Normative Values for Corneal Nerve Morphology Assessed Using Corneal Confocal Microscopy: A Multinational Normative Data Set

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OBJECTIVE
Corneal confocal microscopy is a novel diagnostic technique for the detection of nerve damage and repair in a range of peripheral neuropathies, in particular diabetic neuropathy. Normative reference values are required to enable clinical translation and wider use of this technique. We have therefore undertaken a multicenter collaboration to provide worldwide age-adjusted normative values of corneal nerve fiber parameters.

RESEARCH DESIGN AND METHODS
A total of 1,965 corneal nerve images from 343 healthy volunteers were pooled from six clinical academic centers. All subjects underwent examination with the Heidelberg Retina Tomograph corneal confocal microscope. Images of the central corneal subbasal nerve plexus were acquired by each center using a standard protocol and analyzed by three trained examiners using manual tracing and semiautomated software (CCMetrics). Age trends were established using simple linear regression, and normative corneal nerve fiber density (CNFD), corneal nerve fiber branch density (CNBD), corneal nerve fiber length (CNFL), and corneal nerve fiber tortuosity (CNFT) reference values were calculated using quantile regression analysis.

RESULTS
There was a significant linear age-dependent decrease in CNFD (−0.164 no./mm² per year for men, P < 0.01, and −0.161 no./mm² per year for women, P < 0.01). There was no change with age in CNBD (0.192 no./mm² per year for men, P = 0.26, and −0.050 no./mm² per year for women; P = 0.78). CNFL decreased in men (−0.045 mm/mm² per year, P = 0.07) and women (−0.060 mm/mm² per year, P = 0.02). CNFT increased with age in men (0.044 per year, P < 0.01) and women (0.046 per year, P < 0.01). Height, weight, and BMI did not influence the 5th percentile normative values for any corneal nerve parameter.

CONCLUSIONS
This study provides robust worldwide normative reference values for corneal nerve parameters to be used in research and clinical practice in the study of diabetic and other peripheral neuropathies.
The use of corneal confocal microscopy (CCM) for rapid, noninvasive clinical assessment of corneal nerves has grown substantially in recent years (1). It has proven to be particularly useful as a diagnostic marker for the detection of diabetic neuropathy (2–6) and a range of other peripheral neuropathies (7–10).

However, the clinical translation of CCM has been limited by a lack of normative reference values, allowing investigators to define pathological changes. Furthermore, there is considerable inconsistency in the literature with regard to the density of subbasal nerves in the cornea in control subjects based on the type of instrument, protocol used to acquire images, and definition of corneal nerve structures (3,11). Studies using laser scanning confocal microscopy have reported higher densities of subbasal corneal nerves compared with studies using the tandem scanning confocal microscope and slit scanning confocal microscope, due to differences in the light source, contrast, and resolution of these instruments (11). Furthermore, a range of methods has been used to capture and quantify the nerves and differences in terms of the site and number of images selected and analyzed per subject (12). The majority of studies have defined subbasal nerve density as the total number of major nerves in an area of corneal tissue (no./mm²) (3,13). However, others have presented the data as the number of nerves per image (14) or the total length of the nerves within a frame (15,16) and still referred to it as a density.

Aging and sex may impact peripheral nerve morphology. Corneal sensitivity has been shown to decline with age (17). However, while some relatively small studies have demonstrated no correlation between corneal nerve density and age (4,15,16,18), other earlier studies demonstrated a significant reduction with age (16,19). Using a definition of nerve density that is consistent with our definition of corneal nerve fiber length (CNFL), a study of 85 healthy control subjects showed a linear decline in subbasal nerve density of 0.9% per year (20).

The aim of the present international multicenter collaboration was to establish worldwide age-adjusted normative values of corneal nerve fiber parameters using a common adopted method to capture the images: the same CCM instrument and manual analysis using strict definitions of corneal nerve fiber parameters (3,21).

RESEARCH DESIGN AND METHODS
Six independent international study groups who have previously reported normal values for corneal nerve parameters using the Heidelberg Retina Tomograph (HRT III) Rostock Cornea module (RCM) were invited to provide the coordinating center (Manchester, U.K.) with CCM images of corneal nerves from their healthy subjects who were enrolled and had consented and been studied previously. Corneal nerve images from 343 healthy volunteers were pooled from each academic center (Manchester, n = 139; Brisbane, n = 59; Utah, n = 48; Düsseldorf, n = 42; Calgary, n = 41; and Toronto, n = 14).

Eligibility criteria for inclusion of subjects as control subjects in the original study from each center were good health, age between 5 and 85 years, and lack of neurological signs or symptoms or conditions known to cause neuropathy including diabetes, impaired glucose tolerance, metabolic syndrome, vitamin B₁₂ deficiency, and idiopathic small fiber neuropathy. Subjects with a history of using hard contact lenses and previous refractive surgery were excluded. Data on height, weight, BMI, and HbA₁c were recorded where available. In each center, informed written consent was obtained from all participants prior to their enrolment in the study. All control subjects were recruited and assessed; as part of ongoing clinical studies at each center, they underwent detailed evaluation of neurological symptoms and deficits, quantitative sensory testing, and electrophysiology as well as standard blood tests to exclude other causes of neuropathy, which included serum B₁₂, folate, antinuclear antibody (ANA), and immunoglobulins. This research adhered to the tenets of the Declaration of Helsinki, and local ethics committee approval was obtained in all centers.

Subjects underwent examination with the HRT III Rostock Cornea module in vivo corneal confocal microscope (IVCCM). The subject’s eyes were anesthetized using a drop of 0.4% Benoxinate hydrochloride, and ViscoTears was applied on the front of the eye for lubrication. A drop of viscoelastic gel was placed on the tip of the objective lens, and a sterile disposable Perspex cap was placed over the lens allowing optical coupling of the objective lens to the cornea. The patient was instructed to fixate on a target with the eye not being examined. Several scans of the entire depth of the cornea were recorded by turning the fine focus of the objective lens backward and forward for −2 min using the section mode, which enables manual acquisition and storage of single images of all corneal layers. This provides an en face two-dimensional images with a lateral resolution of −2 μm/pixel and a final image size of 400 × 400 pixels of the subbasal nerve plexus of the cornea from each control subject. This layer is of particular relevance for defining neuropathic changes, since it is the location of the main nerve plexus that supplies the overlying corneal epithelium. Each nerve fiber bundle contains unmyelinated fibers, which run parallel to Bowman layer before dividing and terminating as individual axons in the surface epithelium. Three to six images from the center of the cornea of both eyes were selected and examined in a masked fashion. Three experienced examiners (M.T., M.F., and I.N.P.), masked from the clinical details of the subjects, quantified 1,965 images of all study participants using purpose-written, proprietary software (CCMetrics [M.A. Dabbah, Imaging Science, University of Manchester]). Four corneal nerve parameters were quantified: 1) corneal nerve fiber density (CNFD) (the total number of major nerves per millimeter squared of corneal tissue), 2) corneal nerve branch density (CNBD) (the number of branches emanating from all major nerve trunks per millimeter squared of corneal tissue), 3) CNFL (the total length of all nerve fibers and branches within the area of corneal tissue, and 4) corneal nerve fiber tortuosity (CNFT) (tortuosity coefficient [TC]), which represents the degree of tortuosity from a straight line joining the ends of each main nerve fiber.

Statistical Analysis
Data are presented as mean (SD), Median, or quantile range for men and women by each age decade. Age trends in men and women were investigated separately using simple linear regression analyses. Quantile regression was carried out to determine normative reference values for
CNFD, CNBD, CNFL, and CNFT. This technique allows quantiles to be estimated as a smooth function of age without imposing a parametric distribution, and this method is particularly robust in the presence of outliers.

RESULTS
Of the subjects from six centers that participated in this study, providing IVCCM images of the corneal subbasal nerves from 343 healthy subjects aged between 9 and 82 years old, 172 were male and 171 were female. The demographic results of the participants are presented in Table 1. HbA1c was available for 133 subjects, and BMI, height, and weight were available for 266 subjects (Table 1). HbA1c, height, weight, and BMI did not influence the 5th percentile normative values for CNFD, CNBD, CNFL, or CNFT.

Representative images for each age-group are presented in Fig. 1. Median values for corneal nerve parameters are presented in Table 2 for female and male subjects. Fifth quantile values for CNFD, CNBD, and CNFL and 95th quantiles for CNFT are presented in Table 3. There was a significant linear age-dependent decrease in CNFD (−0.164 no./mm² per year for men, P < 0.01, and −0.161 no./mm² per year for women, P < 0.01). There was no change in CNBD (0.192 no./mm² per year for men, P = 0.26, and −0.050 no./mm² per year for women, P = 0.78). CNFL decreased in men (−0.045 mm/mm² per year, P = 0.07) and women (−0.060 mm/mm² per year, P = 0.02). CNFT increased in men (0.044 per year, P < 0.01) and women (0.046 per year, P < 0.01).

Regression plots for each corneal nerve fiber parameter with lines denoting the median and the 5th and the 95th percentiles are shown in Fig. 2. The findings were similar regardless of sex for CNFD (R² = 0.11, slope loss % per year −0.161, P < 0.0001), CNBD (R² = 0.005, slope loss % per year −0.074, P = 0.55), CNFL (R² = 0.026, slope loss % per year −0.052, P = 0.003), and CNFT (R² = 0.052, slope increase % per year 0.044, P < 0.0001).

CONCLUSIONS
IVCCM has evolved rapidly from a technique predominantly used in clinical research to a diagnostic tool for a variety of ophthalmic and neurological applications (1). It has now emerged as a powerful technique for the study of corneal cellular structure in health and disease in patients (22) and in animal models of diabetic neuropathy (23). The noninvasive nature of this technique has made it an ideal tool to examine all microstructures of the cornea (24).

There is a burgeoning literature on the use of CCM to quantify peripheral neuropathies, in particular diabetic neuropathy (2–6). Thus, CCM has been shown to be an accurate noninvasive method for the early diagnosis of diabetic neuropathy (3,25), idiopathic small-fiber neuropathy (26), Fabry disease (9), hereditary sensory and autonomic neuropathy (8), autoimmune neuropathy (27), and neuropathy associated with Crohn disease and chemotherapy (28). It has been shown to have high reproducibility (29,30) and repeatability (32), with good sensitivity and specificity for the diagnosis of diabetic neuropathy (3,32).

However, before CCM can be adopted for wider clinical use, there is clearly a need to standardize the method of capturing, sampling, and analyzing the images. And there is a need to adopt a global standard approach to quantify corneal nerve morphology using rigorous definitions for each morphologic parameter to enable comparison between different studies. The use of standardized manual analysis software would resolve this problem partially, but of course interobserver differences would remain, based on difference between observers in the recognition of corneal nerve structures (31). This can be overcome with the use of automated image

| Figure 1—Representative CCM images from the subbasal plexus per age-group, yrs, years. |
analysis software that has been developed recently to rapidly quantify corneal nerve pathology (33,34).

As stated earlier, while some relatively small studies have demonstrated no correlation between corneal nerve density and age (4,15,16,18), other earlier studies demonstrated a significant reduction with age (16,19). The disagreement between these previous studies can be potentially explained by the types of corneal confocal microscopes (tandem scanning confocal microscope, slit scanning confocal microscope, and laser scanning corneal confocal microscope) that have been used to acquire images and also the method (volume scan, section scan) used to capture the images as well as the parameters measured. The current data set shows that corneal nerve morphology behaves very similarly to intraepidermal nerve fiber density (IENFD) in terms of an age-dependent decrease in nerve fiber density (35). Furthermore, we show an age-dependent reduction in CNFL and an increase in CNFT but no significant effect on CNBD, presumably from increases as well as decreases in nerve branches due to concomitant degeneration and regeneration, respectively. The possible effects of weight and indeed BMI have been suggested as possible confounders when undertaking longitudinal studies using IENFD (36). Thus, it is reassuring that like IENFD (35), corneal nerve fiber parameters also showed no effect of height, weight, or BMI. A previous study in 64 healthy volunteers showed that CNFL had a broad distribution and was not related to age, although their age range was limited to 39 ± 18 years. Furthermore, multivariate regression analysis demonstrated that HbA1c was the only independent variable accounting for the variation in CNFL (37). Indeed, we also show a broad distribution for corneal nerve parameters, but this is primarily explained by an effect of age. This age effect may be relevant when interpreting recent data showing a more marked reduction in CNFL in patients with type 1 diabetes and paradoxically minimal change in patients with type 2 diabetes and mild neuropathy as opposed to those without and with severe neuropathy (6). Significant changes have been found in patients recently diagnosed with type 2 diabetes (5) and subjects with impaired glucose tolerance (38).

We acknowledge some limitations in the current study including an unequal number of subjects in each group. We also do not have complete data sets for HbA1c or oral glucose tolerance test or data on smoking, alcohol intake, or actual levels of vitamin B12 or folate. However, the latter were checked in each center, and any individuals with an abnormality were excluded.

In conclusion, the current study represents the largest multicenter cohort of healthy control subjects who have undergone CCM using a standardized technique to capture central corneal images with the same model of microscope and rigorous quantification of corneal nerve morphology in one center using a standard definition. We show an age-dependent decrease in CNFD and CNFL with an increase in CNFT. We additionally provide age-dependent normative cutoffs to aid clinicians to identify pathological reductions,
sufficient to identify significant nerve damage and, hence, diagnose peripheral neuropathy. These data provide the basis for wider use of CCM as a diagnostic test for diabetic and other peripheral neuropathies.

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**Author Contributions.** M.T. designed the study, researched data for Manchester, analyzed CCM images and data for all centers, and wrote the manuscript. M.F. and I.N.P. researched data for Manchester and analyzed CCM images for all centers. J.M. was the statistical consultant for the study and analyzed data for all centers. N.P. researched data for Brisbane. A.Z. and D.Z. researched data for Düsseldorf. D.P. and K.R. researched data for Calgary. B.A.P., L.E.L., and V.B. researched data for Toronto. J.R.S. and G.S. researched data for Utah. A.J.M.B. reviewed and revised the manuscript. N.E. reviewed and revised the manuscript. R.A.M. designed the study, supervised the project, and reviewed and revised the manuscript. R.A.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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