

New approach to corneal nerve fibers morphometry in diabetes mellitus on the basis of confocal biomicroscopy

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Aim — to develop a new approach to morphometric analysis of corneal nerve fibers (CNF) and to evaluate their age-related changes in type 1 and type 2 diabetes mellitus patients. **Materials and methods.** The study enrolled 150 participants (300 eyes) aged from 13 to 83 years, of them 37 type 1 diabetes patients (74 eyes), 51 type 2 diabetes patients (102 eyes), and 62 healthy volunteers (124 eyes). All participants were examined with HRT III Rostock Corneal Module (Heidelberg Engineering GmbH). Digital images thus obtained were analyzed with specially developed original software that automatically computes coefficients of CNF orientation symmetry and anisotropy. **Results.** A strong inverse correlation has been found between the coefficient of CNF orientation anisotropy and glycated hemoglobin levels ($r = -0.83, p < 0.001$ and $r = -0.79, p < 0.005$ in patients with type 1 and type 2 diabetes respectively) as well as the duration of the disease ($r = -0.7, p < 0.005$ and $r = -0.72, p < 0.001$, type 1 and type 2 diabetes respectively). On the contrary, the coefficient of CNF orientation symmetry has been shown to be directly correlated with both glycated hemoglobin levels ($r = 0.64, p < 0.005$ and $r = 0.78, p < 0.05$, type 1 and type 2 diabetes respectively) and the duration of the disease ($r = 0.62, p < 0.05$ and $r = 0.73, p < 0.05$, type 1 and type 2 diabetes respectively). Interocular asymmetry in both coefficients was found in diabetic patients but not in the controls. On the basis of the proposed coefficients, age-related changes in corneal nerve fibers orientation have been determined. Normally, the degree of CNF tortuosity increases with age. The rate of this increase is the highest before the age of 35-40. As shown, type 1 diabetes is associated with low coefficients of CNF orientation anisotropy and high coefficients of CNF orientation symmetry as compared to type 2 diabetes of the same age group. **Conclusion.** As suggested by the results, the two proposed coefficients that describe the state of corneal nerve fibers can possibly be used for diagnosis and monitoring of diabetic peripheral neuropathy (DPN). However, to produce enough evidence, further studies should be conducted.

Keywords: corneal nerve fibers, coefficient of corneal nerve fibers orientation anisotropy, coefficient of corneal nerve fibers orientation symmetry, corneal nerve fibers tortuosity, *diabetes mellitus*.

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Diabetes mellitus (DM) is a socially-significant disease associated with high disability rates in the population [1]. There are about 6 million new DM cases registered each year [2, 3]. Diabetic polyneuropathy (DPN) occurs in up to 75% of DM patients and can be classified according to its predominant involvement as either peripheral (spinal and cerebral nerves are mostly affected), or autonomic (vegetative nervous system and, thus, nerve supply of the internal organs is disturbed) [4-11]. Diagnostic options for DM include collection of complaints (burning and/or tingling sensation in limbs, numbness and sensory loss, as well as swelling of feet and chills), skin or sural nerve biopsy of the affected extremity, and electroneuromyography (ENMG) with needle electrodes [12–15].

In recent years there has been much interest in studying of anatomical and physiological features of corneal nerve fibers (CNF), largely due to the supposition made by N. Efron, an Australian scientist, that they may be involved in DPN [16]. Despite the overwhelming number of related publications, the severity of CNF damage in DM and possibilities for its objective interpretation remain a debated issue.

Not so long ago corneal confocal microscopy (CCM) has been introduced enabling detailed investigation of corneal layers and, thus, CNF visualization. This method is in common practice at the Manchester Medical

School (University of Manchester) [17–19]. The following metrics are used to describe the state of CNF: NFD (Nerve Fiber Density) – the number of main nerve trunks, NBD (Nerve Branch Density) – the number of nerve branches, NFL (Nerve Fiber Length) – the total nerve length, NFT (Nerve Fiber Tortuosity) – the coefficient of tortuosity.

There are, however, certain limitations of CCM. For example, a confocal microscope can hardly be pointed to the very same tiny spot in the cornea making the follow up rather difficult. Moreover, the depth of field being rather deep, several layers of CNF are likely to look like one on the screen, thus, producing unreliable NFL, NFD, and NBD values. An additional error comes from the fact that the total length of corneal nerves is actually dependent on their tortuosity. In the latest software this drawback has been partially solved by standardizing NFL for NTF [20]. And yet, the approach is unchanged in the essence: manual selection of nerve fibers remains the main pillar of CCM image interpretation.

The aim of this study was to develop a new approach to morphometric analysis of corneal nerve fibers and investi-

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gate their age- and type 1 and type 2 diabetes-related changes.

Exploration of suitability of the method described below for diagnosis and monitoring of DPN was beyond the scope of the current work. In the future, however, we hope to have the opportunity to implement the method into clinical use and compare its results to ENMG data.

Material and methods

General characteristics of the material are provided in Table 1. The study enrolled 37 type 1 diabetes patients (74 eyes) and 51 type 2 diabetes patients (102 eyes) aged from 13 to 83 years. Exclusion criteria were anterior eye segment pathology and previous incisional or laser eye surgery. The groups were further divided by age. Quantity differences between the subgroups can be explained, first of all, by low prevalence of type 1 diabetes among older adults due to earlier associated mortality [21, 22]. The other reason is that type 2 diabetes is an adult-onset disease (usually manifests after 45 years of age). Patients with so called maturity-onset diabetes of the young (MODY), regarded as a separate entity [23-26], were not included. The control group consisted of 62 conditionally healthy volunteers (124 eyes). Morning and post-prandial glycemia (30 min after the meal) as well as glycosylated hemoglobin levels were taken into account when forming study groups and analyzing results.

Besides conventional ophthalmic examinations (namely, visual acuity testing under standardized lighting conditions, anterior segment slit-lamp biomicroscopy, direct and indirect dilated ophthalmoscopy, perimetry, and tonometry), all participants underwent confocal biomicroscopy using HRT III with Rostock Cornea Module, which works on the principles of laser raster coherent imaging.

Possible relationship between CNF and vitreoretinal changes was not considered in this study.

To assess inter-eye differences in studied parameters, the following adapted formula (designed originally for glaucoma patients [27]) was applied:

$$IAI = \frac{|pOD - pOS|}{|(pOD + pOS)|:2} \times 100\%$$

where *IAI* – *inter-eye asymmetry index*,

pOD – the studied parameter of the right eye,

pOS – the studied parameter of the left eye

It is noteworthy that by using absolute differences instead of relative we were enabled to avoid errors arising from a

unilateral increase of the studied parameter. However, for such a simplified method of IAI computation to work, all the values have to be positive non-zero numbers.

Data analyses were done with PSPP (Linux) and Microsoft Excel 2006 software packages. Distributions were nonparametric and thus, Wilcoxon-Mann-Whitney U-test was performed. Statistical dependences were measured with Spearman's rank correlation coefficient (*r*). Differences were considered statistically significant at $p < 0.05$.

Results and discussion

The new approach to morphometric analysis of CNF consists of objective description of their orientation and computation of coefficients of CNF orientation symmetry and anisotropy. Original software (Liner 1.1) was used for post-processing of confocal microscope images. Detailed mathematical substantiation of the method and its limitations as well as some guidelines to confocal image interpretation have already been published [28].

In contrast to the existing algorithms (CCM Image Analysis Tool), we decided to get rid of manual CNF tracing and thus, made the step of image reading and CNF mapping fully automated. Our software is capable of automated image interpretation and provides the user with a probability map of nerve passage through the area being inspected (**Fig. 1**). An advantage to be mentioned is that nerve fragments within out-of-focus, underexposed, or noisy areas of the image are identified with higher confidence level as compared to visual evaluation.

High software efficiency is due to the following original algorithm. An image is first formalized with nerve fibers regarded as light bands of a certain width against a dark background (**Fig. 2**). The program then compares different nerve fragments to a model one, also referred to as 'ideal nerve fragment'. Taking into account that a real nerve fiber can pass through a particular point of the image at any angle, the model function is incrementally rotated around the original through some angle α , which is between 0° and 180° (**Fig. 3**).

For objective evaluation of similarity between a given confocal image and the generated model function (M), we used a correlation coefficient, which can take values from -1 to 1, higher values corresponding to higher similarity. A comparison drawn between the confocal image and a series of rotated functions provides us with a set of correlation coefficients (negative values are discarded), of which the maximum value indicates the most likely

Table 1. Age and diabetes mellitus distributions among study participants (eyes)

| Group | Age, years | | | Total |
|--------------------|------------|---------|---------|----------|
| | 13–34 | 35–56 | 57–83 | |
| Healthy volunteers | 20 (40) | 20 (40) | 22 (44) | 62 (124) |
| Type 1 diabetes | 31 (62) | 6 (12) | -- | 37 (74) |
| Type 2 diabetes | — | 18 (36) | 33 (66) | 51 (102) |

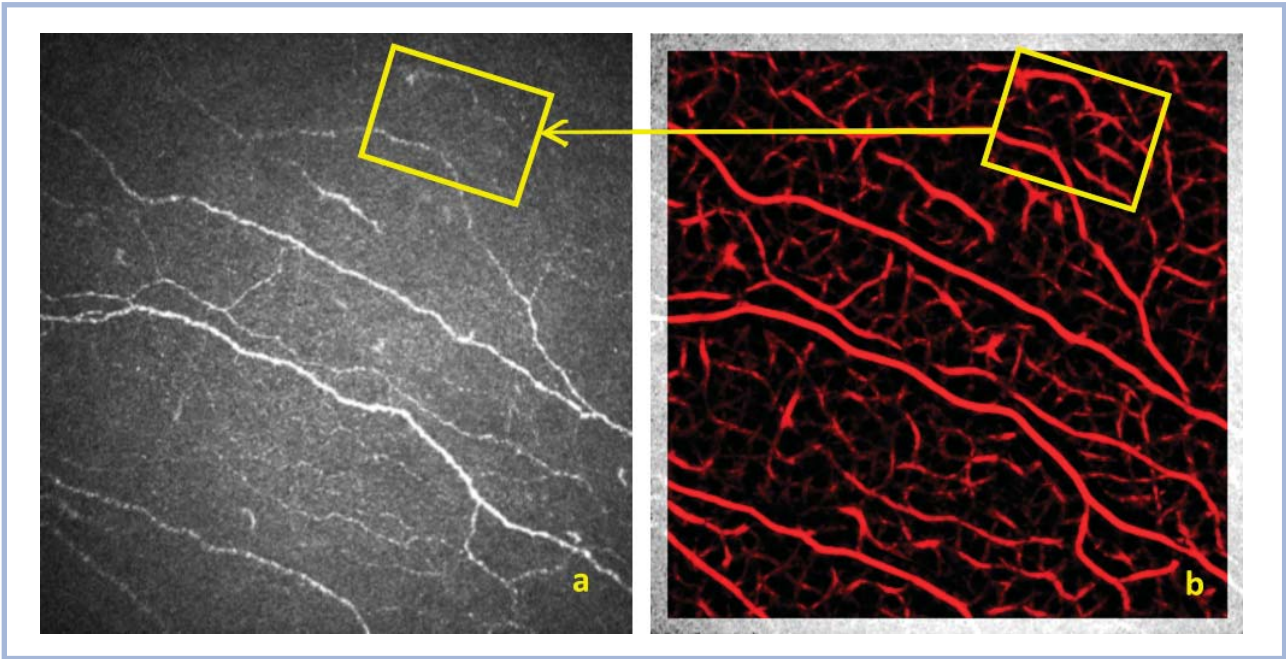


Fig. 1. Initial confocal image (a) and computed probability map of CNF passage (b).
 The developed algorithm allows to identify even those nerve fibers that are almost invisible in the initial image (yellow frame).

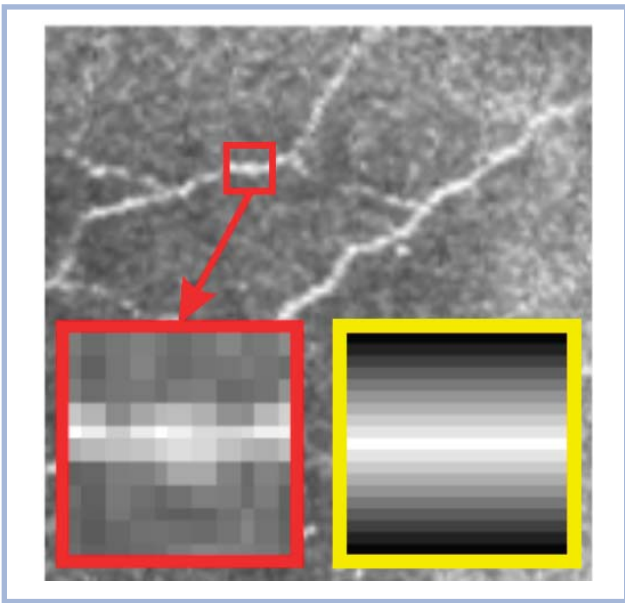


Fig. 2. Comparing a confocal microscope image of CNF to model function.
 The red frame encloses a magnified nerve fragment, the yellow — an 'ideal fragment', which has been generated for comparison.

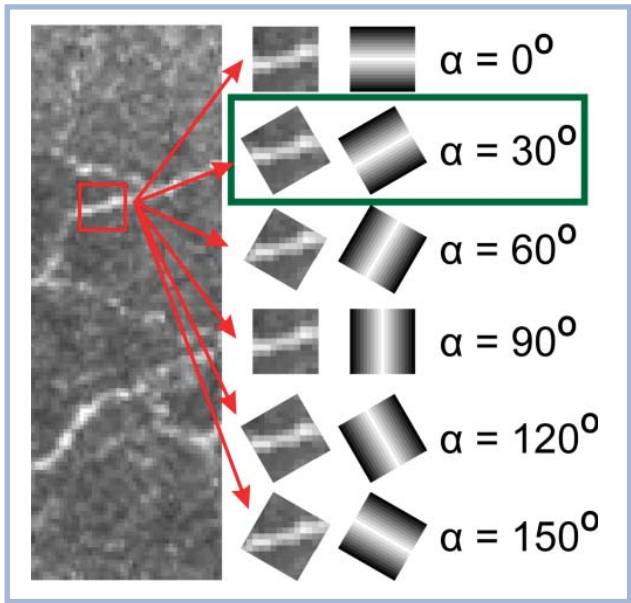


Fig. 3. A series of model functions at various angles α (explained in the text).

angle of nerve passage through this or that point. As a result, the probability of a nerve fiber being present and the angle at which it passes are determined for every point of the image in the form of vectors (**Fig. 4, a**).

Vector magnitudes are then plotted on a rose diagram (**Fig. 4, b**). If most of nerve fibers in a random confocal image are unidirectional, the corresponding rose

diagram spokes will be much longer than the others making the whole diagram look elongated. The shape of the diagram can be quantified by the coefficient of CNF orientation anisotropy ($K_{\Delta I}$) calculated as the ratio of the longest rose diagram spoke to the shortest (**Fig. 5**) and thus, representing the degree of predominance of one of the available fiber orientations.

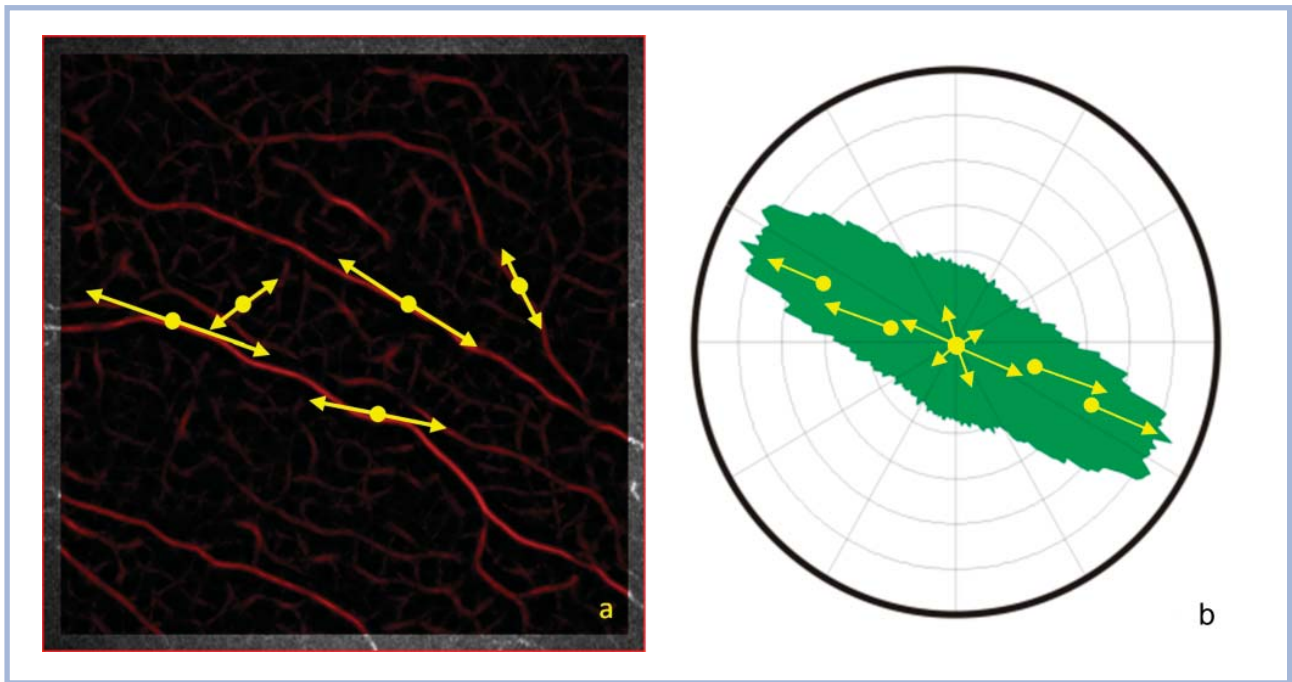


Fig 4. Probability map of CNF passage (vector magnitudes and directions represent probability values of nerve passage and fiber orientations, respectively) (a) and a rose diagram plotted for these data (b).

It is clearly seen that nerve fibers in this confocal image are mostly described by vectors directed from top-left to bottom-right. Probabilities of other fiber orientations are lower and fewer.

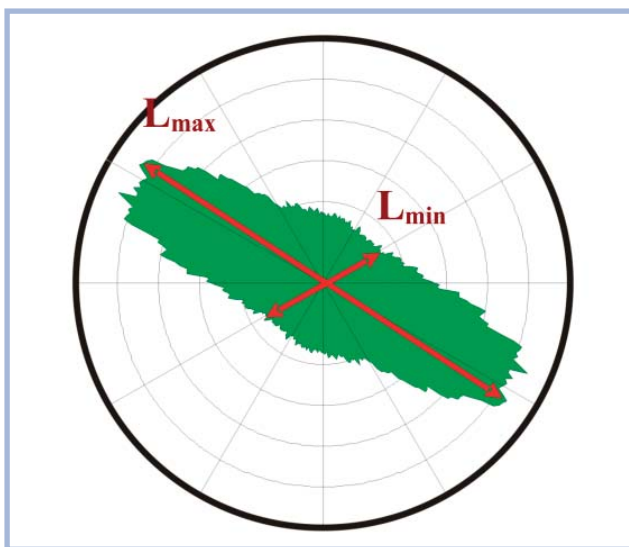


Fig. 5. Calculation principle of the coefficient of CNF orientation anisotropy ($K_{\Delta L}$).

L_{max} и L_{min} — the longest and the shortest rose diagram spokes, respectively (explained in the text).

If, however, there are two or more predominant CNF orientations, the diagram will look distorted, asymmetrical. The degree of distortion can be described as the ratio of areas of the two halves of the diagram divided by its longest spoke. In order to be able to evaluate and compare this characteristic, we introduced another coefficient,

named the coefficient of CNF orientation symmetry (K_{sym}) and calculated as the ratio of the larger half to the smaller. The said algorithm is fully realized in a special software, thus, all the calculations are automated.

Advantages of the new approach to morphometric analysis of CNF include device independence as well as tolerance to low lighting conditions, decreased transparency of the medium, and changing of the imaged spot within the cornea.

Tables 2 and 3 contain mean values of both $K_{\Delta L}$ and K_{sym} determined as described above in healthy volunteers and diabetes patients of different age groups.

In healthy population, the degree of CNF tortuosity increases with age. The rate of this increase is the highest before the age of 35-40. $K_{\Delta L}$ naturally decreases from an average of 3.9 in postpubertal adolescents down to around 2.7 in the elderly, whereas K_{sym} increases from 0.79 to 0.87, respectively (**Fig. 6**). This can be due to impaired trophism of the nerve itself and/or age-related involutinal processes in the organism.

Diabetes mellitus has been found to be associated with a statistically significant decrease in $K_{\Delta L}$ and increase in K_{sym} . Thus, in type 1 diabetes patients aged 13-34 years $K_{\Delta L}$ and K_{sym} were 2.49 ± 0.79 and 0.89 ± 0.11 and in those aged 35-56 years — 2.45 ± 0.63 and 0.94 ± 0.05 , respectively. In type 2 diabetes patients aged 35-56 years $K_{\Delta L}$ was 2.63 ± 0.59 and K_{sym} was 0.88 ± 0.11 . In the oldest age group (type 2 DM, 57-83 year olds) these coefficients were 2.51 ± 0.64 and 0.9 ± 0.07 , respectively.

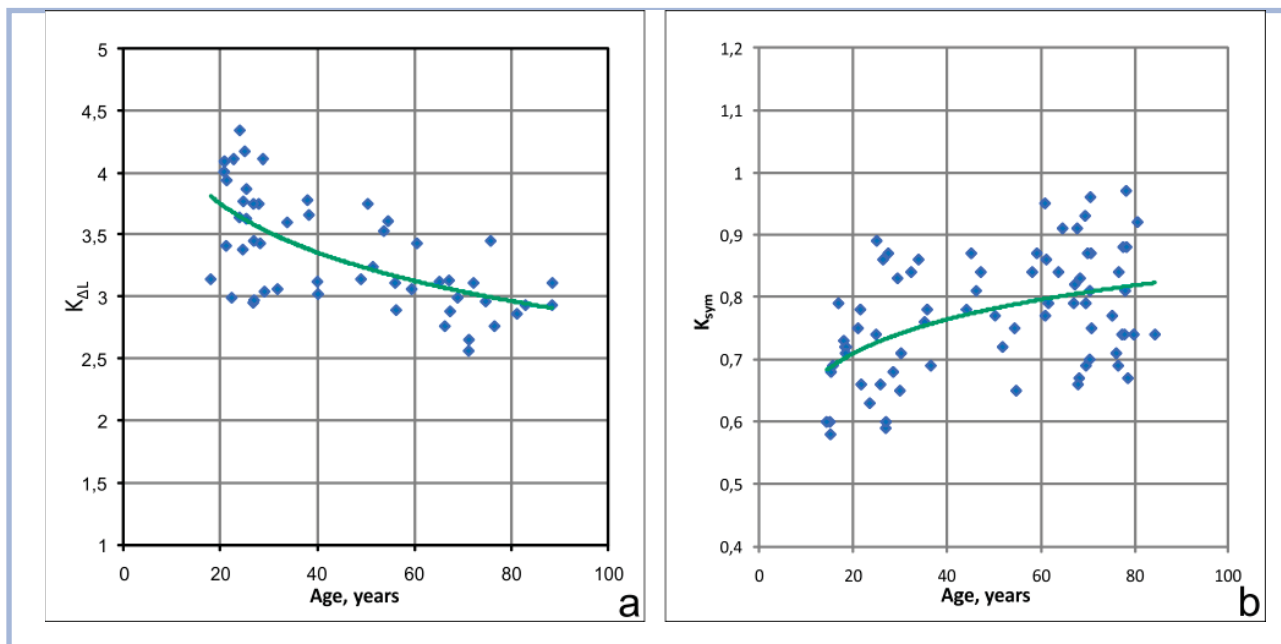


Fig. 6. Dependency of coefficients of CNF orientation anisotropy (a) and symmetry (b) from subject age in healthy population.

Table 2. Mean values of the coefficient of CNF orientation anisotropy ($M \pm \delta$)

| Group | Age, years | | |
|--------------------|------------|-----------|-----------|
| | 13–34 | 35–56 | 57–83 |
| Healthy volunteers | 3.92±0.82 | 3.45±0.85 | 2.71±0.74 |
| Type 1 diabetes | 2.49±0.79 | 2.45±0.63 | — |
| Type 2 diabetes | — | 2.62±0.59 | 2.51±0.64 |

Table 3. Mean values of the coefficient of CNF orientation symmetry ($M \pm \delta$)

| Group | Age, years | | |
|--------------------|------------|-----------|-----------|
| | 13–34 | 35–56 | 57–83 |
| Healthy volunteers | 0.8±0.07 | 0.84±0.11 | 0.87±0.11 |
| Type 1 diabetes | 0.89±0.11 | 0.94±0.05 | — |
| Type 2 diabetes | — | 0.88±0.11 | 0.9±0.07 |

Typical rose diagrams of CNF orientation obtained from healthy and diabetic subjects are presented in **figure 7**. The latter has a distinct isometric shape approximating a circle, which is due to multiple CNF orientations. Normal diagram is markedly elongated representing mostly unidirectional passage of corneal nerve fibers.

We suppose that vascular disorders, accumulated products of glucose anabolism, decreased nerve growth factor level (or activity), oxidative stress, along with genetic abnormalities and hereditary factors may all contribute to changes in $K_{\Delta L}$ and K_{sym} in diabetes patients [29, 30].

A strong inverse correlation has been found between the coefficient of CNF orientation anisotropy and glycated hemoglobin levels ($r=-0.83$, $p<0.001$ and $r=-0.79$, $p<0.005$ in patients with type 1 and type 2 diabetes respectively) as well as the duration of the disease ($r=-0.7$, $p<0.005$ and $r=-0.72$, $p<0.001$, type 1 and type 2 diabetes respectively). On the contrary, the coefficient of CNF orientation symmetry has been shown to be directly correlated with both glycated hemoglobin levels ($r=0.64$, $p<0.005$ and $r=0.78$, $p<0.05$, type 1 and type 2 diabetes respectively) and the duration of the disease ($r=0.62$, $p<0.05$ and $r=0.73$, $p<0.05$, type 1 and type 2 diabetes re-

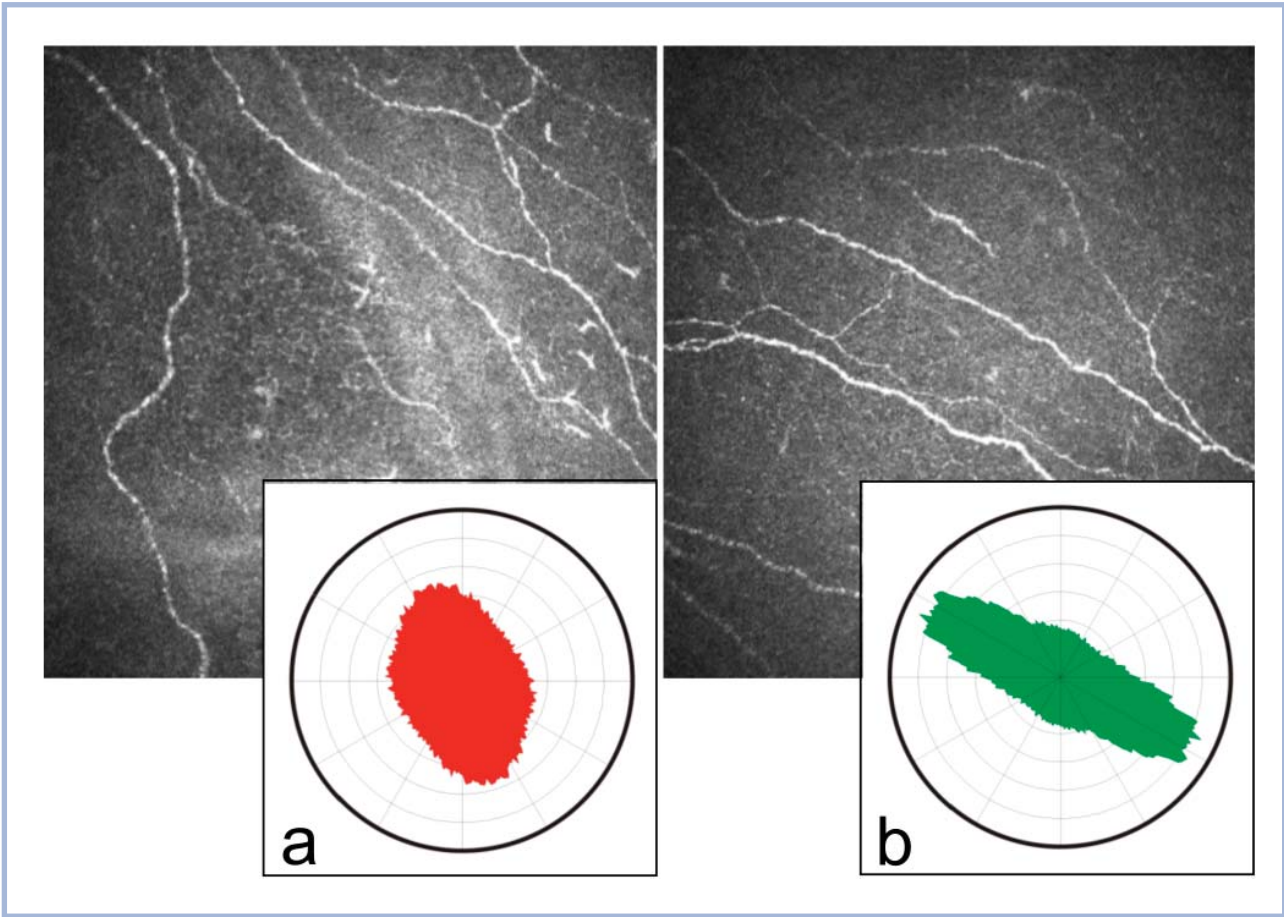


Fig 7. Typical confocal microscope images of corneal nerve fibers in type 1 diabetes (a) and health (b) and corresponding rose diagrams (explained in the text).

spectively). This can be summed up as follows: the higher glycemia (which implies poor compensation of the disease) the lower $K_{\Delta L}$ and the higher K_{sym} . It is possible that high correlation with current glucose levels indirectly points to CNF trophism being involved in diabetes.

Several prospective multicenter studies, namely DCCT (1993-1995), DEKAN (1995), ALADIN 1, ALADIN 2, ALADIN 3 (1995–2000), brought out the key role of glycemia in DPN development. Its occurrence appeared equal in type 1 and type 2 diabetes patients with high glycated hemoglobin levels, despite the differences in disease pathogenesis [6]. In this study, mean values of $K_{\Delta L}$ have been found to be lower in type 1 diabetes patients than in type 2 diabetes patients of the same age group (2.45 ± 0.63 and 2.62 ± 0.59 , respectively), the difference, however, being not statistically significant ($r=0.34$, $p<0.05$). Among variously aged subjects, lower $K_{\Delta L}$ values were also associated with type 1 diabetes, whereas lower K_{sym} values — with type 2 diabetes, despite ‘superimposed’ age-related changes in the latter group. For example, in 57-83 year-old patients with type 2 diabetes $K_{\Delta L}$ and K_{sym} were 2.51 ± 0.64 and 0.9 ± 0.07 , respec-

tively, and in type 1 diabetes patients — 2.49 ± 0.79 and 0.89 ± 0.11 , respectively. A graph of the dependency between the two coefficients and patient age is provided in **figure 8**.

Moreover, considerable inter-eye asymmetry in both coefficients has been shown for diabetes patients, but hardly any — for healthy volunteers (**Fig. 9**). Precisely speaking, in type 1 and type 2 diabetes groups the inter-eye asymmetry indices (*IAI*) were 0.78 ± 0.26 and 0.65 ± 0.14 for $K_{\Delta L}$ and 0.08 ± 0.03 and 0.06 ± 0.03 for K_{sym} , respectively. In the controls, *IAIs* for $K_{\Delta L}$ and K_{sym} were as low as 0.17 ± 0.09 and 0.02 ± 0.01 . As for the weighted mean difference, it did not normally exceed 3% for both coefficients. In addition to that, DM groups have demonstrated a direct correlation between the asymmetry index and the duration of the disease ($r=0.44$, $p<0.05$ for type 1 diabetes, $r=0.35$, $p<0.05$ for type 2 diabetes).

Conclusions

For the first time age-related changes in corneal nerve fibers and those that can well be diabetes-related

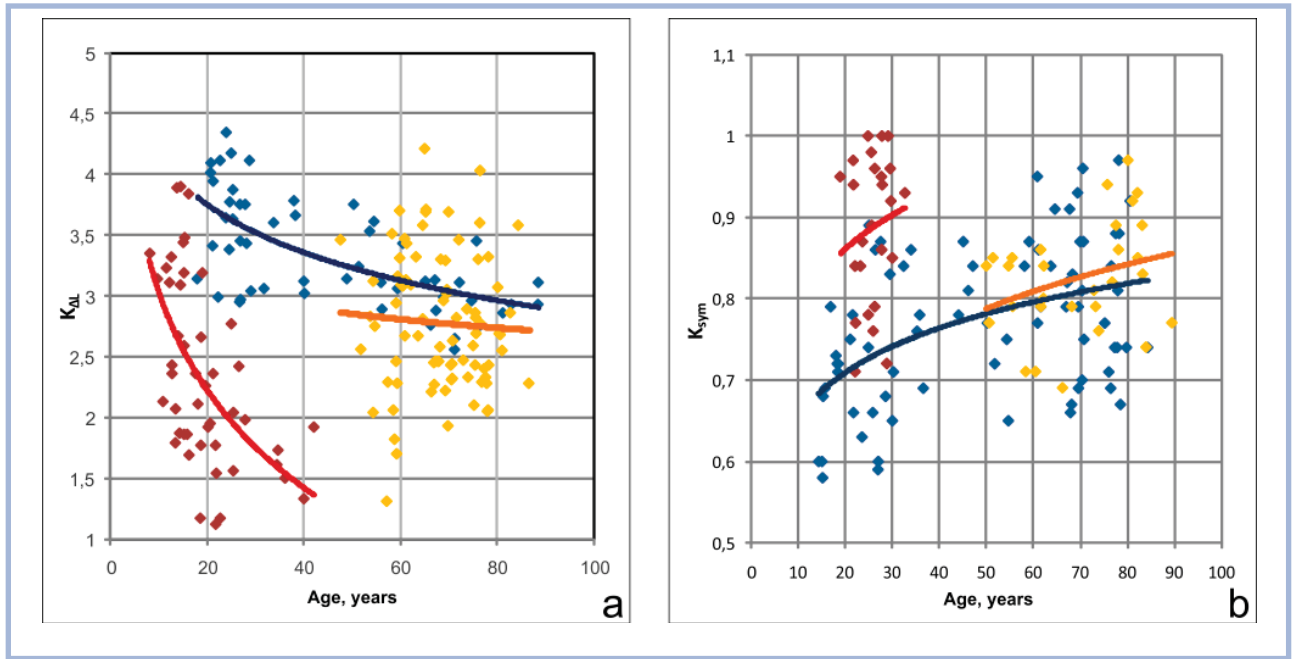


Fig. 8. Distribution of coefficients of CNF orientation anisotropy ($K_{\Delta L}$) and symmetry (K_{sym}) depending on subject age in healthy participants as well as type 1 and type 2 diabetes patients.

Blue dots indicate the norm, red – type 1 diabetes, orange – type 2 diabetes.

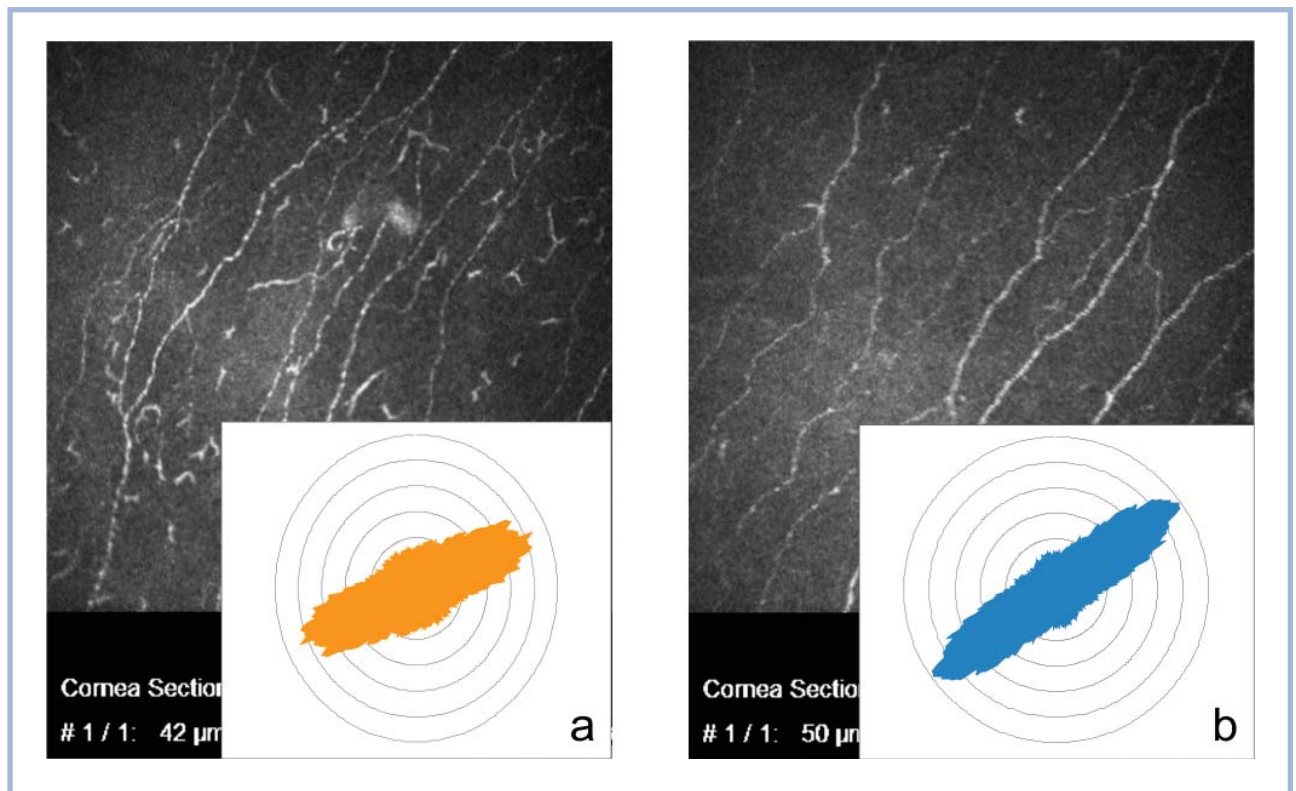


Fig. 9. Confocal microscope images and rose diagrams of CNF orientation obtained from both eyes of a patient with a 9-year history of DM. The diagram from the right eye (a) appears isometric as compared to that of the left eye (b), which is unidirectional. The difference in $K_{\Delta L}$ and K_{sym} between the fellow eyes is 1.36 and 0.04, respectively.

have been studied in a sufficiently large population using an original method of morphometric analysis, which implies assessment of CNF orientation symmetry and anisotropy.

Normally, aging is associated with increased tortuosity and decreased orientation anisotropy of corneal nerve fibers. In diabetes, these trends get more prominent and, moreover, a strong inverse correlation exists between the coefficient of CNF orientation anisotropy, on the one hand, and glycated hemoglobin levels as well as the duration of the disease, on the other. As for the coefficient of CNF orientation symmetry, the correlation revealed is the opposite.

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Study conception and design — S.A., Z.S., I.N.
Acquisition and handling of data — Z.S, I.N., S.M.
Statistical analysis of data — Z.S., I.N.
Drafting of manuscript — Z.S., I.N.
Critical revision — S.A.

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