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Histological Findings in the Eyes of *Abcc6* Knockout Rat Model of Pseudoxanthoma Elasticum

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Purpose: To describe the ocular findings of murine pseudoxanthoma elasticum (PXE) models with ATP-binding cassette subfamily C member 6 (*Abcc6)* gene knockout.

Methods: This experiment was conducted in four *Abcc6*$^{-/-}$ rats and compared with six wild-type *Abcc6*$^{+/+}$ control rats. The animals underwent necropsy at 6 months of age. Histological examination of the eyes was performed.

Results: Histological examination of eight eyes from four *Abcc6*$^{-/-}$ rats revealed multiple nodular foci of calcification in the uvea, sclera, and conjunctiva, focally in perivascular distribution, as well as linear and nodular calcification of Bruch's membrane. Calcific foci were not associated with inflammation in the knockout rats. There was no evidence of calcification in control eyes.

Discussion: The *Abcc6*$^{-/-}$ rat model shows that PXE can affect multiple ocular tissues beyond the calcification in Bruch's membrane noted in human eyes. Nodular calcific foci probably correspond to comet lesions seen in patients with PXE. The presence of ectopic calcium without inflammation distinguishes it from inflammatory calcium deposition in atherosclerosis. Further studies are needed to determine why PXE does not cause inflammatory infiltration.

Translational Relevance: The *Abcc6*$^{-/-}$ murine model may be suitable for studying ocular PXE pathophysiology and ectopic calcification and developing effective therapies.

Introduction

Pseudoxanthoma elasticum (PXE) is a rare inherited disorder that causes progressive ectopic calcification of elastic fibers in the skin, arteries, and eyes. PXE has protean manifestations with the potential for significant morbidity and occasional mortality. Cutaneous manifestations of PXE emerge in childhood or adolescence, and the "characteristic" skin changes consist of small yellow papules on the nape of the neck and in flexural areas such as the axillae, inguinal, popliteal, and periumbilical skin.[1–5] Cardiovascular calcification results in a plethora of pathological manifestations, including diastolic dysfunction, angina pectoris, arterial hypertension, and sudden cardiac failure—often resulting in mortality.[6,7] Interestingly, cardiovascular calcification in PXE does not seem to be associated with inflammation, unlike calcification in atherosclerotic disease and various etiologies of dystrophic calcification.[18] Additional systemic manifestations include gastrointestinal bleeding, intermittent claudication of the arms or legs, and stroke.[4,5,7]

Characteristic ophthalmic findings of PXE are related to calcification at the level of Bruch's membrane. These include angioid streaks, which are cracks in calcified Bruch's membrane that can be associated with subretinal neovascularization and visual loss.[8–10] The multifocal, punctate calcifications in the Bruch's membrane at mid-periphery lend a

mottled appearance to the retinal pigment epithelium (RPE), a finding termed "peau d'orange."[8,9] Other reported ophthalmic manifestations include pattern dystrophy, comet lesions, and optic nerve head drusen.[5,8,9]

Given the severe manifestations of PXE, there is significant emphasis on furthering our understanding of the pathophysiology of this disease. Currently, PXE is mainly a monogenic disease caused by the pathogenic variant in the adenosine triphosphate–binding cassette subfamily C member 6 (*ABCC6*) gene with influence from modifier genes and, interestingly, pseudogenes that have similarity to the PXE gene cause some problems in sequencing.[11,12] The diverse phenotypic manifestations of PXE despite its monogenic inheritance suggest that, in addition to modifier genes, other epigenetic and environmental factors contribute to metabolic alterations of PXE.[12,13] Increasing evidence suggests that defective ABCC6 transporter activity in the liver leads to the mineralization of affected tissues peripherally, resulting in stigmata of PXE.[12] Notably, *Abcc6* is not expressed in the eye. Ocular changes in PXE are a systemic manifestation of local hepatic disease. Animal models with *Abcc6*$^{-/-}$ have been developed and used to study pathomechanics of the mutant gene as well as therapeutic developments to counteract ectopic calcification. These models include mice, rats, and zebrafish.[14–17] Additionally, ectopic calcification is an important manifestation of atherosclerotic disease, so PXE calcification might give us insights into that process as well.[18]

Interestingly, these models have not evaluated the ocular findings extensively. Our study focuses on the characterization of ocular findings in *Abcc6*$^{-/-}$ rats, compared with the eyes of normal rats. The primary goal of the study was to investigate whether this model can replicate the known pathology seen in a human eye with PXE and be suitable for further study of PXE and potential therapies. The second goal is to uncover any additional pathological processes in ocular tissues that may be subclinical and, therefore, not detected in a human eye, shedding light on the potential mechanisms of ocular disease caused by PXE.

Materials and Methods

All protocols were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. The rats had free access to water and food and were maintained in a temperature- and humidity-controlled environment under 12-hour

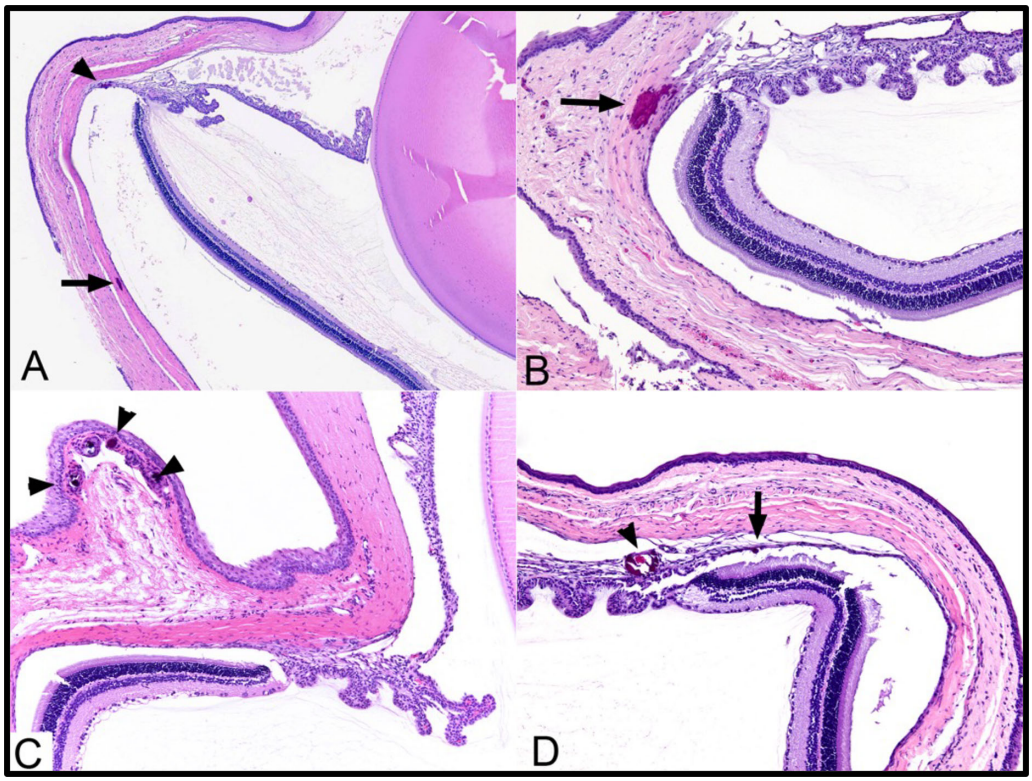

Figure 1. *Abcc6*$^{-/-}$ rat – ocular histopathological findings at low-magnification. (**A**) Foci of peripheral calcification at the level of Bruch's membrane/superficial choroid (arrowhead) and in the sclera posteriorly (arrow). (**B**) Calcification in the anterior sclera extending into ciliary body stroma (*arrow*). (**C**) Multiple calcific foci in the conjunctiva (*arrowheads*). (**D**) Linear and nodular calcification in peripheral Bruch's membrane (*arrow*) and in a ciliary body vessel (*arrowhead*). (All images: stain: hematoxylin and eosin; original magnification ×100).

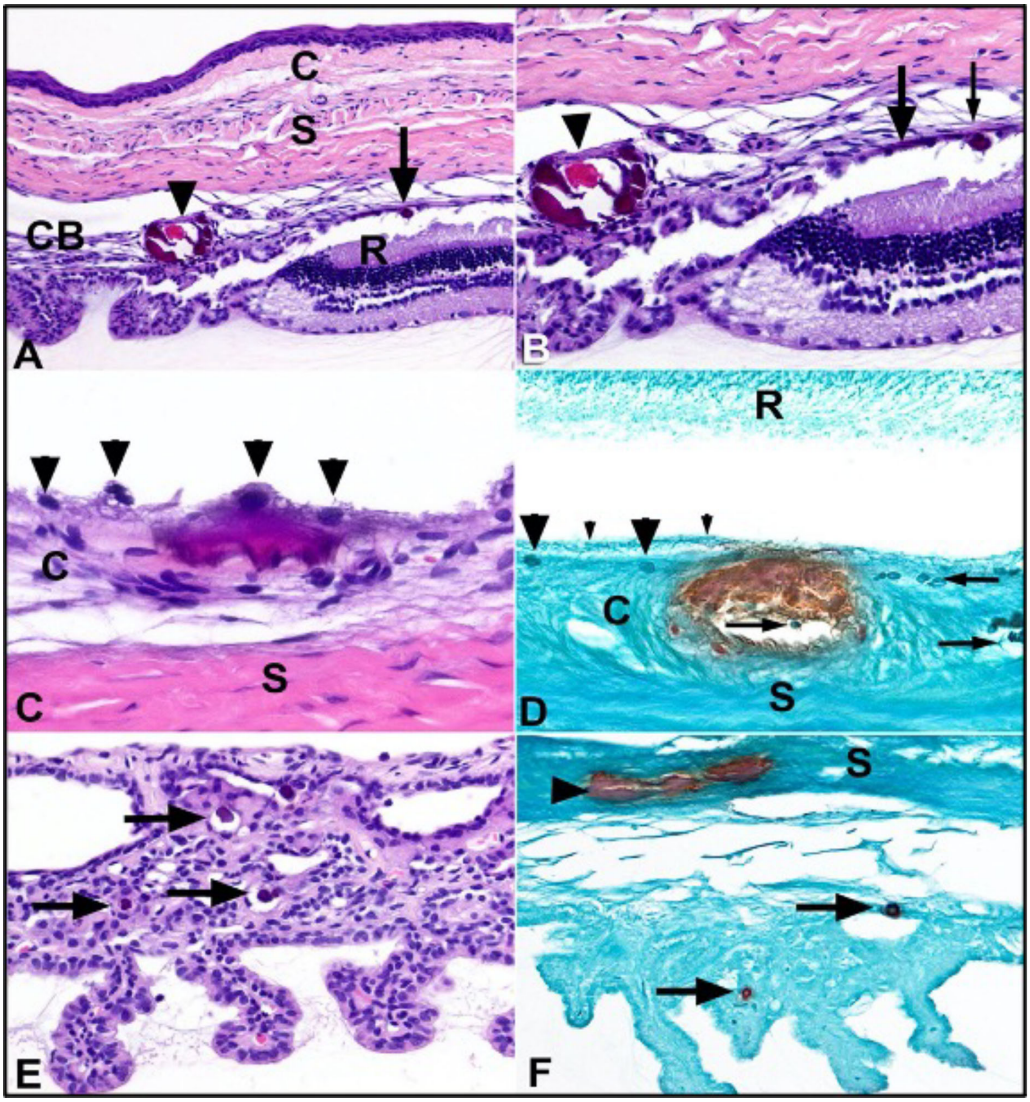

Figure 2. *Abcc6*$^{-/-}$ rat – ocular histopathological findings at high magnification. (**A**) Calcification (*arrowhead*) in the pars plana of the ciliary body (CB). Calcification in Bruch's membrane (*arrow*). Retina (R), sclera (S), and conjunctiva (C). (**B**) Higher magnification highlights purple calcification in the vessel wall (*arrowhead*) with a central red hemorrhage. Linear (*thick arrow*) and nodular (*thin arrow*) purple calcification in peripheral Bruch's membrane. (**C**) Another region of calcification beneath the RPE nuclei (*arrowheads*) and overlying choroidal stroma (C) and sclera (S). Bruch's membrane is indistinct. The calcific focus is likely at the level of Bruch's membrane/inner choroid. (**D**) Alizarin red stain (different eye) highlights red-brown calcification in the choroid (C), above the sclera (S), and beneath RPE nuclei (*big arrowheads*). Artifactual detachment of the overlying retina (R) with photoreceptor outer segment fragments (*small arrows*) focally attached to the RPE. There is a faint linear staining overlying choroidal calcification focus, suggestive of a focal deposit in the Bruch's membrane. The red blood cells in the adjacent choroidal vessels and within the focus of choroidal calcification stain dark green (*arrows*). The presence of red blood cells in calcification suggests choroidal vessel wall involvement. (**E**) Small purple calcifications (*arrows*) in the pars plicata of the ciliary body, some of which seem to be associated with blood vessels. (**F**) Alizarin red stain highlights calcifications in pars plicata (*arrows*) and the overlying sclera (S). (Stain: hematoxylin and eosin [**A**–**C**, **E**], Alizarin red [**D**, **F**]; original magnification ×200 [**A**], original magnification ×400 [**B**, **E**], original magnification ×630 [**C**, **D**], and original magnification ×150 [F]).

light/dark cycles. Environmental enhancements were provided by Laboratory Animal Services at Thomas Jefferson University to promote the psychological well-being of rodents. The enrichments include social housing, blend cob bedding, and plastic polycarbonate structures.

Murine Models of PXE

The *Abcc6*$^{-/-}$ knock-out rat was generated in Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) using zinc finger nuclease and described previously.[16] The rats were maintained on

Rodent diet TD.00442 (Harlan Teklad, Madison, WI), under standard conditions and euthanized at 6 months of age. Four *Abcc6*$^{-/-}$ knock-out rats were used for this study.

Wild-Type Murine Control

Wild-type *Abcc6*$^{+/+}$ Sprague Dawley rats (Charles River Laboratories) were maintained on Rodent diet TD.00442 (Harlan Teklad) under standard conditions and euthanized at 6 months of age. Six *Abcc6*$^{+/+}$ wild-type rats were used for this study.

Complete Necropsy and Histopathological Analysis

After euthanizing the wild-type and *Abcc6*$^{-/-}$ rats by CO_2 asphyxiation followed by thoracotomy, complete necropsies were performed (QL).[19] Eyes were collected and fixed in 10% phosphate-buffered formalin overnight and then transferred and stored in 70% ethanol. Eyes were then embedded in paraffin, cut into 6-µm sections, and stained with hematoxylin and eosin and/or Alizarin red. An experienced ocular pathologist (TM) reviewed all histology slides. Images were acquired with Leica DM 2000 LED microscope using a Leica Microsystems camera (Wetzlar, Germany).

Results

Abcc6$^{-/-}$ Rat Model

Histopathological examination of eight eyes from four *Abcc6*$^{-/-}$ rats revealed multiple foci of calcification in ocular and periocular tissues (Figs. 1–3). In addition to a linear and nodular calcification in Bruch's membrane, most prominent peripherally (Figs. 1A, 1D,

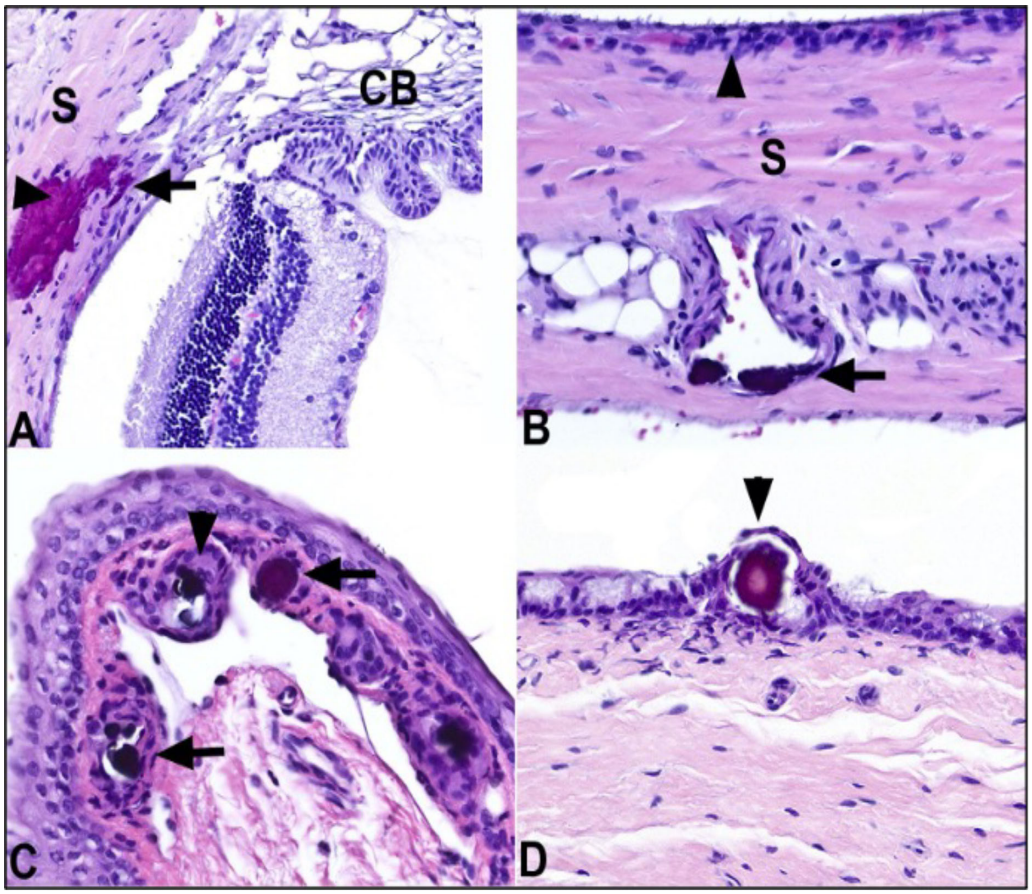

Figure 3. *Abcc6*$^{-/-}$ rat – ocular histopathological findings at high magnification, continued. (**A**) Intrascleral (S) purple irregular calcific deposit (*arrowhead*), which extends to involve the adjacent pars plicata stroma (arrow) of the ciliary body (CB). (**B**) Purple calcification (arrow) in the intrascleral (S) blood vessel, beneath the choroid (*arrowhead*). (**C**) Rounded and irregular purple calcific deposits in the conjunctival substantia propria that seem to be in vascular distribution (*arrows*), focally associated with a foreign body granulomatous response (*arrowhead*). (**D**) A calcific focus (*arrow*) associated with conjunctival epithelium; conjunctival giant cells vs. epithelium. (All images: Stain: hematoxylin and eosin, original magnification ×400).

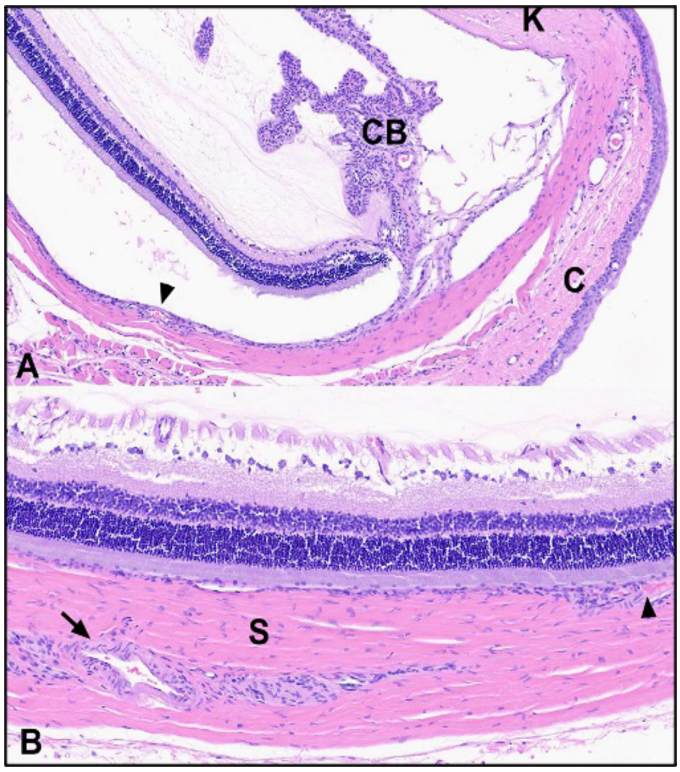

Figure 4. *Abcc6*$^{+/+}$ aged rat – ocular histological findings. (**A**) Unremarkable cornea (K), conjunctiva (C), ciliary body (CB), choroid with large vessel (*arrowhead*), and artifactitiously detached overlying retina with absence of calcification in any of the tissues. (**B**) Higher magnification of the retina and underlying choroid (arrowhead) and sclera (S) with intrascleral blood vessels. There is no evidence of calcification in any of the tissues. (Stain: hematoxylin and eosin; original magnification ×80 [A], original magnification ×100 [B]).

Figs. 2A, B), there were multiple foci of calcification in the uveal tract stroma, predominantly in vascular distribution, presenting as calcific deposits in the ciliary body and choroidal vasculature (Figs. 1D, 2A–F). In one eye (Figs. 1B, 3A), there was a linear stromal calcification in the ciliary body extending from a focus of adjacent scleral calcification. Nodular calcification was also present in the scleral vessels (Fig. 2B) and focally in conjunctival vessels (Fig. 3C). Calcific foci in conjunctival substantia propria were focally associated with a foreign body granulomatous response (Fig. 3C). In one eye, a focus of calcification was directly associated with conjunctival epithelium (Fig. 3D). With the exception of conjunctiva, there was no appreciable inflammatory response associated with calcific deposits. Nodular calcification in Bruch's membrane in one eye was associated with attenuation or loss of the overlying RPE and mild degeneration of the overlying photoreceptor outer segments (Supplemental Fig). However, this finding was subtle and could have been attributed to an artifact. There was no evidence of subretinal neovascularization in any of the eyes.

Abcc6$^{+/+}$ Rat Model

In contrast, histopathological examination of 12 eyes from 6 *Abcc6*$^{+/+}$ rats revealed unremarkable cornea, conjunctiva, uvea, and RPE with no evidence of calcification (Fig. 4).

Discussion

Findings from our study offer insight into the pathophysiology of PXE and suggest that the *Abcc6*$^{-/-}$ murine model may be suitable to improve our understanding of this disease in the human eye. As noted in single-cell RNA sequencing studies, the *Abcc6* gene is not expressed in the eyes of humans and wild-type murine models.[20,21] Table summarizes the current understanding of the histological basis of ocular signs of PXE in humans.[22]

In humans, a recent study showed that there exists a posterior diffuse area of Bruch's membrane calcification.[29] This area has a speckled aspect, which is more commonly visualized as peau d'orange. With age, the Bruch's membrane calcification progresses and confluences, which leads to an area of confluent Bruch's membrane calcification with an increased fundus reflex.[29] In the longitudinal analysis, indocyanine green angiography on patients with PXE in their fourth and fifth decades showed fluorescence that started in the macula and spread centrifugally.[29] In contrast, the peripheral border of peau d'orange does not seem to be age dependent, but rather is a static feature of a patient with PXE.[29] The underlying pathological characteristics of this predetermined area of Bruch's membrane calcification have yet to be established. Additionally, it has been postulated that the comet lesions are areas of focal calcifications.[28]

In the eyes of murine models, our study noted nodular and linear calcification at the level of Bruch's membrane, similar to the calcific deposits in the human eye. However, these calcific foci in a murine model were predominantly peripheral, possibly corresponding with comet signs, which are focal, and the peau d'orange diffuse changes noted in human fundi. We did not observe juxtapapillary calcification associated with angioid streaks seen in the human eye. It is possible that the difference in the mechanical stresses on Bruch's membrane between the human and murine eyes may

Table. The Current Understanding of the Histologic Basis of Ocular Signs of PXE in Humans

Author	Finding(s)	Presumed Mechanism(s)	Implication(s)
Gliem et al.[20]	Abnormal calcification and thickening of the elastic and subsequently collagenous layers of Bruch's membrane.	A deficiency of specific ABCC6 substrates in the blood affects a range of connective tissue sites throughout the body. For reasons yet unknown, Bruch's membrane is a preferred site for these ectopic calcifications.[23,24]	These changes within Bruch's membrane may lead to alterations of the adjacent choriocapillaris as well as the overlying RPE and the neurosensory retina.
	Peau d' orange	An unequivocal pathogenesis has not been established. It is assumed that progressive calcification of the Bruch's membrane is the etiology of this ocular phenotype.[25]	Several authors have noted a patchy transition zone toward the normal-appearing peripheral Bruch's membrane anterior to the equator. This transition zone is perhaps the histopathological correlate for peau d'orange, although there has been no direct evidence to prove this hypothesis.
	Angioid streaks	These occur secondary to breaks within the calcified Bruch's membrane. Larger breaks are often associated with ingrowth of fibrous tissue, RPE cell atrophy, and thinning of the choriocapillaris.	More than 70% of angioid streaks in PXE are associated with choroidal neovascularization, making it a sight-threatening complication.[26]
Murro et al.[25]	Comet lesions	These chorioretinal atrophic spots (named "comet" lesions by JD Gass) are in the mid-periphery, often with their "comet tails" pointing towards the posterior pole.[27] Studies have suggested that degenerate photoreceptors are present over a focal disruption of the RPE–Bruch's membrane.[28]	This finding is considered pathognomonic for patients with PXE, amidst other ocular manifestations. For this reason, especially in young patients, in whom angioid streaks are often not yet present, comet lesions might thus be of significant diagnostic value.

account for this phenomenon.[22] Additionally, differences in Bruch's membrane thickness and composition between the murine and human eyes may account for our observations.[29]

In addition to calcification within Bruch's membrane, we noted foci of calcification in the uveal tract stroma, in the sclera, and in the conjunctiva, predominantly in the vascular distribution in the rat *Abcc6*$^{-/-}$ model. This broadens the spectrum of ocular histopathological changes seen in the murine *Abcc6*$^{-/-}$ model and may be indicative of subclinical pathological changes in the eyes of humans with PXE (Fig. 5).

With the exception of conjunctiva, there was no appreciable inflammatory response associated with calcific deposits. The lack of inflammation near the calcific foci is a remarkable finding. A thorough search of the literature suggested that no histological study

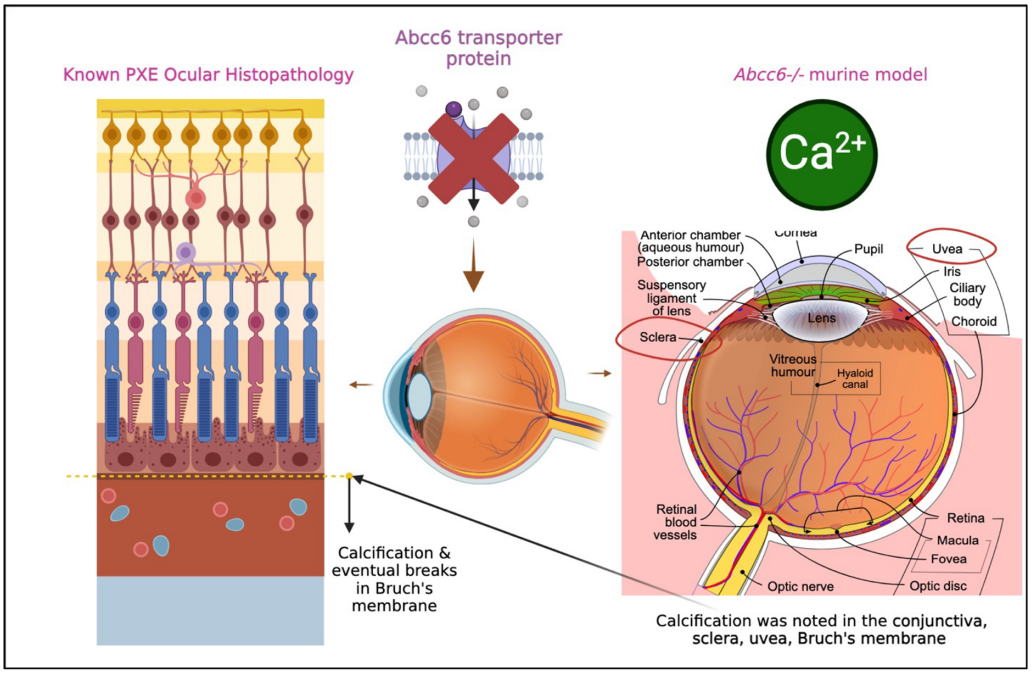

Figure 5. Summary of findings seen in *Abcc6*$^{-/-}$ rat and mouse models in this study.

has so far explicitly shown inflammatory cells in skin biopsies obtained from patients with PXE.[30,31] Similarly, histological, and structural analyses of artery walls of patients with PXE has not detected any immune cells such as those found in vasculitis and or atherosclerosis.[32,33] This finding contrasts with calcification associated with atherosclerosis, where inflammation is commonly observed.[34,35]

To further the current understanding of ectopic mineralization in human patients with PXE in the absence of inflammatory cytokines, a prominent trial was conducted in 2020 wherein the skin/artery inflammation and calcification in patients with PXE were assessed.[18] 18F-sodium fluoride positron emission tomography combined with computed tomography imaging was used to establish that inflammation and calcification were not correlated.[18]

In patients with PXE with nonfunctional ABCC6, the plasma levels of inorganic pyrophosphate are decreased to about 40% of the level in normal healthy individuals.[36,37] Decreased inorganic pyrophosphate levels then allow the growth of calcium hydroxyapatite crystal deposits on the surfaces of the extracellular matrix, particularly the elastic structures.[2,15,36,37] The distinct separation of the two metabolic processes—calcification and inflammation—functions in narrowing therapeutic interventions for patients with PXE. The presence of choroidal neovascularization strengthens the use of antiproliferative agents such as antivascular endothelial growth factor (VEGF) therapies. The pharmaceutical agents, bevacizumab and ranibizumab, antagonists of VEGF, are now a part of clinical recommendations to slow the ocular progression of PXE.[38–40] It should be noted that the VEGF antagonists target only neovascularization, a consequence of calcification of the Bruch's membrane, and they do not antagonize the mineralization process per se.[40–42] In contrast, anticomplement therapies, specifically the downregulation of key cytokines, will arguably not be effective due to the lack of inflammation facilitating the calcification process in PXE.

Limitations

Our study is limited by a relatively small number of rat eyes examined. Histopathology was, in some cases, limited by eccentric sectioning. We did not perform exhaustive serial sectioning of the eyes and flat mount preparations of the RPE and retina, which may have revealed additional pathology. Additionally, this study did not evaluate the eyes *in vivo*, so we cannot correlate the pathological findings with *in vivo* findings.

Conclusions

Overall, our findings suggest that PXE knockout rats develop ectopic calcification in local foci and diffusely in the eye. Additionally, there is no evidence

of an associated inflammatory reaction with these areas of calcification. The comet lesions that were noted by Gass are probably similar to our focal calcific lesions. Further studies can help elucidate how breaks in Bruch's membrane occur and why there are disparities between inflammatory atherosclerotic calcification and PXE calcification.

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43. Figure 5: Created with www.biorender.com.