

# Diabetic Neuropathy Is Characterized by Progressive Corneal Nerve Fiber Loss in the Central and Inferior Whorl Regions

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**Received:** October 1, 2019

**Accepted:** January 20, 2020

**Published:** March 30, 2020

Citation: Ferdousi M, Kalteniece A, Petropoulos I, et al. Diabetic neuropathy is characterized by progressive corneal nerve fiber loss in the central and inferior whorl regions. *Invest Ophthalmol Vis Sci.* 2020;61(3):48. <https://doi.org/10.1167/iovs.61.3.48>

**PURPOSE.** We hypothesized that longitudinal changes in corneal nerve morphology would differ between the central cornea and inferior whorl in relation to other measures of diabetic neuropathy.

**METHODS.** Thirty patients with diabetes (age:  $54.08 \pm 15.86$ , duration:  $23.95 \pm 14.2$ , HbA1c:  $7.51 \pm 1.37$ ) and 19 age-matched healthy controls (age:  $49.47 \pm 13.25$ ) underwent assessment of neuropathy disability score (NDS), vibration perception threshold (VPT), cold (CPT) and warm (WPT) perception thresholds, peroneal motor nerve conduction velocity (PMNCV), corneal nerve fiber density (CNFD), branch density (CNBD), fiber length (CNFL), inferior whorl length (IWL), and the average of CNFL and IWL (ANFL) at baseline and after 1 to 8 years.

**RESULTS.** In patients with diabetes, between baseline and follow-up, there was a significant reduction in CNBD ( $57.72 \pm 30.08$  vs.  $44.04 \pm 23.69$ ;  $P = 0.02$ ), CNFL ( $21.77 \pm 5.19$  vs.  $15.65 \pm 4.7$ ;  $P < 0.0001$ ), IWL ( $24.69 \pm 8.67$  vs.  $14.23 \pm 6.13$ ;  $P < 0.0001$ ), ANFL ( $23.26 \pm 5.53$  vs.  $15.09 \pm 4.48$ ;  $P < 0.0001$ ), and WPT ( $43.56 \pm 4.43$  vs.  $40.78 \pm 4.93$ ;  $P = 0.01$ ), and an increase in VPT ( $12.9 \pm 8.96$  vs.  $13.78 \pm 8.99$ ;  $P = 0.02$ ). There was no significant change in CNFD ( $27.12 \pm 8.2$  vs.  $25.43 \pm 7.11$ ;  $P = 0.2$ ), NDS ( $3.38 \pm 3.35$  vs.  $2.61 \pm 2.8$ ;  $P = 0.08$ ), CPT ( $17.7 \pm 10.59$  vs.  $22.45 \pm 9.23$ ;  $P = 0.06$ ), or PMNCV ( $42.4 \pm 4.21$  vs.  $42.16 \pm 6.3$ ;  $P = 0.2$ ).

**CONCLUSIONS.** There is evidence of corneal nerve loss in patients with diabetes, particularly at the inferior whorl during follow-up.

**Keywords:** corneal nerves, diabetes mellitus, corneal confocal microscopy

Corneal confocal microscopy (CCM) is a rapid noninvasive ophthalmic imaging technique to quantify corneal nerve morphology,<sup>1</sup> and corneal nerve loss is related to the severity of diabetic neuropathy.<sup>2,3</sup> We have recently demonstrated good diagnostic ability of CCM in a large cohort of patients with diabetic neuropathy.<sup>4</sup> The corneal sub-basal nerve plexus is comprised of unmyelinated nerve fibers, which course from the peripheral cornea to the central cornea, and then radiate to the more distal inferior whorl (IW).<sup>5,6</sup> Most studies have shown a loss of corneal nerves in the central cornea.<sup>3,7,8</sup> However, more recent studies in experimental animals<sup>9</sup> and patients with diabetes<sup>10-13</sup>

have shown a reduction in corneal nerves at the IW in patients without diabetic peripheral neuropathy (DPN) and an association with the severity of painful diabetic neuropathy and poorer quality of life.<sup>11</sup> Furthermore, the IW is an anatomically distinct structure, which allows more accurate assessment of longitudinal changes in the sub-basal nerve plexus.<sup>12</sup>

Previously, very few studies have assessed change in corneal nerve morphology longitudinally.<sup>8,14-16</sup> In the current study, we have compared longitudinal changes in corneal nerve morphology in the central cornea and at the IW in relation to other measures of neuropathy.

## METHODOLOGY

### Study Subjects

Thirty patients with type 1 ( $n = 21$ ) or type 2 ( $n = 9$ ) diabetes and 19 age- and sex-matched healthy participants underwent detailed assessment of peripheral neuropathy and CCM at baseline and follow-up while receiving standard of care for diabetes management. We hypothesized that longitudinal changes in corneal nerve morphology would differ between the central cornea and IW in relation to other measures of diabetic neuropathy.

The follow-up visit for each participant ranged between 1 and 8 years, with an average  $3.6 \pm 1.3$  years for patients with diabetes and  $5.0 \pm 1.75$  years for controls. Patients with a history of or any other cause of neuropathy (malignancy, deficiency of B12 or folate, chronic renal, liver failure, connective tissue or systemic disease), current or active diabetic foot ulceration, previous corneal trauma or systemic disease that affects the cornea, surgery and a history of or current contact lens wear were excluded from the study. Each participant provided informed consent, and the research adhered to the tenets of the Declaration of Helsinki and was approved by Greater Manchester East Research Ethics Committee.

### Clinical and Peripheral Neuropathy Assessment

Body mass index (BMI), blood pressure (BP), (glycated hemoglobin) HbA1c, and lipid profile were assessed in each participant. Neuropathy disability score (NDS) was used to quantify vibration, pinprick, temperature perception, and presence or absence of ankle reflexes.<sup>17</sup> Vibration perception threshold (VPT) was evaluated using a Neurothesiometer (Scientific Laboratory Supplies, Wilford, Nottingham, UK), and cold perception threshold (CPT) and warm perception threshold (WPT) were tested on the dorsolateral aspect of the nondominant foot (S1) using a TSA-II NeuroSensory Analyser (Medoc, Ltd., Ramat-Yishai, Israel). Electrodiagnostic studies were undertaken by a consultant neurophysiologist using a Dantec "Keypoint" system (Dantec Dynamics Ltd., Bristol, UK) equipped with DISA temperature regulator to keep the limb temperature at 32°C to 35°C, and peroneal motor nerve conduction velocity (PMNCV) was tested.

### Ophthalmic Assessment

Examinations of the anterior ocular segment using slit-lamp biomicroscopy, and CCM examination using laser scanning CCM HRT III (Heidelberg Retinal Tomograph III Rostock Cornea Module; Heidelberg Engineering, Heidelberg, Germany) were performed for both eyes according to our established protocol.<sup>10,18</sup>

### Corneal Nerves

We selected six images (three per eye) from the central sub-basal nerve plexus and four images from the IW region and manually quantified corneal nerve morphology using CCMetrics (University of Manchester, Manchester, UK). Images were selected by a single expert in a masked fashion taking into account the quality, depth, and variability using an established protocol.<sup>10,19</sup> We quantified five corneal nerve parameters: corneal nerve fiber density (CNFD: total number

of main nerves per square millimeter) ( $\text{no./mm}^2$ ), corneal nerve branch density (CNBD: total number of branches per square millimeter) ( $\text{mm/mm}^2$ ), corneal nerve fiber length (CNFL: total length of main nerves and nerve branches per square millimeter) ( $\text{mm/mm}^2$ ), inferior whorl length (IWL: total length of nerves per square millimeter) ( $\text{mm/mm}^2$ ), and average nerve fiber length ( $\text{ANFL} = \text{CNFL} + \text{IWL}/2$ ) ( $\text{mm/mm}^2$ ).

### Statistical Analyses

The analysis was carried out using SPSS Version 22.0 for Macintosh (IBM Corporation, Armonk, NY, USA). The Shapiro-Wilk test was employed to assess whether the data were normally distributed. Paired and independent *t*-tests (Wilcoxon matched-pair signed-rank test and Mann-Whitney *U* test for nonparametric, respectively) were used to assess differences between the groups. The Pearson correlation coefficient (Spearman for nonparametric) was calculated to assess the correlations between different variables. All data are expressed as mean  $\pm$  SD.  $P < 0.05$  was considered significant. The sample size calculation was measured using G\*Power software Version 3.1.9.4 for Macintosh (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). With the effect size of 0.5,  $\alpha$  of 0.05, and  $\beta$  of 80%, a minimal sample size of 27 subjects was calculated to establish a change. The graphs were created using GraphPad Prism Version 7.0c for Macintosh (GraphPad Software, La Jolla, CA, USA).

## RESULTS

### Clinical and Neuropathy Assessment

**Baseline.** Patients with diabetes and healthy controls were comparable for age ( $54.08 \pm 15.86$  vs.  $49.47 \pm 13.25$ ;  $P = 0.2$ ), sex ( $P = 0.6$ ), ethnicity ( $P = 0.2$ ), and BMI ( $P = 0.1$ ). The mean duration of follow-up in controls was  $5.0 \pm 1.75$  years, and for patients with diabetes it was  $3.6 \pm 1.3$  years (Table 1). High-density lipoprotein (HDL;  $P = 0.8$ ), systolic ( $P = 0.2$ ) and diastolic ( $P = 0.8$ ) BP were comparable between patients with diabetes and controls. HbA1c was higher and total cholesterol and low-density lipoprotein (LDL; all  $P < 0.0001$ ) and triglycerides ( $P = 0.04$ ) were lower in diabetic patients compared with controls. NDS, VPT, and WPT were higher and CPT and PMNCV were lower in patients with diabetes compared with controls (all  $P = 0.001$ ).

**Controls Baseline Versus Follow-Up.** There was a reduction in total cholesterol ( $P = 0.05$ ), but no change in BMI ( $P = 0.8$ ), HDL ( $P = 0.5$ ), LDL ( $P = 0.1$ ), triglycerides ( $P = 0.7$ ), or systolic and diastolic BP (both  $P = 0.9$ ) between baseline and follow-up. NDS ( $P = 0.1$ ), VPT ( $P = 0.5$ ), CPT ( $P = 0.1$ ), WPT ( $P = 0.4$ ), and PMNCV ( $P = 0.3$ ) did not change between baseline and follow-up (Table 1).

**Diabetic Patients Baseline Versus Follow-Up.** There was a significant reduction in total cholesterol ( $P = 0.02$ ) and LDL ( $P = 0.02$ ), but no change in BMI ( $P = 0.08$ ), HbA1c ( $P = 0.2$ ), HDL ( $P = 0.2$ ), triglycerides ( $P = 0.4$ ), systolic ( $P = 0.9$ ) or diastolic ( $P = 0.08$ ) BP. There was a significant increase in VPT ( $P = 0.02$ ) (change per year  $1.12 \pm 2.75$  V), but decrease in WPT ( $P = 0.01$ ) (change per year  $-0.33^\circ\text{C} \pm 2.19^\circ\text{C}$ ), with no change in NDS ( $P = 0.08$ ), CPT ( $P = 0.06$ ), or PMNCV ( $P = 0.2$ ) (Table 1).

TABLE 1. Demographics and Neuropathy Assessment in Healthy Controls and Patients with Diabetes at Baseline and Follow-Up

	Controls Baseline	Controls Follow-Up	P Value	Patients Baseline	Patient Follow-Up	P Value
Age (y)	49.47 ± 13.25	NA		54.08 ± 15.86	NA	
Sex (F/M)	(7/12)			(11/19)		
Ethnicity (Asian/European)	(4/15)			(3/27)		
Duration of diabetes (y)	0 ± 0	NA		23.95 ± 14.2	NA	
Duration of follow-up (y)	5.0 ± 1.75 (1–8 y)			3.6 ± 1.3 (1–6 y)		
HbA1c (%)	5.37 ± 0.42	5.28 ± 0.24	0.08	7.51 ± 1.37 <sup>‡</sup>	7.14 ± 1.07	0.2
BMI (kg/m <sup>2</sup> )	25.76 ± 3.66	27.26 ± 3.02	0.8	28.81 ± 6.69	28.34 ± 5.8	0.08
Cholesterol (mmol/mL)	4.98 ± 0.86	4.65 ± 0.83	0.05	3.95 ± 0.81 <sup>‡</sup>	3.73 ± 0.63	0.02
HDL (mmol/mL)	1.53 ± 0.31	1.61 ± 0.3	0.5	1.5 ± 0.5	1.47 ± 0.49	0.2
Triglycerides (mmol/mL)	1.58 ± 0.94	1.37 ± 0.51	0.7	1.17 ± 0.58 <sup>‡</sup>	1.32 ± 0.94	0.4
LDL (mmol/mL)	2.74 ± 0.76	2.55 ± 0.66	0.1	1.94 ± 0.57 <sup>†</sup>	1.59 ± 0.49	0.02
Systolic BP (mm Hg)	120.21 ± 16.49	121 ± 16.56	0.9	126.2 ± 15.16	128.56 ± 17.32	0.9
Diastolic BP (mm Hg)	68.95 ± 10.64	68 ± 7.37	0.9	68.63 ± 7.85	65.88 ± 8.63	0.08
VPT (V)	6.83 ± 5.53	6.67 ± 4.21	0.5	12.9 ± 8.96 <sup>†</sup>	13.78 ± 8.99	0.02
CPT foot (°C)	27.08 ± 4.33	22.53 ± 7.37	0.1	17.7 ± 10.59 <sup>‡</sup>	22.45 ± 9.23	0.06
WPT foot (°C)	39.27 ± 3.97	41.38 ± 5.28	0.4	43.56 ± 4.43 <sup>†</sup>	40.78 ± 4.93	0.01
PMNCV (m/s)	48.22 ± 4.1	47.25 ± 4	0.3	42.4 ± 4.21 <sup>‡</sup>	42.16 ± 6.3	0.2
NDS (0–10)	0	0	0.1	3.38 ± 3.35 <sup>†</sup>	2.61 ± 2.8	0.08

All data are presented as mean ± SD. NA, not available.

\* *P* < 0.05 compared with controls baseline.

† *P* < 0.01 compared with controls baseline.

‡ *P* < 0.0001 compared with controls baseline.

TABLE 2. Corneal Nerve Parameters in Healthy Controls and Patients with Diabetes at Baseline and Follow-Up

	Controls Baseline	Controls Follow-Up	P Value	Patients Baseline	Patients Follow-Up	P Value
CNFD (no/mm <sup>2</sup> )	31.37 ± 5.31	31.02 ± 5.4	0.9	27.12 ± 8.2 <sup>†</sup>	25.43 ± 7.11	0.2
CNBD (no/mm <sup>2</sup> )	87.53 ± 29.69	68.49 ± 24.95	0.02	57.72 ± 30.08 <sup>†</sup>	44.04 ± 23.69	0.02
CNFL (mm/mm <sup>2</sup> )	26.07 ± 6.42	21.08 ± 4.52	0.01	21.77 ± 5.19 <sup>†</sup>	15.65 ± 4.7	<0.0001
IWL (mm/mm <sup>2</sup> )	33.6 ± 10.11	29.79 ± 8.93	0.1	24.69 ± 8.67 <sup>†</sup>	14.23 ± 6.13	<0.0001
ANFL (mm/mm <sup>2</sup> )	31.04 ± 6.54	26.11 ± 8.02	0.01	23.26 ± 5.53 <sup>‡</sup>	15.09 ± 4.48	<0.0001

All data are presented as mean ± SD.

\* *P* < 0.05 compared with controls baseline.

† *P* < 0.01 compared with controls baseline.

‡ *P* < 0.0001 compared with controls baseline.

### Corneal Confocal Microscopy

**Baseline.** CNFD (no/mm<sup>2</sup>) (27.12 ± 8.2 vs. 31.37 ± 5.31; *P* = 0.004), CNBD (no/mm<sup>2</sup>) (57.72 ± 30.08 vs. 87.53 ± 29.69; *P* = 0.002), CNFL (mm/mm<sup>2</sup>) (21.77 ± 5.19 vs. 26.07 ± 6.42; *P* = 0.03), IWL (mm/mm<sup>2</sup>) (24.69 ± 8.67 vs. 33.6 ± 10.11; *P* = 0.003), and ANFL (mm/mm<sup>2</sup>) (23.26 ± 5.53 vs. 31.04 ± 6.54; *P* = 0.01) were significantly lower in patients with diabetes compared with controls (Table 2, Fig. 1).

**Controls Baseline Versus Follow-Up.** There was a significant reduction in CNBD (*P* = 0.02), CNFL (*P* = 0.01), and ANFL (*P* = 0.01), but no change in CNFD (*P* = 0.9) or IWL (*P* = 0.1) between baseline and follow-up visits (Table 2, Fig. 2).

**Diabetic Patients Baseline Versus Follow-Up.** There was a significant reduction in CNBD (*P* = 0.02), CNFL (*P* < 0.0001), IWL (*P* < 0.0001), and ANFL (*P* < 0.0001), but no change in CNFD (*P* = 0.2) between baseline and follow-up visits (Table 2, Fig. 2).

**Corneal Nerve Parameter Change per Year.** To measure the average rate of corneal nerve loss, we divided the change between the baseline and follow-up visit by the time between the baseline and follow-up visit. The change

per year in CNFL (mm/mm<sup>2</sup>) (2.08 ± 2.02 vs. 0.5 ± 3.35; *P* = 0.02), ANFL (mm/mm<sup>2</sup>) (2.52 ± 1.51 vs. 0.67 ± 2.27; *P* < 0.0001), and IWL (mm/mm<sup>2</sup>) (3.5 ± 2.91 vs. 0.68 ± 2.21; *P* < 0.0001) was significantly higher in patients with diabetes compared with healthy controls (Table 3). The intraclass correlations showed good consistency between the changes per year in CNFL and IWL in patients with diabetes (intraclass correlation coefficient, 0.78; 95% confidence interval, 0.56–0.88; *P* < 0.0001).

### DISCUSSION

There is currently no consensus as to which corneal nerve parameter has optimal sensitivity and specificity for the diagnosis and assessment of progression or benefit after therapeutic intervention in diabetic neuropathy.<sup>20,21</sup> Earlier studies suggested that CNFD<sup>20,21</sup> may be optimal in the diagnosis of DPN, whereas others have shown that CNFL is a more reliable parameter for both diagnosis and prediction of the development of diabetic neuropathy.<sup>22–24</sup> However, longitudinal studies ideally require quantification of the same corneal nerves over time, and as there is significant

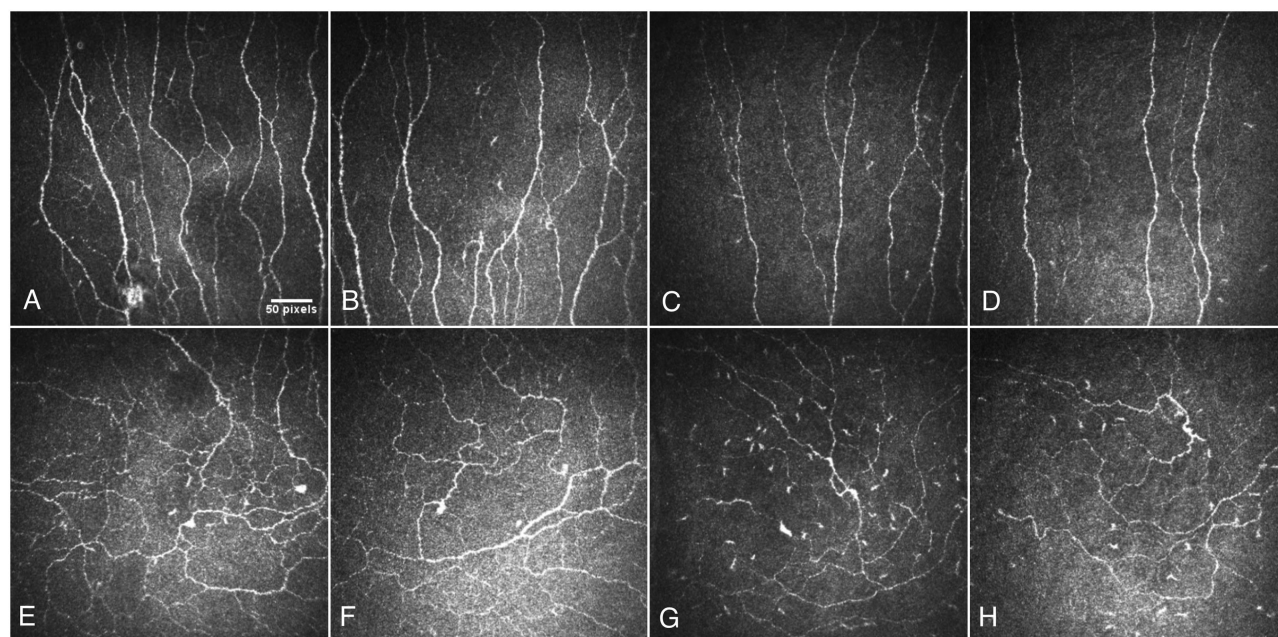


FIGURE 1. CCM images of the central cornea (first row) and IW (second row) in a healthy control at baseline (A, E) and follow-up (B, F), and an age-matched patient with diabetes at baseline (C, G) and follow-up (D, H). Scale bar: 50 pixels.

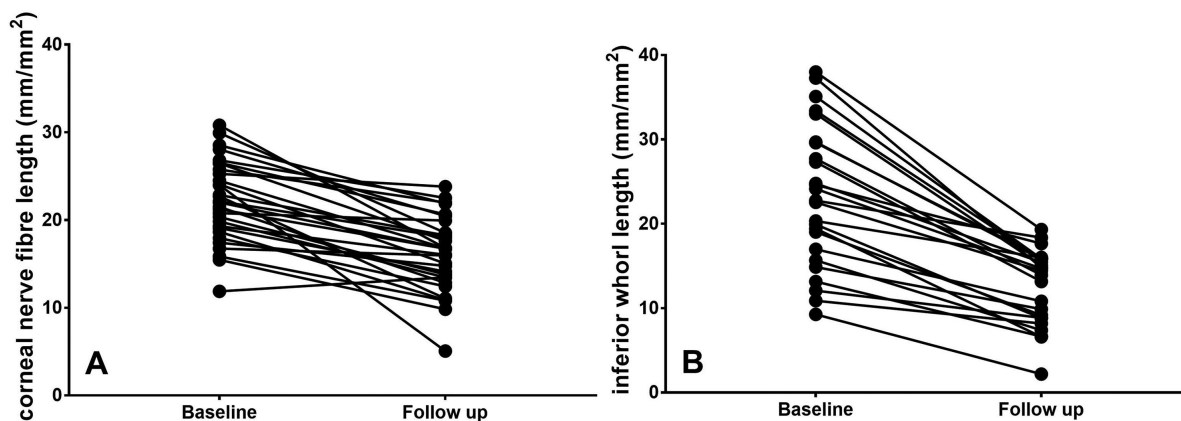


FIGURE 2. (A) CNFL and (B) IWL in patients with diabetes at baseline and follow-up (each dot represents a single patient).

heterogeneity in the central corneal nerve network, it can be difficult to capture the same area.<sup>25</sup>

Quantification of corneal nerve morphology at the IW has several distinct advantages as it assesses an anatomically more distal part of the sub-basal plexus compared with the central nerves,<sup>6</sup> and is therefore more likely to be affected by a dying back neuropathy and it is also a unique landmark

for longitudinal studies. At baseline, all corneal nerve parameters in the central and IW region were reduced in patients with diabetes compared with healthy controls, in agreement with previous studies.<sup>10–12,19</sup> Furthermore, in patients with diabetes a change in more distal nerve fibers as captured by CNBD, CNFL, IWL, and ANFL showed a significant reduction at follow-up, whereas CNFD, a more proximal part of the

TABLE 3. Corneal Nerve Parameter Changes per Year in Healthy Controls and Patients with Diabetes

	Controls Change Per Year	Patients Change Per Year	P Value
CNFD (no/mm <sup>2</sup> )	0.07 ± 0.85	0.28 ± 2.2	0.1
CNBD (no/mm <sup>2</sup> )	3.08 ± 9.21	4.19 ± 7.95	0.9
CNFL (mm/mm <sup>2</sup> )	0.5 ± 3.35	2.08 ± 2.02	0.02
ANFL (mm/mm <sup>2</sup> )	0.67 ± 2.27	2.52 ± 1.51	<0.0001
IWL (mm/mm <sup>2</sup> )	0.68 ± 2.21	3.5 ± 2.91	<0.0001

This table shows the average reduction per year. All data are presented as mean ± SD.

corneal nerve plexus, did not change, indicating that these more distal nerve parameters are more capable of capturing change in CCM over time. Pritchard et al.<sup>8</sup> have also previously demonstrated a reduction in CNFL and CNBD, with no change in CNFD in patients with type 1 diabetes. We also demonstrated a significant reduction in CNFL in healthy controls, consistent with the study of Dehghani et al.<sup>15</sup> who reported a slight reduction in CNFL over 36 months. More recently, we have shown that corneal nerve loss has the greatest predictive value for the development of DPN.<sup>26</sup>

As expected, we also show that the rate of annual decline in CNFL, IWL, and ANFL was significantly higher in patients with diabetes compared with controls.<sup>27</sup> Our previous study also showed a two- to three-fold greater reduction in IWL compared with CNFL in patients with DPN compared with healthy controls.<sup>11</sup>

All other accepted measures of diabetic neuropathy differed from healthy controls. However, over time whereas the controls showed no change, diabetic patients showed a small but significant worsening in VPT with no change in nerve conduction, whereas NDS and CPT showed trends for improvement and WPT improved significantly. This suggests that these measures have significant limitations when assessing longitudinal change, especially in small cohorts of patients with diabetes, especially in early phase 2 clinical trials.

To our knowledge, this is the first longitudinal study comparing corneal nerve loss in the central and IW regions. Our study shows that corneal nerve loss at baseline and over time is greater in the IW compared with the central cornea in controls and patients with diabetes. The reduction in CNFL in control subjects in the present study was greater than that reported by Sharma et al.,<sup>28</sup> but our controls were older, and we assessed CNFL manually.

Cardiovascular risk factors, including glycemic control, BP, and lipids, have been associated with the development of diabetic neuropathy.<sup>29</sup> A large body of experimental and clinical data suggests an important link between dyslipidemia and diabetic neuropathy.<sup>30</sup> A reduction in corneal nerves has been associated with increasing age, HbA1c, and a lower HDL.<sup>14</sup> We have also recently shown that an improvement in corneal nerve morphology after pancreas and kidney transplantation was associated with an improvement in HbA1c and triglycerides.<sup>31</sup> The ADDITION-Denmark study showed that LDL and cholesterol are risk factors for the development of DPN.<sup>32</sup> In the present study, we showed a significant improvement in total cholesterol and LDL attributed to statin treatment, but there was a reduction in all CCM parameters at follow-up.

## CONCLUSIONS

Our study shows greater corneal nerve loss at the IW at follow-up compared with baseline. Furthermore, the minimal change or indeed improvement in accepted measures of diabetic neuropathy questions the utility of these tests in longitudinal and therapeutic studies of diabetic neuropathy.

## Acknowledgments

Supported by the Manchester Biomedical Research Centre and the Greater Manchester Comprehensive Local Research Network. Also supported by the European Union Seventh

Framework Programme FP7/2007-2013 (n°602273), National Institutes of Health (R01DK077903-0101; Bethesda, MD, USA), and Juvenile Diabetes Research Foundation International (27-2008-362). The authors alone are responsible for the content and writing of the article.

Author contributions: MF researched data, performed statistical analysis, and wrote the manuscript; AK researched data, performed statistical analysis, and wrote the manuscript; IP researched data; SA researched data; SD researched data; AM researched data; AJMB reviewed and revised the manuscript; NE reviewed and revised the manuscript; CGF reviewed and revised the manuscript; GL reviewed and revised the manuscript; HS reviewed and revised the manuscript; RAM designed the study and reviewed and revised the manuscript. RAM is the guarantor of this work and, as such, had full access of the data of the study and takes responsibility of the integrity of the data and the accuracy of the data analyses.

Disclosure: **M. Ferdousi**, None; **A. Kalteniece**, None; **I. Petropoulos**, None; **S. Azmi**, None; **S. Dhage**, None; **A. Marshall**, None; **A.J.M. Boulton**, None; **N. Efron**, None; **C.G. Faber**, None; **G. Lauria**, None; **H. Soran**, None; **R.A. Malik**, None

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