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Morphometric stability of the corneal subbasal nerve plexus in healthy individuals: a 3-year longitudinal study using corneal confocal microscopy

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1 figure

3 tables
Abstract

**Purpose:** To examine the age-dependent alterations and the longitudinal course of subbasal nerve plexus (SNP) morphology in healthy individuals.

**Methods:** Sixty-four healthy participants had ocular screening, health and metabolic assessment as well as laser-scanning corneal confocal microscopy examination at baseline and at 12-month intervals for three 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 gender 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 genders. Fifty-two 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:** 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
Introduction

In vivo corneal confocal microscopy (CCM) is a rapid, non-invasive and reiterative technique which enables microstructural evaluation of the human cornea at high resolution. The anatomical location and transparency of the cornea make this tissue structure ideally suited for confocal microscopic assessment. Image acquisition using CCM from different corneal layers and structures helps both clinicians and researchers to extract important information in respect to 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 distinct morphologic attributes such as length of the nerve bundles and their parallel arrangement in relation to the ocular surface. Structural analysis of the SNP has been used to evaluate ocular conditions such as dry eye, ocular allergy and glaucoma, corneal ectasia and dystrophies, the effect of contact lens wear and assessment of nerve regeneration after penetrating keratoplasty and different forms of refractive surgery. Further to ocular applications, CCM has been deployed to assess small nerve fiber pathology induced by several systemic conditions including diabetes, Fabry disease, idiopathic neuropathy and chemotherapy.

Given the utility of SNP evaluation in screening, detection and monitoring of a wide range of systemic and corneal 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 both ex vivo and in vivo techniques. While a number of studies have reported
no significant change in the subbasal nerve morphology with age,\textsuperscript{18-20} others have reported a decrease in nerve density with age\textsuperscript{21-23} and there is also uncertainty as to the age at which SNP structural loss become significant. Furthermore, no data is available concerning the dynamic morphologic changes of corneal nerves in health or disease over time.

The two primary objectives of this study were to investigate: (1) 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 (2) longitudinal changes of CNFL over three years in healthy human corneas.

\textbf{Methods}

\textbf{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, as a part of 4-year LANDMark (Longitudinal Assessment of Neuropathy in Diabetes using novel ophthalmic Markers) study.\textsuperscript{14} Exclusion criteria were: history of corneal surgery, trauma or disease, glaucoma, evidence of corneal compromise, ocular and systemic diseases (e.g. diabetes) 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. Since previous studies investigating the impact of contact lens wear on morphologic changes in subbasal nerves using CCM have failed to demonstrate any impact\textsuperscript{24-26}, we did not exclude contact lens wearers in the present study. 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 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 anaesthetized with a drop of 0.4% benoxinate hydrochloride (oxybuprocaine hydrochloride; Bausch & Lomb, NSW, Australia). Eight central corneal images per participants, displaying in-focus nerves and not overlapping more than 20\%,\textsuperscript{27} were selected by inspection and analyzed using a fully-automated analytical system\textsuperscript{28} to quantify CNFL, which is defined as total length of all nerve fibers in the CCM image (in units of mm/mm\textsuperscript{2}).

Since this study was a 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 procedure. Intraclass correlation coefficient and coefficient of variability for intraobserver were 0.90 and 5.7% and for interobserver were 0.94 and 8.1%, respectively.

**Blood biochemistry and health parameters**

At each visit, blood biochemistry measures (HbA1c and lipid profile) were assayed by a local certified pathology laboratory, 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). Normal distribution of the data was determined with the Kolmogorov-Smirnov test. Quantitative variables are expressed by the mean ± standard deviation (SD) unless otherwise indicated. For the analysis of the categorical variables, the $\chi^2$ test was applied. The independent samples t-test was used to compare age and CNFL between genders. Bivariate correlation was used, as appropriate, for assessment of association of CNFL with alcohol consumption and absolute changes in CNFL with HbA1c. 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 paired sample t-test (normally distributed) and nonparametric Wilcoxon test (not-normally distributed).

To analyze longitudinal data using the linear mixed model (LMM) procedure in the SPSS statistical software, the horizontal data format were 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 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 gender. CNFL was defined as dependent variable. Age (time-varying predictor variable) and gender (time-invariant variable) were specified as covariate and factor, respectively. Age, gender and the gender*age interaction were specified as fixed effects and 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 dependent variable and time which was a variable capturing the order of observation, was defined as repeated variable. We assumed that the correlation between two adjacent CNFL measurements declines across measurement occasions, therefore first order autoregressive covariance structure was chosen. CNFL and gender were considered as dependent variable and factor, respectively. Time and age at enrolment 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 $51.9 \pm 14.7$ years. Age (males: $55.1 \pm 14.0$ years, females: $49.3 \pm 15.0$ years, $p = 0.12$) and CNFL (males: $17.7 \pm 3.6$ mm/mm$^2$, females: $18.2 \pm 3.7$ mm/mm$^2$, $p = 0.62$) did not differ between genders. Four participants (6%) reported to be current smokers with an average 19 cigarettes per
day. CNFL was not significantly different between current smokers and non-smokers (t = 1.3, p = 0.20). Fifty-two participants (81%) reported current alcohol use with an average 5.9 units/week. 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 antidepressants during study period. No association was observed between using antidepressant drugs and mean CNFL at annual visits (independent samples t-test, $p = 0.88$, $p = 0.31$, $p = 0.32$ and $p = 0.86$ at baseline, year 1, year 2 and year 3 visits, respectively).

Participants were divided into three age groups: group 1 aged < 45 years ($n = 19$), group 2 aged 45 - 59 years ($n = 25$) and group 3 aged ≥ 60 years ($n = 20$) (Table 2). There was not a significant effect of age groups 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 was also no correlation between absolute changes in CNFL and HbA$_{1c}$ from baseline to the 36-month visit (Pearson, $r = 0.11$, $p = 0.49$).

LMM1 was deployed to determine the association of age and CNFL. Using backward elimination procedure, fixed effects of gender*age interaction ($F_{(1, 30)} = 0.02$, $p = 0.89$) and gender ($F_{(1, 16)} = 0.04$, $p = 0.85$) were sequentially removed. Type III tests of fixed effects revealed that there was a significant influence of age ($F_{(1, 33)} = 5.67$, $p = 0.02$) on CNFL. 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 Figure 1. LMM2 revealed that the linear effect of time ($F_{(1, 55)} = 0.69$, $p = 0.41$), gender ($F_{(1, 61)} = 1.10$, $p = 0.30$), age at enrolment ($F_{(1, 60)} = 1.13$, $p = 0.29$)
and time*gender interaction ($F_{(1, 55)} = 1.41, p = 0.24$) were not statistically significant.

To further eliminate the potential effect of antidepressants on the longitudinal course of CNFL in healthy participants, LMM2 was repeated excluding participants who were receiving antidepressant therapy during study period. The results were similar to the total cohort with no significant effect of time ($p = 0.47$), gender ($p = 0.25$), age at enrolment ($p = 0.29$) and time*gender 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 pharmacological 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 the SNP parameters and various ocular and systemic pathological conditions or surgical-induced changes, highlights the importance of understanding the natural morphometric behavior 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, our cohort was gender balanced (45% male) and age was not significantly different between genders. The gender of participants was also shown to have no influence on CNFL. While the variability from the mean of CNFL increased with age (Table 2), mean CNFL between the groups was not significantly different. This finding is consistent with those of Patel et al.\textsuperscript{19} who found no significant differences in mean CNFL between three age groups in a cohort of 60 healthy participants. Conversely,
Grupcheva et al\textsuperscript{29} reported a significant difference in mean CNFL between two age
groups (25 ± 5 years vs. 70 ± 5 years) of 50 participants.

Using laser-scanning CCM, a great diversity has been reported in CNFL
quantification in healthy individuals.\textsuperscript{22, 26, 30, 31} The mean central corneal nerve fiber
length in the current study was 18.0 ± 3.6 mm/mm\(^2\) which is similar to that reported
by Wu et al (18.0 ± 4.0 mm/mm\(^2\))\textsuperscript{26}, but lower than those of Niederer et al (20.3 ± 6.5
mm/mm\(^2\))\textsuperscript{22} and Parissi et al (18.6 ± 4.8 mm/mm\(^2\), right eyes and automated
analysis).\textsuperscript{23} Differences in methodologies including number of participants, selected
images, age range and method of CNFL analysis may account for differing results.

One strength of the present study was our consistency in respect to the location of
corneal assessment (central), which entitled a rigid sampling paradigm for the central
region of the cornea, including the number of selected images and the use of an
objective, fully-automated analysis system for image analysis. Employment of a fully-
automated analysis system 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 semi-automated and manual analysis\textsuperscript{28}
and yields results with a high level of reproducibility.

In the current literature, there is some discrepancy among studies as to whether
corneal nerve structure changes with age. While subbasal nerve fiber density has
been reported to reduce with age in an \textit{ex vivo} study in 22 donor corneas aged from
19 to 80 years,\textsuperscript{21} Marfurt et al\textsuperscript{18} using immunohistochemical staining technique found
no significant correlation between CNFL and age in corneas of six donors aged 19 -
78 years. Such a disagreement exists among studies using in vivo CCM as well.\textsuperscript{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 the 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 mm/mm\textsuperscript{2} and -0.05 mm/mm\textsuperscript{2}, respectively. This suggests that 1 mm/mm\textsuperscript{2} reduction in central corneal nerve morphology would require 20 years to take place in normal participants. Although they are cross-sectional, the results of studies by Niederer et al\textsuperscript{22} and Parissi et al\textsuperscript{23} who reported a gradual decline in CNFL with age at a rate of 0.9\% per year and 0.30\% per year, respectively, are in general agreement with our finding. Although marginally non-significant at $\alpha < 0.05$, the estimated covariance of the two random effects in the LMM1 i.e. intercept and age ($\beta = -0.30$, $p = 0.05$) was negative (Table 3), which suggests individuals with high CNFL had a slower linear decrease, whereas individuals with low CNFL had a faster decrease with age. There is also evidence of significant variance in these random effects ($\beta = 0.01$, $p = 0.02$) which indicates that the slopes tend to vary from individual to individual. Apart from HbA\textsubscript{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. 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 gender, yielding a similar longitudinal pattern of CNFL over three years for males and females. It is also worth noting that while neuronal plasticity and regeneration can be influenced by antidepressant treatment, when our analysis was restricted to participants who were not receiving these medications; our results closely resembled those from the total cohort.

No previous study has conducted a longitudinal analysis of corneal nerve morphology in healthy individuals. The results we present allow us to show, for the first time, stability of human corneal nerve morphology as assessed by laser-scanning CCM over a 3-year period. Our study is important in demonstrating: (1) significant association, albeit weak, between CNFL and age and (2) a 3-year morphometric stability of the SNP in healthy individuals. These data provide in vivo evidence for stability of this structural parameter in healthy individuals and add a longitudinal component to the 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 kerato-refractive surgeries and pharmacological 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 this measure 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, \textit{in vivo} wide-field maps of the human SNP have been successfully generated,\textsuperscript{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 \textit{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 constitute a better understanding of SNP in living human cornea in a healthy state, which has implications in investigating the effect of corneal surgery, known transient or chronic alterations as a cause of or secondary to local disease or non-invasive indicator of peripheral neuropathies.
References


### Table 1. Clinical demographic, metabolic and ocular screening measures of study participants at baseline and 36-month visits.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>36 months</th>
<th>p-value (paired t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51.9 ± 14.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>29/35</td>
<td>24/29</td>
<td>-</td>
</tr>
<tr>
<td>HbA1c (%NGSP)</td>
<td>5.4 ± 0.3</td>
<td>5.3 ± 0.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.4 ± 1.2</td>
<td>5.5 ± 1.1</td>
<td>0.86</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.5 ± 0.4</td>
<td>1.5 ± 0.4</td>
<td>0.16</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.4 ± 1.1</td>
<td>3.4 ± 1.0</td>
<td>0.12</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.1 ± 0.5</td>
<td>1.1 ± 0.5</td>
<td>0.22</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>116.0 ± 13.2</td>
<td>116.2 ± 14.0</td>
<td>0.89</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>72.8 ± 6.9</td>
<td>72.1 ± 8.4</td>
<td>0.67</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>170.2 ± 8.7</td>
<td>170.3 ± 8.8</td>
<td>0.66</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>75.7 ± 16.2</td>
<td>75.7 ± 13.7</td>
<td>0.48</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.1 ± 5.1</td>
<td>26.1 ± 4.8</td>
<td>0.67</td>
</tr>
<tr>
<td>Visual acuity (LogMAR)</td>
<td>0.04 ± 0.07</td>
<td>0.03 ± 0.08</td>
<td>0.15*</td>
</tr>
<tr>
<td>Intra-ocular pressure (mmHg)</td>
<td>13.1 ± 2.9</td>
<td>13.3 ± 3.1</td>
<td>0.90</td>
</tr>
</tbody>
</table>

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

*Wilcoxon test

### Table 2. Age and corneal nerve fiber length (CNFL) at baseline in three age groups.

<table>
<thead>
<tr>
<th>Age groups</th>
<th>No. of participants</th>
<th>CNFL (mean ± SD)*</th>
<th>Age (mean ± SD)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: &lt;45 yrs</td>
<td>19</td>
<td>18.6 ± 2.3</td>
<td>33.4 ± 8.7</td>
</tr>
<tr>
<td>Group 2: 45-59 yrs</td>
<td>25</td>
<td>18.1 ± 3.2</td>
<td>53.3 ± 4.4</td>
</tr>
<tr>
<td>Group 3: ≥ 60 yrs</td>
<td>20</td>
<td>17.2 ± 4.9</td>
<td>67.8 ± 3.4</td>
</tr>
<tr>
<td>Total group</td>
<td>64</td>
<td>18.0 ± 3.6</td>
<td>51.9 ± 14.7</td>
</tr>
</tbody>
</table>

* No significant difference among groups (Welch ANOVA statistics = 0.71, p = 0.50);
† Significant difference among groups (one-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 corneal nerve fiber length was examined.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>p-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>20.94</td>
<td>0.89</td>
<td>0.00</td>
<td>19.06 to 22.83</td>
</tr>
<tr>
<td>Age</td>
<td>-0.05</td>
<td>0.02</td>
<td>0.02</td>
<td>-0.09 to -0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>p-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residual</td>
<td>4.25</td>
<td>0.47</td>
<td>&lt;0.001</td>
<td>3.42 to 5.27</td>
</tr>
<tr>
<td>Intercept + age</td>
<td>UN(_{1,1})</td>
<td>9.10</td>
<td>5.45</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>UN(_{2,1})</td>
<td>-0.30</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>UN(_{2,2})</td>
<td>0.01</td>
<td>0.004</td>
<td>0.02</td>
</tr>
</tbody>
</table>

CI: confidence interval; UN: unstructured variance-covariance matrix for random effects
* Dependent variable: corneal nerve fiber length
Figure 1. Quantification of corneal nerve fiber length (CNFL) in healthy participants over 36 months. CNFL did not change over three years follow up (linear mixed model, p = 0.41). Error bars represent mean ± SD.