Keratocyte Density Is Reduced and Related to Corneal Nerve Damage in Diabetic Neuropathy

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Purpose. The purpose of this study was to assess the relationship between corneal keratocyte density (KD) and corneal nerve damage in patients with and without diabetic peripheral neuropathy.

Methods. Eighty-six patients with type 1 and type 2 diabetes and 21 age-matched control subjects underwent assessment of the neuropathy disability score, quantitative sensory testing, electrophysiology, and corneal confocal microscopy and were divided into those without (DN−) ($n = 22$) and with (DN+) ($n = 64$) diabetic neuropathy. Corneal sub-basal nerve parameters and KD in the anterior, mid, and posterior stroma were quantified.

Results. Anterior, mid, and posterior stromal KD were significantly reduced in DN− ($P = 0.02$, $P = 0.009$, $P = 0.01$, respectively) and DN+ (all $P < 0.0001$) subjects compared to controls. Corneal nerve branch density (CNBD) ($P < 0.0001$, $P < 0.0001$) and corneal nerve fiber length (CNFL) ($P = 0.03$, $P < 0.0001$) were significantly reduced in DN− and DN+ subjects, respectively, and corneal nerve fiber density (CNFD) ($P < 0.0001$) was significantly reduced only in DN+ subjects compared to controls. Anterior, mid, and posterior stromal KD correlated significantly with CNFD ($P = 0.008$, $P = 0.005$, $P = 0.01$), CNBD ($P = 0.01$, $P = 0.006$, $P = 0.001$), and CNFL ($P = 0.04$, $P = 0.008$, $P = 0.003$), respectively.

Conclusions. This study demonstrates a reduction in anterior, mid, and posterior KD, which is associated with corneal sub-basal plexus nerve damage in patients with diabetes.

Keywords: corneal confocal microscopy, diabetic peripheral neuropathy, corneal nerves, keratocytes

Corneal confocal microscopy (CCM) is a rapid, noninvasive ophthalmic imaging technique that has been utilized to quantify corneal nerve degeneration and regeneration in a range of neurodegenerative disorders, particularly diabetic neuropathy (DN).[1,2] CCM has also been utilized to show varying degrees of abnormality in the epithelium, stroma, and endothelium of patients with diabetes.[3] We have previously shown that a reduction in epithelial cell density is related to corneal nerve fiber density (CNFD) in patients with diabetes.[4]

The stroma comprises approximately 90% of the corneal volume and contains keratocytes, collagen fibrils, proteoglycans, ions, and interstitial substance.[5] Keratocytes are a population of mesenchymal cells originally derived from neural-crest cells.[6] They are comparable to fibroblasts and can be observed using CCM as hyperreflective spindle or osteoblast-shaped cells.[7] They play a major role in maintaining corneal transparency, mechanical stability, and corneal repair[6] and are capable of synthesizing collagen, glycosaminoglycans, and matrix metalloproteases (MMPs).[8] Keratocytes form a network of dendritic processes that encircle stromal nerve fibers,[9] and a recent experimental study has shown that activated stromal fibroblasts, derived from stromal keratocytes, induce neurite outgrowth by secreting a range of neurotrophins and cytokines.[10] A reduction in corneal keratocyte density (KD) has been implicated in delayed corneal nerve regeneration after LASIK.[11,12] However, previous studies have reported either a reduction or no change in KD in patients with diabetes.[13–15]

The aim of this study was to quantify anterior, mid, and posterior stromal KD in relation to corneal nerve morphology in patients with and without DN.

Methods

Study Subjects

Eighty-six patients with type 1 and type 2 diabetes and 21 age-matched healthy controls underwent assessment of peripheral neuropathy, slit lamp examination, and CCM. Patients were excluded if they had a history of malignancy, another cause of neuropathy, current or active diabetic foot ulceration, deficiency of B12 or folate, chronic renal or liver failure, connective tissue or systemic disease, corneal trauma, systemic disease that involves the cornea, and cystic corneal disorders. Patients with a history of contact lens wear were excluded from the study. Before participation, informed consent was obtained from each participant. The research was approved by North Manchester

Table 1. Clinical Demographics, Neuropathy Assessment, and CCM in Patients (DN– and DN+) Compared to Controls

	Controls, $n = 21$	DN−, $n = 22$	DN+, $n = 64$
Age, y	57.57 ± 2.4	61.66 ± 1.95	63.85 ± 1.31*
Sex, female/male	12/9	8/14	19/45
Diabetes duration, y	N/A	10.47 ± 1.69§	23.24 ± 2.37
T1DM/T2DM	N/A	5/17	24/40
BP systolic, mm Hg	131.19 ± 3.84	132.35 ± 4.1	135.52 ± 2.25
Cholesterol, mM	5.19 ± 0.18	4.61 ± 0.2*§	3.91 ± 0.11‡
BMI, kg/m^2	27.63 ± 1.01	28.12 ± 1.19	30.52 ± 1.07
HbA1c, %	5.70 ± 0.05	6.43 ± 0.22†	7.26 ± 0.2‡
NCCA, mbars	0.63 ± 0.12	0.56 ± 0.12†	1.06 ± 0.18
CNFD, no./mm^2	29.09 ± 1.19	26.54 ± 1.07§	21.27 ± 0.95‡
CNBD, no./mm^2	96.85 ± 4.42	63.6 ± 7.15‡†	47.74 ± 3.43‡
CNFL, mm/mm^2	28.99 ± 0.87	24.7 ± 1.3*§	19.65 ± 0.89‡
AKD, no./mm^2	650.14 ± 29	540.93 ± 26.53*§	425.19 ± 21.2‡
MKD, no./mm^2	405.5 ± 9.12	363.27 ± 12.17‡†	336.56 ± 6.77‡
PKD, no./mm^2	391.21 ± 10.45	351.9 ± 13.29*†	326.77 ± 5.98‡
PMNCV, m/s	46.77 ± 0.81	43.56 ± 0.74§	38.9 ± 0.87‡
SSNCV, m/s	48.48 ± 1.05	45.4 ± 1.02§	40.47 ± 0.82*
PMNA, mV	4.55 ± 0.3	4.7 ± 0.51§	2.78 ± 0.24‡
SSNA, μV	18.52 ± 2.0	9.56 ± 1.23‡	6.45 ± 0.74‡
CT, °C	27.31 ± 0.55	28.05 ± 0.32§	21.99 ± 1.19‡
WT, °C	38.24 ± 0.8	39.5 ± 0.61§	43.51 ± 0.59‡
VPT, V	7.67 ± 1.27	11.53 ± 1.14†	25.09 ± 1.55*

* $P < 0.05$ compared to controls.
† $P < 0.05$ compared to DN+.
‡ $P < 0.01$ compared to controls.
§ $P < 0.01$ compared to DN+.

Research Ethics Committee and adhered to the tenets of Declaration of Helsinki.

Clinical and Peripheral Neuropathy Assessment

All participants underwent assessment of body mass index (BMI), glycated hemoglobin (HbA1c), and total cholesterol. Neurologic deficits were assessed using the neuropathy disability score (NDS), which includes an assessment of vibration perception, pinprick, temperature sensation, and presence or absence of ankle reflexes.[16] Vibration perception threshold (VPT) was assessed using a neurothesiometer (Scientific Laboratory Supplies, Wilfrod, Nottingham, UK). Cold (CTs) and warm (WTs) thresholds were assessed on the dorsolateral aspect of the left foot using a neurosensory analyzer (TSA-II NeuroSensory Analyzer; Medoc, Ltd., Ramat-Yishai, Israel). A consultant neurophysiologist assessed sural sensory nerve amplitude (SSNA), sural sensory nerve conduction velocity (SSNCV), peroneal motor nerve amplitude (PMNA), and peroneal motor nerve conduction velocity (PMNCV) using a nerve conduction testing machine (Keypoint System; Dantec Dynamics Ltd., Bristol, North Somerset, UK), equipped with a temperature regulator (DISA; Dantec, Denmark) to keep the limb temperature constantly at 32° to 35°C.

Ophthalmic Examination

Participants underwent slit lamp examination, noncontact corneal esthesiometry, and CCM. CCM was performed using laser scanning corneal confocal microscopy HRTIII with a corneal module (HRTIII with Rostock Corneal module; Heidelberg Engineering, Heidelberg, Germany) for both eyes following our previously established protocol.[17] The illumination source is a 670-nm diode laser, a class 1 laser system. The size of the laser beam spot was 1 μm in diameter, and the instrument field of view was 400 × 400 μm with a 63× objective lens. Two-dimensional digital images were obtained with a resolution of 10 μm/pixel and a size of 384 × 384 pixels. A charge-coupled device camera attached to the microscope provided live imaging of the cornea and showed the exact location for the examination. Corneal sensation threshold was assessed using a noncontact corneal esthesiometer (NCCA) (Glasgow Caledonian University, Glasgow, Scotland, UK).

Corneal Nerve Assessment. Six images (three images per eye) from the sub-basal nerve plexus were selected for quality, depth, contrast, and location in the cornea according to our previously published protocol.[18] Corneal nerve fiber density (CNFD) (total number of main nerves per square millimeter; no./mm^2), corneal nerve branch density (CNBD) (number of nerve branches per square millimeter; no./mm^2), and corneal nerve fiber length (CNFL) (total length of main nerves and nerve branches per square millimeter; mm/mm^2) were manually analyzed using image analysis software (CCMetrics; M.A. Dabbah, Imaging Science, The University of Manchester, Manchester, UK).

Keratocyte Density. The anterior stroma was defined as the very first high-quality image below Bowman's layer; the posterior stroma was defined as the very first high-quality image just before the endothelium; and the midstroma was an image between the anterior and posterior stromal images. Keratocytes were defined as hyperreflective spindle or osteoblast-shaped nuclei against a dark background[7] and were manually counted using CCMetrics (Imaging Science, The University of Manchester, Manchester, UK). KD (cells/mm^2) was obtained by counting the number of cells per square millimeter.

Inter- and Intraobserver Variability of KD. To measure the intraobserver variability, KD was measured in a subset of images from 15 patients on two separate occasions by observer 1 within a 1-month interval. To assess the interobserver agreement, two observers (observer 1 and 2) analyzed images

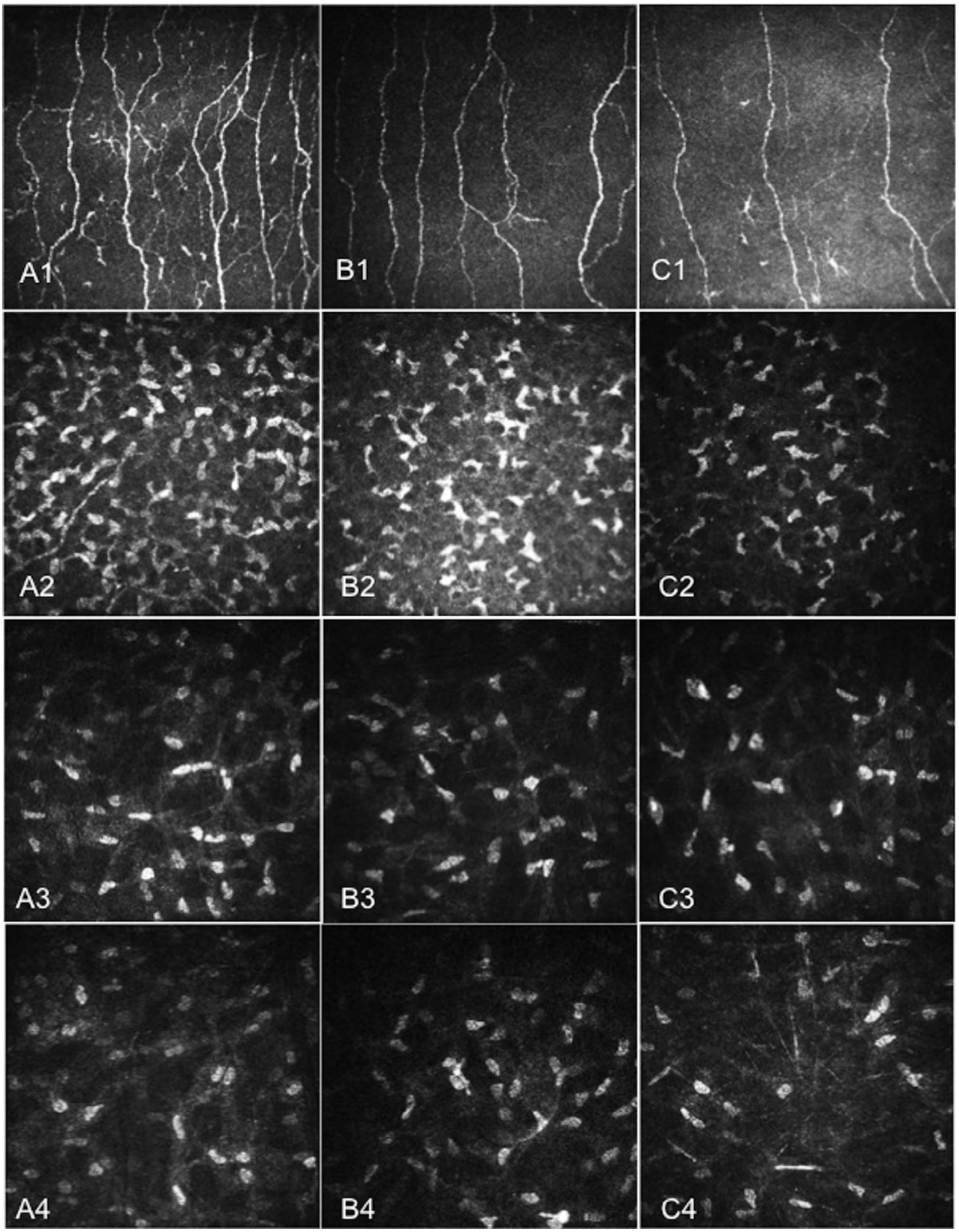

FIGURE 1. CCM images in healthy control (**A1–A4**), patient without DN (**B1–B4**), and patient with DN (**C1–C4**) of the corneal sub-basal nerve plexus (**A1–C1**), anterior stroma (**A2–C2**), midstroma (**A3–C3**), and posterior stroma (**A4–C4**).

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TABLE 2. Pearson Correlation and Significance Between KD and CCM Parameters, Age, Duration of Diabetes, and HbA1c

	CNFD, no./mm^2	CNBD, no./mm^2	CNFL, mm/mm^2	Age, y	Duration Diabetes, y	HbA1c, %
AKD, cells/mm^2	$r = 0.26$ $P = 0.008$	$r = 0.23$ $P = 0.01$	$r = 0.20$ $P = 0.04$	$r = -0.50$ $P < 0.0001$	$r = -0.20$ $P = 0.08$	$r = -0.42$ $P < 0.0001$
MKD, cells/mm^2	$r = 0.27$ $P = 0.005$	$r = 0.27$ $P = 0.006$	$r = 0.26$ $P = 0.008$	$r = -0.32$ $P = 0.001$	$r = -0.23$ $P = 0.04$	$r = -0.19$ $P = 0.07$
PKD, cells/mm^2	$r = 0.24$ $P = 0.01$	$r = 0.32$ $P = 0.001$	$r = 0.29$ $P = 0.003$	$r = -0.37$ $P < 0.0001$	$r = -0.15$ $P = 0.19$	$r = -0.24$ $P = 0.02$

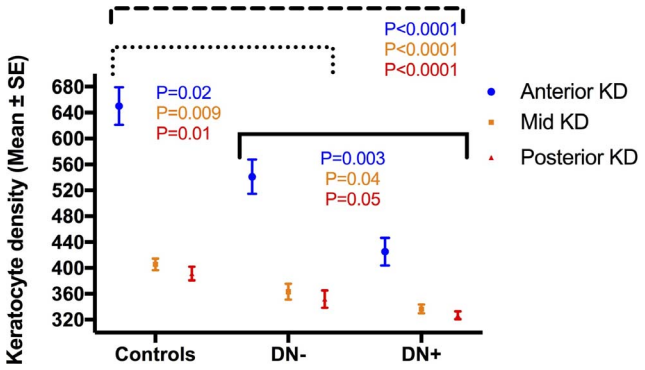

FIGURE 2. Anterior, mid, and posterior stromal KD in healthy controls, diabetic patients without and with DN (DN– and DN+, respectively); *P* values under the *dashed line* compare KD in controls compared to DN+, *P* values under the *dotted line* compare KD in controls compared to DN–; *P* values under the *solid line* compare KD in DN– compared to DN+.

in a subset of images from 15 patients following the same protocol.

Statistical Analysis

Analysis was carried out using statistical software (SPSS, Version 22.0 for Macintosh; IBM Corporation, New York, NY, USA). All data were presented as mean ± SE. Descriptive and frequency statistics were used to describe the data. Independent sample *t*-test (Mann-Whitney *U* test for nonparametric) and 1-way ANOVA (post hoc least significant difference) were used to assess the estimates between groups. For all values, $P < 0.05$ was considered as significant. To assess the inter- and intraobserver agreement, Bland-Altman plots and the intraclass correlation coefficient (ICC) were measured. Pearson correlation (Spearman for nonparametric) was calculated to assess the association between corneal nerve parameters, demographic data, and KD.

RESULTS

Clinical and Peripheral Neuropathy Assessment

BMI and systolic blood pressure were comparable, total cholesterol ($P < 0.0001$) was lower, and HbA1c ($P < 0.0001$) was significantly higher in patients with diabetes compared to control subjects. Total cholesterol ($P = 0.002$) was higher and HbA1c ($P = 0.01$) was significantly lower in DN– compared to DN+ patients, respectively. SSNCV ($P < 0.0001$), SSNA ($P < 0.0001$), PMNCV ($P < 0.0001$), PMNA ($P = 0.002$), and CT ($P = 0.001$) were significantly lower, and WT ($P < 0.0001$) and VPT ($P < 0.0001$) were significantly higher in patients with diabetes compared to control subjects. PMNCV ($P = 0.001$), PMNA ($P < 0.0001$), SSNCV ($P = 0.001$), and CT ($P = 0.001$) were significantly lower, and WT ($P < 0.0001$), VPT ($P < 0.0001$), and corneal sensation threshold ($P = 0.03$) were significantly higher in DN+ compared to DN– patients (Table 1).

Corneal Confocal Microscopy

Corneal Nerves. CNFD ($P < 0.0001$), CNBD ($P < 0.0001$), and CNFL ($P < 0.0001$) were significantly lower in patients with diabetes compared to controls. CNBD ($P < 0.0001$) and CNFL ($P = 0.03$) were significantly lower in DN– patients compared to controls. CNFD ($P = 0.002$), CNBD ($P = 0.02$),

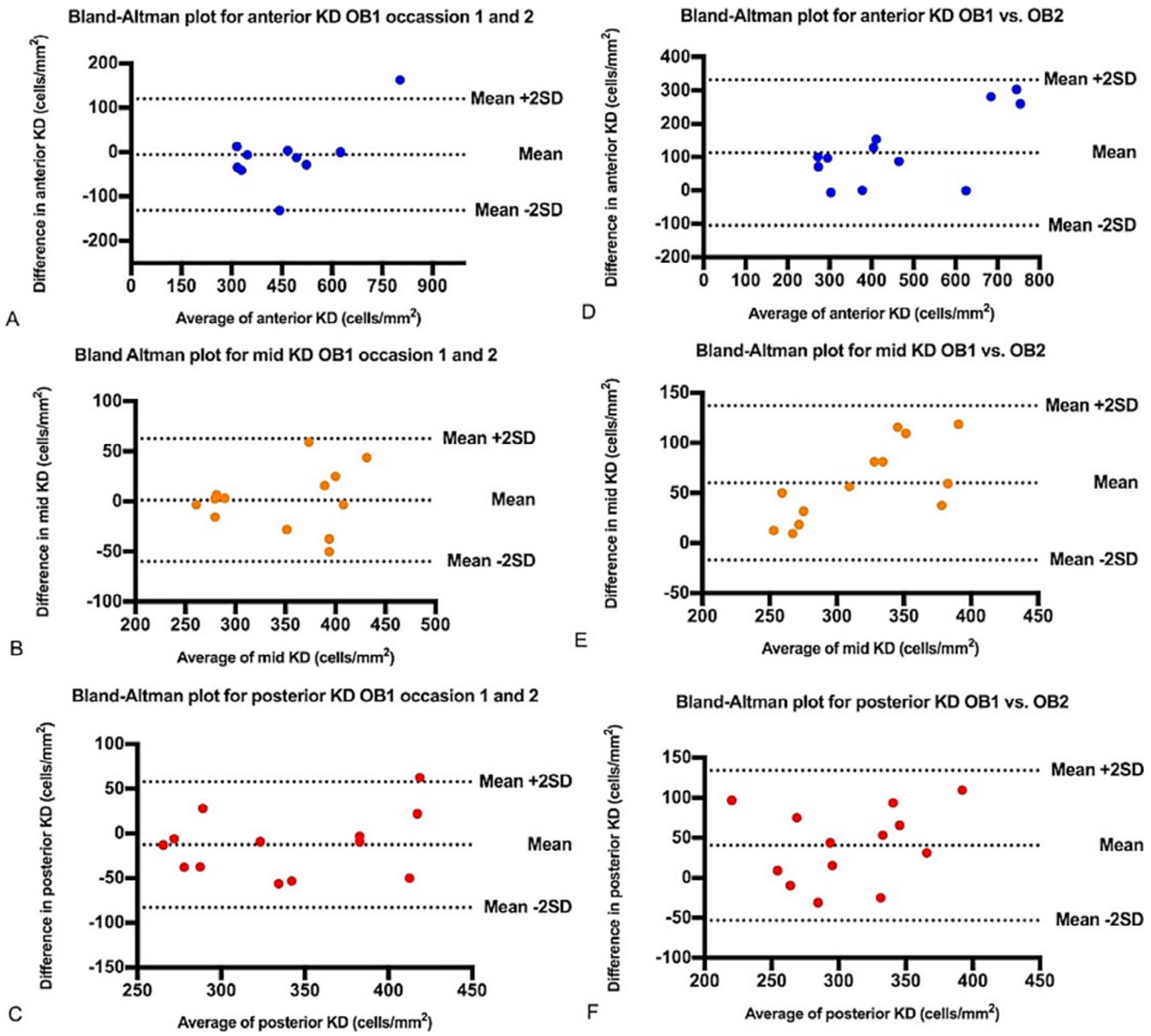

Figure 3. Bland-Altman plots for intraobserver agreement (**A–C**) and interobserver agreement (**D–F**) for anterior, mid, and posterior.

and CNFL ($P = 0.002$) were significantly lower in DN+ compared to DN– patients (Table 1; Fig. 1).

Corneal Keratocytes. Anterior ($P < 0.0001$), mid ($P < 0.0001$), and posterior ($P < 0.0001$) stromal KD were significantly lower in patients with diabetes compared to controls. Anterior, mid, and posterior stromal KD were reduced in DN– ($P = 0.02$, $P = 0.009$, $P = 0.01$, respectively) and DN+ patients (all $P < 0.0001$) compared to controls (Table 1; Figs. 1, 2). In addition, the anterior ($P = 0.003$), mid ($P = 0.04$), and posterior ($P = 0.05$) stromal KD were significantly lower in DN+ compared to DN– groups (Table 1; Figs. 1, 2). Anterior (434.75 ± 38.61 vs. 465.84 ± 19.82, $P = 0.4$), mid (333.09 ± 12.89 vs. 348.27 ± 6.76, $P = 0.3$), and posterior KD (336.7 ± 10.71 vs. 331.22 ± 7.03, $P = 0.6$) did not differ significantly between patients with type 1 and type 2 diabetes.

Correlations With KD

Anterior, mid, and posterior stromal KD correlated significantly with CNFD ($r = 0.26$, $P = 0.008$; $r = 0.27$, $P = 0.005$; $r = 0.24$, $P = 0.01$), CNBD ($r = 0.23$, $P = 0.01$; $r = 0.27$, $P = 0.006$; $r = 0.32$, $P = 0.001$), and CNFL ($r = 0.20$, $P = 0.04$; $r = 0.26$, $P = 0.008$; $r = 0.29$, $P = 0.003$), respectively. Anterior, mid, and posterior stromal KD also correlated negatively with age ($r = –0.5$, $P < 0.0001$; $r = –0.32$, $P = 0.001$; $r = –0.37$, $P < 0.0001$); midstromal KD correlated negatively with the duration of diabetes ($r = –0.23$, $P = 0.04$); and anterior ($r = –0.42$, $P < 0.0001$) and posterior ($r = –0.24$, $P = 0.02$) stromal KD correlated significantly with HbA1c (Table 2).

Intra- and Interobserver Agreement

Excellent intraobserver (Figs. 3A–C) and good interobserver (Figs. 3D–F) agreement was found for anterior (ICC = 0.91 versus ICC = 0.88), mid (ICC = 0.95 versus ICC = 0.81), and posterior KD (ICC = 0.91 versus ICC = 0.77), respectively. There was a significant intraobserver correlation for anterior KD ($r = 0.91$, $P < 0.0001$), mid KD ($r = 0.9$, $P < 0.0001$), and posterior KD ($r = 0.85$, $P < 0.0001$). There was a significant interobserver correlation for anterior KD ($r = 0.83$, $P <$

0.0001), mid KD ($r = 0.81$, $P < 0.0001$), and posterior KD ($r = 0.63$, $P = 0.01$).

Discussion

This study demonstrates a reduction in anterior, mid, and posterior stromal KD in patients with and without DN and excellent intraobserver and interobserver agreements for our method of quantification. Previous studies have shown varying results compared to our study as Frueh et al.[14] found no change in KD in patients with type 1 and type 2 diabetes compared to controls, despite a reduction in stromal nerves. Quadrado et al.[15] also found no significant difference in stromal KD in patients with diabetes compared to control subjects, and a previous study in young patients with type 1 diabetes even demonstrated an increase in posterior KD.[19] However, Bitirgen et al.[13] showed a significant reduction in anterior KD in patients with type 2 diabetes and retinopathy. These differences may be attributed to differing populations studied in relation to age, diabetes type and duration, and examination of different stromal layers. Another concern may be that CCM images may not allow adequate identification of keratocytes; however Patel et al.[20] have validated this technique by comparing KD in CCM images with histologic examination. Contact lens use has also been shown to reduce keratocyte cell density[21] and stromal thinning[22,23]; therefore, patients wearing contact lenses were excluded from this study.

Diabetes is associated with widespread pathology of the cornea, including a reduction in epithelial, keratocyte, nerve, and endothelial cell densities[24,25] attributed to increased advanced glycation.[26] Indeed, in the present study we have shown a significant correlation between KD and both duration of diabetes and HbA1c. We have also shown a significant association between stromal KD and age, which is in agreement with Patel et al.,[20] who also reported a significant correlation between KD and age.

Very few studies have evaluated the relationship between stromal KD and corneal sub-basal nerve plexus morphology. In a recent study, while both intermediate and basal epithelial cell density correlated with CNFD, there was no reduction in anterior KD or correlation with CNFD.[4] We have shown a reduction in the anterior, mid, and posterior KDs even in patients without DN and a further reduction in patients with DN. The greatest loss was in the anterior (29.8%) compared to the mid (15.1%) and posterior (14.8%) KDs, which may be relevant given that this layer is immediately below the sub-basal nerve plexus. Despite the majority of patients in this cohort having mild DN, we still showed a moderate but significant correlation between KD and corneal nerve morphology. It would be of interest to include patients with severe DN as this may further strengthen the association between KD and corneal nerve loss.

A recent experimental study by Yam et al.[10] showed that activated stromal fibroblasts derived from corneal stromal keratocytes induced neurite outgrowth by releasing a range of neurotrophic and proinflammatory factors, including IL-8, IL-15, eotaxin, monocyte chemoattractant protein-1, and the protein RANTES. Several previous studies have shown corneal nerve fiber repair after continuous subcutaneous insulin infusion,[27] simultaneous pancreas and kidney transplantation,[28] and use of the nonerthropoietic peptide Cibinetide.[29,30] However, these studies did not assess a change in KD in relation to nerve repair.

In conclusion, we demonstrate a reduction in anterior, mid and posterior stromal KD that relates to age, duration of diabetes, and glycemic control. This occurs early in patients without DN with a further reduction in those with DN. The modest correlations between KD and corneal nerve morphology suggest a small but significant relationship between keratocytes and corneal nerve integrity.

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