Association of *LOXL1* Polymorphisms With Pseudoexfoliation, Glaucoma, Intraocular Pressure, and Systemic Diseases in a Greek Population. The Thessaloniki Eye Study

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PURPOSE. To investigate the association of the two single-nucleotide polymorphisms (SNPs) in the lysyl oxidase-like 1 (*LOXL1*) gene with pseudoexfoliation syndrome (PEX), pseudoexfoliative glaucoma (PEXG), and primary open-angle glaucoma (POAG) in a Greek population-based setting, from the Thessaloniki Eye study.

METHODS. A total of 233 subjects with successful DNA extraction, PCR amplification, and genotyping were included in the genetic analysis of G153D and R141L SNPs of *LOXL1* gene and classified into four groups: controls ($n = 93$); subjects with PEX ($n = 40$); POAG ($n = 66$); and PEXG ($n = 34$). Multinomial logistic regression was used to test their association with *LOXL1* SNPs with adjustment for covariates. The association of *LOXL1* with IOP (in untreated subjects) and with systemic diseases was explored.

RESULTS. Both *LOXL1* SNPs were present in high frequencies in controls and cases. The G153D was strongly associated with both PEX (odds ratio [OR] = 23.2, $P = 0.003$ for allele G) and PEXG (OR = 24.75, $P = 0.003$ for allele G) and was not associated with POAG ($P = 0.451$). In contrast, the R141L was not associated with PEX ($P = 0.81$), PEXG ($P = 0.063$), or POAG ($P = 0.113$). No association of the G153D with either intraocular pressure (IOP) or systemic diseases was found.

CONCLUSIONS. In the Thessaloniki Eye Study, the G153D SNP of *LOXL1* gene was strongly associated with both PEX and PEXG, whereas the R141L was not associated. No association of the *LOXL1* with IOP or with systemic diseases was found. These findings further support the hypothesis that the *LOXL1* gene contributes to onset of PEXG through PEX. Gene variants of *LOXL1* do not help to identify those with PEX at increased risk for glaucoma development.

Keywords: *LOXL1*, pseudoexfoliation, pseudoexfoliative glaucoma

Pseudoexfoliation syndrome (PEX) is an age-related disorder of the extracellular matrix characterized by production and progressive accumulation of small, white deposits of a fibrillar material in various intraocular and extraocular tissues. This syndrome occurs worldwide; however, the reported prevalence estimates vary considerably.¹

Pseudoexfoliation syndrome is the most common identifiable cause of secondary glaucoma, named pseudoexfoliative glaucoma (PEXG).² Usually, PEXG presents with higher IOP than the primary open-angle glaucoma (POAG) and its prevalence is more IOP-dependent than POAG.³

Only a few large population-based studies have specifically investigated PEX and PEXG prevalence or incidence.^{4–6} The Thessaloniki Eye Study was designed to address the prevalence

of the major eye diseases and disorders, including PEX, in the Greek population of Thessaloniki. The reported prevalence of PEX in subjects aged older than 60 years was 11.9%.⁴ Among those with pseudoexfoliation, only 15% presented with glaucoma.⁴ In the absence of glaucoma, subjects with PEX compared with those without PEX, presented with similar clinical characteristics except that they have higher intraocular pressure (IOP), only by 0.6 mm Hg.⁷ The reasons why some subjects develop PEX currently remain unclear. In addition, it remains unclear why a proportion of subjects with PEX develop glaucoma while the majority of those with PEX do not get glaucoma.

Further pseudoexfoliative material has been also identified by electron microscopy in visceral organs such as lung, heart,

liver, kidney, gallbladder, and meninges. This widespread distribution led to the hypothesis of possible association of PEX with systemic cardiovascular or cerebrovascular diseases. Although there is some evidence in favor of this hypothesis, other studies did not confirm this association and thus, this hypothesis remains controversial.⁷⁻¹⁰

The lysyl oxidase-like 1 (*LOXLI*) gene has been identified as a potential genetic risk factor for both PEX and PEXG since 2007, when a genome-wide association study on pseudoexfoliative individuals from Iceland and Sweden was performed.¹¹ This study demonstrated a strong association (>99% population attributable risk) of PEX and PEXG conferred by three single-nucleotide polymorphisms (SNPs) in the *LOXLI* gene on chromosome 15q24.1. Two SNPs were identified in the first coding exon (rs3825942 or G153D and rs1048661 or R141L) and one within the first intron of this gene (rs2165241). Although the rs2165241 was more significantly associated with PEXG than the two exonic SNPs, this association was not statistically significant after adjusting the results for the two other SNPs.¹¹ Several studies of different ethnic populations have reported associations of *LOXLI* with PEX and PEXG. Many of them did not differentiate between PEX and PEXG. Recently, two clinical-based studies in Greece examined the association of *LOXLI* SNPs with PEX and PEXG and reported conflicting results.^{12,13}

The discovery of common sequence variants in the *LOXLI* gene that confer susceptibility to PEXG, primarily through PEX syndrome, has shed some light in the pathogenesis of the disease.¹⁴ Lysyl oxidase-like 1 is one of five enzymes in the family of lysyl oxidases, which are copper-dependent monoamino oxidases secreted by fibrogenic cells including fibroblasts and smooth muscle cells. These enzymes are involved in the covalent cross-linking of collagen and elastin polymers in extracellular matrix formation. Lysyl oxidase-like 1 is necessary for tropoelastin cross-linking and elastic fiber formation, maintenance, and remodeling.¹⁴

The purpose of the present study is to explore the association of two nonsynonymous *LOXLI* SNPs (G153D and R141L) with PEX, PEXG, and primary open-angle glaucoma (POAG) in a population-based setting using a well-defined, ethnically homogenous population in Thessaloniki, Greece. In addition, in previous studies no information is provided on the potential association of *LOXLI* with IOP or with systemic diseases. This study aims to identify whether the two *LOXLI* SNPs were associated with differences in IOP or with the self-reported history of systemic diseases (diabetes mellitus, cardiovascular disease or cardiovascular surgery) in subjects with PEX, PEXG, POAG, and controls.

METHODS

The Thessaloniki Eye Study is a cross-sectional, population-based study of chronic eye diseases in the Greek population of Thessaloniki. Thessaloniki is the major urban center in Northern Greece and the second largest city in Greece, after Athens. This urban area has a stable and homogenous population with 97.7% of the population identified as being of Greek ethnicity. The study was approved by the Aristotle University Medical School, Ethics Committee and the Institutional Review Board of the University of California, Los Angeles. All study procedures adhered to the principles outlined in the Declaration of Helsinki for research involving human subjects and all participants gave written informed consent prior to their participation.

Details about the recruitment process in the Thessaloniki Eye Study have been previously described.⁴ In brief, 5000 subjects aged 60 years or older were randomly selected in

February 1999 from approximately 321,000 persons registered in the municipality registers of the city of Thessaloniki. Subjects who agreed to participate were invited to the Thessaloniki Eye Study center (Laboratory of Research and Clinical Applications in Ophthalmology at the Aristotle University of Thessaloniki) for an extensive ophthalmologic examination. Clinic-visit examination included measurement of visual acuity using Early Treatment Diabetic Retinopathy Study (ETDRS) charts; Humphrey automated perimetry; Goldmann applanation tonometry (three readings from each eye); gonioscopy; slit-lamp examination (before and after pupil dilation); and fundus biomicroscopy. Details of observation procedures were described elsewhere.⁴

A home-visit eye examination was organized, proposed, and arranged for persons unable to visit the study examination center due to major disability or illness. Home-visit examination included ETDRS visual acuity measurement, Perkins applanation tonometry, slit-lamp examination, and dilated fundus examination.

Prior to clinical examination, a questionnaire was administered including demographic data, ophthalmologic, and medical history. In particular, subjects were queried whether they had ever been diagnosed with hypertension, diabetes mellitus (treated or not with insulin), any cardiovascular disease, heart attack, or they had undergone coronary artery bypass or vascular surgery.

Definitions

Particular attention was given to identify the presence of PEX, which was defined by the presence of pseudoexfoliative material either at the pupil margin or on the lens capsule. Prior to pupil dilation, a detailed high-magnification slit-lamp assessment of the pupil margin was performed. After pupil dilation, the anterior lens surface from each eye was scanned from left to right using a narrow slit-lamp beam and then was examined using a vertical broad slit-lamp beam, looking specifically for early signs of PEX, including pregranular radial lines, as well as established granular deposits. The location of PEX either on the iris, the lens, or both was recorded. The PEX detection and the exact location required consensus agreement between at least two of the three ophthalmologist graders. Dilation was conducted in all patients and even in those with narrow-occludable angle, laser peripheral iridotomy was performed and they were examined later under dilation.

Glaucoma was defined according to specific criteria.⁴ Subjects were classified as having POAG if they had glaucoma and open, normal-appearing anterior chamber angle and absence of other secondary causes of glaucoma in either eye. Subjects were classified as having PEXG if they had glaucoma and presence of pseudoexfoliation in either eye. A consensus agreement between at least two of the three ophthalmologists was required to assign the diagnosis of glaucoma. Three ophthalmologists were responsible for the ophthalmic examination. At least two of the three examined each patient and all diagnoses were in consensus agreement.

Among the 3617 subjects who were deemed eligible, 2554 participated in the study (participation rate: 71%). Of those participating, 2261 (89%) had the clinic-visit examination and 293 (11%) had the home-visit examination.

A blood sample was taken from all subjects with POAG and PEXG and in a consecutive subset of participants without glaucoma. Written consent was obtained before the blood draw. A total of 233 subjects who gave blood samples were included in this analysis. Among them, 93 had neither PEX nor glaucoma in both eyes (controls), 40 had pseudoexfoliation and no glaucoma in either eye (PEX), 66 were diagnosed with POAG, and 34 with PEXG.

TABLE 1. Descriptive Statistics

Characteristics	Control	POAG	PEX	PEXG
<i>n</i> (% sample)	92* (39.9)	65* (28.1)	40 (17.3)	34 (14.7)
Age, y (SD)	68.8 (4.7)	72.6 (6.3)	73.4 (5.6)	74.4 (5.1)
Male, %	41.3	47.7	50.0	47.1
Diabetic, %	17.4	27.7	15.0	11.8
Insulin use, %	3.3	10.8	2.5	0.0
Cardiovascular disease, %	38.0	32.3	32.5	41.2
Cardiovascular surgery, %	9.8	13.8	7.5	8.8
Highest IOP between two eyes, <i>n</i> (SD)	15.4 (3.2)	18.4 (5.4)	15.6 (3.8)	18.7 (6.0)

For those individuals (stratified in controls, POAG, PEX, and PEXG groups) who have been genotyped for *LOXL1* SNPs and also have age, sex, diabetes history, diabetes treated with insulin, cardiovascular disease, coronary surgery, and IOP data recorded (*n* = 231).

* One control subject and one subject with POAG have missing data on covariates and thus were excluded from logistic regression analyses.

Peripheral blood samples were collected from each subject and DNA was extracted using a DNA extraction kit (QIAamp DNA Mini Kit; Qiagen, Venlo, Limburg, Netherlands). Samples of DNA were amplified by PCR as previously described using the sense: 5' GCAGGTGTACAGCTTGCTCA 3' and antisense 5' ACACGAAACCCTGGTCGTAG 3' primers.¹⁵ Polymerase chain reactions (PCRs) were performed in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems, Inc., Foster City, CA, USA). The PCR products were sequenced using the same primers as in PCR amplification in a genetic analyzer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Inc.), using manufacturer's protocol. Analysis of individual sequences was carried out using the AB DNA sequencing analysis software (version 5.2).

Statistical Analysis

All statistical analyses were performed with a statistical software package (Stata version 10.0; StataCorp LP, College Station, TX, USA). Fisher's exact test was used for the genotypic comparison and subjects were grouped as follows: (1) control: subjects without PEX, PEXG, POAG; (2) case 1: subjects with POAG; (3) case 2: subjects with PEX; and (4) case 3: subjects with PEXG. Multinomial logistic regression analysis was conducted in order to examine the association between the two *LOXL1* polymorphisms and POAG, PEX or PEXG, adjusted for the following factors: age, sex, history of diabetes, history of cardiovascular disease, history of cardiovascular surgery, and the higher IOP for the two eyes (possible confounding factors for glaucoma, as identified in a previous report of Thessaloniki Eye study).¹⁶

Logistic regression analysis adjusted for age was also conducted in order to examine the association of the two *LOXL1* SNPs with IOP in controls and cases not receiving IOP-lowering treatment. The association of the *LOXL1* SNPs with the history of the aforementioned systemic diseases was also examined with regression analysis adjusted for age and sex. Hardy-Weinberg equilibrium testing was performed using Mendel version 13.0.^{17,18} Linkage disequilibrium (LD) and haplotype analysis was also performed using Mendel version 13.0.¹⁹ Odds ratios with 95% confidence intervals (CIs) were calculated as measures of each strength of the allele's association with POAG, PEX, and PEXG. The *P* values were not adjusted for multiple testing.

RESULTS

Successful DNA extraction, PCR amplification, and genotyping were carried out for all 233 enrolled subjects. The general characteristics of the participants (mean age, sex, IOP, history of diabetes, insulin use, cardiovascular disease, or cardiovascular surgery) in the four groups are presented in Table 1.

The allelic and genotypic distribution of the G153D (rs3825942) and R141L (rs1048661) polymorphisms in controls, POAG, PEX, and PEXG groups are presented in Table 2. All frequencies were in Hardy-Weinberg equilibrium using a heterozygote-homozygote test (*P* = 0.486 for R141L, *P* = 0.603 for G143D).¹⁸ With regard to the G153D polymorphism, there was a statistically significant difference in subjects with PEX and PEXG compared with controls using either the log-additive allelic model (G versus A allele, *P* < 0.0001 for PEX, *P* = 0.0003 for PEXG) or the recessive model (GG versus AG+AA, odds

TABLE 2. Distribution of Alleles and Genotypes of *LOXL1* SNPs

G153D (rs3825942)	<i>n</i>	Alleles		<i>P</i> Value*	Genotypes			Recessive <i>P</i> Value*	OR (95% CI)
		A	G		A/A	A/G	G/G		
PEXG, <i>n</i> (%)	34	1 (1.5)	67 (98.5)	0.0003	0 (0)	1 (2.9)	33 (97)	0.0001	17.31 (2.59, 725.71)
PEX, <i>n</i> (%)	40	1 (1.2)	79 (98.8)	<0.0001	0 (0)	1 (2.5)	39 (97.5)	<0.0001	20.46 (3.10, 853.82)
POAG, <i>n</i> (%)	66	25 (18.9)	107 (81.1)	0.8830	2 (3)	21 (31.8)	43 (65.1)	1.000	1.02 (0.50, 2.08)
Control, <i>n</i> (%)	93	33 (17.7)	153 (82.3)	Ref group	1 (1.1)	31 (33.3)	61 (65.6)	Ref group	—
R141L (rs1048661)	<i>n</i>	Alleles		<i>P</i> Value*	Genotypes			Recessive <i>P</i> Value*	OR (95% CI)
		T	G		T/T	T/G	G/G		
PEXG, <i>n</i> (%)	34	10 (14.7%)	58 (85.3)	0.1226	0 (0)	10 (29.4)	24 (70.6)	0.1534	1.98 (0.80, 5.15)
PEX, <i>n</i> (%)	40	17 (21.2%)	63 (78.8)	0.6388	1 (2.5)	15 (37.5)	24 (60)	0.2707	1.53 (0.69, 3.46)
POAG, <i>n</i> (%)	66	42 (31.8%)	90 (68.2)	0.1642	8 (12.1)	26 (39.4)	32 (48.5)	1.000	0.96 (0.49, 1.87)
Control, <i>n</i> (%)	93	45 (24.2%)	141 (75.8)	Ref group	3 (3.2)	39 (41.9)	51 (54.8)	Ref group	—

Comparison of controls (reference group) with POAG, PEX, and PEXG groups.

* Fischer's exact test.

TABLE 3. Association of R141L and G153D Polymorphisms With PEX, PEXG, and POAG

	Log Additive (allelic)			Recessive		
	OR	95% CI	P Value	OR	95% CI	P Value
POAG vs. controls						
R141L	0.601	0.321–1.127	0.113	0.693	0.336–1.430	0.322
G153D	0.752	0.359–1.575	0.451	0.821	0.377–1.790	0.621
PEX vs. controls						
R141L	1.347	0.654–2.775	0.81	1.336	0.581–3.072	0.494
G153D	23.203	2.926–183.998	0.003	23.928	3.000–190.790	0.003
PEXG vs. controls						
R141L	2.268	0.955–5.383	0.063	2.274	0.858–6.028	0.098
G153D	24.784	2.928–209.728	0.003	25.055	2.968–211.469	0.003

After adjusting for age, sex, diabetes, cardiovascular disease, cardiovascular surgery, and IOP using multinomial linear regression.

ratio [OR]: 20.46, $P < 0.0001$ for PEX, OR: 17.31, $P = 0.0001$ for PEXG). Subjects with POAG did not differ from controls ($P = 0.883$ and $P = 1.00$, respectively), whereas for the R141L polymorphism, there were no statistically significant differences among the four groups for either the allelic (G versus T) or the recessive models (GG versus TG+TT; Table 2).

The haplotype frequencies in controls were AG, 12.9%; AT, 0%; GT, 24.5%; and GG, 62.7%. A measure of LD between the two SNPs, D prime, was 1.000. Although this is strong evidence for LD, it stems from the fact that one of the haplotypes (AT) is estimated to have frequency 0. The r^2 was 0.0478, which means that knowing the allele at one locus improves slightly the ability to predict the allele at the second locus.

The association of the two polymorphisms with PEX, PEXG, and POAG was further adjusted for age, sex, diabetes, cardiovascular disease, cardiovascular surgery, and IOP using multinomial logistic regression. Both the log-additive model, which assumes the effects of two alleles are independent, and the recessive model were used. Only the G153D polymorphism was associated with PEX and PEXG (Table 3). Subjects with PEX had approximately 24-fold higher odds to carry the G153D polymorphism and subjects with PEXG had 25-fold higher odds to carry the G153D polymorphism, compared with controls, whereas subjects with POAG did not differ from the reference group. No statistically significant differences for the R141L polymorphisms were found when PEX, PEXG, or POAG subjects were compared with the reference group.

In multinomial analysis adjusted for age, the two G153D alleles (A and G) were not associated with IOP differences in controls (mean IOP 15.2 mm Hg for the A allele, 15.8 mm Hg for the G allele, $P = 0.501$) or in POAG subjects not receiving glaucoma treatment (mean IOP 19.2 mm Hg for the A allele, 19.8 mm Hg for the G allele, $P = 0.277$). There was only one untreated PEX and zero untreated PEXG subjects having the A allele, thus we did not run the analysis in these two groups. No association of the two R141L alleles with IOP differences in the four groups was detected (data not shown). Finally, the high risk for PEX and PEXG GG homozygous genotype of G153D was checked to determine whether it was associated with systemic factors like self-reported history of diabetes, diabetes treated with insulin, cardiovascular disease, or coronary surgery. After adjusting for age and sex, no association was found between GG and either diabetes ($P = 0.907$), insulin use ($P = 0.789$), cardiovascular disease ($P = 0.104$), or coronary surgery ($P = 0.934$; data not shown).

DISCUSSION

In our well-established cohort of subjects aged older than 60 years and living in the city of Thessaloniki (Northern Greece),

after adjusting for known risk factors for glaucoma, the G allele of SNP G153D was associated with an odds ratio of approximately 25 with both PEX and PEXG, while the R141L SNP did not present any association. Susceptibility to POAG was not associated with either of the two SNPs. No association of the G153D *LOXL1* SNP was found with either IOP differences or with self-reported systemic diseases. To the best of our knowledge, this is the first study on the association of PEX and PEXG with *LOXL1* coming from a general population-based cohort (not clinical based) and performing adjustment of the results for other covariates. Also this is the first study reporting lack of association of *LOXL1* with the IOP and with systemic diseases.

A meta-analysis was performed in 2010, which aimed to summarize the existing data and also to identify and analyze the consistency and discrepancies of individual studies or population subgroups by stratifying them into four different ethnic groups (Caucasian, Japanese, Chinese, and Indian).²⁰ According to this meta-analysis, there was no statistical difference in any population group or the overall study for any of the three SNPs between PEX and PEXG, suggesting that the *LOXL1* gene may contribute to PEX onset rather than to glaucoma development. The G allele of rs3825942 (G153D) was the common at risk allele for PEX\PEXG, which was consistent in all populations with a total OR of 10.89, and when stratified by ethnic group, the OR was 9.30 in Caucasians, 18.72 in Japanese, 10.97 in Chinese, and 4.17 in Indian populations. In contrast, the T allele of rs2165241 (intronic SNP) and the G allele of rs1048661 (R141L) were risk alleles only in Caucasians with an OR of 3.39 and 2.35, respectively, and both were protective in the Japanese population and not significant in Chinese and Indian populations. Associations of opposite alleles at the same biallelic locus with the same disease has been described in genetic studies and it is called the flip-flop phenomenon.²¹ When the flip-flop phenomenon occurred across different ethnic groups, it could be explained by the population differences. The meta-analysis findings suggest that the rs3825942 is the common disease-associated polymorphisms across different populations and may have functional impacts on the *LOXL1* protein. No association was found between any of the *LOXL1* SNPs with POAG or other types of glaucoma (although the available data on the other types of glaucoma such as pigmentary or angle-closure glaucoma was limited). These results are in accordance with the results of our study, where no difference in the association of the two extronic SNPs between PEX and PEXG was reported. However, in our study only the G allele of rs3825942 was significantly associated with both PEX\PEXG (OR = 23.2 and 24.8, respectively). No association of any SNPs with POAG was found in our study as in the other studies. The lack of association between *LOXL1* and POAG further supports the

hypothesis that *LOXLI* is linked to the pathogenesis of exfoliation but not directly to glaucoma development.

More recently in 2013, two studies on the *LOXLI* association with PEX\PEXG in Greek populations have reported intriguing results.^{13,14} Both of them used clinical-based cohort of subjects with PEX, PEXG, and controls. According to the results of the first study from Athens, only G153D was associated with PEX and PEXG, and R141L was not associated with either PEX or PEXG.¹⁴ The results of this study were in line with the results of our study (coming from the urban area of Thessaloniki, Northern Greece). However, differences in the reported odds ratio can be noticed, since they reported for the allele G of G153D association an OR of 3.52 for PEX and an OR of 3.74 for PEXG, which is smaller than ours (approximately an OR of 23 for PEX and an OR of 25 for PEXG). Partially different were the results from the second study from the region of Epirus.¹³ This study reported an association of G153D SNP with an OR of 2.162 for PEX and an OR of 2.794 for PEXG (much lower than that in our study). According to the results of the adjusted analysis, both *LOXLI* SNPs (G153D and R141L) were associated with PEX and PEXG, while in our study only G153D was associated. Differences among the three studies coming from rather homogenous Greek populations could be explained by the methodological differences in the studies (one population-based, the others clinical-based, the rigorosity of the classification criteria used for cases and controls, or the method of genotyping) but also from true differences in geographic distribution patterns (due to either regional gene pools or environmental influences).

In our study, the high-risk G allele of G153D SNP was quite frequent in all groups (approximately in 100% of both PEX and PEXG and 80% in controls and POAG subjects). Although the G allele of G153D confers a very high population attributable risk, its high frequency in controls makes a genetic test for *LOXLI* of limited clinical use. This test will have a very high sensitivity but a very low specificity, meaning that almost all subjects who will develop PEX can be identified by genetic testing, the test will result in a very high number of false positives though. Furthermore, since the frequencies of *LOXLI* SNPs are similar in PEX and PEXG, this genetic screening would not be able to identify those PEX individuals who are at increased risk of glaucoma. Despite the high prevalence of the *LOXLI* variants in the general population, a much lower proportion of the population develops PEX and an even lower proportion of subjects with PEX develop glaucoma, suggesting that other environmental, genetic or epigenetic factors, that are yet to be identified, may delay, inhibit, or contribute to the development of PEX and PEXG.

This is the first study that tested the hypothesis whether the two alleles of the high risk for PEX\PEXG G153D common sequence variant of *LOXLI* may be associated with differences in IOP. Considering only untreated subjects and after adjusting for age, both the G and A alleles of G153D were not associated with differences in IOP measurements in both controls and POAG subjects. In this context, it is very plausible to assume that *LOXLI* SNPs cannot attribute to the occurrence of high IOP in subjects with PEX and to subsequently development of glaucoma. On the other hand, in our previous report from the Thessaloniki Eye study, subjects with PEX were three times more likely to have glaucoma than subjects without PEX, notably for the same level of presenting IOP when it was higher than 20 mm Hg.³ Based on this finding, we may hypothesize that, in addition to IOP, some other factors may contribute to glaucoma development in subjects with PEX. Whether these factors are genetic (in addition to *LOXLI*), environmental, or epigenetic and how they contribute to the development of glaucoma in subjects with PEX remain to be identified.

The widespread systemic distribution of PEX material in visceral organs like lung, liver, kidney, gallbladder, and cerebral meninges led to the hypothesis that PEX may be associated with systemic comorbidities and comortality. Cardio- and cerebrovascular disease like angina, aortic aneurysm, and dementia have been linked to PEX in the first papers that addressed this issue.^{8,9,22} In contrast, there is compelling evidence from epidemiological population-based and case-controls studies that argue against this association.^{7,11,23–26} In the population-based cohort of Thessaloniki Eye Study, we were unable to confirm any association of PEX with diabetes, hypertension, cardiovascular or cerebrovascular disease, and coronary surgery.⁷ This is the first study that examined and didn't find any association of the high risk for PEX\PEXG G153D SNP of *LOXLI* with any of the aforementioned comorbidities, further supporting the hypothesis that PEX may not be associated with systemic cardiovascular or cerebrovascular diseases.

In the Thessaloniki Eye Study, as a population-based study, the participants were randomly selected from the general population, and therefore selection bias was diminished. Thessaloniki is considered to have a stable and ethnically homogenous population of Greek origin. Genetic homogeneity is of great importance when genetic studies are conducted. One other strength of the present study was the use of a standardized and thorough examination protocol for all the participants in the Thessaloniki Eye Study. Particular attention was given to identify the presence of PEX and the diagnosis of PEXG, and POAG was based on rigorous criteria. By these means, any classification bias was minimized. Age is a strong surrogate for the presence of PEX and one can argue that some of the controls may develop PEX in the future. Although this classification bias exists in all studies involving PEX subjects and controls, it seems unlikely to exist in our study due to the relatively high age of both controls (mean age: 68.8 years) and PEX subjects (mean age: 73.4 years). In addition, we were able to adjust all the results for other known confounders including age, sex, IOP, diabetes, coronary surgery, and cardiovascular disease by using multinomial logistic regression analysis. Most of genetic studies on *LOXLI* have not adjusted their results for potential confounders. Furthermore, this is the first study that ran logistic regression analysis to explore the potential association of the allele G of the G153D *LOXLI* variant with IOP differences in untreated subjects or with systemic diseases among the groups. We were able to perform this analysis given our population-based setting providing a representative random sample and a fair number of previously undiagnosed and untreated glaucoma subjects.

According to the results of our analysis, we confirmed the strong association of G allele of G153D with both PEX and PEXG, as has been widely described in the relevant literature. We did not find a statistically significant association of the R141L common variant with PEX\PEXG. One may suggest that this may be due to the limited sample size in our study. However, our study (with 34 PEXG cases and 93 controls with a 0.758 frequency of G allele of R141L in controls) had 79.8% power to detect an association of 3.592 under the recessive model and 93.7% power to detect an association of 2.98 under the log additive model with the significance level of 0.05. Thus, although it is still possible, it is somewhat unlikely that our inability to detect the association is because our study sample is inadequate. In order to further increase the study sample and since we did not find different association between PEX and PEXG with *LOXLI* SNPs, both PEX and PEXG cases were combined in one group ($n = 74$) and controls and POAG cases were combined in another ($n = 159$). When the two groups were compared, the results did not change significance although a trend was noticed for the R141L SNP (OR: 1.69,

95% CI: 0.92–3.13, $P = 0.0886$ for the recessive model GG versus TT/TG). Another limitation of the analysis of association of *LOXL1* with systemic diseases may be the use of the self-reported history, which may be subject to recall bias. However, there are studies that have shown that the self-reported history on selected chronic diseases is fairly accurate, especially for diabetes and cardiovascular disease.^{27–29}

According to our study findings, G153D and R141L variants of *LOXL1* gene were not heterogenic in PEX and PEXG. Only G153D was strongly associated with PEX/PEXG. No association was found of the high risk G153D allele with increased IOP or with systemic diseases. These findings further support the notion that the *LOXL1* gene contributes to onset of PEX, but its role in PEXG development remains unclear. Under which genetic, environmental, or epigenetic conditions subjects with PEX progress to increased IOP and/or glaucoma development remains unanswered. The detection of yet unidentified genetic and nongenetic factors contributing to this complex disorder called pseudoexfoliation may lead to the better understanding of PEXG pathogenesis and toward the development of more precise screening tools for individuals at risk for pseudoexfoliative glaucoma.

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