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ORIGINAL ARTICLE

Autosomal-dominant myopia associated to a novel *P4HA2* missense variant and defective collagen hydroxylation

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We recently described a complex multisystem syndrome in which mild-moderate myopia segregated as an independent trait. A plethora of genes has been related to sporadic and familial myopia. More recently, in Chinese patients severe myopia (MYP25, OMIM:617238) has been linked to mutations in *P4HA2* gene. Seven family members complaining of reduced distance vision especially at dusk underwent complete ophthalmological examination. Whole-exome sequencing was performed to identify the gene responsible for myopia in the pedigree. Moderate myopia was diagnosed in the family which was associated to the novel missense variant c.1147A > G p.(Lys383Glu) in the prolyl 4-hydroxylase,alpha-polypeptide 2 (*P4HA2*) gene, which catalyzes the formation of 4-hydroxyproline residues in the collagen strands. In vitro studies demonstrated *P4HA2* mRNA and protein reduced expression level as well as decreased collagen hydroxylation and deposition in mutated fibroblast primary cultures compared to healthy cell lines. This study suggests that *P4HA2* mutations may lead to myopic axial elongation of eyeball as a consequence of quantitative and structural alterations of collagen. This is the first confirmatory study which associates a novel dominant missense variant in *P4HA2* with myopia in Caucasian patients. Further studies in larger cohorts are advisable to fully clarify genotype-phenotype correlations.

KEYWORDS

collagen hydroxylation, disease gene identification, genotype-phenotype correlation, myopia, *P4HA2* gene, whole-exome sequencing

1 | INTRODUCTION

Myopia is the most common ocular abnormality worldwide with a high prevalence in Asian (40%-70%) and Caucasian (20%-30%) populations.[1,2] The disease results from the elongation of the eye globe, which causes a distant object's image being formed anteriorly to the retinal plane or, less frequently, by an excessive refraction of light rays due to structural changes of cornea or lens.[3] Severity of myopia is classified in Diopters (D) as low (−0.50 to −2.99D), moderate (−3.00 to −5.99D), and high (≥ −6.00D).[4,5] Phenotypic variability of this disorder has been described in relation with inheritance fashion, age of onset, progression and comorbidity.[6] In fact, high-grade myopia is a main cause of blindness increasing the risk of retinal detachment, macular choroidal degeneration, premature cataract, and glaucoma.[1]

It has been reported that a large proportion of myopia, known as "school- or juvenile-onset myopia," develops and progresses between the ages of 8 and 14 years, whereas, approximately the 10% to 50%

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of adult-onset myopia, from low to moderate clinical degree, manifests during adulthood (18 years or older).[6–10]

Both environmental and genetic factors contribute to the development and heterogeneity of myopia and several family studies suggested a strong genetic background.[11,12] Specifically, genes associated to myopia encode proteins involved in a high spectrum of biological functions, including: neurotransmitters, retinoic acid metabolism, ion channels activity, ocular and central nervous system development, cell cycle and growth and extracellular matrix composition and remodeling.[13–16] For high myopia, 5 genes were associated to autosomal-dominant (*ZNF644*, *CCDC111*, *SCO2*, *SLC39A5* and *P4HA2*)[3,17–21] and 2 genes (*LEPREL1* and *LRPAR1*) to autosomal recessive forms.[22,23]

We recently described a complex multisystem syndrome caused by an autosomal-dominant *LAMA5* mutation in the second and third generations of an Italian family.[24] In this family, followed-up for 20 years, autosomal-dominant mild myopia was also observed as an independent trait. Clinical and genetic investigations on myopia, extended also to the fourth generation of the family, demonstrated that this trait was associated to a novel dominant missense variant c.1147A > G p.(Lys383Glu), in the prolyl 4-hydroxylase, alpha 2 (*P4HA2*) gene. This is the first confirmatory study which associates dominant missense variants in *P4HA2* with myopia (MYP25, OMIM:617238) also in Caucasian patients.

2 | PATIENTS AND METHODS

2.1 | Ethics statements

The research involving human participants has been approved by the Institutional Review Board (IRB) (Università degli Studi della Campania L. Vanvitelli), clinical investigation has been conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent has been obtained from the participants.

Mouse adult tissues were obtained from healthy animals sacrificed in accordance with the recommendations of the European Commission. All the procedures related to animal treatments were approved by Ethic-Scientific Committee for Animal Experiments and Italian Health Ministry.

2.2 | Recruitment of the patients and ophthalmologic evaluation

A fourth-generation Italian pedigree, in which myopia segregated as an autosomal trait, has been observed from 1995 till now (Figure 1A). The following individuals were enrolled in this study: II-4, II-7, II-9 (age ranging 40-43 at time of the first observation) complaining since their second decade of life of reduced distance vision which worsened at dusk; III-14, III-17 and III-20 (age ranging 10-15 at time of the first observation) developed impaired distance vision about 10 years later; III-13 (25-year old at time of the first observation) referred to have reduced distance vision but he refused any instrumental assessment; IV-12 (7-year old, actual age) complaining of eyestrain and pain. II-10, III-7, III-16, III-18 were used as healthy controls for clinical and genetic studies (Figure 1A and Table 1).

All individuals underwent full ophthalmological examination, which included: best-corrected visual acuity measured using the Snellen chart, subjective refraction, slit-lamp anterior segment examination, fundus examination, fundus photography, intraocular pressure measurement via Goldmann applanation tonometry, axial length measurement using ocular biometry (IOLMaster; Carl Zeiss Meditec, Jena, Germany), Goldmann visual field examination, standard electroretinogram (ERG) recorded with a Ganzfield stimulator following the guidelines of the International Society of Clinical Electrophysiology of Vision.[25]

After signed informed consent, DNA was extracted from blood samples of both affected and healthy individuals to perform genetic investigations. All procedures were conducted according to international guidelines and the tenets of the Helsinki Declaration.

2.3 | Whole-exome sequencing (WES) and gene identification strategy

Genomic DNA was extracted from peripheral blood leukocytes of all recruited family members using a standard salting-out procedure. WES was performed on three individuals of the pedigree including 2 affected family members (II-7 and III-17) and 1 healthy individual (III-7) (Figure 1A and Table 1).

Exonic regions were enriched using the Agilent Haloplex Exome kit based on DNA digestion and capture. Exomes were barcoded and sequenced at multiple sites on the Illumina HiSeq1000 platform. Average coverage for all the experiments was 70× and at least 20× for 89% of the target. Paired sequencing reads were aligned to the reference genome (UCSC, hg19 build) using BWA[26] and sorted with SAMtools[27] and Picard (http://broadinstitute.github.io/picard/webcite). Post-alignment processing (local realignment around insertions-deletions and base recalibration), SNV, and small insertions-deletions (ins-del) calling were performed with Genome Analysis Toolkit (GATK)[28] with parameters adapted to the haloplex-generated sequences. The called SNV and ins-del variants produced with both platforms were annotated using ANNOVAR (Parker, CO, USA).[29]

Sequencing data were first filtered to eliminate low-quality score variants, common variants reported in public databases with a minor allele frequency (MAF) > 0.01, neutral variants such as non-coding or synonymous, and variants not shared by affected subjects. SIFT (http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html/), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), Mutation Taster (www.mutationtaster.org/) and likelihood ratio test (LRT) http://evomics.org/resources/likelihood-ratio-test/) were used to assess the deleterious effects of the identified variants. PhyloP (ccg.vital-it.ch/mga/hg19/phylop/phylop.html) was used to measure evolutionary conservation at individual alignment sites. Allele frequency of the annotated variants was obtained from dbSNP (http://www.ncbi.nlm.nih.gov/snp), 1000 Genomes (http://www.ensembl.org/Homo_sapiens/), ExAC (exac.broadinstitute.or) and the ESP6500 version of the Exome Variant Server (evs.gs.washington.edu/).

Assuming a dominant mode of inheritance, we searched for heterozygous rare variants in myopia disease genes. A novel heterozygous missense variant c.1147A > G p.(Lys383Glu) in *P4HA2* gene was identified showing complete segregation with myopia in the family. To search any additional causing variants in further genes, WES data from affected individuals were matched. After that heterozygous variants

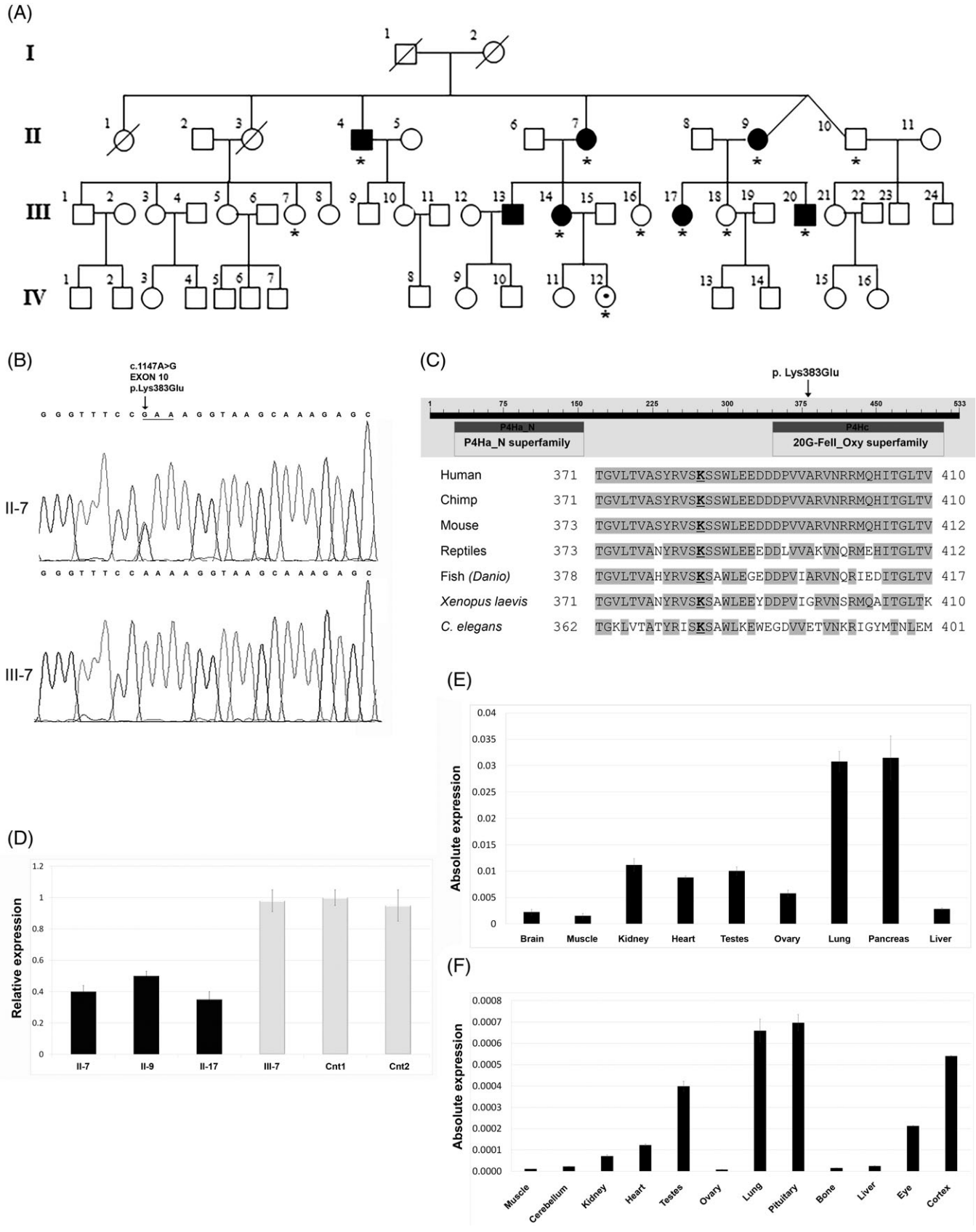

TABLE 1 Clinical features of the family members

ID	Sex	Affected status	Age at onset	Age at last exam	Night blindness	Actual refractive error (DS) RE	Actual refractive error (DS) LE	Axial length (mm) RE	Axial length (mm) LE	Fundus	ERG
II-4	M	A	26	61	U	−1.5*	−1.5*	24.88	24.84	SMD	Normal
II-7	F	A	25	60	A	−3.50	−0.50	26.19	23.97	RE: MD	Affected*
II-9	F	A	21	56	A	−3.50	−5.00	24.41	24.85	MD	Affected*
II-10	M	U	–	56	U	–	–	NP	NP	Normal	Normal
III-7	F	U	–	33	U	–	–	NP	NP	Normal	Normal
III-14	F	A	24	36	U	−2.50	−2.00	NP	NP	Normal	Normal
III-16	F	U	–	27	U	–	–	NP	NP	Normal	Normal
III-17	F	A	23	37	U	−3.50	−3	24.13	23.84	Normal	Normal
III-18	F	U	–	33	U	–	–	NP	NP	Normal	Normal
III-20	M	A	22	28	U	−2.25	−1.50	NP	NP	Normal	Normal
IV-12	F	U	–	7	U	–	–	NP	NP	Normal	Normal

A, affected; affected*, reduced cone and rod response; DS, diopter sphere; ERG: electroretinogram; LE, left eye; MD, myopic dystrophy; NP, not performed; −1.5*, measurement 10 years after myopia refractive surgery; RE, right eye; SMD, slight myopic dystrophy; U, unaffected.

present in the healthy individual, III-7 were subtracted. Fourteen autosomal non-synonymous coding variants in 14 genes, including the c.1147A > G p.(Lys383Glu) missense variant in *P4HA2*, were identified (Table 2). Validation and segregation analysis of these variants was performed by Sanger sequencing (IGA Technology, Udine, Italy). Only the *P4HA2* variant showed a complete segregation with the myopia trait. Other variants in different genes were excluded as potentially pathogenic as they neither segregate with myopia phenotype in our family nor were reported to be associated with ocular disorders. However, their modifying role on the expression of the *P4HA2* variant, in some of the family members, cannot be excluded. PCR amplification and direct sequencing protocols have been previously described.[30,31] Primers for *P4HA2* missense variant detection are listed in Table 3.

2.4 | Fibroblast primary cell cultures

The fibroblast cell lines derived from skin biopsies of the lateral aspect of the right thigh were obtained from the patients II-7, II-9, III-17 and III-20, a healthy family member III-7 and from 2 unrelated healthy controls (Cnt1 and Cnt2). We used cells from patient III-20 because he was affected by myopia only and was negative for mutation in *LAMA5* gene which causes the multisystem syndrome. Epithelial tissue pieces were enzymatically digested for 4 hours at 37°C using collagenase/dispase kit (Roche, Basel, Switzerland), followed by a mechanically disaggregation with knife. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L D-Glucose, 0.11 g/L sodium pyruvate, 10% fetal bovine serum (FBS), 1% Non-Essential Amino Acids (NEAA), 1% L-glutamine, gentamycin 1:200, 1% Pen-Strep and 1% Amphotericin B at 37°C in a humidified CO_2 5% air for about 3 to 4 weeks. Fibroblast cell lines were maintained and used in all the experiments in confluent monolayers.

2.5 | Expression studies

The expression profile of the *P4HA2* gene was evaluated on total RNAs from several adult human (Stratagene, San Diego, California, USA) and mouse tissues. Total RNA from fibroblast cell lines was isolated by using TRIreagent (Sigma-Aldrich, Saint Louis, Missouri USA) protocol. One microgram of total RNA was reverse transcribed with the RevertAid RT Reverse Transcription Kit (Thermo Fisher, Waltham, Massachusetts, USA). Quantitative PCR (qPCR) reactions were performed in triplicate, using gene-specific primers and sybr select master mix for CFX (Life Technology, Carlsbad, California, USA) following the manufacturer's directions. qPCR expression values of the specific genes were normalized vs the expression of the glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) gene. Primers used are listed in Table 3.

The mean values ± standard deviations of 3 independent experiments were analyzed as multiple datasets with ANOVA test for

FIGURE 1 The complete pedigree of the Italian family; mutation analysis and expression studies. (A): Pedigree. Affected family members II-4, II-7, II-9, III-13, III-14, III-17 and III-20 indicated with dark symbols showed mild-moderate myopia. The individual IV-12 is an asymptomatic carrier. Individuals indicated with asterisk underwent clinical, instrumental and genetic examinations. (B) Mutation analysis of the *P4HA2* gene. Arrowhead marks the site of base alteration. The sequences of the affected and unaffected family subjects are shown; the pedigree number is indicated on the left. (C) Evolutionary conservation analysis. Lysine (K) 383, which is highly conserved in vertebrates, is underlined in bold. The catalytic domain at C terminal region with the localization of the p.(Lys383Glu) missense variant is reported in the upper part of (C). (D) *P4HA2* mRNA expression in human fibroblast cell lines from 4 patients (II-7, II-9, III-17 and III-20), 1 healthy family member III-7 and 2 unrelated controls (Cnt1 and Cnt2). A significant reduction of *P4HA2* transcript was observed in mutated cells. (E, F) *P4HA2* mRNA expression in human (E) and mouse (F) tissues. The gene was expressed ubiquitously at low level of expression. Higher amount of transcript was detected in lung and pancreas in human (E) and pituitary gland, lung and testes in the mouse (F). All qPCRs (D-E) were performed in triplicate and normalized to GAPDH mRNA levels. The means ± standard deviation of 3 independent experiments was plotted as histogram representation. Expression is reported as relative expression in (D) and as absolute values in (E) and (F)

TABLE 2 Variants identified through WES analysis

Chr	gene	dchange	pchange	EF	REF	ALT	MAF	SIFT	PolyPhen2	LRT	MT	PhyloP
12	*PMEL*	c.257 T > C	p.(Ile86Thr)	NS	A	G	0	D	D	N	D	C
12	*NOS1*	c.2774 T > A	p.(Phe925Tyr)	NS	A	T	0			D	D	C
12	*EP400*	c.2324A > C	p.(His775Pro)	NS	A	C	0	D		D	N	C
16	*ITGAX*	c.1810G > A	p.(Val604Met)	NS	G	A	0	D	D		N	C
17	*SHPK*	c.1244C > G	p.(Pro415Arg)	NS	G	C	0	D	P	D	D	C
5	***P4HA2***	**c.1147A > G**	**p.(Lys383Glu)**	**NS**	**T**	**C**	**0**	**D**	**D**	**D**	**D**	**C**
1	*CHRM3*	c.1246G > A	p.(Asp416Asn)	NS	G	A	0	T	P	N	D	C
16	*PRSS53*	c.686 T > C	p.(Leu229Pro)	NS	A	G	0		P	U	N	C
17	*TIMM22*	c.37C > G	p.(Pro13Ala)	NS	C	G	0	T		N	D	N
3	*PRRT3*	c.982G > A	p.(Ala328Thr)	NS	C	T	0	T	P	N	N	N
12	*ABCB9*	c.1787G > A	p.(Arg596Gln)	NS	C	T	0	D	D	D	D	C
4	*ADAD1*	c.7A > G	p.(Ser3Gly)	NS	A	G	0	T	B	N	N	N
9	*AK1*	c.257C > A	p.(Thr86Asn)	NS	G	T	0	T	B	N	D	C
1	*TCHH*	c.4888G > A	p.(Glu1630Lys)	NS	C	T	0				N	C

For each gene is reported the chromosomal localization (Chr), the symbol name, the nucleotide (dchange) and amino acid (pchange) change, the type of change (NS: nonsynonymous), the minor allele frequency (MAF), the reference allele (REF) and the alternative allele (ALT), the tolerated or deleterious effect determined through bioinformatics tools (T, tolerated; D, deleterious; P, possible damaging; B, benign), the evolutionary conservation (C, conserved; N, not conserved). EF, exonic function; LRT, likelihood ratio test; MT, mutation taster; WES, whole-exome sequencing; in bold is reported the missense variant identified in the myopia disease gene P4HA2.

multiple comparisons with Bonferroni's corrections. A value of $P < 0.05$ was considered to be statistically significant.

2.6 | Protein extraction and Western blot analysis

Fibroblast cells were collected and washed with cold PBS (2.7 mM KCl, 1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 138 mM NaCl [pH 7.4]). Total proteins were solubilized in lysis buffer [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) [pH 7.5], 150 mM NaCl, 4 mM Ethylenediaminetetraacetic acid (EDTA), 10 mM Na_4PO_7, 2 mM Na_3VO_4, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100 mg of aprotinin/mL, 1 mM leupeptin). Cytoplasmic proteins were extracted from fibroblast cells using 100 to 1000 μL hypotonic buffer (10 mM Tris-HCl [pH 7.5], 2 mM $MgCl_2$, 10 mM $CaCl_2$, 30 mM sucrose, 1% NP40) and ×1 proteinase inhibitor mixture [Sigma-Aldrich, Saint Louis, Missouri USA]). Conditioned medium proteins (normalized vs the number of cells) were precipitated over night by using pre-cooled acetone. Proteins were recovered after centrifugation at 1500*g* at 4°C, separated by SDS-PAGE and transferred onto 0.45-m pore size Immobilon-P membranes (Millipore, Bedford, Massachusetts, USA). The level of expression of P4HA2, β-actin and Collagen Type IV was determined by immunoblot analysis using anti-P4HA2 [sc-161146, Santa Cruz Biotechnology (Dallas, Texas USA), 1:1000]/Rabbit anti Goat HRP-conjugated [Biorad (Hercules, California, USA), 1:2000], anti-COL4A (sc-59 814, Santa Cruz Biotechnology, 1:1000)/goat anti Mouse HRP-conjugated (Biorad, 1:2000), anti-β-Actin (sc-47778, Santa Cruz Biotechnology (Dallas, Texas USA), 1:3000)/goat anti-mouse HRP-conjugated (Biorad, 1:5000).

Densitometric analysis was performed using Scion Image (Ver. 4.0.2; Scion Corporation (Frederick, Maryland USA)). The signals obtained for each protein were normalized vs β-actin (cytoplasmic proteins) and ponceau (medium proteins). The means ± standard deviations of 3 independent experiments were analyzed as multiple data sets with ANOVA test for multiple comparisons with Bonferroni's corrections and were plotted as histogram representation. A value of $P < 0.05$ was considered to be statistically significant.

2.7 | Hydroxyproline assay

Hydroxylated collagen was quantified by using hydroxyproline quantification kit (Sigma-Aldrich MAK008) according to the manufacturer's instructions. The amount of hydroxyproline in 100 μL of fibroblast culture medium of four affected family members (II-7, II-9, III-17 and

TABLE 3 Primers used for mutation analysis and qPCR

Human genes	Forward primer 5′-3′	Reverse primer 5′-3′	MT (°C)	PCR size (bp)
P4HA2-DNA	GGAGAAGGGATGAGCAGTG	AGAGAAAAGAAGACGGAAG	60	533
P4HA2-cDNA(exons 6, 7)	CCACTGATGAGGACGAGATAGG	CATTGCCTGGTACTTGGTTC	60	119
P4HA2-cDNA(exons 9-12)	CTTCAAAGAGGAGGACGAGT	CTCCCACTCCATAATTTGCA	60	295
GAPDH	GGAAGGTGAAGGTCGGAGT	GACAAGCTTCCCGTTCTCAG	60	200
Human genes				
P4ha2	GCTATGCTGTCTTCCAACTG	TCCCTCTTTCTTCCTCTAAC	60	138
Gapdh	TGGAGAAACCTGCCAACTAT	CATACCAGGAAATGAGCTTG	60	198

qPCR, quantitative PCR. For all primer pairs used in qPCR analysis, forward and reverse sequences as well as melting temperature (MT) and PCR sizes are reported.

III-20), 1 related (III-7) and 2 unrelated (Cnt1 and Cnt2) healthy individuals was determined by measuring the absorbance at 560 nm (A_{560}) and compared to the hydroxyproline standard curve. The analysis was conducted in triplicate and for each experiment all samples and standards were run in duplicate in a 96-well plate. Hydroxyproline contents were expressed as μg hydroxyproline/μL. The means ± standard deviations of 3 independent experiments were analyzed as multiple datasets with ANOVA test for multiple comparisons with Bonferroni's corrections and were plotted as histogram representation. A value of $P < 0.05$ was considered to be statistically significant.

2.8 | Histological and immunofluorescence (IF) analyses

Six 2-month-old C57BL/6J inbred mice (10-20 g body weight) were sacrificed after ketamine parenteral injection and then all tissues and organs, including both eyes, were immediately collected and processed for microscopy studies. Specifically, whole eyes were fixed in 1% buffered paraformaldehyde plus 1.25% glutaraldehyde solution for 48 hours and embedded in paraffin for histological studies.[32] Seven micrometer-thick serial sections of the whole eye were cut transverse to the vertical axis of the globe and sliced on superfrost plus microscope slides (Thermo Scientific, Waltham, Massachussetts, USA). After deparaffinization in xylene and rehydration, some sections were stained with hematoxylin and eosin (H&E) and modified Gomori trichrome reactions while others were used for IF evaluation. For antigen unmasking, the slides were immersed in alkaline buffer pH 9 and boiled in microwave vessel for 30′. Thereafter, the sections were incubated in a humidified chamber with blocking buffer (10% FBS, 0.5% MILK, 1% BSA/PBS1X) for 1 hour and then incubated with anti-P4HA2 (sc-161146, Santa Cruz Biotechnology, 1:100)/ Rabbit anti-Goat IgG FITC (F2016, Sigma, 1:200), anti-COL4A (sc-59814, Santa Cruz Biotechnology, 1:100)/sheep anti-Mouse IgG Cy3 (C2181, Sigma, 1:200), anti-COL1A1 (NB600-1054, Novus Biologicals (Littleton, Colorado USA), 1:100)/sheep anti-Rabbit IgG Cy3 (C2306, Sigma, 1:200). Nuclei were detected with DAPI (Thermo Scientific) at the dilution 1:1000. Although the COL4A (sc-59814, Santa Cruz Biotechnology) antibody is recommended for detection of native, non-denatured, human Collagen Type IV, it also stains the mouse collagen type IV in IF analysis. Negative controls were sham incubated without primary antibody. For light microscopy and IF studies, a Nikon Eclipse E800 photomicroscope was used.

3 | RESULTS

3.1 | Clinical description

All the affected individuals had a history of myopia onset at the end of their second decade of life. Mean age at the time of the last clinical evaluation was 39 ± 16 years (age ranging 7 to 61 years). Five subjects (II-10, III-7, III-16, III-18 and IV-12) had uncorrected visual acuity 20/20 with emmetropia in both eyes, 6 patients (II-4, II-7, II-9, III-14, III-17 and III-20) had mild-moderate myopia with a spherical equivalent of −3.05 ± 0.6D in the right eye and −2.4 ± 1.7D in the left eye. Patient III-20 had myopia without the multisystem syndrome.[24] Patient II-4 was subjected to surgical refractive correction and his actual visual acuity is 8 to 9/10.

Mean axial length measurements available for 4 patients (II-4, II-7, II-9, III-17) was in right eye 24.9 mm ± 0.91 and 24.37 mm ± 0.54 in left eye; only patient II-7 had visual acuity in the right eye equal to 20/200 due to an amblyopia and an axial length of 26.19 mm. All subjects were phakic. At fundus examination, three patients (II-4, II-7, II-9) revealed a mild myopic retinopathy.

Intraocular pressure and Goldmann visual field examination were normal. All subjects, except patients II-7 and II-9, disclosed normal scotopic and photopic ERG (Figure 1A and Table 1).

3.2 | *P4HA2* disease gene

The single novel heterozygous missense variant c.1147A > G (p.Lys383Glu) in *P4HA2* gene completely segregated with the myopia trait in II-4, II-7, II-9, III-13, III-14, III-17, III-20 while it was absent in not-myopic examined individuals and in public databases (Figure 1A,B, Table 2). IV-12 (7-year-old) carried the missense variant despite being asymptomatic. Different *P4HA2* mutations have been associated to severe myopia in sporadic and familial cases of Chinese origin.[33]

The P4HA2 enzyme structurally consists of 2 functional domains: a peptide substrate-binding domain and a oxoglutarate/iron-dependent dioxygenase domain.[34] The missense variant identified in our study affects the amino acid lysine (Lys) at 383 which is highly conserved in all species, it was predicted to be deleterious and functionally damaging being close to the catalytic domain of the protein (Figure 1C and Table 2).

P4HA2 was ubiquitously expressed, although at a very low level, in human and in mouse. Tissues with higher absolute expression were lung and pancreas in humans (Figure 1E) and pituitary gland, lung and testes in the mouse (Figure 1F). Interestingly, the eye was the tissue with the fourth level of *P4HA2* expression in the mouse.

IF studies on mouse eye showed intense and seemingly continuous P4ha2 signal in the rod outer and inner segments (ROS and RIS) which was less intense in the outer and inner plexiform layers (OPL and IPL) of the retina (Figure 2B). Intense blots of P4ha2 positivity were also visible at the level of the stroma of the ciliary processes (Figure 2E). In the cornea, a continuous and mild intense P4ha2 reactivity delineated the thin Descemet's membrane, while the Bowman's layer disclosed a faint positivity (Figure 2H).

P4ha2/Col1a1/collagen type IV co-localization studies disclosed overlapping continuous, intense reactions in retina and cornea (Figure 2B-D,J-L) as well as in the stroma of ciliary processes (Figure 2F-H).

A significant reduction of the *P4HA2* transcript was observed in the mutated fibroblast cells as compared to the controls (Figure 1D). Since the c.1147A > G change was located 5 nt from the natural donor splice junction in the exon 9 of the *P4HA2* gene, we excluded by means of a splicing prediction program (http://www.umd.be/HSF/) that the variant may create a novel canonical splicing site in exon 9. No aberrant transcripts were identified in the mutated cells (data not shown), suggesting that the c.1147A >

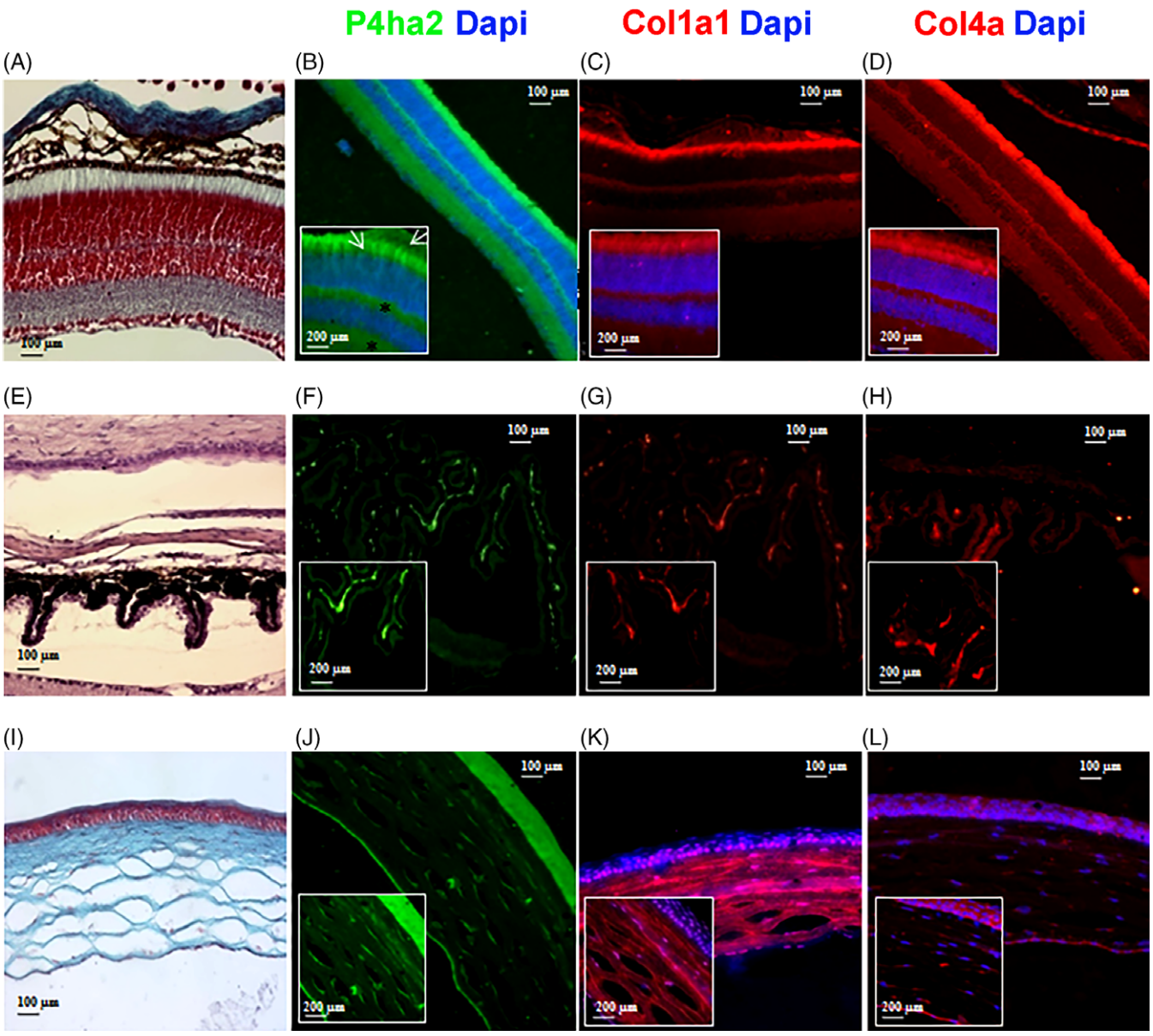

FIGURE 2 Histological evaluation and P4ha2, Col1a1 and Col4A expression in mouse eye. Seven micrometer-thick paraffin cross sections of the whole mouse eye at 2 months of life were analyzed. Gomori trichrome staining of the retina (A; ×20), of the cornea (I; ×20) and H&E staining of the ciliary bodies (E; ×20) were reported. P4ha2 expression was revealed in the rod outer and inner segments (ROS and RIS, white arrowheads), in the plexiform layers (OPL and IPL, black asterisks) of the retina (B; ×20), in the stroma of the ciliary bodies (F; ×20), in Descemet's membrane and Bowman's layer of the cornea (J; ×20). Col1a1 and Col4a immunoreactivity were reported in the retina (C, D; ×20), in the ciliary processes (G, H; ×20) and in the cornea (K, L; ×20). The nuclei were detected with DAPI. All microscope insertions were acquired at ×40

G variant may perturb the mRNA stability which may lead to its premature degradation, as already suggested for the *P4HA2* missense mutations identified in the Chinese patients.[33] This hypothesis is supported by Western blot analysis which showed a significant reduction of P4HA2 protein in mutated cells as compared to controls (Figure 3A). Moreover, our studies first demonstrated that *P4HA2* variants may affect type IV collagen hydroxylation as shown by the significant reduction ($P < 0.05$) of this molecule in culture medium, mirrored by its intra-cytoplasmic accumulation (Figure 3B). These results were further validated by measuring about 40% reduction of hydroxylation activity ($P < 0.05$) in the medium of mutated cells as compared to the controls (Figure 3C).

4 | DISCUSSION

P4HA2 gene has been recently associated to high myopia in sporadic and familial Chinese patients.[33] In this population, a nonsense and a frameshift mutation have been found into the catalytic domain (p. Lys443* and p.Arg451GlyfsTer8) and 3 missense mutations (p. Gln140Arg, p.Ile150Val, p.Glu291Lys) located near the substrate-binding domain.[33] The p.(Lys383Glu) amino acid change is a novel missense variant near the catalytic domain of *P4HA2* and it is the first identified in Caucasian patients. According to the American College of Medical Genetics and Genomics (ACMG) laboratory guideline,[35] this novel missense variant should be classified as "likely pathogenic." In fact, it was absent in public databases was predicted as highly

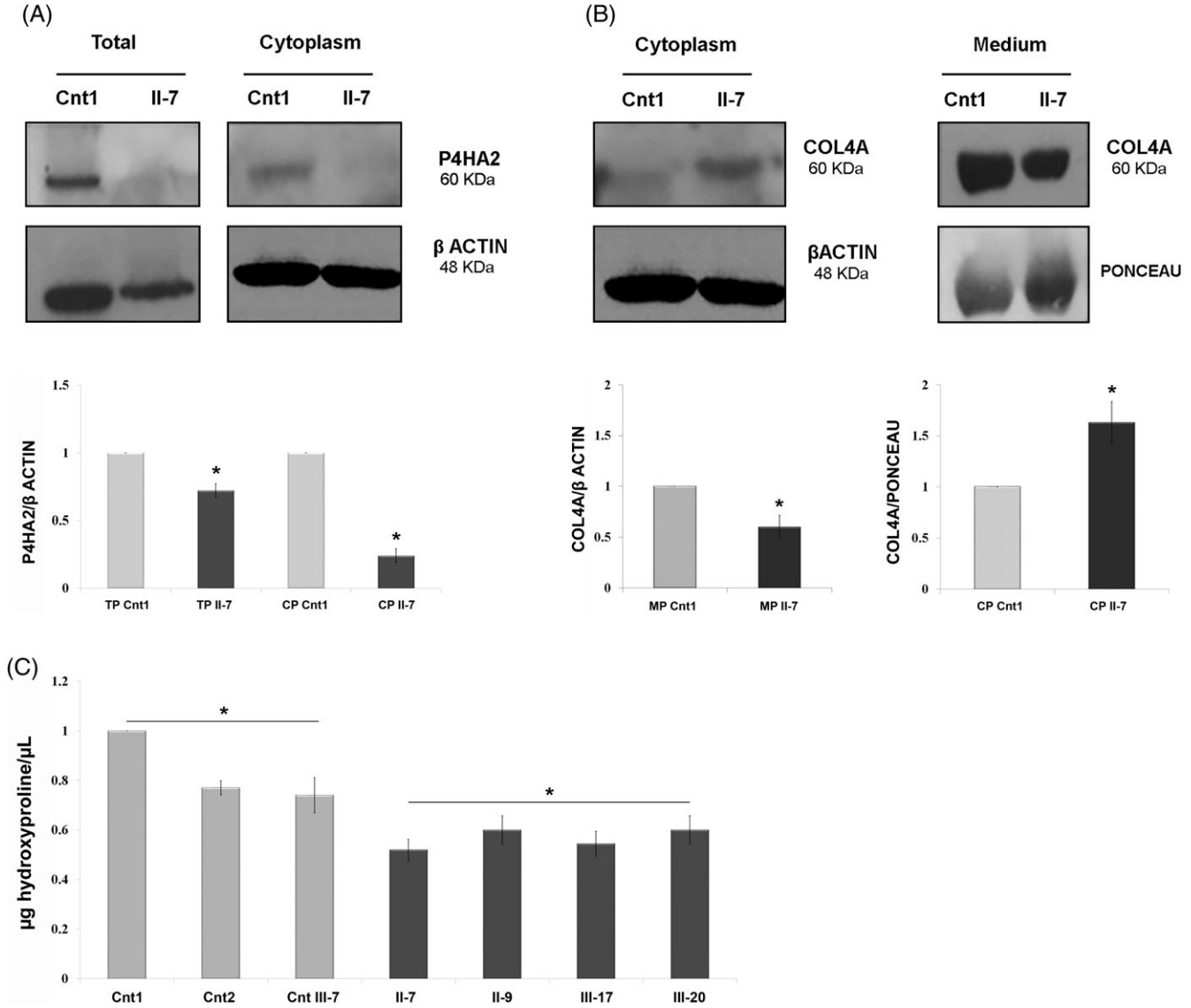

FIGURE 3 Expression analysis of P4HA2 and COL4A in human fibroblast cells. (A) Proteins were stained with anti-P4HA2 (sc-161146, Santa Cruz Biotechnology, 1:1000)/Rabbit anti Goat HRP-conjugated, Biorad, 1:2000) and (B) with anti-COL4A (sc-59 814, Santa Cruz Biotechnology, 1:1000)/goat anti Mouse HRP-conjugated (Biorad, 1:2000). The signals obtained were normalized vs β actin (sc-47778, Santa Cruz Biotechnology, 1:3000/goat anti-mouse HRP-conjugated Biorad, 1:5000) for total and cytoplasmic proteins and vs Ponceau for medium proteins. A value of $P < 0.05$ was considered to be statistically significant and was indicated with the asterisk (*). While a significant reduction of P4HA2 protein was observed in the cytoplasm of the mutated cells (A) a slight accumulation of collagen IV in the cytoplasm and a reduction in the extracellular medium was found (B). (C) Hydroxylation activity was measured as amount of hydroxyproline concentration (µg/µL) in fibroblasts medium of the patients II-7, II-9, III-17 and III-20 as compared to related III-7 and unrelated healthy individuals Cnt1, Cnt2. A significant reduction of hydroxylated collagen was observed in mutated cells

deleterious by all in silico tools, segregated with myopic trait in the family, was located near the catalytic domain of the enzyme and as reported in the Chinese population,[33] missense variants seem to be a common type of alteration in *P4HA2* gene.

In our family, mild-moderate myopia manifested in the late twenties, while mutations identified in Chinese patients all caused severe early-age myopia.[33] The disease phenotype in our family was characterized by bilateral myopia with inter-ocular asymmetry and inter-individual variability with respect to the refractive error and axial length (Table 1). Twenty years follow-up demonstrated very slow progression of myopia in all our patients which however did not improve with age. In particular, II-4 having −7.5D and −5D of refractive error in the right and left eye, respectively, underwent surgery when he was 50 years old. Ten-year post-surgery the residual refractive error increased from −0.5 to −1.5D in both eyes. Following up the IV-12 will allow us to further verify the natural course of *P4HA2*-associated myopia.

The main result of this study is the demonstration of the mechanism by which *P4HA2* variants likely cause myopia. In fact, our data while represent the first confirmation of the association of *P4HA2* gene variants with myopia in Caucasian patients also are the first evidence of the key role of P4HA2 in collagen synthesis. *P4HA2*

encodes the prolyl4-hydroxylase, alpha-polypeptide II enzyme that catalyzes the 4-hydroxylation of proline residues in the Yaa position of the Xaa-Yaa-Gly triplets within collagen strands thus stabilizing the collagen triple helices.[34,36,37] The missense variant c.1147A > G p.(Lys383Glu) identified in our study as well as those found in the Chinese patients all cause a significant decrease of the P4HA2 protein level in cell cultures obtained from patients' tissues, suggesting that they act as loss–of-function mutations. Despite being located 5 nt from the natural donor splice junction in the exon 9, the c.1147A > G variant does not seem to alter the splicing. Although at present we cannot establish the mechanism through which the missense variant causes a reduction of the P4HA2 expression level, we were able to measure about 40% reduction of hydroxylation activity in the medium of mutated cells which resulted in intra-cytoplasmic accumulation and reduced deposition of collagens in our in vitro model. These findings are important since collagens are primary components of the extracellular matrix of the sclera and the reduced hydroxylation of their fibrils likely lead to the typical deformation of the myopic eye.[38] The importance of *P4HA2* in the pathogenesis of myopia is further supported by evidence that its expression is very intense in the mouse eye as demonstrated by our experimental data, while it is almost undetectable in other tissues of both in human and mouse.[39] We first performed expression and localization studies of P4HA2 in mammalian eye where this molecule co-localizes with collagens. The role of the metabolic pathways of P4HA2 and related molecules in human eyes deserve further investigations.

In conclusion, our data confirm the relevance of *P4HA2* in the pathogenesis of myopia also in the late onset form and pave the way to the search of novel molecules acting in the treatment of structural defects of myopic eye.

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Conflict of interest

The authors report no conflicts of interest.

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