

Morphometric Stability of the Corneal Subbasal Nerve Plexus in Healthy Individuals: A 3-Year Longitudinal Study Using Corneal Confocal Microscopy

Cirous Dehghani,¹ Nicola Pritchard,¹ Katie Edwards,¹ Dimitrios Vagenas,¹ Anthony W. Russell,^{2,3} Rayaz A. Malik,⁴ and Nathan Efron¹

¹Institute of Health and Biomedical Innovation, Queensland University of Technology, Queensland, Australia

²Department of Diabetes and Endocrinology, Princess Alexandra Hospital, Woolloongabba, Australia

³School of Medicine, University of Queensland, St. Lucia, Australia

⁴Center for Endocrinology and Diabetes, Institute of Human Development, University of Manchester, Manchester, United Kingdom

Correspondence: Nathan Efron, Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove, Queensland 4059, Australia; n.efron@qut.edu.au.

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PURPOSE. We examined the age-dependent alterations and longitudinal course of subbasal nerve plexus (SNP) morphology in healthy individuals.

METHODS. Laser-scanning corneal confocal microscopy, ocular screening, and health and metabolic assessment were performed on 64 healthy participants at baseline and at 12-month intervals for 3 years. At each annual visit, eight central corneal images of the SNP were selected and analyzed using a fully-automated analysis system to quantify corneal nerve fiber length (CNFL). Two linear mixed model approaches were fitted to examine the relationship between age and CNFL, and the longitudinal changes of CNFL over three years.

RESULTS. At baseline, mean age was 51.9 ± 14.7 years. The cohort was sex balanced ($\chi^2 = 0.56$, $P = 0.45$). Age ($t = 1.6$, $P = 0.12$) and CNFL ($t = -0.50$, $P = 0.62$) did not differ between sexes. A total of 52 participants completed the 36-month visit and 49 participants completed all visits. Age had a significant effect on CNFL ($F_{[1,33]} = 5.67$, $P = 0.02$) with a linear decrease of 0.05 mm/mm^2 in CNFL per one year increase in age. No significant change in CNFL was observed over the 36-month period ($F_{[1,55]} = 0.69$, $P = 0.41$).

CONCLUSIONS. The CNFL showed a stable course over a 36-month period in healthy individuals, although there was a slight linear reduction in CNFL with age. The findings of this study have implications for understanding the time-course of the effect of pathology and surgical or therapeutic interventions on the morphology of the SNP, and serves to confirm the suitability of CNFL as a screening/monitoring marker for peripheral neuropathies.

Keywords: corneal confocal microscopy, subbasal nerve plexus, age, natural history

In vivo corneal confocal microscopy (CCM) is a rapid, noninvasive, and reiterative technique that enables microstructural evaluation of the human cornea at high resolution. The anatomic location and transparency of the cornea make this tissue structure ideally suited for confocal microscopic assessment.¹ Image acquisition using CCM from different corneal layers and structures helps clinicians and researchers to extract important information in respect of alterations induced by various ocular and systemic conditions.

The subbasal nerve plexus (SNP), which is a dense array of nerves located between the corneal basal epithelium and Bowman's layer, is the main corneal nerve structure studied in vivo using CCM as a result of ease of imaging due to its parallel arrangement in relation to the ocular surface, and the presence of distinct morphologic attributes, such as length of the nerve bundles, which are quantified easily. Structural analysis of the SNP has been used to evaluate ocular conditions, such as dry eye, ocular allergy, and glaucoma,²⁻⁵ corneal ectasia, and dystrophies,^{6,7} the effect of contact lens wear^{8,9} and assessment of nerve regeneration after penetrating keratoplasty,¹⁰ and different forms of refractive surgery.^{11,12} The CCM also has

been deployed to assess small nerve fiber pathology induced by several systemic conditions, including diabetes,^{13,14} Fabry disease,¹⁵ idiopathic neuropathy,¹⁶ and chemotherapy.¹⁷

Given the utility of SNP evaluation in screening, detection, and monitoring of a wide range of corneal and systemic neuropathies, it is important to understand how aging might affect this nerve plexus. However, there is inconsistency in the literature with respect to the relationship between age and neural morphometric change in the SNP using ex vivo and in vivo techniques. While a number of studies have reported no significant change in subbasal nerve morphology with age,¹⁸⁻²⁰ others have reported a decrease in nerve density with age²¹⁻²³ and there also is uncertainty as to the age at which SNP structural loss become significant. Furthermore, to our knowledge no data are available concerning dynamic morphologic changes of corneal nerves in health or disease over time.

The two primary objectives of this study were to investigate the relationship between age and corneal nerve fiber length (CNFL), which is the most standardized, generally adopted, and frequently reported SNP morphometric parameter obtained

from CCM; and longitudinal changes of CNFL over three years in healthy human corneas.

METHODS

Study Participants

Following approval from the research ethics committee of Queensland University of Technology (Queensland, Australia) and obtaining written informed consent, 64 healthy participants were enrolled. Participants were recruited from the community in Brisbane, Australia, who were nondiabetic control participants in the 4-year Longitudinal Assessment of Neuropathy in Diabetes using novel ophthalmic Markers (LANDMark) study.¹⁴ Exclusion criteria were history of corneal surgery, trauma or disease, glaucoma, evidence of corneal compromise, ocular and systemic diseases that might have adversely affected the cornea, and history of neuropathy. These criteria were reassessed at each annual visit.

All participants underwent assessment of visual acuity, slit-lamp biomicroscopy, and tonometry, and all corneas were confirmed to be within clinical norms. Four participants were current soft contact lens wearers and were asked to refrain from contact lens wear on the day of examinations. Contact lens wearers were not excluded from the present study, because previous investigations of the impact of contact lens wear on morphologic changes in subbasal nerves using CCM have failed to demonstrate any impact.²⁴⁻²⁶ All participants were observed at baseline and the examinations continued at 12-month intervals over three years for a total of four visits. The study was conducted in accordance with the tenets of the Declaration of Helsinki.

Corneal Confocal Microscopy and Image Analysis

At each visit, all participants underwent corneal confocal microscopy examination approximately at the corneal apex using the Heidelberg Retina Tomograph III with Rostock Corneal Module (Heidelberg Engineering GmbH, Dossenheim, Germany). One eye (on the side of hand dominance) was selected and anesthetized with a drop of 0.4% benoxinate hydrochloride (oxybuprocaine hydrochloride; Bausch & Lomb, New South Wales, Australia). Eight central corneal images per participant, displaying in-focus nerves and not overlapping more than 20%,²⁷ were selected by inspection and analyzed using a fully-automated analytical system²⁸ to quantify CNFL, which is defined as total length of all nerve fibers in the CCM image (in units of mm/mm²).

Since this study was part of a larger project designed to investigate the utility of ophthalmic markers of neuropathy in diabetic and healthy individuals, we sought to determine intra- and interobserver variability of CNFL measurement in a study of 16 and 11 participants, respectively. Participants underwent CCM examination on two occasions (same observer for intraobserver and different observers for interobserver variability) on the same day of examination followed by automated CNFL quantification using the above procedures. Intraclass correlation coefficient and coefficient of variability for intra-observer differences were 0.90 and 5.7%, and for interobserver differences they were 0.94.

Blood Biochemistry and Health Parameters

At each visit, blood biochemistry measures (HbA_{1c} and lipid profile) were assayed by a local certified pathology laboratory (Sullivan Nicolaides Pathology, Queensland, Australia), and clinical measures (height, weight, and blood pressure) were assessed by a research nurse.

Statistical Analysis

Statistical analysis of the data was performed using SPSS (version 21; SPSS, Inc., Chicago, IL, USA). Normal distribution of the data was determined with the Kolmogorov-Smirnov test. Quantitative variables are expressed by the mean \pm SD unless otherwise indicated. For the analysis of the categorical variables, the χ^2 test was applied. The independent samples *t*-test was used to compare age and CNFL between sexes. Bivariate correlation was used, as appropriate, for assessment of association of CNFL with alcohol consumption and absolute changes in CNFL with HbA_{1c}. Welch ANOVA was used to test the CNFL difference among age groups at baseline visit. Differences in characteristics from baseline visit to year-3 visit were assessed using the paired sample *t*-test (for normally distributed data) and nonparametric Wilcoxon test (for non-normally distributed data).

To analyze longitudinal data using the linear mixed model (LMM) procedure in the SPSS statistical software, the horizontal data format was converted to vertical structure; thus, there were four rows per participant corresponding to the four measurements collected over time on each participant. The relationship between age and CNFL, and the changes of CNFL over a 3-year period were examined by fitting two linear mixed models with restricted maximum likelihood estimation. The first model (LMM1) contained CNFL, age at each annual visit, and sex. The CNFL was defined as a dependent variable. Age (time-varying predictor variable) and sex (time-invariant variable) were specified as covariate and factor, respectively. Age, sex, and the sex \times age interaction were specified as fixed effects, and the Type III method of sums of squares was used. In the random effects dialog box, unstructured covariance type was chosen and age was entered in the model.

The assessment of linear change of CNFL over time (36 months) was carried out by fitting the second model (LMM2), in which CNFL was specified as a dependent variable, and time, which was a variable capturing the order of observation, was defined as a repeated variable. Correlation between two adjacent CNFL measurements was assumed to decline across measurement occasions; therefore, a first order autoregressive covariance structure was chosen. The CNFL and sex were considered as dependent variable and factor, respectively. Time and age at enrollment were assigned as covariates.

RESULTS

The demographic and clinical data of participants at baseline and 36-month visits are given in Table 1. A total of 64 participants completed the baseline visit and 52 completed the 36-month visit. The baseline cohort included 29 males and 35 females ($\chi^2 = 0.56$, $P = 0.45$). Mean age was 51.9 ± 14.7 years. Age (males, 55.1 ± 14.0 years; females, 49.3 ± 15.0 years; $P = 0.12$) and CNFL (males, 17.7 ± 3.6 mm/mm²; females, 18.2 ± 3.7 mm/mm²; $P = 0.62$) did not differ between sexes. Four participants (6%) reported to be current smokers with an average 19 cigarettes per day. The CNFL was not significantly different between current smokers and nonsmokers ($t = 1.3$, $P = 0.20$). A total of 52 participants (81%) reported current alcohol use with an average 5.9 units/wk. No significant correlation was found between alcohol consumption (units/week) and CNFL (Spearman's Rho, $r_s = -0.09$, $P = 0.53$) at baseline visit. Nine participants were taking antidepressant medications during study period. No association was observed between the use of antidepressant drugs and mean CNFL at annual visits (independent samples *t*-test, $P = 0.88$, 0.31, 0.32, and 0.86 at baseline, and year 1, 2, and 3 visits, respectively).

TABLE 1. Clinical Demographic, Metabolic and Ocular Screening Measures of Study Participants at Baseline and 36-Month Visits

Parameter	Baseline	36 mo	P Value, Paired <i>t</i> -Test
Age, y	51.9 ± 14.7	-	-
Sex, male/female	29/35	24/28	-
HbA _{1c} , %NGSP	5.4 ± 0.3	5.3 ± 0.4	<0.01
Total cholesterol, mmol/L	5.4 ± 1.2	5.5 ± 1.1	0.86
HDL, mmol/L	1.5 ± 0.4	1.5 ± 0.4	0.16
LDL, mmol/L	3.4 ± 1.1	3.4 ± 1.0	0.12
Triglycerides, mmol/L	1.1 ± 0.5	1.1 ± 0.5	0.22
Systolic blood pressure, mm Hg	116.0 ± 13.2	116.2 ± 14.0	0.89
Diastolic blood pressure, mm Hg	72.8 ± 6.9	72.1 ± 8.4	0.67
Height, cm	170.2 ± 8.7	170.3 ± 8.8	0.66
Weight, kg	75.7 ± 16.2	75.7 ± 13.7	0.48
BMI, kg/m ²	26.1 ± 5.1	26.1 ± 4.8	0.67
Visual acuity, logMAR	0.04 ± 0.07	0.03 ± 0.08	0.15*
IOP, mm Hg	13.1 ± 2.9	13.3 ± 3.1	0.90

Values shown are mean ± SD, or counts for categorical variables.
* Wilcoxon test.

Participants were divided into three age groups: group 1, aged <45 years ($n = 19$); group 2, aged 45 to 59 years ($n = 25$); and group 3, aged ≥60 years ($n = 20$, Table 2). There was no significant effect of age group on CNFL (Welch ANOVA, $P = 0.50$).

Apart from a clinically insignificant decline in HbA_{1c} ($P < 0.01$) over 36 months, there were no significant changes to health, metabolic, or ocular screening measures (Table 1). There also was no correlation between absolute changes in CNFL and HbA_{1c} from baseline to the 36-month visit (Pearson, $r = 0.11$, $P = 0.49$).

The LMM1 was deployed to determine the association of age and CNFL. Using a backward elimination procedure, fixed effects of sex*age interaction ($F_{[1,30]} = 0.02$, $P = 0.89$) and sex ($F_{[1,16]} = 0.04$, $P = 0.85$) were sequentially removed. Type III tests of fixed effects revealed that there was a significant influence of age on CNFL ($F_{[1,33]} = 5.67$, $P = 0.02$). Estimates of fixed effects and covariance parameters are presented in Table 3.

The natural history of CNFL over the 36-month observation period is depicted graphically in the Figure. The LMM2 revealed that the linear effects of time ($F_{[1,55]} = 0.69$, $P = 0.41$), sex ($F_{[1,61]} = 1.10$, $P = 0.30$), age at enrollment ($F_{[1,60]} = 1.13$, $P = 0.29$), and time*sex interaction ($F_{[1,55]} = 1.41$, $P = 0.24$) were not statistically significant. To eliminate further the potential confounding effect of antidepressant drugs on the

TABLE 2. Age and CNFL at Baseline in Three Age Groups

Age Groups	No. of Participants	CNFL, mm/mm ² , mean ± SD*	Age, y, mean ± SD†
Group 1: <45 y	19	18.6 ± 2.3	33.4 ± 8.7
Group 2: 45-59 y	25	18.1 ± 3.2	53.3 ± 4.4
Group 3: ≥60 y	20	17.2 ± 4.9	67.8 ± 3.4
Total group	64	18.0 ± 3.6	51.9 ± 14.7

* No significant difference among groups (Welch ANOVA statistics = 0.71, $P = 0.50$).

† Significant difference among groups (1-way ANOVA, $F = 172.8$, $P < 0.001$).

TABLE 3. Estimates of Fixed Effects and Covariance Parameters From Linear Mixed Model 1 in Which the Relationship of Age and CNFL was Examined

Parameter	Estimate	SE	P Value	95% CI
Estimates of fixed effects*				
Intercept	20.94	0.89	0.00	19.06 to 22.83
Age	-0.05	0.02	0.02	-0.09 to -0.01
Estimates of covariance parameters*				
Residual	4.25	0.47	<0.001	3.42 to 5.27
Intercept + age				
UN _(1,1)	9.10	5.45	0.09	2.81 to 29.42
UN _(2,1)	-0.30	0.15	0.05	-0.61 to 0.00
UN _(2,2)	0.01	0.004	0.02	0.00 to 0.02

CI, confidence interval; UN, unstructured variance-covariance matrix for random effects.

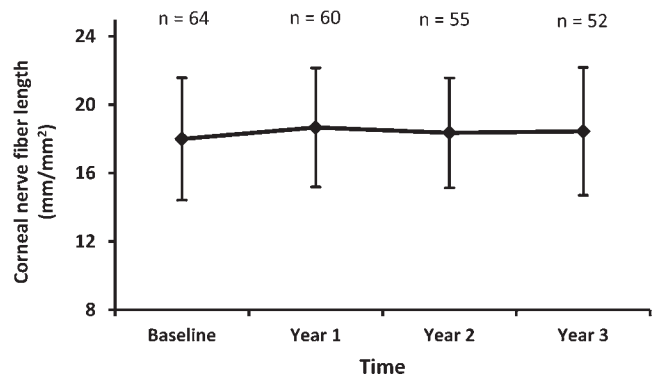
* Dependent variable, CNFL.

analysis of data relating to the longitudinal course of CNFL in healthy participants, LMM2 was repeated excluding participants who were receiving antidepressant therapy during the study period. The results were similar to those for the total cohort, with no significant effect of time ($P = 0.47$), sex ($P = 0.25$), age at enrollment ($P = 0.29$), and time*sex interaction ($P = 0.16$).

DISCUSSION

The feasibility of assessing corneal nerve morphology via CCM and the promising role of these structural parameters as an indicator of corneal nerve recovery following surgical and pharmacologic intervention, and the potential for screening for peripheral neuropathies, has led to an increase in the scope of this approach. An increasing number of studies showing a relationship between quantitative analysis of SNP parameters, and various ocular and systemic pathologic conditions or surgical-induced changes, highlights the importance of understanding the natural morphometric characteristics of the SNP over time.

In this longitudinal prospective study, participants were followed over 36 months with repeated monitoring of ocular, health, and CNFL measures. At baseline, the cohort was sex balanced (45% male) and age was not significantly different between sexes. The sex of participants also was shown to have no influence on CNFL. While the variability from the mean of CNFL increased with age (Table 2), mean CNFL between the

**FIGURE.** Quantification of CNFL in healthy controls over 36 months. The CNFL did not change over three years of follow up (linear mixed model, $P = 0.41$). Error bars represent mean ± SD.

groups was not significantly different. This finding is consistent with those of Patel et al.¹⁹ who found no significant differences in mean CNFL between three age groups in a cohort of 60 healthy participants. Conversely, Grupcheva et al.²⁹ reported a significant difference in mean CNFL between two age groups (25 ± 5 vs. 70 ± 5 years) of 50 participants.

Using laser-scanning CCM, a great diversity has been reported in CNFL quantification in healthy individuals.^{22,26,30,31} The mean central CNFL in the current study (18.0 ± 3.6 mm/mm²) is identical to that reported by Wu et al.²⁶ (18.0 ± 4.0 mm/mm²), but slightly lower than the findings of Niederer et al.²² (20.3 ± 6.5 mm/mm²) and Parissi et al.²³ (18.6 ± 4.8 mm/mm²). Differences in methodologies, including number of participants, selected images, age range, and method of CNFL analysis, may account for these small discrepancies.

A strength of the present study was consistency in respect to the location of corneal assessment (central), which was facilitated by an optimized sampling paradigm for the central region of the cornea that involved selection of a prescribed number of centrally-located images with minimum overlap.²⁷ As well, employment of an objective, fully-automated image analysis system²⁸ facilitated reliable and objective quantification of CNFL, which was important for ascertaining the natural course of this CCM measure. It has been demonstrated that fully-automated analysis of CNFL obtained from laser-scanning CCM images agrees very well with semiautomated and manual analysis,²⁸ and yields results with a high level of reproducibility.

In the current literature, there is some discrepancy among ex vivo studies as to whether corneal nerve structure changes with age. While subbasal nerve fiber density has been reported to reduce with age in an ex vivo study of 22 donor corneas aged from 19 to 80 years,²¹ Marfurt et al.,¹⁸ using an immunohistochemical staining technique, found no significant correlation between CNFL and age in corneas of six donors aged 19 to 78 years. Such a disagreement exists among studies using in vivo CCM as well.^{19,20,22,23}

The usual design employed in previous studies reporting the effect of age on corneal nerve morphology has been cross-sectional, in which measurements are made on participants of various ages and the detected differences are attributed to the effect of age. However, such results do not necessarily reflect real age changes. A longitudinal design with serial measurements in the same individuals over time allows true age changes for individuals to be determined. The findings of the current study (LMM1, Table 3) showed that there was a significant linear decrease in CNFL with age. The mean estimated initial status (at birth) and the linear change rate (per year) of CNFL for the total group were 20.94 and -0.05 mm/mm², respectively. This suggested that 1 mm/mm² reduction in central corneal nerve morphology would require 20 years to take place in healthy participants. The cross-sectional studies of Niederer et al.²² and Parissi et al.²³ reported a gradual decline in CNFL with age at a rate of 0.9% and 0.30% per year, respectively, which overestimate the finding our longitudinal study reported here (0.05 mm/mm² per year).

Although marginally nonsignificant at $\alpha < 0.05$, the estimated covariance of the two random effects in the LMM1; that is, intercept and age ($\beta = -0.30$, $P = 0.05$) was negative (Table 3), which suggested individuals with high CNFL had a slower linear decrease, whereas individuals with low CNFL had a faster decrease, with age. There also is evidence of significant variance in these random effects ($\beta = 0.01$, $P = 0.02$), indicating variation among individuals in the rate of change of CNFL.

Apart from HbA_{1c} with a minor (0.1% NGSP), but statistically significant difference, the average of all clinical, metabolic, and ocular screening measures remained stable from baseline to 36-month visit. The LMM2 showed that in this 3-year longitudinal study, CNFL appeared to be stable as a

function of time. The relationship of time with CNFL change did not vary depending on sex, yielding a similar longitudinal pattern of CNFL over three years for males and females. It also is worth noting that, while neuronal plasticity and regeneration can be influenced by antidiabetic treatment,^{32,33} when our analysis was restricted to participants who were not receiving these medications, our results closely resembled those from the total cohort.

To our knowledge, no previous study has reported a longitudinal analysis of corneal nerve morphology in healthy individuals. The results presented here demonstrate, for the first time to our knowledge, stability of human corneal nerve morphology as assessed by laser-scanning CCM over a 3-year period. These findings are important in demonstrating a significant, albeit weak, association between CNFL and age, and the 3-year morphometric stability of the SNP in healthy individuals. These data provided in vivo evidence for stability of this structural parameter in healthy individuals and added a longitudinal perspective to consider alongside the results of cross-sectional studies demonstrating the dependence of this parameter with age. The outcomes of this study may improve the ability of clinicians and researchers to understand the time-course of central corneal reinnervation following interventions, such as keratorefractive surgeries and pharmacologic treatment, and will assist in the interpretation of longitudinal studies using CNFL assessment as a screening/monitoring marker for peripheral neuropathies.

Although we found stability of CNFL over a 36-month follow up period, this finding might not apply to CNFL changes over longer time periods. Furthermore, these findings are limited to nerve changes in the central cornea, and may not be applicable to other more peripheral regions of the human SNP. More recently, in vivo wide-field maps of the human SNP have been generated successfully,^{34,35} which might be useful to provide insights into changes in the entire SNP, if this procedure were to be deployed in longitudinal studies.

In conclusion, the current longitudinal in vivo CCM study confirms a slight reduction in CNFL as a function of age, while there was no significant dynamic morphologic change over 36 months. The data of this longitudinal study provide a better understanding of the SNP in the living human cornea in a healthy state, which has implications in investigating the effect of corneal surgery, transient or chronic alterations as a cause of, or secondary to, local disease, or peripheral neuropathies, using CNFL as a noninvasive biomarker.

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