

# Corneal and Retinal Neuronal Degeneration in Early Stages of Diabetic Retinopathy

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**PURPOSE.** To examine the neuronal structural integrity of cornea and retina as markers for neuronal degeneration in nonproliferative diabetic retinopathy (NPDR).

**METHODS.** Participants were recruited from the broader Brisbane community, Queensland, Australia. Two hundred forty-one participants (187 with diabetes and 54 nondiabetic controls) were examined. Diabetic retinopathy (DR) was graded according to the Early Treatment Diabetic Retinopathy Study (ETDRS) scale. Corneal nerve fiber length (CNFL), corneal nerve branch density (CNBD), corneal nerve fiber tortuosity (CNFT), full retinal thickness, retinal nerve fiber layer (RNFL), ganglion cell complex (GCC), focal (FLV) and global loss volumes (GLV), hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>), nephropathy, neuropathy, and cardiovascular measures were examined.

**RESULTS.** The central zone ( $P = 0.174$ ), parafoveal thickness ( $P = 0.090$ ), perifovea ( $P = 0.592$ ), RNFL ( $P = 0.866$ ), GCC ( $P = 0.798$ ), and GCC GLV ( $P = 0.338$ ) did not differ significantly between the groups. In comparison to the control group, those with very mild NPDR and those with mild NPDR had significantly higher focal loss in GCC volume ( $P = 0.036$ ). CNFL was significantly lower in those with mild NPDR ( $P = 0.004$ ) in comparison to the control group and those with no DR. The CNBD ( $P = 0.094$ ) and CNFT ( $P = 0.458$ ) did not differ between the groups.

**CONCLUSIONS.** Both corneal and retinal neuronal degeneration may occur in early stages of diabetic retinopathy. Further studies are required to examine these potential markers for neuronal degeneration in the absence of clinical signs of DR.

**Keywords:** diabetes, retinopathy, retinal thickness, ganglion cell complex, optical coherence tomography

Diabetic retinopathy (DR), the major ophthalmic complication of diabetes mellitus (DM), is well recognized for its insidious nature with fewer or no symptoms in early stages. DR has been traditionally defined as a microvascular complication based on the first clinically observable vascular signs on ophthalmoscopy; however, there is accumulating evidence that there is neuroretinal damage that is evident prior to the appearance of clinical signs of DR and is associated with visual compromise.<sup>1</sup> Therefore, a detailed evaluation of neuronal measures in relation to retinal vascular findings will broaden our understanding of this potentially sight-threatening condition. In this regard, optical coherence tomography (OCT) has been demonstrated as a noninvasive, repeatable, and effective technique<sup>2</sup> that can detect subtle neuroretinal degeneration in people with diabetes prior to the appearance of clinical signs of DR.<sup>3-16</sup> OCT is capable of quantifying structural changes at specific retinal layers and is being utilized in the monitoring of therapeutic interventions

and in predicting the prognosis of retinal complications in diabetes. In addition to neuroretinal complications, recent studies have documented diabetes-related neural degeneration in the cornea in relation to the presence<sup>17-20</sup> and severity<sup>21</sup> of peripheral neuropathy, with the help of corneal confocal microscopy (CCM). CCM is capable of demonstrating neuronal regeneration after intervention such as pancreatic or kidney transplantation<sup>22</sup> and in predicting the future development of diabetic neuropathy.<sup>23</sup> Interestingly, CCM-derived corneal nerve fiber length (CNFL), corneal nerve fiber density (CNFD), and corneal nerve branch density (CNBD) are reduced in patients with diabetes and no DR and are further compromised with the severity of DR.<sup>24-26</sup> However, it is not known if these corneal neuronal and retinal neuronal “markers” can differentiate patients with early stages of DR. In this report, we specifically investigate the utility of OCT and CCM in persons with nonproliferative diabetic retinopathy (NPDR) in comparison to nondiabetic individuals.



## MATERIALS AND METHODS

The study received ethics approval from the Queensland University of Technology, Princess Alexandra Hospital, and Mater Hospital Human Research Ethics Committees and was conducted according to the tenets of the Declaration of Helsinki as revised in 2008. Individuals with type 1 diabetes were recruited from Princess Alexandra Hospital, Mater hospital, and the broader Brisbane community. Two hundred forty-one participants (54 nondiabetic controls and 187 with diabetes) were examined. Participants provided written informed consent prior to enrolment. Concurrent assent was provided by both guardian and the participant for those aged between 14 and 18 years. Participants who had their 18th birthday during their enrolment or follow-up in the study were asked to re-assent to participate as an adult, that is, without parental consent. In this instance, the participants were asked to sign and date the consent document(s) for the study. The type of diabetes was ascertained from general practitioner reports. Information about the duration of diabetes was by self-report.

Participants underwent medical evaluation that included body mass index (BMI), blood pressure (BP), total cholesterol and triglyceride levels, and the urine albumin/creatinine ratio (ACR).

### Ophthalmic Screening

Participants underwent visual acuity assessment, slit-lamp biomicroscopy examination, intraocular pressure measurement, and a three-field fundus photography.

Individuals with refractive error greater than  $\pm 6.00$  diopter (D) sphere or astigmatism greater than  $\pm 3.00$  D cylinder were excluded. Individuals with cataract that prevented a good view of the posterior segment with fundus photography or OCT, laser treatment for DR and/or macular edema, diagnosis or reasonable suspicion of glaucoma from optic nerve head appearance, intraocular pressure above 22 mm Hg, and previously diagnosed neurologic condition that might affect retinal nerve fibers (e.g., Parkinson's disease<sup>27</sup> or multiple sclerosis<sup>28</sup>) were excluded.

### Corneal Confocal Microscopy

A uniform protocol was followed when performing CCM for all study participants. The cornea of the hand-dominant side was selected for confocal microscopic examination and anesthetized with a drop of 0.4% benoxinate hydrochloride (oxybuprocaine hydrochloride; Bausch & Lomb, North Ryde, NSW, Australia). The Heidelberg Retina Tomograph III coupled with Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany) was utilized. Participants were required to fixate on a light target adjacent to the CCM module with the eye not being examined, but with both eyes wide open. In the eye to be examined, gentle contact was made on the central cornea by the applanation cap of the CCM. A side-mounted charge-coupled device camera allowed the examiner to ensure that the central region of cornea was being examined. Multiple images of the subbasal nerve plexus were captured from the central cornea in the "section" mode. The captured images were stored digitally, and the best eight images of the corneal nerve plexus that demonstrated distinct and focused nerves which did not overlap more than 20%<sup>29</sup> were included for the analysis.

A fully automated segmentation technique (CCMetrics<sup>30</sup>) was used to quantify the CNFL, CNBD, and CNFT. The CNFL represents the sum of length of all corneal nerve fibers and branches within the area of the corneal tissue. The CNBD

represents the total number of all branches emanating from major nerves within the area of the corneal tissue. The CNFT represents the degree of tortuosity from a straight line joining the ends of each main nerve fiber.<sup>31</sup> Detailed methodology has been described elsewhere.<sup>32</sup>

### Retinopathy Assessment

Diabetic retinopathy (defined as ophthalmoscopically visible signs only) was graded according to the Early Treatment Diabetic Retinopathy Study (ETDRS) scheme<sup>33</sup> by an ophthalmologist who was masked to the details of the participant. Diabetes but no DR was defined as an ETDRS = 10. An ETDRS of 20 was defined as very mild NPDR (microaneurysms only) and an ETDRS of 35 (hard exudates, cotton-wool spots, and/or mild retinal hemorrhages) was defined as mild NPDR. Nondiabetic participants were considered as the control group. Accordingly, 121 participants were identified as having diabetes but no DR, 39 with very mild NPDR, and 27 with mild NPDR.

Hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>) of all participants was measured on the day of the ophthalmic examination. The eye on the side of the dominant hand was tested unless contraindicated by the above eligibility criteria, in which case the eye on the nondominant side was tested.

Spectral-domain OCT (RTVue, Model RT-100, ver.4.0; Fremont, CA, USA) was used to examine full retinal and inner retinal thickness. Full retinal thickness is measured along 12 radial lines, each 6 mm long, centered at the fovea and averaged at three regions. The outermost region is the perifoveal zone that has an inner circle of diameter 3 mm and an outer circle of diameter 6 mm. The middle parafoveal zone has an inner circle of diameter of 1 mm and an outer circle of diameter 3 mm. The innermost zone is that within the circle of diameter 1 mm that includes the fovea. The protocol facilitates measurement of volume data (as mm<sup>3</sup>) within the central 1 mm, the middle parafovea (as hemispheres and as quadrants), and the outer perifovea (as hemispheres and as quadrants), within the measured 6 × 6-mm zone at the macula.

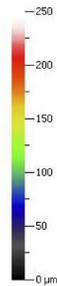
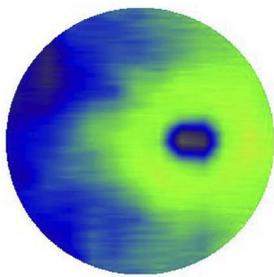
The retinal nerve fiber layer (RNFL) thickness was measured around the optic nerve head, and the ganglion cell complex (GCC) thickness was measured at the macula. The RNFL thickness, which reportedly comprises axons of the ganglion cells, was measured along a circle of 3.45 mm diameter centered at the optic nerve head. The GCC is a composite of the inner plexiform layer, ganglion cell layer, and nerve fiber layer and is measured along 15 vertical lines and 1 horizontal line, covering a zone of 7 × 7 mm that is centered at 1 mm temporal to fovea. The pattern-based GCC parameters, namely, focal loss volume (GCC FLV) and global loss volume (GCC GLV), were assessed. These parameters have been explained elsewhere<sup>34</sup> and are additionally summarized below. Figure 1 shows the GCC thickness, deviation, and significance maps for an individual. The thickness map displays the thickness of the GCC for each pixel in a pseudocolor-coded fashion. Warmer colors represent thicker regions and cooler colors represent relatively thinner regions.

The difference map is calculated, wherein the thickness at each pixel is compared with that of an age-matched normative database and the difference is displayed in a color-coded fashion. The warmer colors represent thicker areas in relation to the in-built age-matched normative database, while cooler colors indicate relatively thinner areas. The significance map shows the significance of the difference in comparison to the normative database.

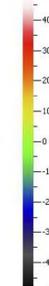
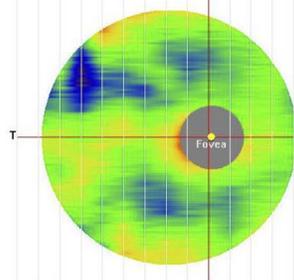
The GLV indicates the global loss in GCC volume over the entire GCC map. The percentage decrease in thickness at each

## Ganglion cell complex maps

## Thickness map



## Deviation map



## Significance map

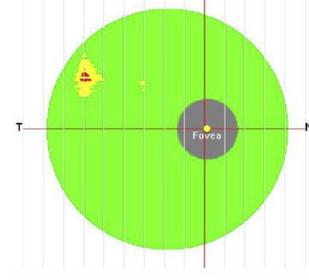


FIGURE 1. Thickness map, deviation and significance maps of the ganglion cell complex of an individual.

pixel compared to the machine's normative database is calculated. The resulting map is known as the fractional deviation map, where the pixels with values  $< 0$  are summed up and divided by the entire GCC area to give GLV (%).

The FLV indicates the focal loss in GCC volume over the entire GCC map. The thickness value at each pixel is compared to that of an age-matched normative database to give a pattern map. This map for the subject is then compared with the average pattern map from the age-matched database. The difference between the two maps is the pattern deviation map, which is color coded and assigned significance values for the deviation. The pixel values in the pattern deviation map with values  $< 0$  in fractional deviation map are summed with those for a  $p < 5\%$  from pattern deviation map and divided by the total area to give FLV.

The parameter GCC GLV is analogous to the mean deviation of the visual field plot that indicates a general depression in the visual field sensitivity. The GCC FLV, on the other hand, is analogous to the pattern standard deviation of the visual field plot. The GCC FLV represents the local depression in the map that is adjusted or corrected for the overall depression of the topography that is visible only in depth. The overall thicknesses in the central zone, parafovea, perifovea, RNFL, GCC, and pattern-based GCC parameters were considered for analysis.

The OCT was performed by one of four operators (SS, CD, NP, KE), but all followed a uniform protocol in that images with improper segmentation, poor definition of boundaries, artifacts, or blurry images due to poor fixation, and images with blink artifacts were an indication to repeat the scan during image acquisition. Further, the scans included in this study were manually checked by a single operator (SS). Images with any such errors (less than five eyes in the overall study sample) were not included for the study.

### Neuropathy Assessment

Individuals underwent neuropathy evaluation and were classified as with or without neuropathy according to a modified neuropathy disability score (NDS). The NDS criteria involved neurologic examination of three sensory modalities, namely, vibration perception, sharp/blunt sensation, temperature sensation, and ankle reflex. A score of 0 is given for a normal response and 1 for an abnormal response for each individual test component. The ankle reflex was assessed using a reflex hammer, with the scores being 0 for normal, 1 for reinforcement, and 2 for absent. Each foot can have maximum

score of 5, resulting in a total score of 10 for both feet. An NDS  $\geq 3$  is designated as neuropathy and higher scores indicate advanced degrees of neuropathy.<sup>35</sup>

Peroneal, tibial, and sural nerve conduction velocities (CV) and amplitudes in the lower limb on the hand-dominant side were assessed as a part of neurophysiological examination using a Neuropack S1 EMG/Evoked Potential Measuring System (Nihon Kohden, Tokyo, Japan). Symptoms of the participants were assessed using a diabetic neuropathy symptom score (DNSS) questionnaire, and the outcome was recorded on a scale from 0 to 4 where a score greater than 0 indicates the presence of neuropathy.<sup>36</sup>

Thresholds for heat, cold, heat pain, cold pain, and vibration sensation were measured on the dorsolateral aspect of the foot using the Medoc Quantitative Sensory Analyser testing (QST), Model TSA-II (Medoc Advanced Medical Systems, Ramat Yishai, Israel).

### Statistical Analysis

Statistical software for Social Sciences (SPSS) (released 2008, SPSS Statistics for Windows, Version 17.0; Chicago, IL, USA) was used for the analyses. A Kolmogorov-Smirnov test was used to assess the normality of data distribution. A  $\chi^2$  test was utilized to compare proportions. An ANOVA test was utilized to test for significant overall differences among normally distributed data, followed by a Tukey's test only for those variables that were significant with ANOVA test. The GCC FLV and GCC GLV were not normally distributed; therefore, group differences were assessed using a Kruskal-Wallis test and further differences were assessed using a Mann-Whitney  $U$  test. A  $P$  value of  $< 0.05$  was considered statistically significant.

### RESULTS

Table 1 shows the clinical characteristics in the controls, diabetes with no DR, and diabetes with very mild and mild NPDR groups. The HbA<sub>1c</sub> levels, duration of diabetes, total cholesterol, BMI, QST cold sensation threshold, warm sensation threshold average, warm induced pain threshold average (degrees), vibration threshold average, peroneal motor nerve CV (PMNCV), monofilament number of points felt of 3, NDS (0–10), and DNS total score were significantly different between the groups. Among these significant variables, a majority of the differences were attributable to all three DM groups in comparison to the control group. HbA<sub>1c</sub> and total cholesterol differed significantly between the no DR group and mild NPDR

**TABLE 1.** Clinical Characteristics in the Controls (No Diabetes) and in Those With No DR and Early Stages of Nonproliferative DR

Characteristics	Controls, A		Diabetes With no DR, B		Diabetes With Very Mild NPDR, C		Diabetes With Mild NPDR, D		Group Diff. P Value	Post Hoc Sig. Diff.
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Age	49.3	14.4	49.0	16.1	53.8	12.9	49.5	11.9	0.343	
HbA <sub>1c</sub> , %	5.4	0.3	7.7	1.3	7.8	1.3	8.8	1.7	<b>&lt;0.001</b>	A vs. B,C,D B vs. D
Duration of diabetes	0.0	0.0	12.9	11.0	24.7	13.6	21.7	12.6	<b>&lt;0.001</b>	B vs. C,D
Triglycerides, mM	1.0	0.4	1.3	0.9	1.5	0.9	1.5	1.4	0.089	
Total cholesterol, mM	5.4	1.2	4.4	0.9	4.6	0.9	4.6	1.4	<b>&lt;0.001</b>	A vs. B,C,D B vs. D
BP resting systolic, mm Hg	122.3	15.7	125.3	16.1	126.8	12.7	128.7	14.8	0.227	
BP resting diastolic, mm Hg	77.9	9.7	76.7	8.6	76.2	6.9	78.3	8.8	0.818	
BMI, kg/m <sup>2</sup>	26.3	5.4	28.1	6.1	29.4	4.8	30.8	6.6	<b>0.001</b>	A vs. B,C,D
Urine alb/creat ratio, mg/mmol	9.2	7.4	10.3	7.2	9.2	5.4	9.6	4.6	0.682	
QST cold sensation threshold average, °C	28.8	2.6	27.0	5.3	23.6	9.3	23.4	8.7	<b>0.001</b>	A vs. B,C,D
QST warm sensation threshold average, °C	37.3	3.8	38.4	4.8	39.9	4.8	40.6	5.0	<b>0.012</b>	A vs. C,D B vs. D
QST warm induced pain threshold average, °C	47.2	3.0	47.3	3.2	47.4	2.9	48.6	2.1	<b>0.044</b>	A vs. D B vs. D
QST vibration threshold average, Hz	6.8	8.6	10.7	15.5	20.4	23.3	17.7	24.9	<b>&lt;0.001</b>	A vs. B,C,D B vs. C,D
QST cold induced pain threshold average, °C	10.3	9.3	11.5	9.6	11.9	10.6	8.0	9.1	0.287	
Peroneal CV ankle to FH, m/s	49.3	5.8	46.5	5.5	42.7	6.1	41.2	6.9	<b>&lt;0.001</b>	A vs. B,C,D B vs. C,D
Monofilament no. of points felt of 3	3.0	0.1	2.8	0.6	2.6	0.8	2.2	1.2	<b>&lt;0.001</b>	A vs. B,C,D B vs. D
Neuropathy disability score, 0–10	0.3	0.6	1.1	1.6	2.1	2.6	3.4	3.6	<b>&lt;0.001</b>	A vs. B,C,D
DNSS total score	0.1	0.3	0.5	0.9	0.6	1.1	0.8	1.2	<b>0.005</b>	A vs. B,C,D

FH, fossa head; BP, blood pressure; QST, quantitative sensory testing; CV, conduction velocity; DNSS, diabetic neuropathy symptom score. Significant *P* values in bold.

groups. Among the neuropathy-related measures, QST measures, PMNCV, and monofilament exam were significantly different in mild NPDR in comparison to the no DR group.

Table 2 summarizes the retinal thickness and corneal nerve morphology measures in those with no diabetes (controls), diabetes with no DR, diabetes with very mild NPDR, and diabetes with mild NPDR. The central zone ( $P = 0.174$ ), parafoveal thickness ( $P = 0.090$ ), perifovea ( $P = 0.592$ ), RNFL ( $P = 0.866$ ), GCC ( $P = 0.798$ ), and GCC GLV ( $P = 0.338$ ) did not differ significantly between the groups. However, GCC FLV was significantly different between groups ( $P = 0.036$ ). In comparison to the control group, those with diabetes and very mild NPDR, as well as those with mild NPDR, had significantly

higher focal loss in GCC volume (Fig. 2). With regard to the corneal nerve parameters, the CNFL was significantly different between the groups ( $P = 0.004$ ). The group differences were attributable to those with diabetes and mild NPDR in comparison to the control group as well as in comparison to those with no DR. The CNBD ( $P = 0.094$ ) and CNFT ( $P = 0.458$ ) did not differ between the groups (Fig. 3).

To assess if participants with DR also have diabetic polyneuropathy (DPN) and vice versa, we examined the proportion of people with and without DR in relation to those with and without DPN. Among those with no DPN ( $n = 144$ ), 41 (28.4%) had very mild or mild NPDR. Among those with no DR ( $n = 121$ ), 14 (11.6%) had mild DPN (Table 3).

**TABLE 2.** Ophthalmic Measures in the Controls (No Diabetes) and in Those With No DR and Early Stages of Nonproliferative DR

Measures	Nondiabetic Controls, A			Diabetes With no DR, B			Diabetes With Very Mild NPDR, C			Diabetes With Mild NPDR, D			Group Diff. P Value	Post Hoc Sig. Diff.
	<i>n</i>	Mean	SD	<i>n</i>	Mean	SD	<i>n</i>	Mean	SD	<i>n</i>	Mean	SD		
Thickness in central zone, with fovea, $\mu\text{m}$	53	254	25	121	246	26	39	245	29	26	251	20	0.174	
Thickness in parafovea, $\mu\text{m}$	53	317	15	121	314	16	39	309	20	26	309	15	0.09	
Thickness in perifovea, $\mu\text{m}$	53	274	14	121	274	14	39	271	15	26	271	17	0.592	
RNFL thickness, $\mu\text{m}$	54	105	11	121	105	11	39	106	13	27	103	14	0.866	
GCC thickness, $\mu\text{m}$	53	98	7	121	98	8	39	97	10	26	97	9	0.798	
GCC FLV, %*	53	0.21	0.72	121	0.34	0.78	39	0.54	1.98	26	0.88	3.2	<b>0.036*</b>	A vs. C,D
GCC GLV, %*	53	3.97	3.85	121	3.88	4.37	39	5.04	4.7	26	5.13	4.95	0.338	
CCM CNBD, mm <sup>2</sup>	53	83	57	106	69	51	35	71	55	25	51	44	0.094	
CCM CNFL, mm/mm <sup>2</sup>	53	21	6	106	19	6	35	18	7	25	15	7	<b>0.004</b>	D vs. A,B
CCM CNFT tortuosity units	53	0	0	106	0	0	35	0	0	25	0	0	0.458	

SD, standard deviation; RNFL, retinal nerve fibre layer; GCC, ganglion cell complex; FLV, focal loss volume; GLV global loss volume; CCM, corneal confocal microscopy; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length; CNFT, corneal nerve fibre tortuosity. Significant *P* values in bold.

\* *P* value by Kruskal-Wallis and post hoc by Mann-Whitney *U* test.

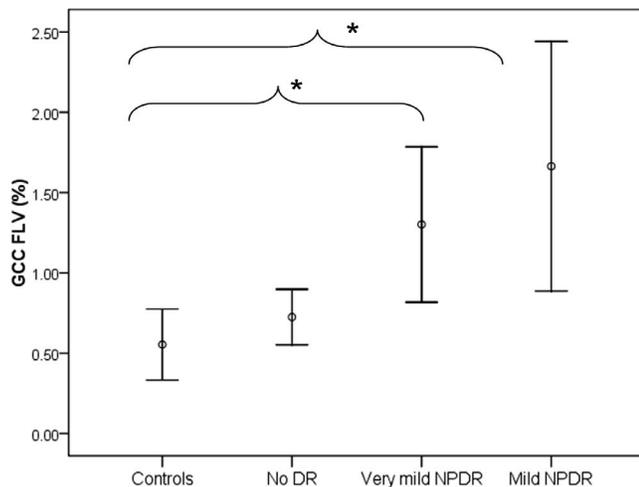


FIGURE 2. GCC FLV in no DR and in stages of DR. Marker indicates mean retinal thickness in  $\mu\text{m}$ . Error bars are 95% confidence intervals. Asterisk indicates significant differences.

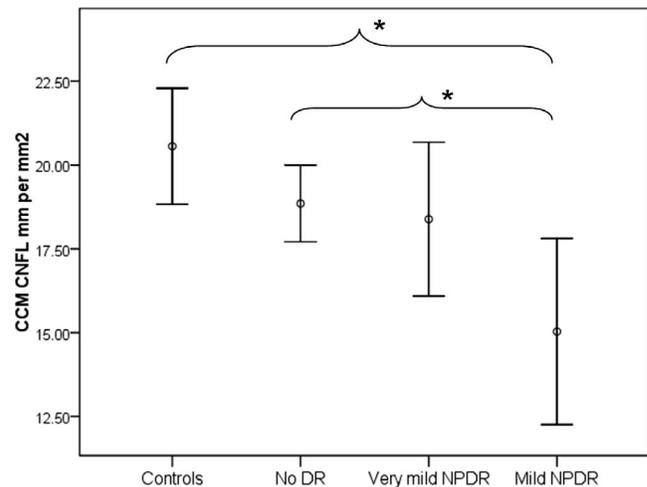


FIGURE 3. CNFL in no DR and in stages of DR. Marker indicates mean retinal thickness in  $\mu\text{m}$ . Error bars are 95% confidence intervals. Asterisk indicates significant differences.

## DISCUSSION

Our study sought to examine the corneal and retinal morphology as measures of corneal and retinal neuronal degeneration in participants with no DR and in very mild and in mild NPDR. We observed that there is both corneal and retinal neuronal degeneration in early stages of DR; CNFL is significantly lower in patients with mild NPDR in comparison to those with no diabetes and diabetes with no DR. We observed that there are pockets of ganglion cell volume loss in patients with very mild and mild NPDR in comparison to nondiabetic participants. This further substantiates the concept that diabetes is a multisystem disorder affecting multiple ophthalmic systems and that DR involves vascular damage and neuronal damage. We intended to explore the neural and vascular link in early-stage DR. Our study highlights that neuronal damage in both cornea and retina may occur during early stages of DR. However, these neuronal signs may be initiated by subtle vascular changes.<sup>37</sup> Therefore, it is not clear as to which comes first: the chicken or the egg.

The CNFL showed a decreasing trend with the severity of NPDR. Although CNBD and CNFT showed a similar trend, they were not statistically significant. This could mean that CNFL is a better marker than CNBD or CNFT in early stages of DR. With regard to corneal nerve morphology, previous studies<sup>38,39</sup> reported that the corneal nerve morphology is significantly compromised in patients with diabetes but no DR and in those with DR in comparison to nondiabetic participants; these studies concluded that CCM is able to detect corneal neuronal changes in the absence of clinical signs of DR. The observation that corneal nerves are affected in diabetes, given that the cornea is avascular, provides an interesting dimension to the theory of neural and microvascular link. The proposition that corneal neuropathy may be a vascular complication of diabetes may be subject to debate in view of the corneal nerve deficits. However, a likely ophthalmic proposition for corneal neuropathy as a microvascular complication in diabetes could be that the cornea is also nourished by the aqueous,<sup>40</sup> which derives its nutrients from the blood supply to the ciliary body. If the vessels supplying the ciliary body are compromised in diabetes, the corneal nerves may not be properly nourished and hence may be subject to insult. Another explanation could be that the pathogenesis in diabetes and hence neuropathy involves several theories, among which ischemia (the obvious vascular component) is only one.<sup>41</sup> Hyperglycemia, advanced

glycation end products, sorbitol pathway, and oxidative stress<sup>42</sup> are also implicated in the pathologic changes in the nerves in diabetes. Accumulation of polyols (particularly sorbitol) in the nerves in the aldose reductase pathway is activated by hyperglycemia,<sup>43</sup> resulting in increased sorbitol formation. Accumulation of sorbitol and fructose leads to reduced nerve myoinositol, decreased sodium-potassium ATPase activity,<sup>44</sup> alteration in protein kinase C subunits, and slowed nerve CV in the peripheral nerves in diabetes.<sup>45</sup> It is likely that a similar mechanism occurs in the corneal nerves.

We followed well-accepted and validated protocols that have been previously demonstrated to be robust and repeatable. Thus quantification of corneal nerve morphology using CCM has been reported to be reproducible/repeatable<sup>46-48</sup> and can be quantified using a manual (CCMetrics),<sup>30</sup> semiautomated (NeuronJ)<sup>49</sup> or fully automated technique (ACCMetrics).<sup>50</sup> The performance of each of the above three techniques has been reported to be highly and significantly correlated (correlation coefficient ranging from 0.87 to 0.98,  $P < 0.01$ ). The three techniques demonstrate good agreement and superior ability to detect an abnormality in CNFL. Of these, the fully automated analysis has been reported to be better than the manual or semiautomated methods as it is faster while retaining objective evaluation and reliability.<sup>51</sup> Additionally, the application of a standardized approach as in the current study has recently been shown to have high intra- and interobserver reproducibility for all corneal nerve parameters.<sup>52</sup>

We observed that there are focal losses in GCC volume (GCC FLV) in the macular area in very mild and mild NPDR in the presence of comparatively normal GCC and RNFL thickness. GCC FLV is similar in concept to pattern standard deviation of the Humphrey visual fields test, which indicates the local depressions and potholes in the entire GCC map after correcting for the overall depression of the topography of GCC thickness map. We hypothesize that these focal losses occurring in ganglion cells may be more easily detected in the macular area because they are multilayered in thickness. The focal losses in GCC volume show a worsening trend from nondiabetic individuals to no DR to mild stages of DR; we hypothesize that the loss may worsen in moderate and severe NPDR and PDR and may manifest as a significant reduction in the thickness of GCC or RNFL in further stages of DR. One of the earliest changes in DR is reported as the breakdown of blood-retinal barrier and compromise to neuroretinal layers

**TABLE 3.** Proportion of Individuals With and Without Diabetic Neuropathy per NDS Criteria in Early Stages of DR

Groups	Control	DM no DPN	Mild DPN	Moderate DPN	Severe DPN	Total
Controls, <i>n</i>	54	0	0	0	0	54
% within controls, ETDRS	100.0	0.0	0.0	0.0	0.0	100.0
% within severity of NDS	100.0	0.0	0.0	0.0	0.0	22.4
No DR, <i>n</i>	0	103	14	4	0	121
% within controls, ETDRS	0.0	85.1	11.6	3.3	0.0	100.0
% within severity of NDS	0.0	71.5	53.8	30.8	0.0	50.2
Very mild NPDR, <i>n</i>	0	28	5	6	0	39
% within controls, ETDRS	0.0	71.8	12.8	15.4	0.0	100.0
% within severity of NDS	0.0	19.4	19.2	46.2	0.0	16.2
Mild NPDR, <i>n</i>	0	13	7	3	4	27
% within controls, ETDRS	0.0	48.1	25.9	11.1	14.8	100.0
% within severity of NDS	0.0	9.0	26.9	23.1	100.0	11.2
Total	54	144	26	13	4	241
% within controls, ETDRS	22.4	59.8	10.8	5.4	1.7	100.0
% within severity of NDS	100.0	100.0	100.0	100.0	100.0	100.0

ETDRS, Early Treatment Diabetic Retinopathy Study; DPN, diabetic peripheral neuropathy; NDS, neuropathy disability score; NPDR, nonproliferative diabetic retinopathy.

well before ophthalmoscopic signs of DR. The retina predominantly consists of three types of cells, namely, neurons, glial cells, and blood vessels. Antonetti et al.<sup>53</sup> hypothesized that even prior to the development of vascular signs, the neurons, glial tissue, and the neurovascular link may be affected in diabetes due to altered blood-retinal barrier, and/or an inflammatory process and an accelerated death of ganglion cells, amacrine cells, bipolar cells, and photoreceptor cells. Therefore, the focal loss in GCC may reflect degeneration of any type or combination of neural or glial cell involvement.<sup>54</sup> Inflammatory process has been proposed as the link between neuronal degeneration and microvascular complications. Another hypothesis is that the retinal neuronal degeneration may initiate an autoregulatory response by the retinal vasculature leading to vasodilation and formation of microaneurysms,<sup>42</sup> indicating that neuronal degeneration may induce the formation of retinal vascular complications in diabetes.

We also examined for the likely role of age on retinal thickness parameters. The four groups (Table 1) did not differ in terms of mean age ( $P = 0.343$ ). Especially the mean age of participants in the control group, diabetes with no DR, and diabetes with mild NPDR was similar (49–49.5 years). The group with diabetes with very mild NPDR had a mean age of 53.8 years, which represents a difference of 4.3 years from the other groups. While there was no statistically significant difference in age between the groups, the clinical significance of this age difference of 4 years in very mild NPDR compared to the other three groups is not clear and may still confound the results.

A large body of literature<sup>3–16</sup> has accrued in demonstrating neuroretinal degeneration in early stages of DR that worsens with the severity of DR. However, this has been predominantly reported as reduced thickness of the RNFL or that of GCC layers. Our study presents novel findings in that we propose that the loss in GCC probably begins as focal changes that may become widespread to involve reduction in the GCC thickness later.

Our study outcomes indicate that the CNFL is reduced in participants with DM and mild DR when compared to nondiabetic participants as well as in those with diabetes and no DR. With regard to retinal measures, neuronal degeneration is identifiable in very mild NPDR and mild NPDR when compared with that of nondiabetic participants. In contrast, a previous study observed that CNFL is reduced in patients with diabetes but no DR also.<sup>58</sup> In addition, we observed no

significant loss in GCC FLV in those with diabetes but no DR, when previous studies<sup>12,13,15</sup> have reported neuroretinal degeneration in those with no DR also. A likely explanation for this difference could be that the neuronal and microvascular changes have been reported to have a varying presentation across individuals<sup>42</sup> and therefore may present with varying results. Another likely explanation for this difference could be the number of fields photographed. The signs of DR may be widespread, and therefore the corresponding GCC loss may also be widespread; however, OCT is able to detect this focal loss in GCC volumetric measure by examining the macular area. The RTVue-100 has been utilized in the current and several previous studies that have evaluated GCC parameters to discriminate the eyes of subjects with glaucoma from those of healthy subjects. Apart from the GCC thickness, the pattern-based GCC parameters are well-established parameters in the diagnosis of glaucoma and show high sensitivity (at fixed specificity) in identifying both early and advanced glaucoma. For instance, the GCC GLV can detect preperimetric glaucoma, while the GCC FLV can detect advanced glaucoma.<sup>55</sup> These protocols were originally designed for the detection of glaucoma. However, these two parameters have also shown promise in other disease conditions such as age-related macular degeneration,<sup>56,57</sup> and in our previous work we have demonstrated a higher GCC FLV (greater focal loss in GCC volume) in diabetic neuropathy, irrespective of the presence or absence of DR.<sup>58</sup> However, newly available technologies that can produce up to 100,000 A-scans per second may provide in-depth information and are worth investigating.

In our study, grading of DR was performed with dilated three-field 45° fundus photography, wherein the optic nerve head and the macula, center of the macula and temporal to macula, were examined. With a greater number of fields, a significantly greater number of lesions may be detected in the superior and the inferior fields beyond the arcades and may alter the classification of DR and therefore have varied results. In addition, the scanning protocols are limited to the scanned area in and around the macula. Therefore, any loss outside this zone is not known. Therefore, the study results must be interpreted as those within the scanned area. Nevertheless, some of the earliest alterations are reported to be breakdown of blood-retinal barrier and alterations to the neurosensory retinal function.<sup>42</sup> The initial lesions along with endothelial proliferation, pericyte damage, and microaneurysms have been

identified to be focal and in the posterior pole<sup>59</sup> and are therefore more likely to align with our study results. In addition, a decrease in the density of deep capillary plexus at the macular area has been noted in patients with no or a mild stage of DR.<sup>60</sup> Changes in the capillary plexus and therefore the blood supply and nutrients may affect the normal integrity and/or functioning of the ganglion cells and other neural cells in the retina; these are associated with diabetes-induced accelerated ganglion cell death and likely involve bipolar cells and also photoreceptors.<sup>42</sup> Therefore, the number of fields photographed is less likely to affect our study results.

Although it appears that neuronal/neuroretinal degeneration is one of the earliest signs of DR, our previous study<sup>58,61</sup> and a recent study<sup>62</sup> have demonstrated that the neuronal degeneration in diabetes could be a sign of DPN rather than retinopathy. Our study shows that there are individuals with DPN but essentially no clinical signs of DR and also individuals with no DPN but with DR and individuals with DPN and DR. The results from our study suggest that both complications could coexist or exist in the absence of the other during the early stages. Diabetic neuropathy shares many common risk factors<sup>63</sup> with DR, namely, greater age, gender predilection, higher glycemic levels, longer duration of diabetes, higher systolic BP, and dyslipidemia and has been proposed as a microvascular complication<sup>64</sup> and/or a neural complication<sup>65</sup> of diabetes. There is a possibility that either condition may occur first, or coexist. This again is a novel finding of this study and one that is entirely plausible.

With regard to the clinical characteristics in the stratified groups, we observed an increasing trend with higher HbA<sub>1c</sub>, duration of diabetes, and a higher BMI. In addition to the above findings, HbA<sub>1c</sub> and total cholesterol were significantly different in the mild NPDR group in comparison to the diabetes but no DR group. A majority of the neuropathy-related measures were significantly worse in participants with diabetes compared to the nondiabetic participants, which is expected. For instance, the peroneal CV and number of points detected with monofilament are lower in the diabetic participants in comparison to the nondiabetic group, with a trend for worsening in no DR, very mild, and mild NPDR. Similarly, NDS and DNSS show an increasing or worsening trend from control group to mild NPDR group. Similar to ophthalmic measures, the clinical variables, namely QST warm sensation and warm pain thresholds, vibration threshold, personal CV, and number of monofilament points detected, also differ in those with mild NPDR compared to those with diabetes but no DR. A majority of the clinical measures are common to both DR and DPN and therefore have common mediating pathways that lead to neuronal degeneration in the eye as well as in the peripheral nerves.

Our findings demonstrate that (1) both corneal and retinal neuronal degeneration occur in very early stages of NPDR; (2) CNFL may be a better marker than CNBD and CNFT for detecting corneal neuronal degeneration in mild NPDR compared with controls and those with diabetes but no DR; (3) a specific pattern of loss in volume in GCC layers occurs in very early stages of NPDR, in the presence of a comparatively normal GCC/RNFL layer thickness; (4) as we hypothesized, focal losses in GCC volume may precede a decrease in thickness of GCC/RNFL layers; (5) DR and DPN, both being complications of diabetes and sharing several common risk factors, can occur independent of each other and may be associated with the neuronal changes evident in the eye; (6) the plausibility of occurrence of ophthalmic neuronal degeneration may likely be due to coexisting DPN. Although such focal losses can be better documented in longitudinal studies, in this cross-sectional study, we present novel findings that the well-established neuronal degeneration in DR reported in the

literature may be local/focal neuronal degeneration in early stages of DR and could later manifest as a reduction in GCC thickness. We believe our data provide a basis for future longitudinal studies to explore the progression of these focal/local neuronal changes with the development and progression of DR.

Diabetes is a multisystem disorder affecting several sensory nerves supplying the eye. Over the past few years, CCM and OCT have been identified as biomarkers in the diagnosis, prediction, and progression of neurodegenerative disorders such as multiple sclerosis, Parkinson disease, and Alzheimer's disease.<sup>66</sup> The current study has expanded the already impressive role of OCT and CCM in demonstrating the presence of neuronal degeneration in very early stages of DR.

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