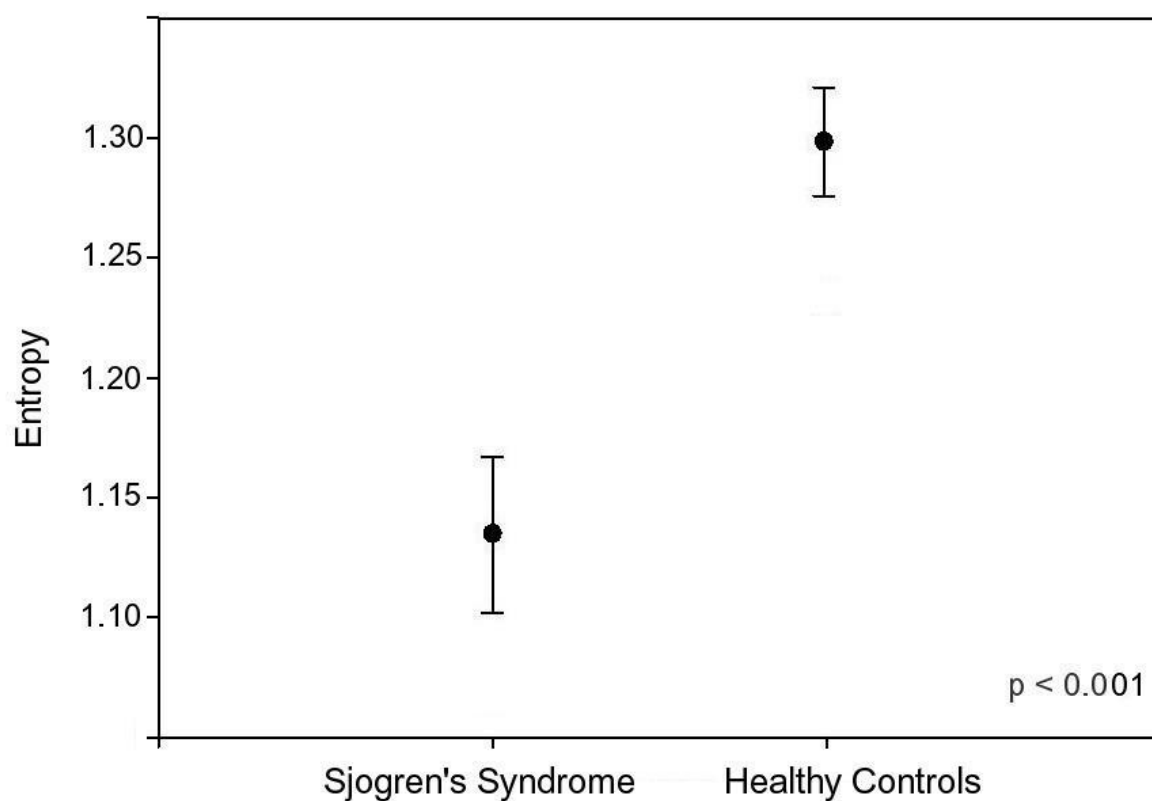


**Figure 1.** *Healthy subject. Images of the corneal nerve fibers after in vivo laser confocal microscopy (top, left); single-pixel contours of the corneal nerve fibers images extracted by a Canny edge filter (top, right); The log log plot and its linearity (bottom,  $p < 0.001$ ): the corneal nerve fiber distribution is fractal. Fractal analysis may be performed. Entropy is the slope of the best fit among 1 and 100 pixels.*

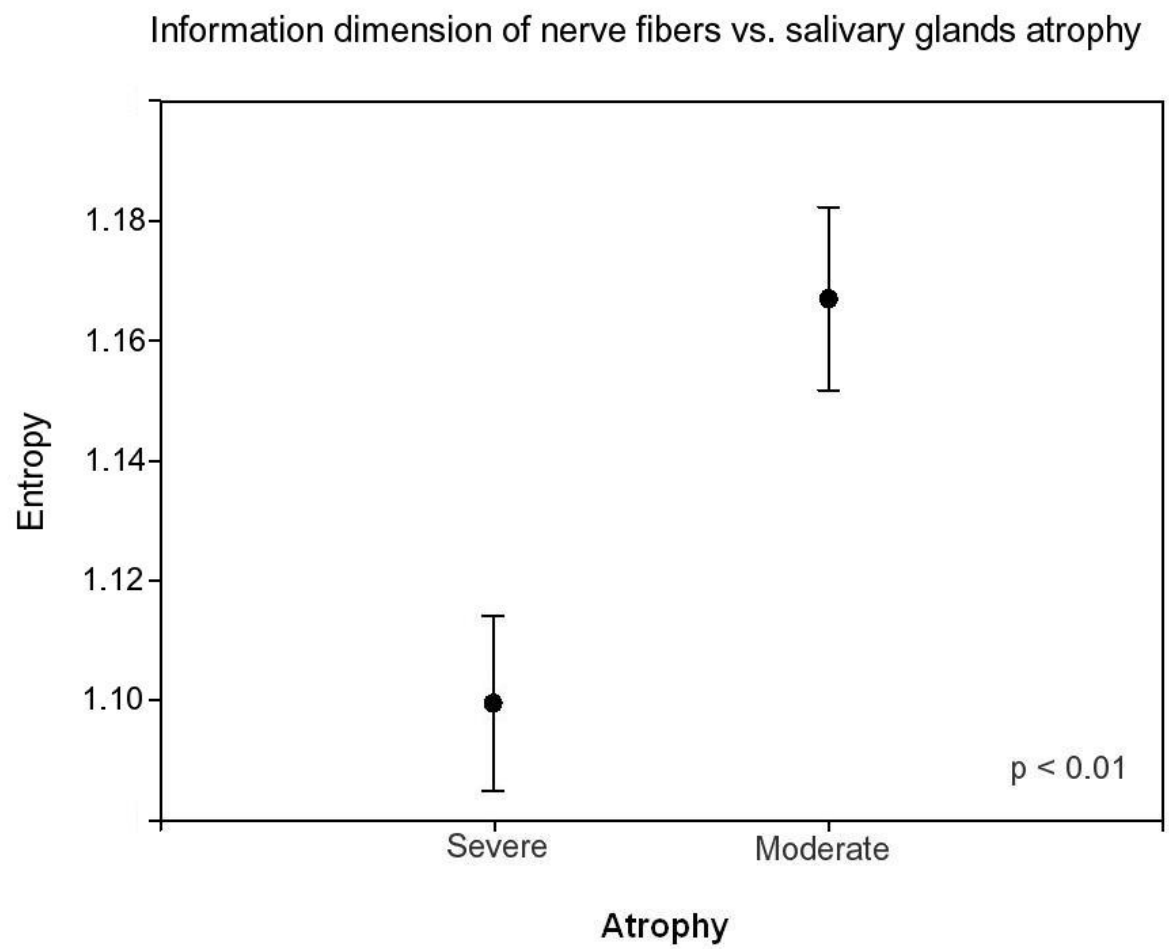


**Figure 2.** Sjogren Syndrome patient. Images of the corneal nerve fibers after in vivo laser confocal microscopy (top, left); single-pixel contours of the corneal nerve fibers images extracted by a Canny edge filter (top, right); The log log plot and its linearity (bottom,  $p < 0.001$ ): the corneal nerve fiber distribution is fractal. Fractal analysis may be performed. Entropy is the slope of the best fit among 1 and 100 pixels. Note that the slope in the patient is lower than in the healthy subject (see fig.1)

### Information dimension of nerve fibers in SS and Controls



**Figure 3.** Entropy evaluation of the images of the nerve corneal distribution observed by laser scanning confocal microscopy statistically distinguishes between Sjogren s syndrome patients and healthy subjects showing a reduction of the entropy of corneal nerve distribution in the patients



**Figure 4.** Entropy of the corneal nerve fibers correlates with salivary gland atrophy degree in the SS patients.