The pattern of the inferocentral whorl region of the corneal subbasal nerve plexus is altered with age

Reza A. Badian a, **, Mattias Andréasson b, c, d, Per Svenningsson b, c, d, Tor Paaske Utheim e, f, Neil Lagali f, g, h

a Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway
b Center for Neurology, Academic Specialist Center, Stockholm, Sweden
c Department of Neurology, Karolinska University Hospital, Stockholm, Sweden
d Department of Clinical Neuroscience, Karolinska Institute, Stockholm, Sweden
e Department of Ophthalmology, Oslo University Hospital, Oslo, Norway
f Department of Ophthalmology, Sorlandet Hospital Arendal, Arendal, Norway
g Department of Ophthalmology, Institute for Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden
h Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway

ARTICLE INFO

Keywords:
Cornea
Subbasal nerve plexus
Inferocentral whorl
In vivo confocal microscopy
Diabetes mellitus
Parkinson’s disease
Aging

ABSTRACT

Purpose: To describe the pattern of the nerves in the inferocentral whorl region of the human corneal subbasal nerve plexus (SBNP) in health and diseases known to affect the subbasal nerves.

Methods: Laser-scanning in vivo confocal microscopy (IVCM) was used to image the SBNP bilaterally in 91 healthy subjects, 39 subjects with type 2 diabetes mellitus (T2DM), and 43 subjects with Parkinson’s disease (PD). Whorl regions were classified according to nerve orientation relative to age and health/disease status.

Results: Of 346 examined eyes, 300 (86.7%) had an identifiable whorl pattern. In healthy subjects, a clockwise nerve orientation of the whorl was most common (67.9%), followed by non-rotatory or ‘seam’ morphology (21.4%), and counterclockwise (10.7%). The clockwise orientation was more prevalent in healthy subjects than in T2DM or PD (P < 0.001). Healthy individuals below 50 years of age had a predominantly clockwise orientation (93.8%) which was reduced to 51.9% in those over 50 years (P < 0.001). Age but not disease status explained whorl orientation in T2DM and PD groups. Moreover, whorl orientation is bilaterally clockwise in the young, but adopts other orientations and becomes asymmetric across eyes with age. Finally, we report reflective ‘dot-like’ features confined to the whorl region of the subbasal plexus, sometimes appearing in close association with subbasal nerves and present in 84–93% of examined eyes regardless of disease status, eye or sex.

Conclusion: Subbasal nerves in the inferocentral whorl region are predominantly clockwise in young, healthy corneas. With aging and conditions of T2DM and PD, counterclockwise and non-rotatory configurations increase in prevalence, and bilateral symmetry is lost. Mechanisms regulating these changes warrant further investigation.

1. Introduction

In vivo confocal microscopy (IVCM) has in recent years become increasingly utilized as a non-invasive imaging technique for the examination of the cornea in health and disease [1]. IVCM is well-suited for clinical imaging of the ultrathin corneal subbasal nerve plexus (SBNP) plane, to enable high-contrast and high-resolution examination of the corneal innervation [1–3], as an indicator of the health status of the cornea and/or of the status of the peripheral nervous system. Studies using IVCM have shown that the SBNP is affected in different ocular and systemic conditions such as diabetes mellitus [4–7], keratoconus [8], dry eye disease [9], multiple sclerosis [10], corneal dystrophy [11], corneal refractive surgery [12–14], and following corneal transplantation [15].

While subbasal nerves have primarily been analyzed quantitatively by measuring parameters such as nerve density, branching, and tortuosity [16,17], it has also been recognized that nerves are not distributed uniformly throughout the SBNP but are typically organized in a pattern.

* Corresponding author. Department of Ophthalmology, Institute for Biomedical and Clinical Sciences, Faculty of Medicine, Linköping University, 581 83, Linköping, Sweden.
** Corresponding author. Department of Medical Biochemistry, Oslo University Hospital, 0424, Oslo, Norway.
E-mail addresses: rezabadian@gmail.com (R.A. Badian), neil.lagali@liu.se (N. Lagali).

https://doi.org/10.1016/j.jtos.2021.08.015
Received 20 October 2020; Received in revised form 16 August 2021; Accepted 21 August 2021
Available online 25 August 2021
1542-0124/© 2021 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
In an early seminal study, nerves of the SBNP were observed by IVCM examination to orient towards the inferocentral area of the cornea (approximately 1–2 mm inferior and nasal to the corneal apex, located in the lower nasal quadrant), in a vortex or a whorl-like pattern [3]. Further studies showed that the whorl pattern was altered in keratoconus [18] and that the normal SBNP is a highly dynamic structure with continuous centripetal movement of branches of the whorl [19]. Given that the nerves of the SBNP spiral in towards the whorl region, the mean density of subbasal nerves in the whorl region has been reported to be significantly higher than in the central cornea [3,20,21]. The orientation of the whorl [3,18], and the central corneal innervation has been suggested to be asymmetric between right and left eyes in healthy individuals, based on a study of 6 healthy individuals, (3 males and 3 females, mean age ± SD: 30.5 ± 5.8 years), where a clockwise orientation of the whorl was observed in all eyes [22]. However, these were all younger subjects and it is not known whether increasing age has any effect on the whorl pattern in health and disease. Also, in some cases, a counterclockwise configuration of the nerves in the whorl region has also been described [21,23]. Recently, a third, ‘linear seam’ morphology was also described in animal corneas [24].

In the context of disease, subbasal nerves in moderate keratoconus differ from the healthy cornea, and have been reported to be horizontally oriented at the apex, and curvilinear in the whorl region [18]. In a study examining two subjects with diabetes using wide-field mosaic depictions of the central 3 mm of the SBNP, the authors claimed the whorl and its surrounding area appeared to have the greatest loss of nerve fibers [25], Utsunomiya and colleagues, however, in a larger study found that nerve density in the whorl region was elevated in both healthy and in diabetic patients compared to nerve density in other areas of the SBNP [26]. Similarly, we previously reported that in type 2 diabetes mellitus (T2DM) the subbasal nerve density in the whorl region was higher than in the central cornea and insensitive to the presence of T2DM [27].

Given that the SBNP and in particular the whorl region is dynamic, and given that nerve density comparisons across individuals are subject to substantial variability [28], we hypothesized that apart from nerve density, the architecture and orientation of nerves in the whorl region can change with degenerative disease. To further investigate this, it was necessary to first establish the orientation of the whorl region in normal healthy eyes, as only a few studies with a relatively small number of eyes have reported anatomical details of the whorl region. In a recent report, Marfurt et al. described a meta-analysis of the 20 whorl formations in the human corneal SBNP described to date in the literature; of these had a clockwise orientation and one had a counterclockwise orientation [24]. It was postulated that this IVCM data could possibly be skewed by the small number of corneas represented and potential bias in reporting of accepted whorl patterns [24]. In that study, Marfurt et al. investigated the mammalian cornea of 6 species and noted an ‘imaginary linear seam’ pattern of nerve convergence instead of a whorl in a small percentage of density, the architecture and orientation of nerves in the whorl region to substantial variability [28], we hypothesized that apart from nerve density in relation to small fiber neuropathy [6,36–41]. Although the focus of prior studies in PD and T2DM groups has been primarily to quantify subbasal nerve density, the pattern of nerves in the whorl region, which may be a sensitive parameter for disease-related changes, has not been studied.

2. Methods

2.1. Subjects and recruitment

This study comprised previously reported groups of healthy subjects and subjects with T2DM [27,42], where IVCM examinations were conducted including the whorl region of the SBNP. Further, a group of healthy control subjects and subjects with Parkinson’s disease (PD) were recruited for examination at the outpatient clinic at Center for Neurology and the Department of Neurology, Karolinska University Hospital, Stockholm, Sweden, as previously described [31]. A diagnosis of probable PD was confirmed according to established clinical criteria set by the Movement Disorders Society [43]. PD subjects with a known diagnosis of diabetes mellitus, rheumatoid arthritis, polynephropathy, renal failure, iron deficiency anaemia or heavy alcohol consumption were excluded. Ophthalmic exclusion criteria included prior eye surgery, previous corneal trauma, use of topical eye drop medication, and contact lens wear. Dry eye disease (DED) was excluded on the basis of reported symptoms and/or use of topical treatment for DED. All subjects in the study gave written informed consent to participate, with examination protocols approved by the regional research ethics boards in Linköping (healthy subjects, ref. no. M172-09), in Umeå (control and T2DM subjects, ref. no. 2013-21-31 M), and in Stockholm (control and PD subjects, ref. no. 2018/264-31/2).

2.2. In vivo confocal microscopy

IVCM was conducted as previously described [42,44]. Briefly, the laser-scanning IVCM system Heidelberg Retinal Tomograph with Rostock Cornea Module (HRT3-RCM, Heidelberg Engineering, Heidelberg, Germany) was used for in vivo examination of human corneas as per manufacturer instructions. The confocal depth was set to image the SBNP layer by adjusting a motorized joystick-driven depth control unit. With the nerves of the subbasal plexus visible in the real-time display of the microscope, images of the plexus were automatically recorded at 3 to 8 frames/sec as the operator adjusted the field of view to locate the inferocentral whorl region. In most cases, the subjects were instructed to lift their gaze, in order to bring the inferocentral region into view. Fine depth adjustments were made to maximize the visibility of the whorl region during acquisition, to enable later analysis of the predominant orientation of the subbasal nerves. A single, experienced examiner (NL) performed all IVCM examinations, and bilateral examinations were conducted.

2.3. Assessment of subbasal nerve plexus images

In multiple single-frame and wide-area mosaic images of the corneal SBNP, the characteristic configuration of the whorl region was identified, where possible. In cases where mosaic images were available, the convergence region of inwardly radially-oriented nerves was used to identify the inferocentral whorl region, and in cases where no clear spiral organization could be detected, to determine if another convergence pattern of nerves was present. Subsequently, the orientation of the whorl region was recorded for each mosaic or from a sequence of still images. Mosaics were typically composed of several hundred raw images each; however, only a few images per eye in the correct apical...
location are required to identify and confirm the orientation of the whorl region. Assessments were made by two experienced observers (RB and NL) masked to the group identity of the subjects. Orientation of the whorl region was classified into one of three types based on the path of subbasal nerves moving towards the whorl center: clockwise (CW), counter-clockwise (CCW), or non-rotatory/seam (NR), with the latter representing a horizontal fold-like convergence, in accordance with definitions used in prior studies [21–23]. The presence of dot-like features in the whorl region was assessed by examining a single 400 × 400 μm image frame centred on each whorl region, either by cropping a larger mosaic to this region, or by selection of an IVCM image frame centred on the whorl region and with good nerve visibility. The percentage of dot-like features in apparent contact with subbasal nerves was assessed by two experienced observers independently [45] and masked to the health status of the subject, by performing counts of the number of dot-like features with and without visible contacts to subbasal nerves in the whorl region, dividing the number of dots in apparent contact with nerves by the total number of visible dots. The percentages across both observers were averaged for each whorl region. Large differences between observers (>50%) were discussed among the observers to achieve a consensus in identification of dot-like features with nerve contacts. In such cases, a consensus was achieved.

### 3. Results

#### 3.1. Subject characteristics

The total number of subjects included in the study was 173 (346 eyes). Of these, 91 subjects were healthy control subjects. The remainder of the group consisted of subjects with different pathologies; 39 subjects with type 2 diabetes mellitus (T2DM) and 43 subjects with Parkinson’s disease (PD). An overview of the group characteristics is given in Table 1. In IVCM data from all groups, a proportion of eyes did not have a record of the whorl region or the region could not be definitively found or identified. In total, 300 of the 346 eyes (86.7%) had IVCM images of the whorl region suitable for analysis. The average age of the total study population was 60.3 ± 17.9 years, where T2DM and PD groups had a similar mean age (69.2 years), while healthy subjects were generally younger (53.1 ± 21.2 years). ANOVA analysis revealed T2DM and PD groups were age-matched, while each disease group had significantly older subjects relative to the healthy group (Table 1) (P < 0.001) for both. Females constituted 38.3% of the entire study population.

#### 3.2. Whorl orientation with disease, sex and age

Distribution of whorl orientation (clockwise, counter-clockwise and non-rotatory) in the healthy and disease groups and with respect to eye is indicated in Table 1. In the entire study group, prevalence of clockwise, counter-clockwise and non-rotatory orientations were 57%, 16%, and 27%, respectively (all eyes). The clockwise orientation was most frequent overall, and most frequent in each healthy or disease subgroup.
For right and left eyes separately, the same pattern was observed, with clockwise orientation generally being the most prevalent in the entire group and in the disease subgroups. Three-way Chi-square analysis revealed that the clockwise orientation was significantly more frequent in healthy eyes than would be expected by chance, for both left and right eyes (Table 1). The frequency of clockwise orientation, however, in T2DM and PD groups relative to other orientations was not significant, regardless of eye. Results stratified by eye are given in Table 1.

Due to the non-equal number of male and female subjects, a sub-analysis based on sex was conducted. The distribution of whorl orientations within each group (healthy, T2DM, PD or all subjects combined) did not differ based on sex (Table 2). Considering the age of the subjects, however, younger subjects were overrepresented in the healthy control group. Therefore, a subgroup analysis was performed for the healthy control group consisting of a total of 91 subjects (Table 3). Dividing the healthy group into subjects aged under 50 years and those aged 50 years and above, resulted in a mean age of the older healthy group (69.1 ± 3.7 years) nearly identical to the T2DM and PD groups in this study. The age of the younger healthy group was 27.0 ± 6.4 years. Considering the distribution of whorl orientations among young and old healthy subjects, a dramatic difference was noted. Almost all young subjects had a clockwise orientation (93.8%), whereas only 51.9% in the older group had a clockwise orientation, irrespective of whether eyes were pooled or analyzed separately (Table 3). The difference in distribution of orientations between young and older healthy subjects was highly significant.

To determine whether this age difference alone could account for the difference in orientation distributions between healthy subjects and those with degenerative disease, the older healthy group which had the same mean age as the disease groups was used for comparison. Neither the T2DM nor the PD group had a distribution of whorl orientation that differed from the older group of healthy subjects. Conversely, both T2DM and PD group distributions differed significantly from the younger healthy subjects considering all eyes. The analysis was repeated considering each eye separately, with similar results, indicating no significant distribution differences between T2DM or PD groups from the older healthy group and a highly significant difference in distribution from younger healthy subjects (Table 4).

### 3.3. Bilateral whorl orientation

Considering bilateral data from the same subject and categorizing into one of the six combinations of bilateral whorl orientation (Table 5), the young healthy group (consisting of 78 subjects with bilateral whorl data available) had a distribution of combinations that differed significantly from the older healthy group (P < 0.001). Comparing the degenerative disease groups to the older healthy group, no significant difference in distribution of whorl orientation was detected for T2DM (32 subjects with bilateral data) or PD (25 subjects with bilateral data). However, in both disease groups, the bilateral distribution of combinations differed significantly from the younger healthy subjects (P < 0.001 for both). Interestingly, although a clockwise orientation of the whorl in both eyes of the same subject was the most prevalent combination in healthy subjects (52.6% overall, or 93.1% in young and 28.6% in older healthy subjects), the most prevalent combinations in disease groups were asymmetric: clockwise/non-rotatory in T2DM subjects (31.3%) and clockwise/counterclockwise (40.0%) in PD subjects. In cases of older individuals, the paths of a few individual subbasal nerves

**Table 2**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Healthy Controls</th>
<th>Type 2 Diabetes Mellitus</th>
<th>Parkinson’s Disease</th>
<th>Total (all subjects)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>101</td>
<td>67</td>
<td>37</td>
<td>31</td>
</tr>
<tr>
<td>Female</td>
<td>60.1</td>
<td>39.9</td>
<td>54.4</td>
<td>45.6</td>
</tr>
</tbody>
</table>

**Whorl orientation**

- **% of eyes (no. of eyes)**
  - Clockwise: 70.3 (71)
  - Counterclockwise: 12.9 (13)
  - Non-rotatory: 16.8 (17)
  - P-value (Male vs. Female): 0.03
  - Dot-like features present in whorl (% of eyes): 84.2
    - P-value (Male vs. Female): 0.67

**Table 3**

Subgroup analysis of healthy subjects in this study with identifiable whorl regions, grouped by age. Values in **bold** indicate significance.

<table>
<thead>
<tr>
<th>Healthy Controls</th>
<th>Type 2 Diabetes Mellitus</th>
<th>Parkinson’s Disease</th>
<th>Total (all subjects)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>35</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>No. of eyes examined</td>
<td>64</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>% female</td>
<td>34.3</td>
<td>41.8</td>
<td></td>
</tr>
<tr>
<td>Mean age (years, ± SD)</td>
<td>27.0 ± 6.4</td>
<td>69.1 ± 3.7</td>
<td></td>
</tr>
</tbody>
</table>

**Whorl orientation**

- **% of eyes (no. of eyes)**
  - Clockwise: 93.8 (60)
  - Counterclockwise: 1.6 (1)
  - Non-rotatory: 4.7 (3)
  - P-value (<50 years vs. ≥50 years): < 0.001
  - Dot-like features present in whorl (% of eyes): 87.3
    - P-value (<50 years vs. ≥50 years): 0.67

**Table 4**

Differences in distribution of whorl orientations in the younger (<50 years) and older (≥50 years) healthy subjects relative to disease groups, for all eyes in a pooled analysis and for right (RE) and left eyes (LE) analyzed individually. Values presented are Chi-square P-values. Values in **bold** indicate significance.

<table>
<thead>
<tr>
<th>Healthy Controls</th>
<th>Type 2 Diabetes Mellitus</th>
<th>Parkinson’s Disease</th>
<th>Total (all subjects)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All eyes</td>
<td>&lt; 0.001</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>&lt; 0.001</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>T2DM (RE/LE)</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.87</td>
</tr>
<tr>
<td>PD (RE/LE)</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.52</td>
</tr>
</tbody>
</table>
adjacent to the whorl region (outside the apex) were sometimes observed to adopt a counterclockwise orientation, even where the predominant orientation where nerves terminate in the whorl region at the corneal apex was clockwise (Fig. 1).

3.4. Dot-like features in the whorl region

Many of the whorl regions contained bright, hyper-reflective, rounded dot-like features of unknown identity or origin, that were confined to the central whorl region in most cases. The dot-like features were distinct entities that did not have a dendriform shape but were circular, and were differentiated from perforation sites of the stromal nerves through Bowman’s layer [21,46] by their smaller, rounded shape, and localization to the whorl center (Fig. 2).

These dot-like features were present in all groups regardless of age, sex, right/left eye, or health status, with 88.0% of all subjects with identifiable whorl regions exhibiting the features (Tables 1 - 3).

The proportion of eyes with dot-like features did not vary across groups nor with respect to sex in healthy, T2DM, PD or all subjects combined (Tables 1, 2). Moreover, the proportion of eyes with dot-like features did not change, regardless of group, when only unilateral data (right or left eyes only) was considered (Tables 1, 3), being always present in a proportion of about 90%. Although the proportion was consistent across groups, the density of dot-like features within a given whorl region could vary substantially, even among young, healthy individuals. Some subjects exhibited a whorl region with many closely spaced dots, while others exhibited only a few dots and still others lacked dots completely (Fig. 2). Upon closer inspection, in some cases it appeared that the dot-like features were in direct contact with the subbasal nerves in the whorl region, forming one or more connection points with subbasal nerves (Fig. 3). When changing the focus depth to the basal epithelial layer a distance of 4 μm anterior to the subbasal

---

**Table 5**

Frequency of the six bilateral combinations of whorl orientation in study subjects arranged by group. Values indicate percentages with actual number of subjects in parentheses. Abbreviations: CW: clockwise; CCW: counterclockwise; NR: non-rotatory. Values in bold indicate significance.

<table>
<thead>
<tr>
<th>Bilateral combination</th>
<th>Healthy &lt;50 years</th>
<th>Healthy ≥50 years</th>
<th>Type 2 Diabetes Mellitus</th>
<th>Parkinson’s Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW/</td>
<td>52.6 (41)</td>
<td>93.1 (27)</td>
<td>28.6 (14)</td>
<td>21.9 (7)</td>
</tr>
<tr>
<td>Symmetric</td>
<td></td>
<td></td>
<td></td>
<td>16.0 (4)</td>
</tr>
<tr>
<td>CW</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
<td>6.3 (2)</td>
<td>4.0 (1)</td>
</tr>
<tr>
<td>CCW</td>
<td>14.1 (11)</td>
<td>3.4 (1)</td>
<td>20.4 (10)</td>
<td>9.4 (3)</td>
</tr>
<tr>
<td>NR/</td>
<td></td>
<td></td>
<td></td>
<td>28.0 (7)</td>
</tr>
<tr>
<td>NR</td>
<td>16.7 (13)</td>
<td>0.0 (0)</td>
<td>26.5 (15)</td>
<td>18.8 (6)</td>
</tr>
<tr>
<td>CCW</td>
<td>11.5 (9)</td>
<td>3.4 (1)</td>
<td>16.3 (10)</td>
<td>31.3 (10)</td>
</tr>
<tr>
<td>Asymmetric</td>
<td></td>
<td></td>
<td></td>
<td>8.0 (2)</td>
</tr>
<tr>
<td>CW/</td>
<td>5.1 (4)</td>
<td>0.0 (0)</td>
<td>8.2 (4)</td>
<td>12.5 (4)</td>
</tr>
<tr>
<td>Symmetric</td>
<td></td>
<td></td>
<td></td>
<td>4.0 (1)</td>
</tr>
<tr>
<td>NR</td>
<td>12.5 (10)</td>
<td>8.0 (2)</td>
<td>16.3 (10)</td>
<td>31.3 (10)</td>
</tr>
<tr>
<td>CCW</td>
<td>31.3 (10)</td>
<td>8.0 (2)</td>
<td>16.3 (10)</td>
<td>31.3 (10)</td>
</tr>
<tr>
<td>NR</td>
<td>25.0 (19)</td>
<td>25.0 (19)</td>
<td>25.0 (19)</td>
<td>25.0 (19)</td>
</tr>
</tbody>
</table>

P-value vs. Healthy ≥50 years vs. Healthy <50 years

Table 5: Frequency of the six bilateral combinations of whorl orientation in study subjects arranged by group. Values indicate percentages with actual number of subjects in parentheses. Abbreviations: CW: clockwise; CCW: counterclockwise; NR: non-rotatory. Values in bold indicate significance.

---

**Fig. 1.** Bilateral whorl orientation in left (left column) and right (right column) eyes of the same subject. (A, B) Clockwise whorl orientation in both eyes of a healthy 23-year-old female subject. Note the dot-like features (arrows). (C, D) Non-rotatory orientation of converging subbasal nerves in the inferocentral region of a healthy 68-year-old male subject. A ‘seam-like’ line (arrows) is evident where nerves appear to terminate. (E, F) Asymmetric whorl orientation in eyes of a 70-year-old male subject with type 2 diabetes mellitus. In (E), nerves adopt a counterclockwise path (arrows) but do not form a complete whorl. In (F), a clear clockwise orientation of nerve paths is observed (white arrows); however, some nerves adjacent to the whorl region adopt a counterclockwise curvature (black arrows) before curving back towards the clockwise pattern as they terminate at the apex. (G, H) Asymmetric whorl orientations in a 68-year-old male with Parkinson’s disease. In (H), although the whorl has an overall clockwise orientation, nerves entering the inferocentral region appear to adopt a counterclockwise orientation multiple times (white arrows), each time doubling back to finally adopt a clockwise orientation. Scale bars: (A, B) 100 μm, (C – H) 200 μm.
plexus, a dot-like feature apparently connected to a subbasal nerve was still visible, indicating that the dot-like feature was not confined to a single plane but potentially had a three-dimensional structure. Moreover, dot-like features were present regardless of clockwise, counterclockwise or non-rotatory whorl orientation (Fig. 3). Quantifying dot-like features by two independent observers to determine apparent connections with subbasal nerves indicated about two-thirds of the dot-like features had visible connections with subbasal nerves in the whorl region in healthy individuals, which was significantly greater than the approximately 40% of dot-like features in subjects with systemic disease (P < 0.001, Table 1).

4. Discussion

Although the inferocentral whorl region in the human cornea is the most recognizable feature of the SBNP, surprisingly few studies have investigated the orientational characteristics of this region. The orientation of subbasal nerves in the whorl being either clockwise or counterclockwise has been reported by Marfurt et al. and He et al. in groups of healthy individuals or subjects with pathologies [21,23]. A more complete description of the prevalence of whorl orientations in a larger group of human subjects, however, is still lacking [24]. Analysis of the whorl region in 300 human eyes in this study led to the striking finding that although the clockwise whorl orientation was most prevalent, there was a substantial change in distribution of whorl orientations with aging in healthy subjects. While almost all whorls are clockwise in young healthy subjects, in subjects over the age of 50 years the orientation changes, with a substantial proportion of eyes exhibiting either counterclockwise or non-rotatory orientations.

In this study with a retrospective cross-sectional design, the findings strongly suggest that the orientation of the nerves in the whorl region changes with age, with the clockwise orientation transforming into a non-rotatory or counterclockwise orientation over time in a substantial proportion of individuals. Detailed IVCM images of the inferocentral region in this study in older individuals indicated that groups of nerves appeared to change their predominant orientation to a counterclockwise direction even while the main whorl orientation was still clockwise. It is therefore plausible that over time, given the dynamic nature of the SBNP [19], the orientation of the entire whorl region could change as regenerating axons replace earlier ones with a different path. This loss of clockwise orientation in older subjects was also observed in groups of older subjects with degenerative disease such as T2DM or PD, indicating a more general phenomenon likely independent of the specific degenerative disease.

Another finding in this study was that whorl orientations are not binary in humans (clockwise or counterclockwise) but a third, non-rotatory orientation exists, and importantly, both eyes of the same subject do not necessarily have the same orientation (presence or absence of bilateral symmetry). In particular, as the cornea ages, this symmetry appears to be lost. Likewise, the symmetry was lost in subjects with degenerative disease.

Prior studies in disease populations such as PD and T2DM have focused on quantification of subbasal nerve density. An IVCM study by Podgorny et al., with 26 PD patients (mean age = 63 years) and 22 healthy controls (mean age = 63 years) reported significant reduction in subbasal nerve density and length in PD [29]. Another IVCM study by

Fig. 2. Variability in density of dot-like features in the central whorl region in young, healthy subjects. (A) Whorl region in the left eye of a 19-year-old male subject with a dense distribution of dot-like features. (B) Right eye of the same subject as in (A) indicates a similarly high density of dot-like features. (C) 20-year-old male subject with only a few dot-like features (arrows). (D) 25-year-old female subject lacking dot-like features in the central whorl region. All images 400 x 400 μm.
Fig. 3. Dot-like features in appearing to be contact with subbasal nerves in the whorl region in young, healthy subjects. (A) In a 26-year-old male, a dot-like feature was observed with two apparent connection points to adjacent subbasal nerves (arrows). (B) In a 23-year-old male subject, two dot-like features appeared to be connected to subbasal nerves at multiple points (arrows). (C) In a 24-year-old male subject, a dot-like feature in the whorl region (arrow) appeared connected to an adjacent subbasal nerve. (D) 4 μm anterior to the subbasal plexus in the basal epithelial layer, the same dot-like feature in (C) was observed again (arrow), potentially indicating its three-dimensional structure. The dot-like features (arrows) were present regardless of (E) clockwise orientation, 23-year-old female, (F) counterclockwise whorl orientation, 33-year-old male. (G) in contrast to the younger healthy subjects, a 68-year-old female with PD had non-rotatory orientation, but still with dot-like features present (arrows). Note that some but not all dot-like features in the images appear to be in contact with nerves. All images 400 x 400 μm.
observed localized to the whorl in this single subject [19], although the cornea, a concentration of reflective white dot-like features could be also studied the migration of subbasal nerves [19]. In published nerves in the whorl region may be a sensitive indicator of disease that could be assessed in addition to standard quantitative analysis of nerve density. More studies are warranted, specifically to examine the whorl orientation in health and disease. Likewise, the apparent age dependence of the orientation would be important to consider in future studies examining orientation patterns.

A study of patients using overnight orthokeratology lenses demonstrated alterations to the SBNP and whorl region [20], and increased variability of subbasal nerve orientation has also been described following LASIK [47]. Here, however, we report for the first time that healthy subjects without ocular disease or interventions, likely experience alterations in the whorl region with aging. What is it about aging, that leads to breakdown of the clockwise pattern and causes subbasal nerves to adopt a counterclockwise or non-rotatory (or ‘seam’) configuration? Presumably nerve guidance cues or signals, that establish and maintain a constant migration of subbasal nerves towards the whorl region, are somehow altered with age. The consequences of this alteration, beyond the anatomic change in the SBNP, however, are unknown, and require further study. The results of this study also suggest that factors regulating nerve guidance in the cornea, such as specific proteins [48], neuro-stimulatory substances and/or neurotransmitters either in the cornea or tear film, may change with age. Eyelid pressure and intraocular pressure changes with age may also play a role. These hypotheses warrant further investigation and could have consequences for corneal diseases associated with nerves and aging.

A limitation of this study is the retrospective, cross-sectional design. Ideally, longitudinal study of the whorl region in subjects over an extended period (e.g., in the years before and after the putative transition age) would be required to definitively demonstrate changes in nerve orientation in the whorl region of a single subject. Nevertheless, the age-related changes observed here strongly suggest that alterations of the whorl orientation occur with age. The age threshold where these changes begin to manifest, however, is not yet clear. The present study was not designed to evaluate detailed age-related changes in the whorl; further studies with defined age intervals and equal-sized subgroups are therefore warranted. Likewise, further studies will be needed to investigate the influence of demographic factors such as ethnicity or lifestyle factors, or the potential impact of eye disease.

Another novel finding in this study was the presence of dot-like features in the whorl region of the SBNP. Patel and McGhee, who first studied the inferocentral whorl pattern of subbasal nerves by IVCM [3], also studied the migration of subbasal nerves [19]. In published images of the whorl region taken over a two-year period from the same cornea, a concentration of reflective white dot-like features could be observed localized to the whorl in this single subject [19], although the dot-like features were not specifically identified. In anatomic studies of ex vivo human corneas, it was shown that bulb-like thickenings were present where sub-epithelial nerves penetrated Bowman’s layer to give rise to subbasal nerves [21,46]. These ‘perforation sites’, however, are also visible in confocal microscopy images [49,50], are distributed throughout the central and peripheral cornea, and are not localized to the whorl region. Nor does the morphology of the perforation sites match the dot-like features of the whorl, with the latter not appearing to be a point of clear origination or termination of subbasal nerves. Moreover, while perforation sites were clearly visible in corneal tissue specimens stained with acetylcholinesterase [46], the dot-like features were not discernible in the whorl region in that ex vivo study. Nor do the dot-like features appear to be anastomoses of subbasal nerves, which occur both in the central and peripheral cornea and can be detected with acetylcholinesterase staining [21].

It is also unlikely that the dot-like features are a type of dendritic cell, since they are located exclusively in the whorl region; if they migrated to the whorl region, at least a few of these features would be found outside the whorl region, which we did not detect in this study. Instead, the dot-like features appear to be bulbous thickenings, which appear in many cases to be connected to one or more nerve segments, and these thickenings at least in some cases may extend upwards a few microns into the basal epithelial layer. Why these dot-like thickenings are almost always circular in form is unknown; likewise, their localization to the whorl region and their variability in density across different subjects is also unknown. Although we cannot determine with certainty whether all dot-like features are connected to nerves due to the difficulty in visualizing all fine nerve fibers in IVCM images, we suggest their association with corneal nerves in the whorl region. Differences in nerve connections across healthy and disease groups could be due to the difficulty of visualizing small interconnecting nerves in the whorl region in diseases such as T2DM and PD. Alternatively, the reduction of such connections may potentially represent a pathological process. Further evidence is required to definitively determine if some dot-like features appear independently of corneal nerves, and whether such connections could be impacted by systemic disease.

The dots had a nearly 90% prevalence among subjects in this study regardless of age, sex, or health status. It is unknown whether there would be a physiologic or biological consequence for the 10% of subjects lacking these dot-like features. Future studies should aim to elucidate the detailed anatomy of the dot-like features. The lack of dot-like features in published images of ex vivo stained corneas, however, suggests that they could be prone to rapid postmortem degeneration. Further studies are warranted to investigate the characteristics of these dot-like features, but we believe this to be the first report specifically describing and comparing these features of the whorl region anatomy between healthy and disease groups. One hypothesis, based on the location and anatomic nature of the dot-like features, is that they could be synaptic end bulbs of the subbasal nerves, which are known to trigger neurotransmitter release. Another possibility is that the dot-like features are of leukocytic origin, possibly a type of dendritic cell [45] localized to the whorl region. Further, more detailed studies, however, are required to investigate these hypotheses. Generally, parameters related to the pattern of the whorl region of the SBNP have not been a focus in clinical studies examining subbasal nerves by IVCM; however, the results presented here offer baseline data that could be useful for future studies. The whorl region and its anatomic features could be a sensitive indicator of aging, disease, normal homeostasis or pathology.

Declaration of competing interest

None of the authors has any conflict of interest, financial or otherwise to declare, pertaining to the subject matter or conduct of this study.

Acknowledgement

This study received funding from Hofgren’s fond, NEURO Sweden.

References


The Ocular Surface 22 (2021) 204–212


Lum E, Golebiowski B, Swarbrick HA. Mapping the corneal sub-basal nerve plexus

Kallinikos P, Berhanu M, O Niederer RL, Perumal D, Sherwin T, McGhee CN. Corneal innervation and cellular


Kallinikos P, Berhanu M, O Niederer RL, Perumal D, Sherwin T, McGhee CN. Corneal innervation and cellular


Kallinikos P, Berhanu M, O Niederer RL, Perumal D, Sherwin T, McGhee CN. Corneal innervation and cellular


Kallinikos P, Berhanu M, O Niederer RL, Perumal D, Sherwin T, McGhee CN. Corneal innervation and cellular