

In Vivo Corneal Confocal Microscopy and Histopathology of Keratitis Fugax Hereditaria From a Pathogenic Variant in *NLRP3*



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- **PURPOSE:** To apply in vivo corneal confocal microscopy (IVCM) to study the pathogenesis of keratitis (keratoendotheliitis) fugax hereditaria, an autosomal dominant cryopyrin-associated periodic keratitis, associated with the c.61G>C pathogenic variant in the *NLRP3* gene, in its acute and chronic phase, and to report histopathologic findings after penetrating keratoplasty.
- **DESIGN:** This was an observational case series.
- **METHODS:** The study population included 6 patients during an acute attack, 18 patients in the chronic phase, and 1 patient who underwent penetrating keratoplasty. Interventions included Sanger sequencing for the *NLRP3* variant c.61C>G, a clinical examination, corneal photography, IVCM, light microscopy, and immunohistochemistry. Our primary outcome measures included IVCM and histopathologic findings.
- **RESULTS:** During the acute attack, hyperreflective cellular structures consistent with inflammatory cells transiently occupied the anterior to middle layers of the corneal stroma. Other corneal layers were unremarkable. With recurring attacks, central oval stromal opacities accumulated. IVCM revealed that they contained long, hyperreflective, needle-shaped structures in the extracellular matrix. Using light microscopy, the anterior half of the stroma displayed thin and finely vacuolated lamellae, and keratocytes throughout the stroma were immunopositive for syndecan.
- **CONCLUSIONS:** The acute attacks and chronic stromal deposits mainly involve the anterior to middle layers of the corneal stroma, and the disease is primarily a keratitis rather than a keratoendotheliitis. IVCM shows that inflammatory cells invade only the stroma during an acute attack. IVCM and light microscopic findings suggest that the central corneal opacities represent gradual depo-

sition of extracellular lipids. The disease could make a good in vivo model to study activation of the *NLRP3* inflammasome in cryopyrin-associated periodic syndromes. (*Am J Ophthalmol* 2020;213:217–225. © 2020 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

KERATOENDOTHELIITIS FUGAX HEREDITARIA (Mendelian Inheritance in Man 148200), initially called keratitis fugax hereditaria (KFH), is a cryopyrin-associated periodic keratitis that predominantly, if not exclusively, affects the cornea.^{1–3} It is inherited in an autosomal dominant pattern and is emerging as relatively frequent in Finland.³ The episodic attacks begin at the median age of 11 years and recur 1 to 6 times per year. The attack is characterized by unilateral pain, pericorneal injection, and photophobia. It usually lasts 1 to 2 days, but vision can remain blurry for several weeks. The attacks become milder and decrease in frequency during middle age. Repeated episodes result in bilateral horizontally oval central stromal opacities in approximately half of patients, some of whom experience permanently reduced vision. The patients have no systemic symptoms or signs during the acute episodes.³

We recently discovered that KFH is associated with a heterozygous pathogenic variant c.61G>C in the *NLRP3* gene.³ The *NLRP3* protein is expressed mainly by macrophages but also in corneal tissue.^{4,5} This variant corresponds to the amino acid substitution p.Asp21His in *NLRP3*, also known as cryopyrin, a component of the inflammasome. The inflammasome is a large multiprotein complex that plays a crucial role in innate immunity, and can be activated by various stimuli, including microbes. Other known mutations in *MWS NLRP3* cause 3 monogenic, autoinflammatory, cryopyrin-associated periodic syndromes: familial cold autoinflammatory syndrome (MIM 120100), Muckle-Wells syndrome (MIM 191900), and chronic infantile neurological cutaneous articular syndrome (MIM 607115), also known as neonatal-onset multisystem inflammatory disease.⁶ The 3 syndromes share corneal phenotypes with KFH.^{7,8}

During acute attacks, a pseudoguttata-like corneal phenotype was observed with specular microscopy,^{2,3}



Supplemental Material available at AJO.com.

Accepted for publication Feb 1, 2020.

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leading to the term keratoendotheliitis, but all patients had normal endothelial cells between attacks.³ In some patients, an anterior chamber reaction was observed. After several episodes, corneal stromal opacities frequently evolve. The corneal thickness is slightly increased during acute attacks, but the thickness will eventually decrease after opacities have developed.³

To elucidate the nature and pathogenesis of the acute episodes and of the persistent corneal opacities in KFH, we performed *in vivo* corneal confocal microscopy (IVCM) in 6 patients during an acute attack and in 12 patients, some of whom had typical horizontally oval corneal stromal opacities, during the quiet phase. Moreover, we identified 1 patient carrying the pathogenic variant who in the past had undergone bilateral penetrating keratoplasty because of typical chronic stromal opacities.

METHODS

- **PATIENTS:** Patients who had genetically confirmed KFH were eligible to join this observational cohort study that was approved by the institutional review board of the Operative Section of the Helsinki University Hospital and that followed the tenets of the Declaration of Helsinki. We obtained written informed consent from all participants. In addition, one of us scanned the records of 2197 surgeries of 1673 patients who had undergone any type of keratoplasty between January 1, 1995, and December 31, 2015, and had the removed corneal disk archived in the Ophthalmic Pathology Laboratory, Helsinki University Hospital, Finland, for corneal opacities consistent with KFH. We identified 1 female patient with preoperative anterior segment photographs that were consistent with this diagnosis and who had her left eye operated in 1998 and the right eye in 2004.

- **GENETIC ANALYSIS:** The *NLRP3* variant c.61G>C (rs200154873; GenBank: NM_004895.4) was confirmed by Sanger sequencing in all participants, including the patient who had undergone penetrating keratoplasty in the past, as described earlier.³

- **CLINICAL EXAMINATION:** We performed a comprehensive ophthalmic examination of 18 patients. The Standardization of Uveitis Nomenclature (SUN) Working Group definitions were used to grade anterior chamber cells.⁹ We also performed anterior segment photography, noncontact specular microscopy (EM-3000; Tomey, Nagoya, Japan), and Fourier domain, swept source anterior segment optical coherence tomography (SS-1000 CASIA; Tomey).

- **IN VIVO CONFOCAL MICROSCOPY:** After instilling a drop of 1% tetracaine hydrochloride (Minims Tetracaine Hydro 1%, Bausch & Lomb, Laval, Quebec, Canada) to

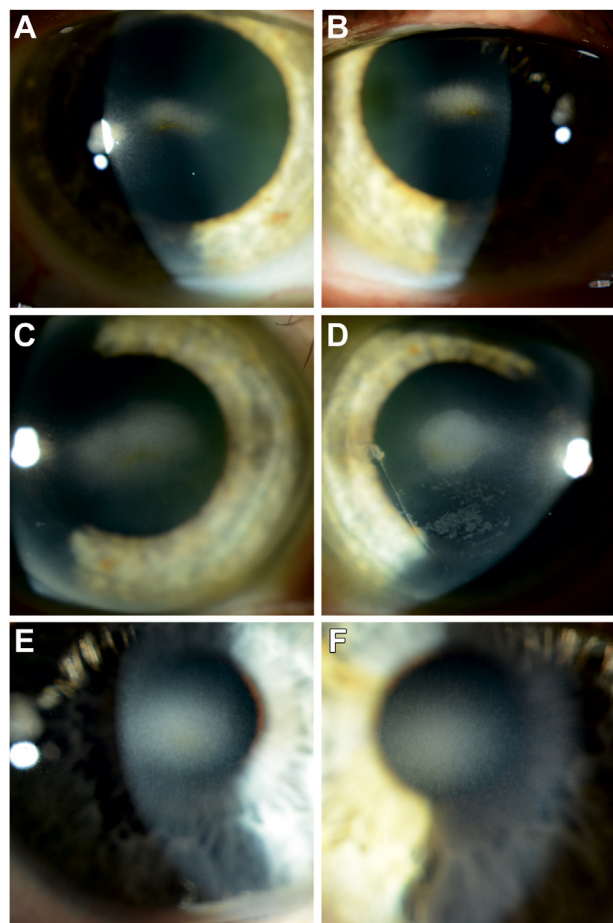


FIGURE 1. The central corneal stromal opacities in the right and left eye of 3 new patients with keratitis fugax hereditaria. (A and B) Patient 15-01 (56 years of age, best corrected visual acuity [BCVA] 20/40 OU). (C and D) Patient 15-04 (68 years of age, BCVA 20/32 OD, 20/40 OS). (E and F) Patient 17-01 (46 years of age, BCVA 20/20 OD, 20/16 OS).

the lower conjunctival sac, corneal images were obtained with the Heidelberg Retina Tomograph 3 equipped with the Rostock Cornea Module (HRT III RCM; both from Heidelberg Engineering GmbH, Dossenheim, Germany) according to the manufacturer's instructions. We imaged the central cornea along the sagittal axis so that we successfully captured the epithelium, subepithelial neural plexus, anterior stroma, posterior stroma, and endothelium.

- **OPHTHALMIC PATHOLOGY:** The archived formalin-fixed, paraffin-embedded specimens were cut at 5 μ m and routinely stained with hematoxylin–eosin, periodic acid–Schiff, Masson trichrome, Congo red, and Oil Red O stains. They also underwent immunoperoxidase staining using primary mouse monoclonal antibodies against vimentin and CD34 (QBEnd/10; Roche Diagnostics, Mannheim, Germany), an intermediate filament and a transmembrane phosphoglycoprotein, respectively, present on normal

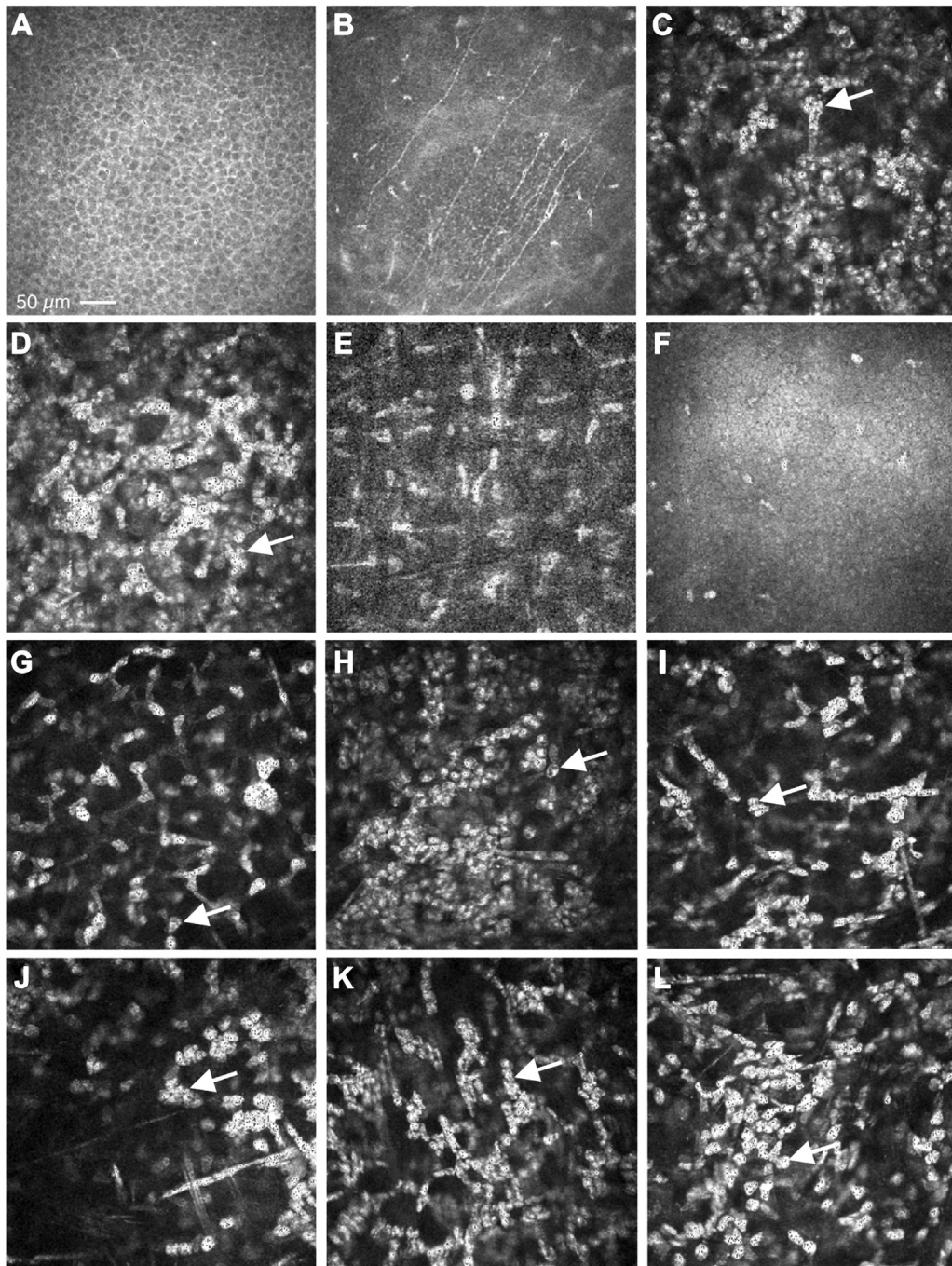


FIGURE 2. In vivo corneal confocal microscopy findings in keratitis fugax hereditaria. The left eye of patient 5-02 (15 years of age, best corrected visual acuity 20/20 in both eyes) during an acute attack (A through F). The central cornea shows normal epithelium (A), hyperreflective dendrite-like Langerhans cells at the level of the basal cells and the Bowman layer (B), accumulation of hyperreflective small roundish cellular structures increasing from the anterior to the middle stromal layers (C through E), and an endothelium with only subtle endothelial gaps and blebs (F). A few hyperreflective small roundish cellular structures are seen just anterior to the endothelium. Imaging of 6 other patients (G through L; 5-04 OS, 5-02 OD, 20-01 OD, 15-03 OD, 7-05 OD, and 16-01 OD, 14-31 years of age) during an acute attack showing identical accumulation of hyperreflective small roundish cellular structures in varying numbers in the middle stromal layers (arrows). In some eyes, they arrange in rows (I), and in some corneas isolated needle-like hyperreflective structures are present (J).

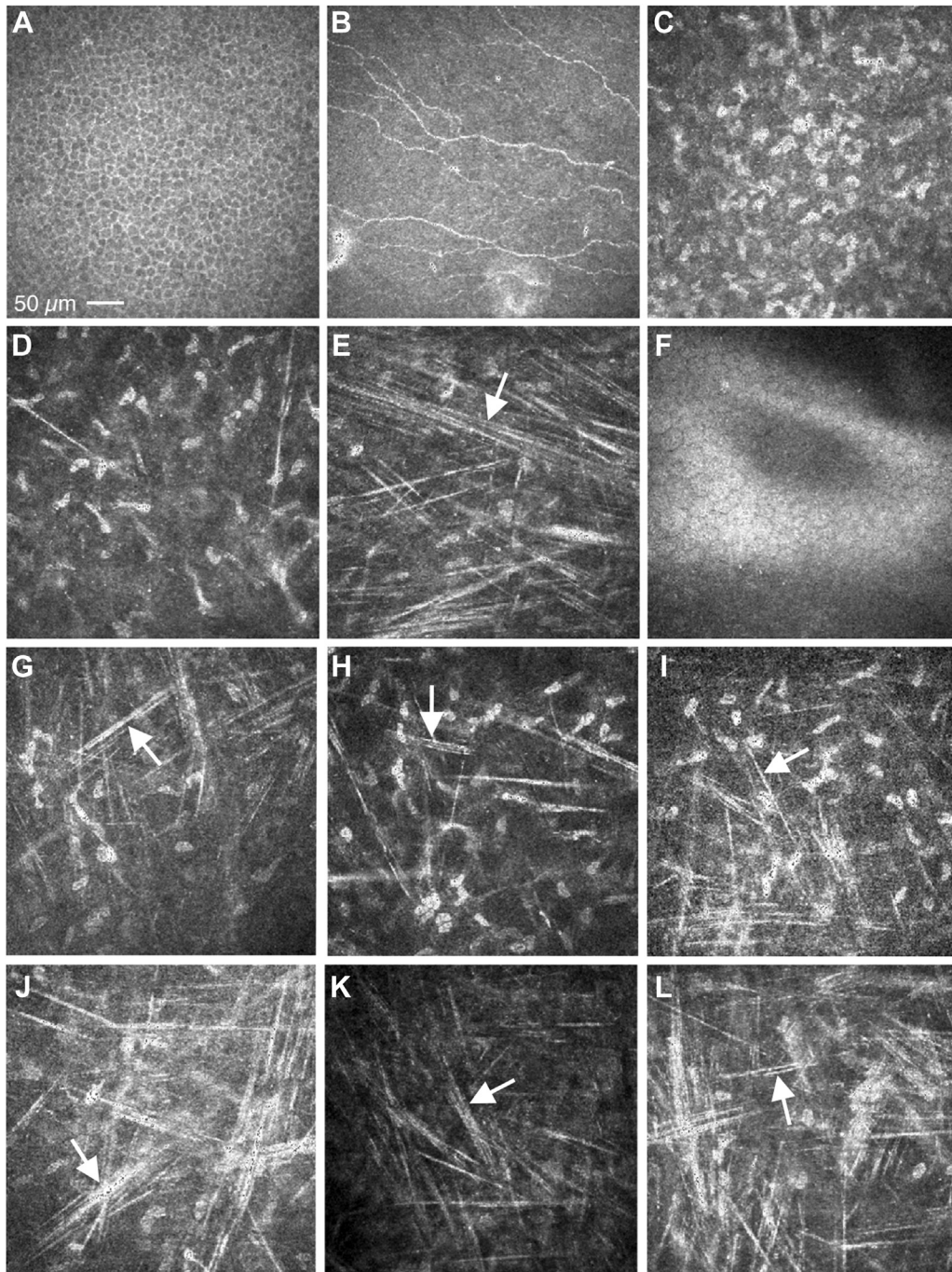


FIGURE 3. In vivo corneal confocal microscopy findings in keratitis fugax hereditaria. The left eye of patient 17-01 (46 years of age, best corrected visual acuity [BCVA] 20/16) during a quiet phase. The central cornea shows normal epithelium (A), hyperreflective dendrite-like Langerhans cells, and normal nerves at the level of the basal cells and the Bowman layer (B), normal anterior stroma (C), hyperreflective needle-like structures in the middle stromal layers (D and E), and endothelium with some polymegethism (F). Imaging of 6 other patients (15-01 OD [G], 7-05 OD [H], 16-04 OS [I], 1-04 OS [J], 1-04 OD [K], and 17-01 OD [L], 18-69 years of age) shows variable numbers of identical hyperreflective needle-like structures in the middle stromal layers.

keratocytes, syndecan-1 (CD138; B-A38, Roche), a transmembrane heparan sulfate proteoglycan present on normal corneal epithelial cells^{10,11} and reactive keratocytes, α -smooth muscle actin (1A4; Dako, Glostrup, Denmark)

present in smooth muscle and myofibroblasts, CD163 (10D6; Novocastra, Newcastle upon Tyne, United Kingdom), a membrane protein present on M2 macrophages, and CD3 (2GV6; Ventana Medical Systems,

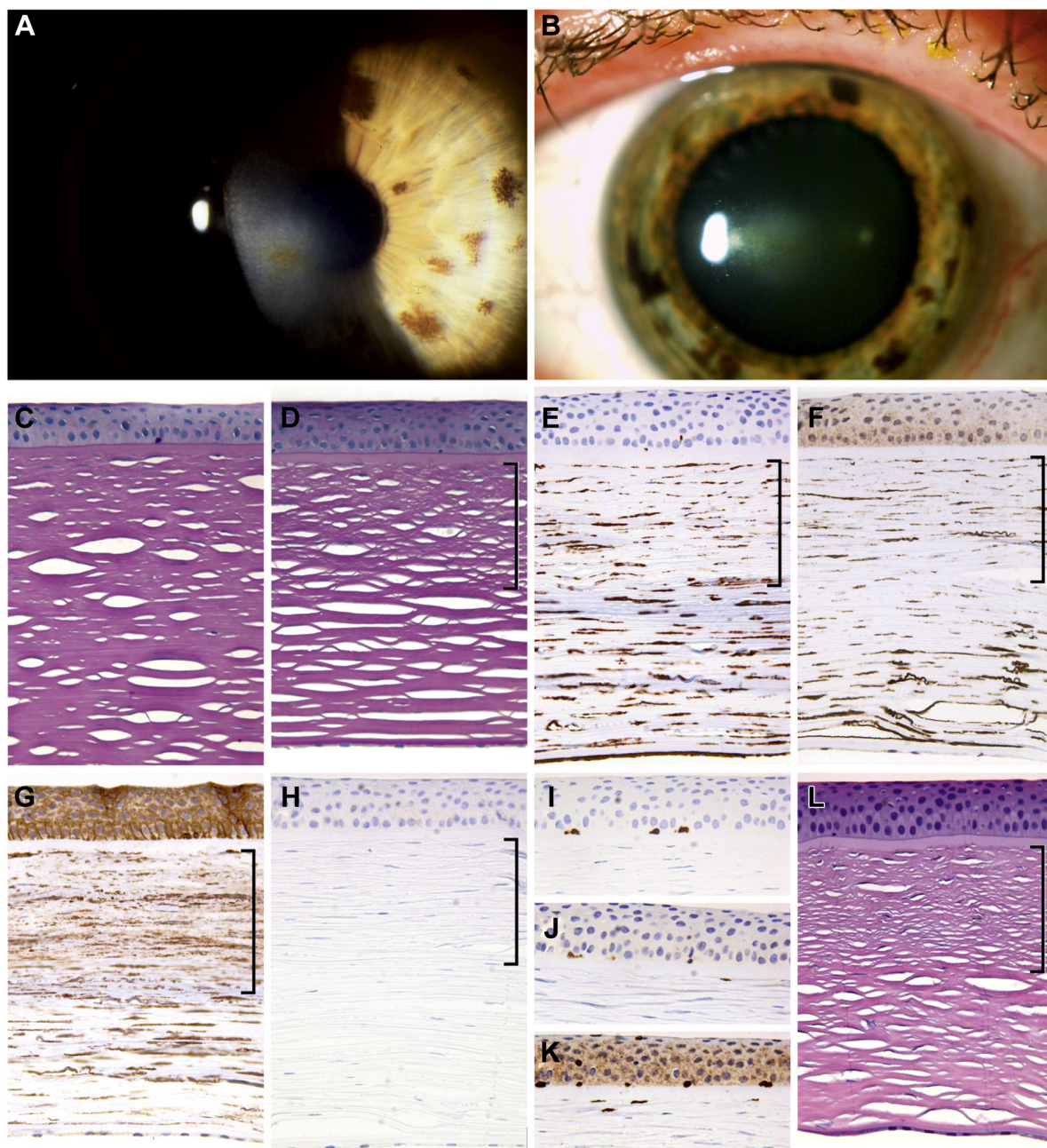


FIGURE 4. Histopathologic findings in keratitis fugax hereditaria in 2 corneal disks obtained at penetrating keratoplasty from 1 patient. (A) The right cornea with a typical oval central opacity and an incidental iron line in 1992, when the patient was 41 years of age. (B) The opacity is unchanged in 2004 at the time of the keratoplasty, when the patient was 53 years of age. The peripheral (C) and central (D) part of the disk has a normal epithelium, an intact Bowman layer, and relatively thin stromal lamellae. In the central part (D) the lamellae of the anterior half of the stroma are thin, finely vacuolated (bracket in D through H identifies this layer), the posterior lamellae are relatively thicker, and the endothelium is normal. Stromal keratocytes are somewhat unevenly immunopositive for vimentin (E) and CD34 epitope (F) and they have acquired syndecan-1 (G; CD138) as a reactive change. Note granular staining in the vacuolated layer. (H) No smooth muscle actin-immunopositive myofibroblasts are present. Scattered T cells reside intraepithelially and subepithelially and, less frequently, under the Bowman layer and between the anteriormost stromal lamellae as identified with antibodies to CD3 (I), CD4 (J), and CD8 (K). The corneal button from penetrating keratoplasty of the left eye (L) shows identical histopathology with thin finely vacuolated anterior half of stromal lamellae (bracket). Periodic acid-Schiff stain (C and D), immunoperoxidase staining (E through K), and hematoxylin-eosin stain (L). (C through L) Original magnification, $\times 190$.

Tucson, AZ, USA), CD4 (rabbit monoclonal SP35; Cell Marque, Rocklin, CA, USA), and CD8 (4B11; Novocastra), cell surface proteins present on naïve T-cells, helper T-cells and macrophages, and cytotoxic T-cells and natural killer cells, respectively.

RESULTS

OF THE 18 PATIENTS IMAGED, 7 WERE DESCRIBED IN OUR PREVIOUS genetic study.³ Here we add 5 new families, 3 of which had several affected members (Supplemental Table 1 and Supplemental Figure 1, Supplemental Material available online at AJO.com). Six patients, including 3 new ones, had bilateral central horizontally oval corneal opacities (Figure 1). All patients harbored the variant c.61G>C in *NLRP3*.

- **ACUTE ATTACK:** We imaged 6 patients within 1 to 2 days after the onset of symptoms during an acute attack of keratitis. All of them reported typical symptoms: a unilateral foreign body sensation that transformed into pain, followed by blurry vision. None reported systemic symptoms. Clinically, reduced visual acuity, conjunctival injection, and corneal haze were present. Most patients had cells in the anterior chamber, corresponding to SUN 0.5+, but patient 15-03 had SUN 2+ anterior uveitis. Specular microscopy showed cornea pseudoguttata as reported earlier.^{2,3} During the attack, corneal thickness was slightly increased (45 μm) compared with the fellow eye, as measured using corneal tomography.

IVCM showed a normal epithelium with some hyperreflective dendritiform elements at the level of the basal epithelial cells and the Bowman layer (Figure 2, A and B). We imaged numerous hyperreflective small roundish bodies 10-15 μm in diameter in the middle stromal layers of the central cornea, most probably representing infiltrating inflammatory cells (Figure 2, C and D). The posterior stroma and endothelium appeared to be essentially normal, although subtle endothelial gaps and blebs were seen (Figure 2, E and F). We also documented identical migration to the middle stromal layers of putative inflammatory cells in varying numbers in all 6 patients from 5 families that we imaged (5-02, 5-04, 7-05, 15-03, 16-01, and 20-01) during their acute attack (Figure 2, G through L). The cells were often arranged in clusters or rows. Most keratocyte nuclei appeared relatively normal, although some were hyperreflective. Isolated needle-shaped bodies were found in some corneas (Figure 2, J). We documented similar cells in *Pseudomonas* keratitis, supporting their inflammatory nature (Supplemental Figure 2; Supplemental Material available at AJO.com).

- **CHRONIC PHASE:** We imaged 18 patients with IVCM in the chronic phase, including the 6 patients imaged

during an acute attack. Again, the epithelium and subepithelial nerves were normal with hyperreflective dendritiform elements at the level of the Bowman layer (Figure 3, A and B). Coinciding with the central horizontally oval opacity, we imaged 50- to 200- μm -long highly reflective, strictly linear, needle-shaped elements toward the middle stromal layers (Figure 3, C through E). The nuclei of keratocytes appeared to be relatively normal. The endothelial cells could display polymegathism (Figure 3, F). Similar needle-shaped structures were present in some patients with clinically clear corneas, but to a lesser degree. Three patients had only a few of these structures (patients 3-03, 16-01, and 16-04). We detected at least some of these structures in most (67%) patients (Figure 3, G through L).

- **OPHTHALMIC PATHOLOGY:** After 4 decades of typical symptoms and consequent development of typical bilateral central horizontally oval corneal stromal opacities, a female patient underwent penetrating keratoplasty of her left and right eye at the age of 48 and 53 years, respectively. We have not encountered any other patient who would have had corneal surgery. During the preceding decade, her opacities had remained essentially stationary (Figure 4, A and B). The removed corneal disks showed a normal epithelium and an intact 12- μm thick Bowman layer with solitary leukocytes under the basal epithelial cell layer (Figure 4, C and D). The stroma was thin, leading to central corneal thicknesses of 320 μm and 270 μm in the right and left eye, respectively. Corresponding to the central opacity, the anterior half of the stromal lamellae were thinner than those in the anterior peripheral part of the disk (Figure 4, C) and in the deeper central stroma (Figure 4, D) and finely vacuolated, whereas only minor vacuoles could be discerned in the peripheral and deep stromal lamellae that also were relatively thin. The Descemet membrane was 3 μm thick and thin for the patient's age. We counted on average 38 nuclei of endothelial cells per millimeter, which is typical for the patient's age.

Periodic acid-Schiff, Masson trichrome, and Congo red stain revealed no stromal deposits. Oil Red O staining was technically inadequate because frozen sections were not available. Immunostaining of stromal keratocytes for vimentin and the CD34 epitope (Figure 4, E and F) was somewhat uneven compared with that typically seen in normal cornea. The keratocytes were also immunolabeled for syndecan-1, and the reaction was more pronounced and granular in the anterior central stroma with thin vacuolated lamellae (Figure 4, G). We detected no α -smooth muscle actin-immunopositive myofibroblasts or CD163-immunopositive macrophages (Figure 4, H). No polymorphonuclear leukocytes were present. A few scattered T cells were found both within and beneath the epithelium and among the anterior most stromal lamellae reacted with antibodies against CD3, CD4, and CD8 (Figure 4, I through K).

DISCUSSION

IN THIS FIRST IVCN STUDY OF THE CRYOPYRIN-ASSOCIATED periodic corneal inflammation known as keratitis or KFH, 6 patients who carried the variant c.61G>C in *NLRP3* that leads to Asp21His substitution in cryopyrin showed during their acute attack invasion of the corneal stroma by hyperreflective structures that we interpreted as inflammatory cells. Detection of inflammatory cells using IVCN is based on the morphology and size of such hyperreflective structures.¹² Cryopyrin is a crucial component of the NLRP3 inflammasome that, when activated, leads to release of interleukin-1 β . The symptoms and signs, the transient corneal haze, and the accompanying anterior chamber reaction support the identification of the hyperreflective structures as inflammatory cells. This interpretation is further supported by a recent study in which experimental activation of the NLRP3 inflammasome recruited neutrophils to the mouse cornea, a reaction that was markedly weaker in NLRP3 knockout mice. Such cells in our patients were absent between attacks, and we detected only a few scattered T-lymphocytes and no polymorphonuclear leukocytes in 2 penetrating keratoplasty specimens from 1 patient between attacks.

In corneal autoimmune diseases, inflammatory cells are usually located in the limbal region or in the peripheral cornea, whereas in KFH, an autoinflammatory disease, we observed putative inflammatory cells from the periphery to the central stroma. Currently, it is unknown what triggers the inflammatory reaction and homes the cells to the corneal stroma, and how they disappear after the attack. In a rabbit model of corneal injury, inflammatory cells also disappear almost completely in 24 hours after injury.¹³ Classification of inflammatory cells is not possible using IVCN either.¹⁴ The NLRP3 protein is mainly expressed in monocytes and macrophages,¹⁵ which we did not detect in the penetrating keratoplasty specimens in the quiet phase. It has also been reported that corneal cells express cryopyrin.⁵

In contrast to the pseudoguttata appearance of the corneal endothelium as has been observed by specular microscopy,^{2,3} IVCN documented uninvolved corneal endothelium during acute attacks. The pseudoguttata-like phenotype is most probably the result of a masking effect caused by the stromal clusters of inflammatory cells. The term keratoendotheliitis^{2,3} therefore might be misleading, and we recommend reverting to the initial description KFH¹ like we have done in the present report. While we cannot exclude that secondary endotheliitis might be present in some patients, the endothelium is not the predominant site of inflammation.

Central stromal opacities develop after repeated attacks, as seen in 12 of 18 c.61G>C variant carriers imaged between attacks. These faint to distinct, slowly progressive opacities reduce light transmittance, and visual acuity decreases accordingly.³ The opacities bear resemblance to

the disk-like central opacities in Schnyder corneal dystrophy without crystals.¹⁶ The fine vacuolization of the anterior half of the central corneal stroma is seen both in Schnyder corneal dystrophy and in lipid keratopathy from various causes, and would be consistent with deposition of intrastromal lipid in KFH. Finally, the needle-shaped structures found in the extracellular matrix of the middle stromal layers by IVCN closely resemble those described in some patients with Schnyder corneal dystrophy.^{17–20}

Using IVCN, similar needle-shaped structures have been described in several corneal diseases without lipid deposition as well, including microbial keratitis,²¹ Mooren ulcer,¹² and chemical injury,²² and postoperatively after penetrating keratoplasty,²³ Descemet stripping automated endothelial keratoplasty,²⁴ femtosecond laser-assisted keratoplasty,²⁵ corneal crosslinking,²⁶ and wound repair after laser in situ keratomileusis.²⁷ In these contexts, needle-shaped elements have generally been interpreted as activated keratocytes.²⁸ Other authors have proposed that the needle-like structures might represent either lipofuscin¹⁸ or disorganized extracellular material.²⁵ This theory would be supported by our observation that keratocytes in KFH were prominently immunolabeled for syndecan-1, which is a sign of activation. Interestingly, syndecan also has been implicated as a negative regulator of leukocyte-mediated inflammatory responses.²⁹ However, the studies mentioned mostly show single, faint, scattered needle-shaped structures and not such dense aggregates that we found. Moreover, we found no evidence of a scarring reaction in the form of myofibroblasts and macrophage infiltration.

We favor the theory that in the chronic phase of KFH the needle-shaped elements might correspond to hyperreflective extracellular matrix around degenerated collagen fiber bundles associated with deposition of lipid. These changes would originate from repeated release of inflammatory cytokines and from waves of disintegrating leukocytes. Such degenerative changes would gradually lead to thinning of corneal lamellae, especially in the anterior stroma. This theory finds support from the unusual granular syndecan deposition in the anterior corneal stroma.

The cornea is transparent because of its highly specialized ultrastructure where the collagen lamellae have a parallel 3-dimensional arrangement and uniform spacing between fibers. Interfibrillar spacing and proteoglycan content of the extracellular matrix is even more critical to corneal transparency. Stromal debris causes corneal haze and extracellular matrix backscatter is seen in IVCN.³⁰ Another theory is that stem cell-like keratocytes may have a role in structural adaptation by minimizing light scatter in the cornea. When keratocytes change from quiescent to activated ones, their biochemical homeostasis changes as well, and this increases light scatter.^{31–34} The former process appears more likely in KFH. However, it is unclear why the opacities develop

only in the center of the cornea. Proximity to the limbal circulation might somehow offer protection or clearance.

Our data confirm that KFH is a cryopyrin-associated periodic corneal autoinflammation mainly involving anterior to middle stromal layers. We also demonstrate that corneal endothelium is not notably affected, and therefore the term keratoendotheliitis is not correct. After repeated attacks, corneal opacities develop that by ICCM consist of hyperreflective needle-shaped structures and may reflect both degenerated stromal extracellular matrix and deposition of lipid. Currently, we do not know why the patients carrying the variant p.Asp21His in NLRP3 only have symptoms in the anterior segment of the eye, and why other tissues are not affected as they are in other cryopyrin-associated periodical syndromes from pathogenic variants in *NLRP3*.^{7,8,35,36} Because of its transparency and avascularity, the cornea and KFH could potentially make a good in vivo human model to study

the activation, regulation, and tissue specificity of the NLRP3 inflammasome.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

JONIA. TURUNEN: CONCEPTUALIZATION, INVESTIGATION, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Annamari T. Immonen:** Investigation, Writing - original draft, Visualization, Project administration. **Reetta-Stiina Järvinen:** Investigation. **Sabita Kawan:** Investigation. **Pauliina Repo:** Investigation, Visualization. **Anna Korsbäck:** Investigation. **Olli Ala-Fossi:** Investigation. **Aino M. Jaakkola:** Investigation. **Anna Majander:** Investigation. **Minna Vesaluoma:** Investigation, Writing - review & editing. **Tero T. Kivelä:** Conceptualization, Writing - review & editing, Supervision.

ALL AUTHORS HAVE COMPLETED AND SUBMITTED THE ICMJE FORM FOR DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST. Funding/Support: Supported by The Eye and Tissue Bank Foundation and The Eye Foundation, Helsinki, Finland. Financial Disclosures: Dr Turunen received lecture fees from Thea Finland and Blueprint Genetics Finland. Dr Kivelä received lecture fees from Santen Finland. Drs Immonen, Järvinen, Kawan, Repo, Korsbäck, Ala-Fossi, Jaakkola, Majander, and Vesaluoma have no financial disclosures or conflicts of interest.

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