Classification of Posterior Polymorphous Corneal Dystrophy as a Corneal Ectatic Disorder Following Confirmation of Associated Significant Corneal Steepening

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IMPORTANCE The identification of steep corneal curvatures in a significant percentage of patients with posterior polymorphous corneal dystrophy (PPCD) confirms this previously reported association and suggests a role for the ZEB1 protein in keratocyte function.

OBJECTIVE To determine whether PPCD is characterized by significant corneal steepening.

DESIGN, SETTING, AND PARTICIPANTS Cross-sectional study at university-based and private ophthalmology practices of 38 individuals (27 affected and 11 unaffected) from 23 families with PPCD.

EXPOSURE Slitlamp examination and corneal topographic imaging were performed for individuals with PPCD and unaffected family members. Saliva or blood samples were obtained from each individual for DNA isolation and ZEB1 sequencing. Corneal ZEB1 expression was measured using immunohistochemistry.

MAIN OUTCOMES AND MEASURES Percentage of individuals affected with PPCD and controls with an average keratometric value greater than 48.0 diopters (D) in each eye; the mean keratometric value averaged for both eyes of individuals with PPCD and controls; and the correlation of ZEB1 mutation with keratometric value.

RESULTS ZEB1 coding region mutations were identified in 7 of the 27 affected individuals. Ten of the 38 individuals (26.3%) had average keratometric values greater than 48.0 D OU: 10 of 27 individuals with PPCD (37.0%); 6 of 7 individuals with ZEB1 mutations (85.7%) and 4 of 20 individuals without ZEB1 mutations (20.0%) and 0 of 11 unaffected individuals (P = .04 for unaffected vs affected individuals; P = .004 for individuals with PPCD with vs without ZEB1 mutation). The mean keratometric value of each eye of affected individuals (48.2 D) was significantly greater than that of each eye of unaffected family members (44.1 D) (P = .03).

AFFECTED INDIVIDUALS WITH ZEB1 MUTATIONS demonstrated a mean keratometric value of 53.3 D, which was significantly greater than that of affected individuals without ZEB1 mutations (46.5 D; P = .004). Fluorescence immunohistochemistry demonstrated ZEB1 expression in keratocyte nuclei.

CONCLUSIONS AND RELEVANCE Abnormally steep corneal curvatures are identified in 37% of all individuals with PPCD and 86% of affected individuals with PPCD secondary to ZEB1 mutations. ZEB1 is present in keratocyte nuclei, suggesting a role for ZEB1 in keratocyte function. Therefore, ZEB1 may play a role in both corneal stromal and endothelial development and function, and PPCD should be considered both an endothelial dystrophy and an ectatic disorder.

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Posterior polymorphous corneal dystrophy (PPCD; OMIM 122000) is an autosomal dominant corneal endothelial dystrophy characterized by well-described corneal endothelial abnormalities. Although the corneal endothelial dystrophies have traditionally been considered isolated disorders of the corneal endothelium, each has been associated with extraocular abnormalities: PPCD with abdominal hernias and Alport syndrome,1-3 and both Fuchs endothelial corneal dystrophy (OMIM 613267) and congenital hereditary endothelial dystrophy (OMIM 217700) with hearing loss.4-6 Poster"
crose for cryoprotection. Immunodetection of ZEB1 (ab87280; Abcam Inc) and CD34 (keratocyte marker; 3569; Cell Signaling Technology) was performed using a standard immunohistochemistry protocol. In brief, sections were rehydrated in phosphate-buffered saline (PBS) and 0.3% Triton X-100, washed twice in PBS, and blocked in PBS and 0.05% polysorbate 20 supplemented with 1% bovine serum albumin and 10% normal serum. The sections were then incubated overnight, with each primary antibody diluted 1:100 in blocking buffer, washed once in PBS and 0.05% polysorbate 20, and then washed twice in PBS. The sections were subsequently incubated with the secondary antibody (Alexa Fluor 488 or 594; Life Technologies), diluted 1:500 in blocking buffer, washed once in PBS and 0.05% polysorbate 20, and then washed twice in PBS and mounted with Vectashield (Vector Laboratories Inc) aqueous mounting medium containing 4',6-diamidino-2-phenylindole (DAPI). A negative control consisting of a species-specific normal IgG (Jackson ImmunoResearch) was used at the same concentration as each primary antibody. Fluorescence imaging was performed on an epifluorescence Zeiss microscope (Axio Imager.A2; Carl Zeiss).

Statistical Analyses
The Fisher exact test was used to compare the percentages of affected and unaffected individuals with PPCD who had average keratometric values greater than 48.0 D in each eye and the percentages of affected individuals with and without ZEB1 mutations who had average keratometric values greater than 48.0 D in each eye. The t test was used to determine the significance of the difference in the mean keratometric value between the eyes of affected individuals and the eyes of unaffected family members and between the eyes of affected individuals with ZEB1 mutations and the eyes of affected individuals without ZEB1 mutations. P < .05 was considered to be statistically significant.

Results
Corneal Topographic and Slitlamp Imaging
Corneal topographic imaging was performed for 38 individuals (27 affected and 11 unaffected) from 23 of the 45 families with PPCD recruited to date. Ten of the 38 individuals (26.3%) for whom corneal topographic imaging was performed demonstrated average keratometric values greater than 48.0 D in each eye, including a significantly greater percentage of individuals with PPCD (27.0% [10 of 27 affected individuals]) compared with unaffected individuals (0% [0 of 11 unaffected individuals]) (P = .04, determined by use of the Fisher exact test). Corneal topographic imaging was performed for affected family members of 2 of these 10 individuals, who all demonstrated average keratometric values greater than 48.0 D in each eye as well. The mean (SD) keratometric value of each eye of affected individuals measured 48.2 (5.8) D, which was significantly greater than the mean (SD) keratometric value of 44.1 (2.2) D for unaffected family members (P = .03, determined by use of the t test).

Slitlamp examinations of the 10 individuals with PPCD who demonstrated average keratometric values greater than 48.0 D in each eye revealed characteristic clinical features of keratoconus in both eyes of only 2 individuals (Table 1). In both of these patients, corneal topographic imaging demonstrated central corneal steepening, consistent with keratoconus. In addition, imaging of the posterior corneal surface for 1 patient using slit-scanning topography (Orbscan; Bausch & Lomb) demonstrated significant elevation of the posterior corneal profile compared with a best-fit sphere (OD, 0.172 mm; OS, 0.078 mm), the apex of which corresponded in location to the thinnest portion of the cornea in each eye (OD, 354 μm; OS, 403 μm). A third patient did not demonstrate any clinical features of keratoconus on slit-lamp biomicroscopy (Figure 1) but did demonstrate inferior nasal steepening on corneal topographic imaging (Figure 2).

Although corneal topographic imaging was not performed, an insufficient number of individuals were recruited from each family in which an individual with PPCD and steep corneal curvature was identified to confirm that all affected relatives with PPCD had steep corneas as well. However, in both families in which 2 affected individuals underwent corneal topographic imaging, both individuals had steep corneas. In addition, in the family in which an unaffected individual underwent corneal topographic imaging, the individual did not demonstrate a steep cornea in either eye.

ZEB1 Coding and Promoter Region Screening
Screening of the ZEB1 coding region demonstrated nonsense mutations in 7 of the 27 affected individuals (25.9%), including 6 of the 23 probands (26.1%). Screening of the ZEB1 promoter region in each affected individual did not reveal any presumed pathogenic sequence variants.32 A significantly greater percentage of affected individuals with a ZEB1 mutation (85.7% [6 of 7 individuals]) demonstrated average keratometric values greater than 48.0 D in each eye compared with affected individuals without a ZEB1 mutation (20.0% [4 of 20 individuals]) (P = .004, determined by use of the Fisher exact test). In addition, the mean (SD) keratometric value of each eye of affected individuals with a ZEB1 mutation, 53.3 (4.8) D, was significantly greater than that of each eye of affected individuals without a ZEB1 mutation, 46.5 (5.0) D (P = .004, determined by use of the t test).

Clinical Course and Management
The mean age of the 10 individuals with PPCD and average keratometric values greater than 48.0 D in each eye at the time of the initial slitlamp examination and corneal topographic imaging was 37.7 years (range, 12–66 years). Three of the individuals were younger than 30 years of age when first examined, but serial topographic images are not available for any of the 3 individuals to evaluate for progressive corneal steepening. In addition, 2 of the 3 individuals underwent penetrating keratoplasty in either 1 or both eyes, thus not permitting detection of changes in the native corneal curvature. Overall, 7 of the 10 affected individuals with steep keratometric values (70.0%) underwent a corneal transplant in 1 or both eyes, a significantly higher percentage than the 15.9% (10 of 63) of affected individuals with either unknown keratometric val-
ues or average keratometric values less than 48.0 D in each eye who required a corneal transplant (P < .001). For 6 of the 7 individuals, a corneal transplant was performed for visually significant corneal edema, and for 1 of the 7 individuals, the indication was corneal ectasia and central corneal scarring.

**Immunohistochemical Detection of ZEB1 in the Corneal Stroma**

To determine whether ZEB1 is expressed in the corneal stroma, fluorescence immunodetection of keratocytes in an eye bank cornea was performed using an antibody to CD34, a type I transmembrane glycoprophosphoprotein that is typically present in stromal fibroblasts (Figure 3). Antibodies directed against ZEB1 demonstrated that the protein is expressed by the stromal keratocytes, as well as by the corneal epithelial and endothelial cells. In each cell type, expression was localized to the nucleus, identified with the DAPI stain, as can clearly be seen in the merged images (Figure 3). Isotype and secondary only controls showed either no or low and diffuse background staining (data not shown).

**Discussion**

Although traditionally considered an isolated disorder of the corneal endothelium, PPCD has now been shown to be associated with significantly steeper mean keratometric values, as previous investigators have indicated in smaller, uncontrolled series. We defined an abnormally steep corneal curvature as having a mean keratometric value greater than 48.0 D in each eye because this value is approximately 3 SDs above the mean (SD) keratometric value of 43.97 (1.54) D described in normal individuals.16,33 Thirty-seven percent of individuals with PPCD and 86% of individuals with PPCD3 demon-

### Table 1. Clinical Features of Patients With Posterior Polymorphous Corneal Dystrophy and Steep Corneal Curvature (>48.0 D OU)

<table>
<thead>
<tr>
<th>Patient No./Sex/Eye</th>
<th>Simulated Keratometry, D</th>
<th>Keratoconus</th>
<th>Topographic Features</th>
<th>ZEB1 Mutation</th>
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*Individual depicted in Figures 1 and 2.

Individuals are related (patient 7 is the mother of patient 8).
strated corneal curvatures that were greater than 48.0 D in both eyes, whereas no unaffected individuals demonstrated corneal curvatures that were greater than 48.0 D in both eyes. In addition, the mean keratometric value averaged for both eyes of individuals with PPCD (48.2 D) is approximately 3 SDs above the population mean, and for individuals with PPCD3, the mean keratometric value (53.3 D) is 6 SDs above the population mean. Therefore, PPCD should be considered a corneal ectatic disorder, along with keratoconus, keratoglobus, and pellucid marginal degeneration. Although the corneal ectasias are characterized by corneal stromal thinning, because a corneal transplant was performed for visually significant stromal edema in the majority of individuals with steep keratometric values, we are not able to correlate decreased corneal stromal thickness with steep corneal curvature. In addition, because we had not performed corneal topographic imaging on patients with PPCD until recently, we do not have serial topographic images of affected individuals to assess for progression. However, we plan to do so going forward, in order to determine whether the abnormally steep corneal curvature associated with PPCD is progressive throughout life, or whether it stabilizes at a particular age in affected individuals, as is typically the case with the other corneal ectatic disorders.

An argument may be made that PPCD itself is not a corneal ectatic disorder but, instead, presents in association with corneal steepening because it shares a common genetic basis with keratoconus. Supporters of this argument would point to the studies reporting mutations in VSX1 purported to play a role in the development of PPCD.

Figure 1. Slitlamp Photomicrographs of Individual With Posterior Polymorphous Corneal Dystrophy

Confluent areas of gray-white opacification are noted at the level of Descemet membrane (A), and scattered endothelial vesicles are seen with retroillumination against the red reflex (B). Screening of ZEB1 revealed the novel mutation: p.(Gln884Argfs*37).

Figure 2. Corneal Topographic Images of Individual With Posterior Polymorphous Corneal Dystrophy 3

The affected individual shown in Figure 1 demonstrates inferior temporal steepening in each cornea, with average keratometric values measuring 50.39 D in the right eye (R) and 48.61 D in the left eye (L). No clinical features of keratoconus were noted on slitlamp biomicroscopic imaging of either cornea.
just as many studies have been published either not substantiating or refuting a role for VSX1 in both PPCD and keratoconus.27,31,34-44 In addition, multiple genome-wide linkage and association studies on keratoconus have not identified the involvement of either the PPCD1 locus on chromosome 20, containing VSX1, or the PPCD3 locus on chromosome 10, containing ZEB1.39-45,55 Given this, and the association of PPCD with corneal steepening in the absence of clinical features of keratoconus described in our report and others,7,13,15,16,20 significant evidence exists to suggest that PPCD is associated with corneal steepening independent of an association with keratoconus (Table 2).

Clarification of the association of PPCD with steep corneal curvature will require further elucidation of the genetic factors that influence corneal curvature, as well as the role that ZEB1 plays in both corneal endothelial and stromal development and function. To date, we have examined and collected DNA from 73 affected individuals with PPCD. Seventeen of these 73 individuals (23.3%) have required a corneal transplant, and this group of individuals includes 9 of the 26 individuals with ZEB1 mutations (34.6%). The fact that this percentage is twice that of the affected individuals without ZEB1 mutations (17.0% [8 of 47 individuals]) is suggestive that ZEB1 haploinsufficiency results in a more severe endothelial dysfunction than the yet to be identified protein dysfunction associated with non-ZEB1 PPCD, such as PPCD1. Similarly, the significantly greater mean keratometric value of the affected individuals with a ZEB1 mutation compared with that of the affected individuals without a ZEB1 mutation is additional evidence of a more severe clinical phenotype associated with PPCD3 and indicates an association

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Table 2. Previous Reports of Posterior Polymorphous Corneal Dystrophy Associated With Abnormalities of Corneal Curvature

*Topographic and histopathologic features but no clinical features of keratoconus.
*Keratometric values provided for only 1 of the 7 patients.
*One individual had undergone a corneal transplant in 1 eye, and a preoperative keratometric value was not available.
*One individual demonstrated topographic features but no clinical features of keratoconus.

Figure 3. Immunodetection of ZEB1 in Normal Donor Cornea

A. A cross-section of the cornea probed with anti-ZEB1 (red, 594 nm) and anti-CD34 (green, 488 nm) demonstrates ZEB1 expression in the epithelium, stroma, and endothelium. Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI) (objective, original magnification ×10). B. Higher magnification views of the indicated sections of the cornea demonstrate that ZEB1 is present in the nuclei of epithelial cells, stromal keratocytes, and endothelial cells (oil objective, original magnification ×100).
between more severe endothelial dysfunction and more pronounced corneal steepening.

We acknowledge several limitations of our study, one of which is the use of the need for a corneal transplant as an indicator of the degree of endothelial dysfunction. As we have indicated that a significantly greater percentage of patients with average keratometric values greater than 48.0 D in each eye than with average keratometric values less than 48.0 D required a corneal transplant, this suggests that the difference is due to the presence of corneal ectasia as the indication for transplantation in the former group. However, a corneal transplant was performed for visually significant corneal edema, not corneal ectasia, in all but one of these patients. The suggestion that the degree of endothelial dysfunction is greater in eyes with more pronounced corneal steepening is an association that will require more detailed clinical characterization in a larger number of affected individuals to definitively demonstrate. Another limitation of our study is that keratometry and ZEB1 screening data were available and analyzed for 37.0% (27 of 73) of all recruited affected individuals from just over one-half (23 of 45) of the families recruited to date. Obviously, individuals with a more severely affected phenotype may be more likely to participate in research studies than those with more mild manifestations of a disease, a selection bias that may affect the study findings.

Although PPCD is clearly a corneal endothelial dystrophy, we believe that our report and the previous reports listed in Table 2 contain sufficient evidence to consider PPCD to be a corneal ectatic disorder as well. As truncating mutations in ZEB1 account for approximately one-third of the cases of PPCD, and as the genetic basis of the other two-thirds remains to be elucidated, an understanding of the genetic basis of the endothelial dysfunction and corneal steepening that characterize PPCD begins with an analysis of ZEB1 expression and function. We have previously demonstrated ZEB1 expression in the corneal endothelium and present evidence of its expression in the corneal stroma in the present report. In PPCD3, we have demonstrated decreased expression of ZEB1 and increased expression of collagen, type IV, alpha 3 (COL4A3; OMIM 120070) in the corneal endothelium, leading to the proposed pathogenesis underlying the endothelial cell abnormalities observed in affected individuals and in Zeb1 heterozygous and Zeb1-null mice. We are currently investigating whether ZEB1 and COL4A3 also demonstrate inversely related corneal stromal expression levels in PPCD3 and whether the interaction of ZEB1 with other proteins expressed in the corneal stroma that contain E2-box motifs (to which ZEB1 binds) and/or are the product of genes implicated in the determination of corneal curvature (such as FRAP1 and PDGFRα) may be involved in the pathogenesis of the significant corneal steepening.

### Additional Contributions

We thank Cosimo Mazzotta, MD, for providing unpublished keratometry data from a patient that he and his colleagues previously reported with PPCD and steep corneal curvature.

### REFERENCES

6. Zeb1
7. ZEB1
8. ZEB1
9. ZEB1