Culver et al. *Cibinetide improves corneal nerve fiber abundance in patients with sarcoidosis-associated small nerve fiber loss and neuropathic pain*

**Supplementary Materials.**

In addition to the major anatomical features of the corneal sub-basal nerve plexus (nerve fiber number, length, and branching), a number of complex structural details are captured by corneal confocal microscopy (CCM). These include nerve bundle thickness (reflecting a variable number of C-fibers contained within) and prominent periodic beading (varicosities containing the mitochondria necessary for normal nerve function). ¹ Neuropathic disease processes, e.g., diabetes, significantly alter the corneal nerve morphology in a variety of major (e.g., fiber loss and decreased branching), as well as more subtle ways (e.g., increased tortuosity, thinning, and change in bead size and periodicity). ², ³ To date a variety of methodologies have been employed for quantifying CCM images of the sub-basal nerve plexus, ranging from manual, to semi- and fully-automated procedures which have focused primarily on major features of the nerve fiber net.

Automated image analysis provides the advantages rapid data reduction, which makes a larger image sample size more practicable, while reducing the influence of potential observer bias. Algorithms employed are based on preliminary image processing with the goal to maximize the signal to noise ratio, followed by target (nerve fiber) detection. Each of these processes modulate the information contained in each image to a variable extent. In general, although prominent features of the image are typically preserved, image processing degrades fine detail, e.g., small structural differences in the corneal nerve fiber net. Therefore, different analysis algorithms do not produce numerically identical outputs. Formal assessment of the agreement of current published methods has shown that although each give somewhat different actual values, the methods generally agree with little bias, are highly correlated, and function well to discriminate between normal subjects and those with mild to severe SNFL. ⁴

The fully automated program ACCmetrics ⁵ has been widely employed for analyzing CCM data and was designed to provide individual estimates of corneal nerve fiber density, length, and branching. Although an estimate of fiber width is also generated by ACCmetrics, it is estimated using a complex series of filtering and Gaussian curve-fitting routines. Area is calculated from the width of representative cross-sections of the fiber times length, rather than direct quantification of pixels occupying the area of the identified structure. Additionally, although the ACCmetrics approach improves target fidelity, it also degrades or ignores fine anatomical details such as nerve fiber beading.

As our study has focused on potential longitudinal changes in the corneal nerve fiber net, we have approached this problem of quantification with the hypothesis that determining the number of pixels in the relatively unprocessed image comprising the corneal nerve fiber...
network will sensitively assess small scale variability. To accomplish this, using FIJI (ImageJ) the edge of each 384 X 384 pixel standard image from the Heidelberg Corneal Confocal Microscope is first cropped by 2 pixels to reduce edge effects, resulting in an analyzed area of 0.158342 mm². The algorithm then creates a mask representing nerve fibers by applying a Guassian filter to enhance target contrast, adjusts the detection threshold based on background brightness, followed by use of the eigenvalues of the Hessian matrix to identify tubular nerve fiber structures. Corneal nerve fiber area (CNFA) is defined as the image area (µm²) covered by the mask. To convert these units to µm²/mm² divide by 0.158342.

To directly compare the performance of ACCmetrics and the FIJI based automated algorithm, CCM images obtained from 101 subjects with and without neuropathy were analyzed and compared using both routines. The data were obtained following informed consent and approval of the Ethics Committee of the University of Manchester, Manchester, UK. The study group consisted of 20 normal subjects, and 81 patients with diabetes (59 males; median age of 52 years). The diabetes patients were classified by neuropathic disability score (NDS) and placed into groups: NDS=0-2 (no neuropathy; n=20), 3-5 (mild neuropathy; n=21), 6-8 (moderate neuropathy; n=19), or 9-10 (severe neuropathy; n=21). A total of 579 images (5-6 per individual) were processed using each automated method and then averaged for each subject.

The results (Figure S1) show high correlation (all statistically significant at the p<0.0001 level) for both the within method comparison of ACCmetrics-derived variables (CNFD: corneal nerve fiber density; CNFL: corneal nerve fiber length; CTBD: corneal total branch density), as well with CNFA.

### Multivariate Correlations

<table>
<thead>
<tr>
<th></th>
<th>CNFD (n/mm²)</th>
<th>CNFL (mm/mm²)</th>
<th>CTBD (n/mm²)</th>
<th>CNFA µm²/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNFD</td>
<td>1.0000</td>
<td>0.9622</td>
<td>0.7956</td>
<td>0.8929</td>
</tr>
<tr>
<td>CNFL</td>
<td>0.9622</td>
<td>1.0000</td>
<td>0.8727</td>
<td>0.9271</td>
</tr>
<tr>
<td>CTBD</td>
<td>0.7956</td>
<td>0.8727</td>
<td>1.0000</td>
<td>0.8624</td>
</tr>
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<td>CNFA</td>
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<td>1.0000</td>
</tr>
</tbody>
</table>
The table summarizes that, as expected, assessment of complex features using CNFA correlates well with each of the ACCmetrics variables, and best with CNFL. It is also clear from the analysis that a substantial proportional bias does not exist between the two methods. To evaluate this in more detail, the relationship between CNFL (ACCmetrics) and CNFA (FIJI) was examined using Passing-Bablok linear regression, which is appropriate for comparing methods exhibiting different variances and compared to the results of non-linear curve fitting. The results show (Figure S2) that although the relationship is fitted well by Passing-Bablok regression, especially for patients with neuropathy (dotted line), the clear right shift towards CNFA for higher corneal nerve fiber abundance is better approximated by an exponential function (solid line). Specifically, the Passing-Bablok parameters are slope 0.00048 (95% CI: 0.00044, 0.00053) with intercept of 1.91. The splaying of graph to the right can be explained by the observation that the nerve fiber bundles of normal individuals are typically thicker than those of subjects with neuropathy. Since CNFL is not sensitive to varying fiber width, whereas the FIJI protocol is, FIJI assigns relatively greater values.
A full comparison of these analysis methods, including the sensitivity and specificity for determination of presence and degree of neuropathy will be published elsewhere.

**Figure S2.**

![Graph showing corneal nerve fiber quantification](image)

- normal
- no neuropathy
- mild neuropathy
- moderate neuropathy
- severe neuropathy

\[ CNFL = 34.51(1 - \exp(-2.182^{5} \times \text{CNFA})) \]

- Passing-Bablok Regression

**Corneal nerve fiber quantification of sarcoidosis patients using Accmetrics.**

To compare data from the sarcoidosis population derived using the fully automated analysis program ACCmetrics versus CNFA as determined using FIJI, the same images obtained at baseline and day 28 were analyzed using each method. Statistical significance was assessed using linear regression analysis with baseline value as a covariate. The results show that as expected, CNFD (Fig S3A) did not show significant change, with the Least Square Mean (LSM) estimate for the placebo, 1 mg, and 4 mg groups approximating zero, and the 8 mg group increased by ~ 1.5 fibers/mm². Although CNFL (Fig S3B) showed similar, small increases for the 4 and 8 mg groups compared to placebo and 1 mg, these also were not significant. In contrast, the LSM estimates of CNBD (Fig S3C) were increased significantly compared to placebo in both the 4 mg and 8 mg groups. Additionally, the 4 mg group exhibited a strong trend for an increase with respect to the 1 mg group.
These results underscore the fact that since CNFD is calculated from integer values only, the data distribution is discrete and is therefore characterized by limited resolution and dynamic range. In this data set the range of values possible for CNFD ranged up to 9 main fibers per image. Therefore, only 10 values were possible (0 fibers/0.16 mm²; 1 fiber/0.16 mm²,...,9 fibers/0.16 mm²). In contrast, CNFL is calculated on the basis of number of pixels within the program-generated estimated length of each fiber. This approximates a continuous distribution and more sensitively assessed for change having a range of up 4080 µm per image (i.e., at the resolution of the analysis of 1.04 µm/pixel, a total of 3923 pixels). However, as a one-dimensional variable, the dynamic range is less than that of CNFA, which as a two-dimensional variable was about 2.5-fold greater with a range of 10,452 µm² per image (9,633 pixels). The observation that CNBD, although also a discrete variable with a range of 0 to 23 branches on main nerve fibers/image, showed significant differences for the two higher dosage groups suggests strongly that increased branching is a key characteristic of early nerve fiber regrowth. Notably, the results of prior study of patients with diabetes-related small nerve fiber loss who undergo curative pancreatic transplantation exhibited a significant increase in CNBD at 6 months, followed only 12 months later by significant increases in CNFD and CNFL.  

SUPPLEMENTARY REFERENCES.


