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ORIGINAL ARTICLE

Recessive multiple epiphyseal dysplasia and Stargardt disease in two sisters

Leonardo Gatticchi[1] | **Dominika Vešelényiová**[2] | **Jan Miertus**[3,4] | **Paolo Enrico Maltese**[4] | **Elena Manara**[5] | **Alisia Costantini**[4] | **Sabrina Benedetti**[4] | **Darina Ďurovčíková**[6] | **Juraj Krajcovic**[2] | **Matteo Bertelli**[4,5]

[1]Department of Experimental Medicine, Laboratory of Biochemistry, University of Perugia, Perugia, Italy

[2]Department of Biology, Faculty of Natural Sciences, University of Ss. Cyril and Methodius, Trnava, Slovakia

[3]Génius n.o, Trnava, Slovakia

[4]MAGI's Lab, Genetic Testing Laboratory, Rovereto, Italy

[5]MAGI Euregio, Bolzano, Italy

[6]Institute of Genetics and Molecular Medicine, Faculty of Medicine, Slovak Healthcare University, Bratislava, Slovakia

Correspondence
Paolo Enrico Maltese, Magi's Lab, Via Delle Maioliche 57/D, 38068 Rovereto, Italy.
Email: paolo.maltese@assomagi.org

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ABSTRACT

Background: The rapid spread of genome-wide next-generation sequencing in the molecular diagnosis of rare genetic disorders has produced increasing evidence of multilocus genomic variations in cases with a previously well-characterized molecular diagnosis. Here, we describe two patients with a rare combination of skeletal abnormalities and retinal dystrophy caused by variants in the *SLC26A2* and *ABCA4* genes, respectively, in a family with parental consanguinity.

Methods: Next-generation sequencing and Sanger sequencing were performed to obtain a molecular diagnosis for the retinal and skeletal phenotypes, respectively.

Results: Genetic testing revealed that the sisters were homozygous for the p.(Cys653Ser) variant in *SLC26A2* and heterozygous for the missense p.(Pro68Leu) and splice donor c.6386+2C>G variants in *ABCA4*. Segregation analysis confirmed the carrier status of the parents.

Conclusion: Despite low frequency of occurrence, the detection of multilocus genomic variations in a single disease gene-oriented approach can provide accurate diagnosis even in cases with high phenotypic complexity. A targeted sequencing approach can detect relationships between observed phenotypes and underlying genotypes, useful for clinical management.

KEYWORDS
ABCA4, rMED, *SLC26A2*, STGD1

1 | INTRODUCTION

Since the beginning of the post-genomic era, clinical use of genome-wide screening for disease-associated genes has become part of the diagnostic workup, particularly for rare genetic disorders. Increasing evidence shows the frequency of multilocus genomic variants in cases with a previously well characterized molecular diagnosis (Balci et al., 2017; Farwell et al., 2015; Posey et al. 2016, 2017; Retterer et al., 2016; Yang et al., 2013, 2014). Large-scale NGS has revealed that 4.9% of individuals in an outbred population have two or more genomic variants (Posey et al., 2017), whereas in a Turkish cohort (with a higher degree of consanguinity) 12% of the subjects had multiple molecular diagnoses (Karaca et al., 2018). This high genotypic variability contributes to the complexity of phenotypes, often misleading clinicians from recognizing the real burden of the underlying genotypes. From a clinical point of view, the

presence of additional disease features extending beyond those previously described for a single disease gene variant is referred to as phenotypic expansion (Miertuš et al., 2020). Conversely, the blending of two overlapping disease phenotypes caused by variants in two or more functionally linked genes may be classified erroneously as a new disease manifestation, rather than a more complex form of a common disease. Moreover, patients with multiple molecular defects, each of which can be attributed to a single genetic variant and which individually affect different organ systems, may occur among clearly distinguished phenotypes. Populations with high rates of consanguineous marriages are more prone to multiple disease-associated variants and may show a wider spectrum of phenotypes due to intrafamilial clinical variability. However, cases with de novo variants in consanguineous populations have also been reported (Al-Qattan et al., 2015; Fahiminiya et al., 2014; Ratbi et al., 2007).

Here, we describe two sisters with rare inherited Mendelian disorders affecting two distinct organ systems: autosomal recessive multiple epiphyseal dysplasia, a collagen disease resulting in skeletal development anomalies, and Stargardt disease, which causes a distinctive form of retinal dystrophy.

Autosomal recessive multiple epiphyseal dysplasia (rMED; OMIM #226900) is a bone disorder caused by genetic variants in the sulfate transporter solute carrier family 26 (SLC26A2; Jackson et al., 2012). It belongs to a group of cartilage and bone diseases termed osteochondrodysplasias. Fifty-nine different variants in the *SLC26A2* gene have been linked to four, currently incurable, osteochondrodysplasias of varying severity: perinatal lethal achondrogenesis type 1B (ACG1B; OMIM #600972), atelosteogenesis type 2 (AO2; OMIM #256050), and the nonlethal rMED and diastrophic dysplasia (DTD; OMIM #222600) (Gualeni et al., 2013; Karniski, 2004). These osteochondrodysplasias share many clinical features, such as normal to mildly short stature, joint pain, club feet, shortening of limbs, waddling gait, early-onset hip joint osteoarthritis, cleft palate, and ear deformities (Bonafe et al., 2008; Hinrichs et al., 2010; Kausar et al., 2018; Mäkitie et al., 2003, 2015). However, phenotypes may vary according to the type of genetic variant (Czarny-Ratajczak 2010). SLC26A2 is a plasma membrane sulfate/chloride antiporter responsible for inorganic sulfate uptake and proteoglycan sulfonation (Forlino et al., 2005; Park et al., 2014; Pecora et al., 2006). Ablation of *SLC26A2* in murine chondrocytes was recently reported to impair extracellular deposition of collagen (ColII and ColIX) and cartilage formation (Zheng et al., 2019).

Stargardt disease (STGD1; OMIM #248200) is the most common inherited macular dystrophy, with an estimated prevalence of 1 in 8000–10000 individuals (Tanna et al., 2017). It is an autosomal recessive disease caused by variants in the gene encoding photoreceptor-specific retinal-specific phospholipid-transporting ATPase, ABCA4 (Rivera et al., 2000; Tsybovsky et al., 2010). ABCA4 plays an essential role in the retinal visual cycle, participating in removal of vitamin A byproducts (bisretinoids) from photoreceptor cells. Loss of function variants in the *ABCA4* gene lead to accumulation of bisretinoids (e.g., A2E, vitamin A dimer) in lipofuscin granules in retinal pigment epithelium (RPE) cells, as well as to pigment epithelial atrophy and subsequent photoreceptor involvement (Charbel Issa et al., 2013; Rotenstreich et al., 2003; Sparrow et al., 2012). Accumulation of vitamin A-derived toxic metabolites leads to formation of reactive oxygen species (ROS) an oxidative stress, which promotes RPE degeneration through activation of the endoplasmic reticulum stress pathway (Li et al., 2015). *ABCA4* is a large gene with 50 exons and more than 900 reported variants (Jiang et al., 2016; Oldani et al., 2012; Salles et al., 2017; Sangermano et al., 2018; Schulz et al., 2017; Zernant et al., 2014). A wide spectrum of manifestations with variable prognosis is, therefore, associated with *ABCA4* variants, including bilateral loss of central vision, dyschromatopsia, and macular atrophy with deposition of yellow-white lipofuscin flecks in the RPE (Fujinami et al., 2015). The type of *ABCA4* variant determines the diversity of disease outcomes and onsets, with severer forms developing in early childhood. Missense variants generally have better prognoses, milder forms, and later onsets in young adult individuals. In contrast, loss of function variants cause rapid progressive damage to retinal function and structure (Tanaka et al., 2018). Other combinations of pathogenic alleles of *ABCA4* also cause cone-rod dystrophy and retinitis pigmentosa-like phenotypes, while single-copy alleles have been suggested to be an underlying risk factor for age-related macular degeneration (Cremers et al., 2020; Rivera et al., 2000). Although clinical diagnosis is often delayed due to variability in disease phenotypes, genetic testing together with fundus fluorescein angiography and autofluorescence imaging make it possible to detect specific retinal defects even in the early stages of STGD1 onset (Abed et al., 2018; Fujinami et al., 2015; Lois et al., 2001). Summaries of the clinical and genetic data of the two patients are reported.

2 | MATERIALS AND METHODS

2.1 | Editorial policy and ethical considerations

This study is a retrospective description of two cases that does not require ethics committee approval. Written informed consent to the use of anonymized genetic and clinical results was obtained from the patients or their parents in the case of minors. The research observed the principles of the Declaration of Helsinki.

2.2 | Diagnostic sequencing methods

Blood samples were processed at MAGI's laboratories for genomic DNA extraction using standard protocols. *SLC26A2* was Sanger sequenced using the 3730xl DNA Analyzer (Applied Biosystems). For molecular diagnosis of the retinal phenotype, genetic screening by next-generation sequencing (NGS) was performed on a panel of genes involved in retinal dystrophy and macular degeneration: *ABCA4* (RefSeq: NM_000350), *ELOVL4* (RefSeq: NM_022726), *PRPH2* (RefSeq: NM_000322), *CNGB3* (RefSeq: NM_019098), and *PROM1* (RefSeq: NM_006017). Fifty nanograms of genomic DNA was simultaneously fragmented and tagged by Nextera transposon-based shearing technology and library enrichment was performed by Nextera Rapid Capture Enrichment (Illumina). Massive parallel sequencing was performed using Illumina MiSeq with a paired-end protocol and a reads length of 150 bp. Nucleotide alterations of interest were confirmed by Sanger sequencing, when target region coverage was less than 10 reads. The sequences obtained were aligned with the human reference sequence GRCh38 (https://www.ncbi.nlm.nih.gov/grc/human) using Burrows–Wheeler Aligner software (0.7.17-r1188). Mean coverage was 185.5× and at least 25× for 99.7% of the target regions. All coding target regions were sequenced with a mean coverage of 10×. Reads alignment was obtained with SAMtools (1.6) and duplicates were removed using Sambamba software. Variant calling from NGS data was performed by GATK Unified Genotyper and BCF tools (1.6) software. Annotation and analysis of variants were performed with VEP software (version 91) and the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP). The main transcript of each gene of interest was

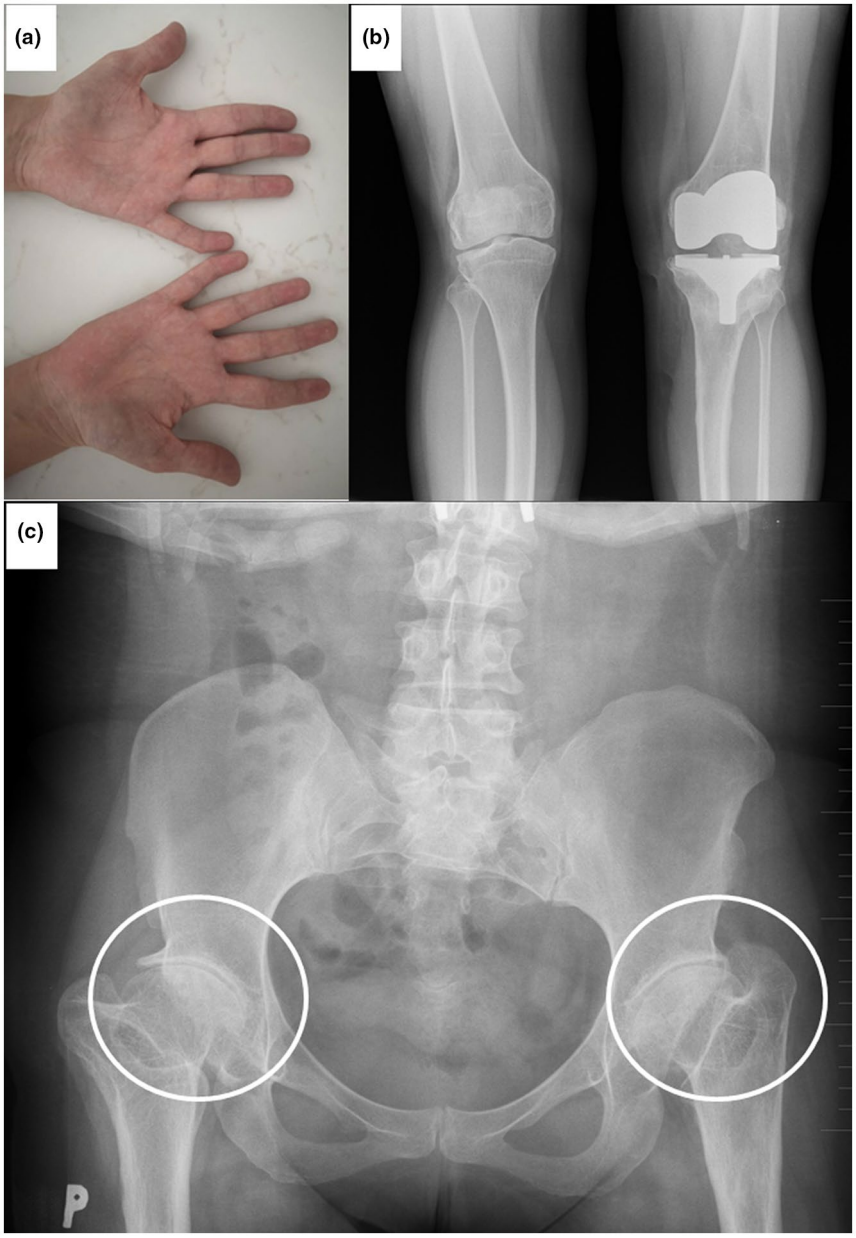

FIGURE 1 Skeletal and radiological findings of the proband: (a) Abnormal shape of fifth and second fingers of both hands. Fifth fingers do not show typical clinodactyly, but resemble disrupted interphalangeal joint(s). Dermatoglyphics appear slightly shallower than normal. (b) X-ray of left knee joint replacement. (c) X-ray showing hip dysplasia (white circles)

identified from the APPRIS (RefSeq107) database. Sanger sequencing on PCR products from genomic DNA was then used for the family segregation study.

3 | RESULTS

Clinical data of the proband was recorded during clinical evaluation at the Medical Genetics Office of the nonprofit organization Génius. Other medical records available to date were collected retrospectively at the same facility. Detailed pretest genetic counseling was provided to the sisters and their parents, and informed written consent was obtained to make the genetic causes of osteochondrodysplasia and retinal dystrophy in their family available anonymously for research and publication.

The proband (female, 26 years) was born prematurely at week 35 week to consanguineous parents. Birth weight and length were 1.930 g and 42 cm. The baby showed respiratory distress and had an APGAR score of 1/3/6. She had an episode of anemia of unknown origin at 1 month of age, treated with whole blood transfusion. The child was diagnosed with hip and knee dysplasia at age 3 years. She underwent many orthopedic operations to correct bone deformities, such as femoral head and hip joint replacement, treatment of knee instability and scoliosis. Anthropometric and radiographic skeletal manifestations are reported in Figure 1.

Due to defective production of growth hormone (GH), the patient was diagnosed with dwarfism and treated with recombinant GH from age 8 years. Severe osteoporosis led to upper limb fracture that required orthopedic surgery.

The younger sister (age 20 years) has a similar phenotype with bone development defects, and a history of 10 different surgical procedures, including bilateral hip replacement and removal of a warm thyroid nodule. Unlike the elder sister, she did not require GH replacement therapy. Both patients showed generalized osteoporosis/osteopenia and poor joint flexibility and stability with pain and swelling, particularly of the hips and knees. Their clinical and genetic data is summarized in Table 1. A concomitant retinal phenotype, consisting in an early-onset maculopathy with progressive visual dysfunction, was observed at 6 and 7 years in the elder and younger sisters, respectively, with more serious manifestations in the proband.

Additional features such as hypercholesterolemia, eosinophilia of unknown origin, and sticky platelet syndrome type II were observed in the proband.

To confirm clinically suspected autosomal recessive multiple epiphyseal dysplasia (rMED), genetic screening was performed with the proband's genomic DNA. A homozygous variant of the *SLC26A2* gene at cytogenetic location 5q32 was detected (*SLC26A2*: NM_000112.3: ex3: c.1957 T > A: NP_000103.2: p.(Cys653Ser)). The p. Cys653Ser variant is known to be pathogenic and caused by a missense substitution at residue 1957 in exon 3 of *SLC26A2* (Kausar et al., 2018; Mäkitie et al., 2003). Segregation analysis showed that both parents were heterozygous carriers of the variant, while the younger sister was homozygous for the same variant.

TABLE 1 *SLC26A2* Comparison of phenotypes

	Proband	Sister
Mutation	*SLC26A2* p.[(Cys653Ser)];[(Cys653Ser)]	
Overlap of phenotypes	Poor joint flexibility	
	Poor joint stability	
	Pain and swelling of joints, mostly hips and knees	
	Osteoporosis/osteopenia	
Current age	26 years	20 years
Age of onset	3 years	9 years
Number of orthopedic operations	24	11
Joint replacements	Left knee	Both hips
Densitometry	Area: Hip	
Total area (cm^2)	35.73	32.36
BMC (g)	24.03	11.39
T-Score	−3.2	−5
Z-score	−3.2	−3.3
Densitometry	Area: Lumbar spine	
Total area (cm^2)	27.14	40
BMC (g)	26.9	19.34
T-Score	−1	−5
Z-score	−0.7	−1.7

Note: Dual energy X-ray absorptiometry (DEXA) scores are reported as T-scores and Z-scores. The T-score is a comparison of the subject's bone density with that of a healthy 30-year-old of the same sex. The Z-score is a comparison of the subject's bone density with that of an average person of the same age and sex. The density of these bones is then compared with an average index based on age, sex, and size. The resulting comparison is used to determine risk of fractures and the stage of osteoporosis (if any).

To identify possible genetic defects underlying the retinal phenotype, we used an NGS approach with a specific gene panel. A molecular diagnosis of Stargardt/fundus flavimaculatus disease was obtained for both patients on the basis of two compound heterozygous variants in the *ABCA4* gene at 1p22.1: a maternally inherited missense variant (*ABCA4*: NM_000350.2: ex3: c.203C>T: NP_000341.2: p.(Pro68Leu); dbSNP rs62654397) and a paternally inherited splice donor variant (*ABCA4*: NM_00035.2: int46: c.6386+2C>G; dbSNP rs61753043). The p.(Pro68Leu) variant results in substitution of a highly conserved amino acid of the protein, whereas the c.6386+2C>G variant alters a splice donor site in the *ABCA4* gene. Both variants are known to be pathogenic and have been associated with Stargardt disease (Jaakson et al., 2003; Rivera et al., 2000; Schulz et al., 2017) and choroidal dystrophy (Bertelsen et al., 2014). Segregation analysis

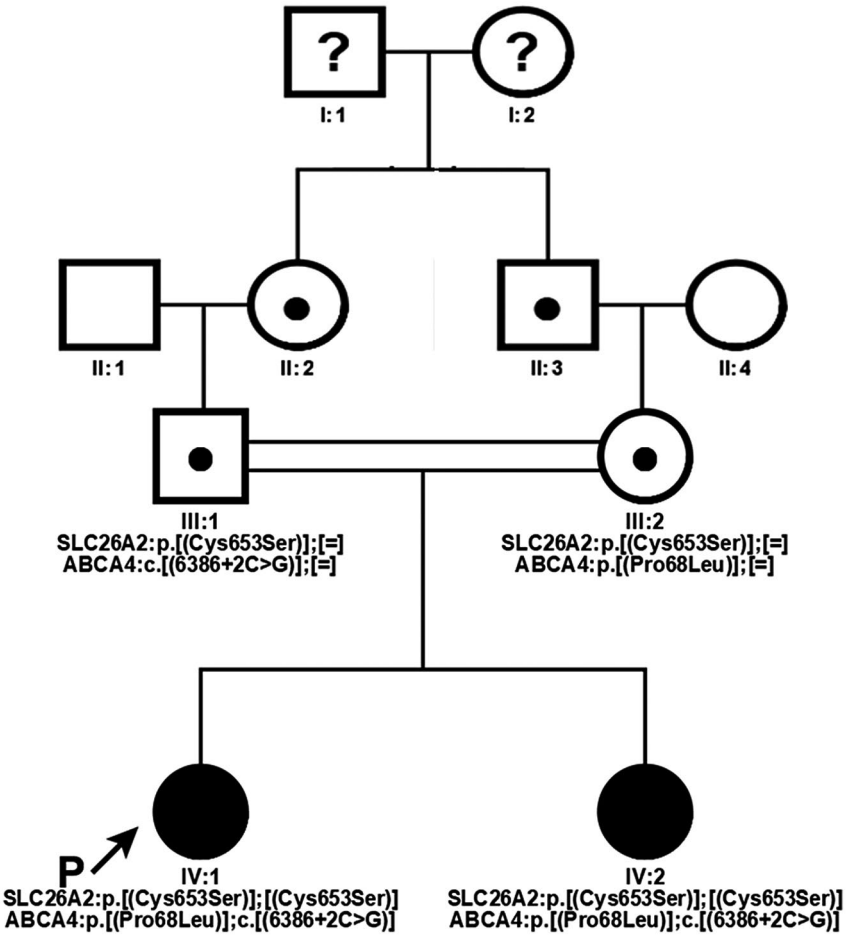

FIGURE 2 Family pedigree showing consanguinity of the proband's parents. The osteochondrodysplasia genotype, derived from a homozygous variant in the *SLC26A2* gene and compound heterozygous variants in the *ABCA4* gene, is responsible for the retinal phenotype.

of the variants using standard Sanger sequencing confirmed an autosomal recessive inheritance pattern (Figure 2).

Retinal disease progressed over the years. At the last evaluation, visual acuity was 1/50 and 2/50 for both eyes in the proband and the sister, respectively. Fundus examination showed severe macular atrophy (Heidelberg Spectralis, Heidelberg Engineering; Figure 3). Multifocal ERG responses were severely reduced at all rings in both eyes (Retimax instrument (CSO; Figure 4).

4 | DISCUSSION

The introduction of NGS for the diagnosis of rare genetic disorders has led to the discovery of multiple genetic variants underlying cases with atypical clinical manifestations or expanded phenotypes of well-known diseases (Chong et al., 2015; Jehee et al., 2017). Here, we described two sisters with a rare combination of skeletal abnormalities and macular dystrophy caused by variants in *SLC26A2* and *ABCA4*, respectively, in a family with parental consanguinity. Carrier status was confirmed by study of the parents' DNA.

The detection of two distinct Mendelian disorders affecting different organ systems has important implications for patient care and helps clinicians establish personalized therapeutic approaches. Genetic studies showed that both patients were homozygous for the p.(Cys653Ser) variant in *SLC26A2*, responsible for autosomal recessive multiple epiphyseal dysplasia (rMED), and compound heterozygous for variants in the *ABCA4* gene, which causes Stargardt disease (STGD1). The p.(Cys653Ser) variant in *SLC26A2* is a known pathogenic variant associated with diastrophic dysplasia and rMED (Rossi & Superti-Furga, 2001). Biochemical characterization of this variant showed reduced sulfate transport activity due to lower expression of the transporter on the cell surface (Karniski, 2004). The clinical manifestations of p.(Cys653Ser) are relatively mild compared to other inherited forms of *SLC26A2*-related skeletal dysplasias, although affected subjects often require recurrent surgery for skeletal abnormalities (Hinrichs et al., 2010; Mäkitie et al., 2003). A recent study described a new role of *Slc26a2* in collagen deposition in the extracellular matrix (Zheng et al., 2019). Defective collagen secretion of $Slc26a2^{-/-}$ chondrocytes promotes intracellular retention of collagen, resulting in activation of fibroblast growth factor receptor 3 (FGFR3) signaling, which ultimately triggers the ATF6 arm of the unfolded protein response. Activating variants of FGFR tyrosine kinase activity play a pivotal role in the development of skeletal

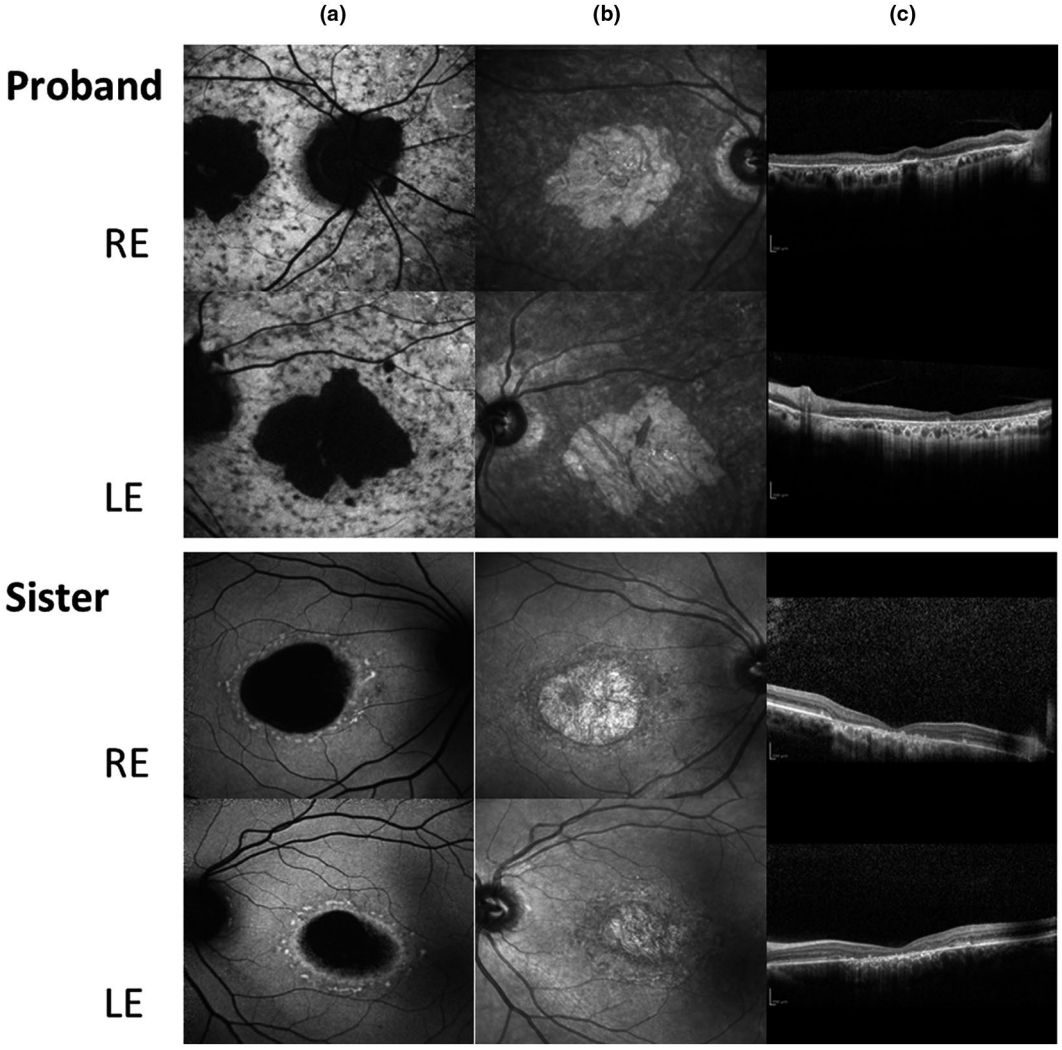

FIGURE 3 Proband and sister: (a) Autofluorescence images showing hypofluorescent macular area due to absence of the RPE. (b) Infrared and (c) OCT images showing macular atrophy in both eyes

dysplasias by negatively regulating chondrocyte proliferation in cartilage (Foldynova-Trantirkova et al., 2012). Inhibition of FGFR3 signaling is the only therapeutic option that has been investigated for osteochondrodysplasia due to *SLC26A2* variants.

The p.(Pro68Leu) variant in *ABCA4* has already been described as a deleterious variant associated with Stargardt disease (Rivera et al., 2000) and generalized choriocapillaris dystrophy (Bertelsen et al., 2014). The c.6386+2C>G variant in *ABCA4* was identified for the first time by Jaakson et al. (2003); functional characterization confirmed its pathogenicity due to alteration of a splicing donor site in exon 46 and lack of protein translation (Schulz et al., 2017). Several therapeutic strategies for Stargardt disease are currently being studied, including pharmacological approaches, gene replacement therapy, stem-cell therapy, and dietary antioxidant supplements. The precise genetic definition of a disease is important, as it can facilitate access to clinical trials and to specific therapies as soon as they become available.

The clinical diagnosis of complex disorders in which two or more genotypes contribute features is challenging for physicians. In this case, rMED was correctly suspected and genetic sequencing was directed at a specific gene, *SLC26A2*. As the ocular features were not correlated with the systemic disorder, additional genetic test for retinal dystrophies and macular degenerations was performed defining the maculopathy as being associated with Stargardt disease. Curiously, contrary to what may happen in the offspring of consanguineous parents where the risk of multiple homozygosity is known, in this case, the disease was caused by two distinct compound heterozygous *ABCA4* variants. Two genetic analyses sufficed to solve this case, but the picture could clearly have been more complex and clinical and genetic diagnosis more complicated.

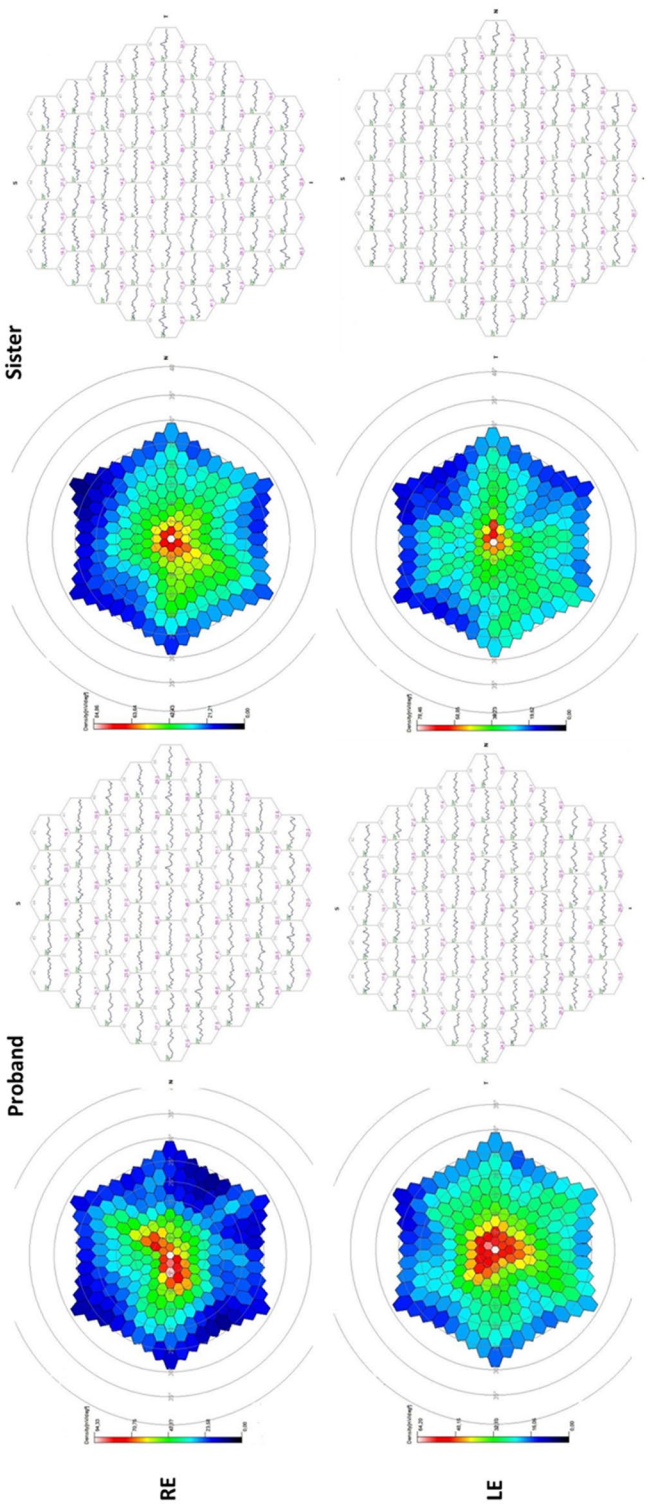

FIGURE 4 mfERG contour maps and response arrays showing significant loss of amplitude in both eyes of the proband and her sister

In conclusion, the rapid evolution of genome-wide NGS platforms can have major implications in the diagnosis of rare genetic disorders with multilocus genomic variations with respect to a single disease gene-oriented approach, and can provide accurate diagnosis even in cases with a high degree of phenotypic complexity. A targeted sequencing approach can reveal intimate relationships between observed phenotypes and underlying genotypes, enabling medical research aimed at better therapeutic options.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

LG made substantial contributions to the conception of the work and was a major contributor to writing the manuscript. DV was a major contributor to writing the manuscript. JM performed genetic counseling and clinical examinations, collected the patient data, and was a major contributor to writing the manuscript. PM was a major contributor to writing the manuscript. EM was responsible for clinical interpretation of the test results and substantially revised the text. AC performed the genetic testing, interpreted the genetic data, and substantially revised the text. SB performed the genetic testing, interpreted the genetic data, and substantially revised the text. DD performed clinical evaluation of the proband and was a major contributor to writing the manuscript. JK made substantial contributions to the conception of the work and substantially revised the text. MB made substantial contributions to the conception of the work and substantially revised the text. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

Paolo Enrico Maltese https://orcid.org/0000-0002-1974-4937
Elena Manara https://orcid.org/0000-0002-1176-3359

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