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Quantitative analysis of corneal nerve fibers in type 2 diabetics with and without diabetic peripheral neuropathy: Comparison of manual and automated assessments

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ABSTRACT

Aims: To examine and compare fully-automated and manually measured corneal nerve fiber parameters in type 2 diabetes mellitus (T2DM) patients with and without diabetic peripheral neuropathy (DPN).

Methods: A total of 128 T2DM subjects and 24 healthy controls underwent neuropathy assessment and bilateral corneal confocal microscopy (CCM). Five representative nerve fiber images were selected for each participant and analyzed manually and with fully-automated software. Corneal nerve fiber length (CNFL), branch density (CNBD), and fiber density (CNFD) were examined.

Results: Manual and full-automated methods for the whole cohort were significantly positive correlated for CNFL, CNBD and CNFD ($r = 0.818, 0.845, 0.457$, all $P < 0.001$). Analysis of agreement between the two measurements using Bland-Altman method showed a bias of 2.05 mm/mm^2 (95% limits of agreement: −2.03 mm/mm^2, 6.13 mm/mm^2), 1.62 no./mm^2 (95% limits of agreement: −17.92 no./mm^2, 21.17 no./mm^2), and 16.0 no./mm^2 (95% limits of agreement: −0.14 no./mm^2, 32.14 no./mm^2) for CNFL, CNBD and CNFD respectively. A progressive decrease in manual and full-automated CNFL, CNBD and CNFD accompanied with the occurrence of DPN, The fully-automated method slightly underestimated corneal nerve fiber parameters.

Conclusions: This study demonstrated strong correlations between manual and fully-automated CNFL and CNBD, but not CNFD. Fully-automated corneal nerve fiber parameter quantification may be a fast, objective way to detect DPN.

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1. Introduction

Diabetic peripheral neuropathy (DPN) is the most common chronic complication of DM with an incidence of up to 50% [1]. Early diagnosis and intervention are essential for the prevention and treatment of DPN. Small nerve fibers are usually the first to be damaged [2,3] and repaired [4,5] in DPN. Currently, clinically used DPN screening methods (e.g., ankle jerk reflex, 10 g Semmes-Weinstein monofilament examination (SWME), vibration sensation, and nerve electrophysiology) are mainly used to assess myelinated and large fiber neuropathy, and most of the patients diagnosed with DPN using these tests are no longer in the early stage [6]. Quantitative sensory testing (QST) and intraepidermal nerve fiber density (IENFD) measurements are commonly used to detect small fiber neuropathy, but these are subjective and invasive [7]. Therefore, there is an urgent clinical need for new techniques that detect small fiber injury in the early stage of DPN.

Corneal confocal microscopy (CCM) is a relatively new, non-invasive technology that can visualize corneal nerves and accurately detect small fiber neuropathy [8]. Recent studies have shown that CCM can be used to quantitatively analyze corneal sub-basal l nerves in DM patients for early diagnosis and disease assessment of DPN [9,10]. Currently, quantitative analysis corneal nerve parameters from images obtained by CCM is mostly based on manual and semiautomatic measurements [11–13]. Although both methods have high reproducibility by using the same set of ground rules, they are time consuming, laborious, and require considerable expertise. Therefore, fully-automated, objective, reproducible, quantitative corneal nerve fiber analysis techniques are needed for DPN evaluation.

ACCMetrics is a fully-automated corneal nerve analysis software program that was developed by investigators from the University of Manchester [14]. It has the advantages of being easy to learn and use. Several previous studies have examined correlations between manual and fully-automated techniques [15–17]. However, fewer than 100 samples have been evaluated using fully-automated analysis and studies have been mostly limited to type 1 DM patients. Additionally, fully-automated and manually measured corneal nerve parameters needed to evaluate DPN have not been examined and compared in a Chinese population. Therefore, this study examines and compares corneal nerve quantitative measurements made with both manual and fully-automated techniques in subjects with T2DM. The repeatability of each type of measurement was also examined.

2. Methods

2.1. Study population

In the present study, we included 128 patients with type 2 diabetes mellitus (T2DM) and 24 healthy controls. All subjects had written informed consent to participate in the study. Inclusion criteria include the following: (1) established T2DM diagnosed according to the 1999 World Health Organization diagnostic criteria (fasting plasma glucose $\geq$ 7.0 mmol/L and/or 2-h plasma glucose $\geq$ 11.1 mmol/L). Exclusion criteria included: (1) a history of wearing corneal contact lenses or eye trauma or surgery; (2) acute infectious disease or a history of cerebral infarction, connective tissue disease, cervical and lumbar lesions (such as nerve root compression, spinal stenosis or degeneration of the cervical and lumbar spine); (3) other causes of peripheral neuropathy (i.e., vitamin B12 or folic acid deficiency, Guillain-Barré syndrome, drug-induced neurotoxicity or nerve injury induced by renal or liver insufficiency as a result of metabolic poisoning). The details of our study design have been previously reported [18].

2.2. Demographic, medical and laboratory data

Data were collected on subjects' age, gender, height, body weight, T2DM duration. All participants underwent measurement of body mass index (BMI), waist circumference, systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting blood glucose levels (FBG), glycated hemoglobin levels (HbA1c), total cholesterol levels (TCh), and triglyceride levels (TG).

2.3. CCM examination

Laser scanning CCM was conducted using the Heidelberg Retina Laser Tomograph II (HRTII) with Rostock cornea module (Heidelberg Engineering GmbH, Heidelberg, Germany). Laser wavelength was 670 nm; field of vision, 384 $\times$ 384 μm; transverse and vertical resolution, 1 μm; and magnification, 800×. The right eye of each subject was selected as the eye to be tested. Before the examination, precautions were explained to the subject in detail. The eye to be examined was first topically anesthetized with 4% iobuca hydrochloride. The subjects were then seated in front of the operating platform, and their chins and foreheads were fixed by chin and forehead rests under the microscope. The subjects were asked to focus their eyes on the indicator light. Focal plane depth was pre-assigned as 0 mm. Examination siteswere the central cornea and the cornea 2 mm interior to the limbus. The lens was pulled until it made contact with the cornea, after which the focal plane was rotated to alter its depth. At least 100 frames of images were obtained during each examination.

2.4. Image analysis

The 5 clearest and most representative nerve fiber images were manually selected based on image depth, focal position, and contrast. Two nerve fiber image analysis methods were used for image analysis, and the following parameters were calculated: (1) CNFL (corneal nerve fiber length, mm/mm^2): the combined length of all nerve fibers per square millimeter, (2) CNBD (corneal nerve branch density, no./mm^2): the number of branch nerves sent by the nerve trunk per square millimeter and (3) CNFD (corneal nerve fiber density, no./mm^2):

the number of all nerve fibers per square millimeter. All analyses were performed by a single experienced researcher.

Manual analyses were performed using the Simple Neurite Tracer plugin of the Fiji high-definition image analysis software package (based on ImageJ, National Institutes of Health, Bethesda, MD). Manual measurements are denoted with a subscripted M (i.e., $CNFL_M$, $CNBD_M$, and $CNFD_M$).

Fully-automated measurements were calculated using the ACCMetrics analysis software package (MA Dabbah, Imaging Science, University of Manchester, Manchester, UK), which automatically analyzes nerve fiber images. Corneal nerve fiber quantification with fully automatic analysis software involves two steps. First, CCM images were enhanced and neural fibers were identified. Second, corneal nerve fiber morphology was quantified. Automatic measurements are denoted with a subscripted A (i.e., $CNFL_A$, $CNBD_A$, and $CNFD_A$).

2.5. DPN diagnosis

DPN was assessed using the modified diagnostic criteria released by the Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes (NEURODIAB) in 2009 [19] and Consensus Statement of the joint 8th International Symposium on Diabetic Neuropathy in Toronto, Canada. According to these criteria, patients were diagnosed with DPN if they (1) had definite medical history of diabetes; (2) developed neuropathy at or after diagnosis of diabetes; (3) had clinical symptoms and signs consistent with DPN; (4) presented abnormal findings on at least two of the tests mentioned in the supplement (temperature sensation, vibration sensation, ankle jerk reflex, SWME, nerve electrophysiology); and (5) did not have nerve root compression, spinal stenosis, cervical or lumbar spine degeneration, cerebral infarction, Guillain-Barre´ syndrome, severe arteriovenous lesions, drug-induced neurotoxicity or renal or liver insufficiency.

2.6. Statistical analysis

Data are presented as mean ± standard deviation. ANOVA method or a non-parametric counterpart, Kruskal-Wallis were used to assess differences between groups depending on normality of the data. Chi squared analyses were used to assess frequencies of gender. Between-method correlations and agreements were assessed using Spearman's rank correlation coefficients and Bland-Altman analyses, respectively. Statistical analyses were performed using Stata version 13.0 and GraphPad Prism version 6.0 statistical software. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Subject characteristics

The demographic and clinical characteristics of patients with diabetes and control subjects are summarized in Table 1. There was no statistically significant difference among groups in gender, BMI, waist circumference, or TG. However, diabetic subjects with DPN (70.2 ± 7.3 years) were significantly older than control subjects (68.6 ± 5.2 years, $P < 0.001$) and diabetic subjects without DPN (67.12 ± 6.01, $P < 0.001$). Additionally, diabetes duration was significantly greater in subjects with DPN (12.6 ± 7.3 years) than in subjects without DPN (9.8 ± 7.1 years, $P < 0.001$). Both FBG ($P < 0.001$) and HbA1c ($P < 0.001$) levels were significantly higher in diabetic patients compared with control subjects with no difference between T2DM patients with and without DPN.

3.2. Correlations and agreements between manual and fully-automated methods

Manual and fully-automated CNFL ($r = 0.818$), CNBD ($r = 0.845$), and CNFD ($r = 0.457$) measurements were significantly and positively correlated (all $P < 0.001$) (Fig. 1a–c). Analysis of agreement between the two measurements using Bland-Altman method (Fig. 1d-f) showed a bias of 2.05 mm/mm^2 (95% limits of agreement: −2.03 mm/mm^2, 6.13 mm/mm^2) for CNFL, 1.62 no./mm^2 (95% limits of agreement: −17.92 no./mm^2, 21.17 no./mm^2) for CNBD, and 16.0 no./mm^2 (95% limits of agreement: −0.14 no./mm^2, 32.14 no./mm^2) for CNFD.

3.3. Comparison of corneal nerve fiber parameters in subjects with and without diabetic peripheral neuropathy

The $CNFL_M$ was significantly higher in control subjects than in T2DM subjects with ($P < 0.001$) and without ($P = 0.012$) DPN. Additionally, $CNFL_M$ in the DPN group was significantly lower than in the non-DPN group ($P = 0.010$). The $CNBD_M$ was also significantly higher in control subjects than in diabetic subjects with ($P < 0.001$) and without ($P = 0.036$) DPN. Additionally, $CNBD_M$ was significantly lower in the DPN group than in the non-DPN group ($P = 0.006$). There was a downward trend in $CNFD_M$ among the three groups, but the difference was not statistically significant ($P = 0.298$).

There was no statistically significant difference between the control and non-DPN groups in $CNFL_A$ (13.37 ± 3.65 vs. 14.66 ± 2.31 mm/mm^2, $P = 0.103$) or $CNBD_A$ (32.96 ± 19.30 vs. 36.20 ± 12.87 branches/mm^2, $P = 0.136$). However, both $CNFL_A$ and $CNBD_A$ were significantly lower in the DPN group than in either the control (both $P < 0.001$) or non-DPN groups ($P = 0.018$, and 0.002, respectively). Additionally, $CNFD_A$ was significantly higher in the control group than in both the non-DPN ($P = 0.012$) and DPN ($P < 0.001$) groups. All results are presented in Table 2.

4. Discussion

CCM is a relatively new, non-invasive technology that can detect small fiber neuropathy accurately in patients with diabetes. The commonly used CCM nerve fiber analysis methods at present are time consuming, laborious, and depend on the professional level of analysts. Therefore, to overcame this problem, several research groups have developed fully automated nerve fiber analysis software [14,20,21]. This study examined and compared measurements of quantitative corneal nerve fiber parameters using manual and automated CCM image analysis. All manually and automatically

Table 1 – Characteristics of the study populations.

	Controls (N = 24)	Non-DPN(N = 49)	DPN(N = 79)	P value
Male/female	9/15	18/30	46/33	0.059
Age (years)	68.63 ± 5.19	67.12 ± 6.01	70.15 ± 7.34[a,b]	<0.001
Diabetes duration (years)	–	9.79 ± 7.09	12.58 ± 7.28[b]	<0.001
BMI (kg/m^2)	25.41 ± 3.57	24.56 ± 2.93	25.38 ± 3.40	0.418
Waist circumference (cm)	86.38 ± 11.71	87.68 ± 8.28	88.80 ± 9.01	0.477
FBG (mmol/L)	5.97 ± 1.54	8.14 ± 2.41[a]	8.83 ± 2.69[a]	<0.001
HbA1c (%)	5.88 ± 0.82	7.07 ± 0.96[a]	7.94 ± 1.86[a]	<0.001
Tch (mmol/L)	4.89 ± 0.91	5.39 ± 1.07	4.91 ± 1.23[b]	0.030
TG (mmol/L)	1.62 ± 0.89	2.00 ± 0.90	1.93 ± 1.43	0.054

Data are presented as means ± standard deviations or n.

Abbreviation: BMI, body mass index; HbA1c, glycosylated hemoglobin; FBG, fast blood glucose; TC, total cholesterol; TG, triglycerides.

[a] Compared to healthy controls.

[b] Compared to T2DM patients without DPN.

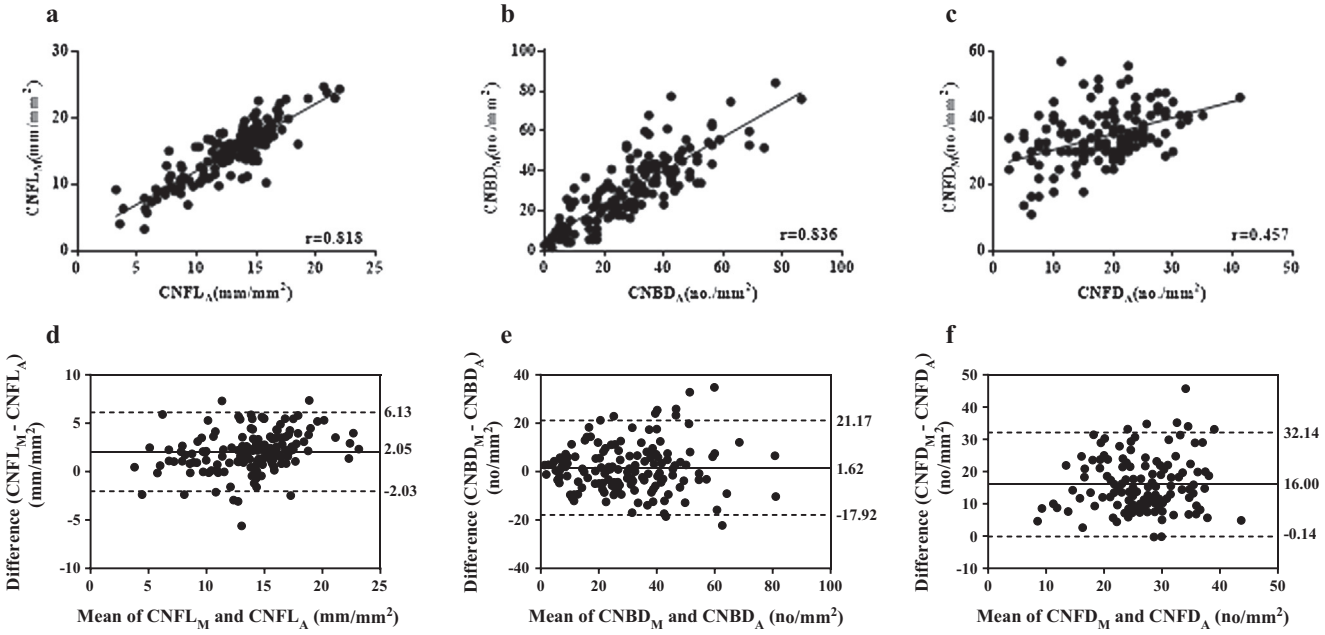

Fig. 1 – Correlations and agreements between manual and fully-automated methods of CNFL, CNBD and CNFD quantification in the 152 study participants. The first row contains plots of CNFL$_M$ against CNFL$_A$ (a), CNBD$_M$ against CNBD$_A$ (b) and CNFD$_M$ against CNFD$_A$ (c), r refers to Spearman's rank correlation coefficient. The second row contains Bland-Altman plot for CNFL (d), CNBD (e) and CNFD (f) indicating the level of agreement between full-automated and manual measurements. The continuous lines indicate the mean difference (=bias). The dashed lines indicate the 95% limits of agreement.

Table 2 – Corneal nerve morphology in controls and in T2DM with and without diabetic peripheral neuropathy (DPN).

CCM paremeters	Controls	Non-DPN	DPN	P value
CNFL$_M$ (mm/mm^2)	17.81 ± 3.19	15.48 ± 3.66[a]	13.60 ± 4.15[a,b]	<0.001
CNBD$_M$ (no./mm^2)	41.48 ± 16.50	33.02 ± 17.60[a]	25.03 ± 15.95[a,b]	<0.001
CNFD$_M$ (no./mm^2)	35.32 ± 5.55	35.68 ± 7.64	33.51 ± 8.96	0.298
CNFL$_A$ (mm/mm^2)	14.66 ± 2.31	13.37 ± 3.65	11.92 ± 3.51[a,b]	0.005
CNBD$_A$ (no./mm^2)	36.20 ± 12.87	32.96 ± 19.30	23.66 ± 15.60[a,b]	<0.001
CNFD$_A$ (no./mm^2)	23.18 ± 5.77	18.98 ± 7.21[a]	16.88 ± 7.39[a]	0.002

Data are presented as means ± standard deviations.

Abbreviation: CNFL, corneal nerve fiber length; CNBD, corneal nerve branch density; CNFD, corneal nerve fiber density.

[a] Compared to healthy controls.

[b] Compared to T2DM patients without DPN.

measured nerve parameters were significantly correlated. Additionally, our results show that both manual and fully-automated measurements can effectively distinguish between subjects with and without DPN. Our results are consistent with those of prior studies [8–10], which also showed a progressive decline in corneal nerve fiber parameters as DPN developed.

In our study, the Bland-Altman plots showed high agreement in CNFL and CNBD values and weak agreement in CNFD values between manual and fully-automated measurements. Fully automatic and manual analyses show significant correlations among CNFL, CNBD and CNFD detection; These findings are in agreement with prior studies. Schaldemose et al. [22] found that the two analytical methods have strong correlations and reported that the correlation coefficients of CNFL and CNBD between the two methods were 0.92 and 0.86, respectively. Chen et al. [21] reported that the correlation coefficients of CNFL, CNBD, and CNFD between the two methods were 0.86, 0.70, and 0.86, respectively.

Studies have shown that CNFD can accurately reflect damage of small nerve fibers and fairly reflect repair of nerve fibers [23,24]. Some studies have shown that manual analysis of CNFD has the best reproducibility [13]. Our study also found a significant positive correlation of CNFD between manual and automated measurements, but the correlation was weaker than those of CNFL and CNBD. The mean CNFD difference was as large as 15.9 no./mm^2, which reflected poorer consistency.

The current study found that automated corneal nerve fiber measurements were slightly lower than corresponding manual measurements. This is consistent with the findings of Dehghani et al. [15], who reported a mean CNFL difference between measurements of 0.9 mm/mm^2, and Ostrovski et al. [17] And Pacaud et al. [25] who found that manual parameter measurements were consistently larger than automated ones (mean CNFL difference of 3.4 and 6.7 mm/mm^2, respectively). This discrepancy may have been caused by incorrect marking of nerve fiber trajectories in manual analyses that resulted in corneal nerve fiber parameter overestimation [21]. Furthermore, fully-automated analyses do not detect nerve fibers with low background contrast [16]. Therefore, fully-automated measurements can underestimate corneal nerve fiber parameters However, fully-automated analyses greatly reduce image analysis time, making it more feasible to use corneal nerve fiber measurements in the clinical setting. Petropoulos et al. [16] found that automated analyses reduced single CCM image analysis time from 2 to 7 min for manual assessment to 10–22 s.

This study had several limitations. First, it had a relatively small sample size (152 subjects). Second, single image examination time was not examined for either automated or manual measurements. Therefore, further prospective studies that include a larger number of subjects are needed to better understand how CCM image analyses can help diagnose and stage DPN in diabetic patients. In conclusion, this is the first study to show strong correlations between fully- automated and manual CCM corneal nerve fiber analyses in a Chinese Han population. Both manual and automated measurements can effectively distinguish between subjects with and without DPN. Although fully-automated analyses slightly underestimate corneal parameters, it has overwhelming advantages of speed, objectivity and consistency over the manual analysis method in analyzing CCM nerve fiber images, which is beneficial when promoting the use of CCM to detect DPN both in clinic and multi-centrals.

5. Author contributionsxx

Qingchun Li and Ying Zhong wrote the manuscript. Ruiyun Zhang, Qi Zhang, Hangping Zheng and Lijin Ji collected the data. Tiansong Zhang, Wanwan Sun and Xiaoming Zhu analyzed the data. Shuo Zhang and Xiaoxia Liu revised the study and manuscript. Qian Xiong and Bin Lu conducted the study design and quality control. All authors read and approved the final manuscript.

6. Declarations of interest

No potential conflicts of interest relevant to this article were reported.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2019.03.039.

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