

# Non-syndromic inherited retinal diseases in Poland: Genes, mutations, and phenotypes

Anna M. Tracewska,<sup>1</sup> Beata Kocyla-Karczmarewicz,<sup>2</sup> Agnieszka Rafalska,<sup>3</sup> Joanna Murawska,<sup>4</sup> Joanna Jakubaszko-Jabłońska,<sup>3,5,6</sup> Małgorzata Rydzanicz,<sup>7</sup> Piotr Stawiński,<sup>7</sup> Elżbieta Ciara,<sup>2</sup> Beata S. Lipska-Ziętkiewicz,<sup>8,9</sup> Muhammad Imran Khan,<sup>10,11</sup> Frans P.M. Cremers,<sup>10,11</sup> Rafał Płoski,<sup>7</sup> Krystyna H. Chrzanowska<sup>2</sup>

(The last two authors contributed equally to this study.)

<sup>1</sup>Lukasiewicz Research Network – PORT Polish Center for Technology Development, Wrocław, Poland; <sup>2</sup>Children's Memorial Health Institute, Warsaw, Poland; <sup>3</sup>Department of Ophthalmology, Wrocław Medical University, Wrocław, Poland; <sup>4</sup>Department of Ophthalmology, University Clinical Centre, Gdańsk, Poland; <sup>5</sup>Department of Paediatric Traumatology and Emergency Medicine, Wrocław Medical University, Wrocław, Poland; <sup>6</sup>SPEKTRUM Ophthalmology Clinic, Wrocław, Poland; <sup>7</sup>Department of Medical Genetics, Medical University of Warsaw, Warsaw, Poland; <sup>8</sup>Centre for Rare Diseases, Medical University of Gdańsk, Gdańsk, Poland; <sup>9</sup>Department of Biology and Medical Genetics, Clinical Genetics Unit, Medical University of Gdańsk, Gdańsk, Poland; <sup>10</sup>Department of Human Genetics, Radboud University Medical Center, Nijmegen, The Netherlands; <sup>11</sup>Donders Institute for Brain, Cognition and Behavior, Radboud University Medical Center, Nijmegen, The Netherlands

**Purpose:** Inherited retinal diseases (IRDs), encompassing many clinical entities affecting the retina, are classified as rare disorders. Their extreme heterogeneity made molecular screening in the era before next-generation sequencing (NGS) expensive and time-consuming. Since then, many NGS studies of IRD molecular background have been conducted in Western populations; however, knowledge of the IRD mutational spectrum in Poland is still limited. Until now, there has been almost no comprehensive analysis of this particular population regarding the molecular basis and inheritance of IRDs. Therefore, the purpose of this study was to gain knowledge about the type and prevalence of causative variants in the Polish population.

**Methods:** We recruited 190 Polish families with non-syndromic IRDs, including Stargardt disease (STGD), retinitis pigmentosa (RP), cone- and cone-rod dystrophy (CD/CRD), achromatopsia, and congenital stationary night blindness. A pool of molecular inversion probes was used, which targeted 108 genes associated with non-syndromic IRDs known in 2013. We applied filtering for known variants occurring with an allele frequency >0.5% in public and in-house databases, with the exception of variants in *ABCA4*, when the frequency filter was set to 3.0%. Hypomorphic p.(Asn1868Ile) was added manually. In the case of novel missense or splicing variants, we used in silico prediction software to assess mutation causality.

**Results:** We detected causative mutations in 115 of the 190 families with non-syndromic IRD (60.2%). Fifty-nine individuals with STGD, RP, and CD/CRD carried causal variants in *ABCA4*. Novel single nucleotide variants were found in *ABCA4*, *CEP290*, *EYS*, *MAK*, and *CNGA3*. The complex allele c.[1622T>C;3113C>T], p.[Leu541Pro;Ala1038Val] was found in 33 individuals with *ABCA4*-associated disorders, which makes it the most prevalent allele in the Polish population (17% of all solved cases). Diagnosis was reevaluated in 16 cases.

**Conclusions:** Previously, there were no comprehensive reports of IRDs in the Polish population. This study is the first to indicate that the most common IRDs in Poland are *ABCA4*-associated diseases, regardless of the phenotype. In Polish patients with RP, the second most prevalent causal gene was *RHO* and the third *RPGR*, while there were not as many mutations in *EYS* as in Western populations. The number of initial erroneous diagnoses may be the result of limited access to diagnostics with advanced tools, such as electroretinography; however, it is necessary to raise awareness among Polish ophthalmologists of rare IRDs. Additionally, it must be emphasized that in some cases genetic analysis of the patient is necessary to achieve an accurate diagnosis.

Inherited retinal diseases (IRDs) are classified by the European Committee as rare disorders, affecting fewer than 1 in 2,000 individuals (EUROIRDs). IRDs are many separate

conditions, which can be distinguished by different ages of onset, progression rates, and the primary involvement of different photoreceptors: rods or cones.

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Correspondence to: Anna Tracewska, Lukasiewicz Research Network - PORT Polish Centre for Technology Development, Cell Analysis Laboratory, Stabłowicka 147, Wrocław, Dolnośląskie 54-066, Poland; Phone +48 71 734 77 77; email: [asiemiatkowska@gumed.edu.pl](mailto:asiemiatkowska@gumed.edu.pl)

Previously, there were no comprehensive reports of IRDs within the Polish population. The sole incidence of retinitis pigmentosa (RP) is said to be approximately 1 in 4,000 individuals. Thus, the estimated number of patients with IRD in Poland with 38 million inhabitants is higher

than 10,000 if we account for phenotypes rarer than RP, such as Stargardt disease (STGD, 1 in 8,000–10,000), cone and cone-rod dystrophy (CD/CRD, 1 in 20,000), and Leber congenital amaurosis (LCA, 1 in 50,000) [1]. On both sides of this spectrum of rod and cone involvement lie achromatopsia (ACHM) and congenital stationary night blindness (CSNB), which are rarer and are believed to be non-progressive in most cases. According to the Retinal Information Network (RetNet), more than 250 genes have been implicated in all forms of hereditary retinal disease. Approximately 60% of these genes account for non-syndromic IRDs; thus, the genetic diagnostic tools are expensive and difficult to use. Even today, in the era of next-generation sequencing (NGS), as in Poland genetic screening of IRD genes is, for the most part, not covered by health insurance, the tests are usually not offered by physicians.

There have been many NGS studies of the molecular background of these diseases in Western populations. However, knowledge of the IRD variant spectrum in Poland remains limited. Reports of pathogenic alterations in the Polish population in literature are scarce [2-8]. Our aim was to gain knowledge about the type and prevalence of causative variants in the Polish population. This information will serve as a basis for devising cost-effective population-specific genetic tests.

## METHODS

**Patient recruitment:** This study was approved by the local Bioethics Committee and the work has been carried out in accordance with the Declaration of Helsinki and ARVO statement on human subjects. Proper written informed consent, as well as data processing agreements with a clause for using the data in accordance with the General Data Protection Regulation EU Act, was obtained from all the participants.

Given the rarity of the diseases, the material was gathered in two groups. A group of 82, mostly pediatric, patients was recruited at Children's Memorial Health Institute (CMHI), where affected individuals from the whole of Poland are admitted. The other, adult group was gathered by local ophthalmologists in six Polish cities: Wrocław, Warsaw, Gdańsk, Kraków, Poznań, and Szczecin. After positive identification of a causal or possibly causal alteration, we recruited affected (74) and unaffected (365) family members to determine familial cosegregation (Appendix 1). Family history was assessed in each case to determine the inheritance pattern of IRD. Overall, the cohort consisted of 69 children (less than 18 years old at recruitment) and 121 adults.

**Ophthalmological analyses:** The patients underwent a full ophthalmological evaluation, including visual acuity testing, color vision testing, autorefractometry, tonometry, perimetry, and dilated fundus exam. Color digital photographs were taken to document changes in the fundus. Optical coherence tomography, fluorescein angiography, and electroretinography (ERG) were performed whenever feasible.

**Genetic analyses:** Four to 10 ml of blood were drawn into BD Vacutainer® EDTA tubes (Becton Dickinson, Franklin Lakes, NJ) to prevent coagulation. DNA from fresh or frozen blood was extracted using an automated method on a MagNA Pure 24 System (Roche, Basel, Switzerland) or a QIASymphony robot with QIASymphony DSP DNA Kits (Qiagen, Hilden, Germany). Additionally, for the purpose of segregation testing, DNA was self-collected with buccal swabs by the proband's family members.

DNA from buccal swabs was isolated with the QIASymphony DNA Investigator Kit (Qiagen). The DNA concentration was measured with the Quant-iT™ dsDNA Assay Kit, broad range, using a NanoDrop 3300 spectrofluorometer (ThermoFisher Scientific, Waltham, MA). The samples were then normalized to 20 ng/μl, and the targeted next-generation sequencing (tNGS) library was prepared using the molecular inversion probes (MIPs) technique targeting 108 genes involved in the pathogenesis of IRDs, as described elsewhere [9]. Paired-end sequencing (2 × 100 bp) with custom primers was performed on a HiSeq 1500 system (Illumina, San Diego, CA) with the rapid run mode, according to the manufacturer's protocol. FASTQ files were generated with bcl2fastq software (Illumina). We then analyzed the FASTQ data with the SeqNext module of the SeqPilot software (JSI Medical Systems, Ettenheim, Germany). The initial setting of the minor allele frequency (MAF) frequency filter in public databases was <0.5%, with subsequent manual identification of causative variants having higher frequency in global populations. We confirmed all causal and probably causal mutations, as well as performed familial segregation using Sanger sequencing in all amplicons, excluding the most common *ABCA4* (gene ID: 24; OMIM: 601691) complex allele alterations, c.1622T>C, p.(Leu541Pro), and c.3113C>T, p.(Ala1038Val), where we applied a restriction fragment length polymorphism (enzymes *TspRI* and *BseYI*, respectively; New England Biolabs, Ipswich, MA). We inspected novel splice variants using four in silico prediction programs incorporated into the AlaMut Visual Splicing Effects module: SpliceSiteFinder-like (SSFL), MaxEntScan (MES), NNSPLICE, and GeneSplicer (Biosoftware, 2014; Interactive Biosoftware, Rouen, France).

Unsolved patients from whom we could obtain informed participation consent for at least their parents underwent exome sequencing (ES) according to the protocol described by Ryzanicz et al. [10].

## RESULTS

Out of 206 recruited families (86 at CMHI and 120 at other institutes), all probands and three affected family members underwent tNGS. This group was further divided, because systemic involvement was later found in ten subjects, three individuals were reevaluated to have retinal post-inflammatory changes, and two patients were proved to have optic nerve atrophy. An additional family withdrew from the study. All of these probands were excluded from the non-syndromic cohort (Figure 1).

A group of 190 individuals with non-syndromic IRD was selected for this study. Within this cohort, the initial clinical diagnosis was altered, sometimes with the aid of genetic results, in 16 persons. In one case, after genetic screening, the diagnosis changed from RP to choroideremia.

Ninety-nine probands were diagnosed with RP, 48 with STGD, 16 with CD/CRD, one with bestrophinopathy, one with choroideremia, ten with LCA, nine with ACHM, and three with CSNB (Appendix 2). In the case of three probands, the diagnosis was uncertain as the phenotype was borderline (two had STGD or CRD and one CSNB or RP). Age of onset presented a wide spectrum from birth to 65 years old (median = 8), and the median age at diagnosis was 11 (Appendix 3). At the time of recruitment, patients ranged in age from 1 to 71 years old (median = 27). The median difference between age of onset and age at diagnosis was 2 years (mean = 5 years). Patients with RP experienced the longest delay in diagnosis; in one case as long as 29 years (Figure 2). On average, a patient had to wait for a genetic resolution of the disease for 17.8 years (median = 12.5 years).

Most probands (123, about 65%) were isolated cases; however, 32 families had apparent dominant inheritance pattern, and 35 were recessive (within this group, X-linked recessive inheritance was possible in 14 families). After genetic testing, eight of the families with dominant inheritance were found to display a recessive, pseudodominant inheritance pattern due to mutations in *ABCA4* (results published in Tracewska et al. [11]), one in *CNGA1* (Gene ID 1259, OMIM: 123825), and one in *EYS* (Gene ID 346007, OMIM 612424). The X-linked recessive pattern was confirmed by finding causal variants in eight families, of whom six had variants in *RPGR* (Gene ID: 6103 OMIM:

312610), one had a variant in *CACNA1F* (Gene ID: 778, OMIM: 300110), and one had a mutation in *CHM* (Gene ID: 1121, OMIM: 300390). One additional isolated male patient appeared to have a pseudoheterozygous mutation in *RP2* (Gene ID: 6102, OMIM: 300757), an X-linked gene. This change was not present in the mother, and most probably represents an early embryonic somatic mutation, present in the peripheral blood and the buccal swab-derived DNA of the patient. Novel single nucleotide variants were found in *ABCA4* (c.(2653+1\_2654-1)(\*1\_?)del, p.(Gly885Valfs\*71) [12]), *MAK* (Gene ID: 4117, OMIM: 154235), *CNGA3* (Gene ID: 1261, OMIM: 600053; c.1379T>C, p.(Leu460Pro), *EYS* ((c.(7723+1\_7724-1)(8071+1\_8072-1)dup), and *CEP290* (Gene ID: 80184, OMIM 610142; c.250+2T>C, p.[=,Glu84Glyfs\*10,Ser42Phefs\*2]. An additional novel variant in *IMPG2* (Gene ID: 50939, OMIM: 607056), c.1963del, p.(Met655Trpfs\*46), was found heterozygously without an allelic counterpart. Two probands who were negative in the MIPs study were subjected to ES, and mutations were found in insufficiently covered regions of *C21ORF2* (Gene ID: 755, OMIM: 603191) and *NR2E3* (Gene ID: 10002, OMIM: 604485).

Within the isolated cases group, 67 (presumably) solved cases displayed defects in genes involved in autosomal recessive, seven autosomal dominant (two, de novo, three, no contact with the father or father deceased, and two with incomplete penetrance), and one X-linked recessive inheritance pattern (de novo). Dominant de novo mutations could not, however, be fully proven as such, as performing marker analysis for paternity exclusion was not feasible. In two cases homozygous for *CNGA3* c.847C>T, p.(Arg283Trp) and *C21ORF2* c.33\_34ins16, p.(Ala12Serfs\*60), 242 and 254, respectively, the father lacked the variant. Assuming correct paternity in the family, it might have been allelic dropout in PCR in the father's DNA sample, which happened in several cases and was mostly remedied with the use of different primer pairs. Alternatively, a loss of heterozygosity in the child could be an explanation. As in the previous case, paternity could not be tested.

In eight (presumably) solved cases, proving variant segregation was impossible due to the lack of contact with family members, refusal to participate, or loss of contact with the proband. Altogether, eight results were deemed inconclusive, some with more convincing pathogenicity clues (Figure 1). The most common variants, found in 33 individuals, were mutations in *ABCA4* belonging to a complex allele: c.[1622T>C;3113C>T], p.[Leu541Pro;Ala1038Val]. It is by far the most prevalent allele in the Polish population, accounting for approximately 17% of all solved cases (Figure 3). All variants are presented in Appendix 4.

### DISCUSSION

The targeted NGS panel allowed us to successfully determine the cause or probable cause of the disease in 60% of the cases. In this cohort consisting of patients of Polish origin, we found many novel variants (however, only six remain unreported in genomic variant databases at the time

of this publication). Out of 123 isolated patients, 77 were solved, and four presented a variant of unknown significance (VUS): *IMPDH1* (Gene ID: 3614, OMIM: 146690) c.968A>G, p.(Lys323Arg); *PRPF8* (Gene ID: 10594, OMIM: 607300) c.5792C>T, p.(Thr1931Met; VUS), which might still be pathogenic (lack of family members to confirm segregation);

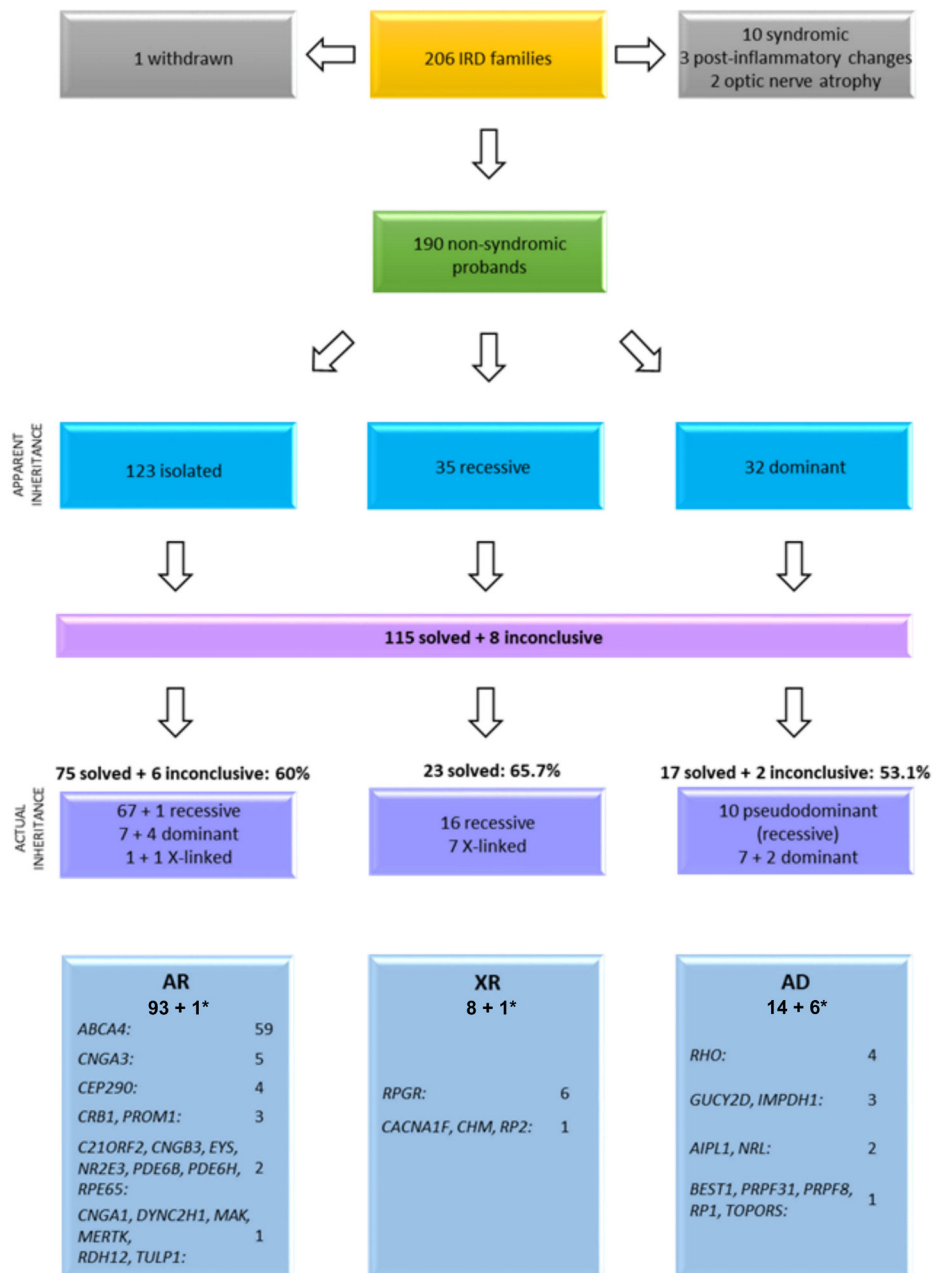


Figure 1. The initial number of IRD index cases versus final diagnoses and mutated genes. Shown are inheritance patterns and genes with pathogenic alterations in the Polish population. The bottom boxes represent actual inheritance in individuals grouped from all types of apparent inheritance. \*, inconclusive; AR, autosomal recessive; XR, X-linked recessive; AD, autosomal dominant.

*CEP290* c.223A>G, p.(Lys75Glu), a VUS found with the pathogenic c.1984C>T, p.(Gln662\*) on the other allele; and *CACNA1F* c.847T>C, p.(Cys283Arg), in which case there was no other affected family member to confirm pathogenicity with cosegregation, and therefore, it was classified as a VUS). An additional inconclusive result, *AIPL1* (Gene ID: 23746, OMIM: 604392) c.737A>C, p.(Tyr246Ser), was found in a dominant family in whom all tested members were

affected with RP, parents and two children. The presumably pathogenic allele came from the father, but functional studies would be required to actually determine its impact on the protein. Another change in *NRL* (Gene ID: 4901, OMIM: 162080), c.242\_244del, p.(Gln81del) may be benign, but the patient's daughter (untested) developed local retinal pigment clumping, which is consistent with the *NRL* phenotype and its mode of inheritance.

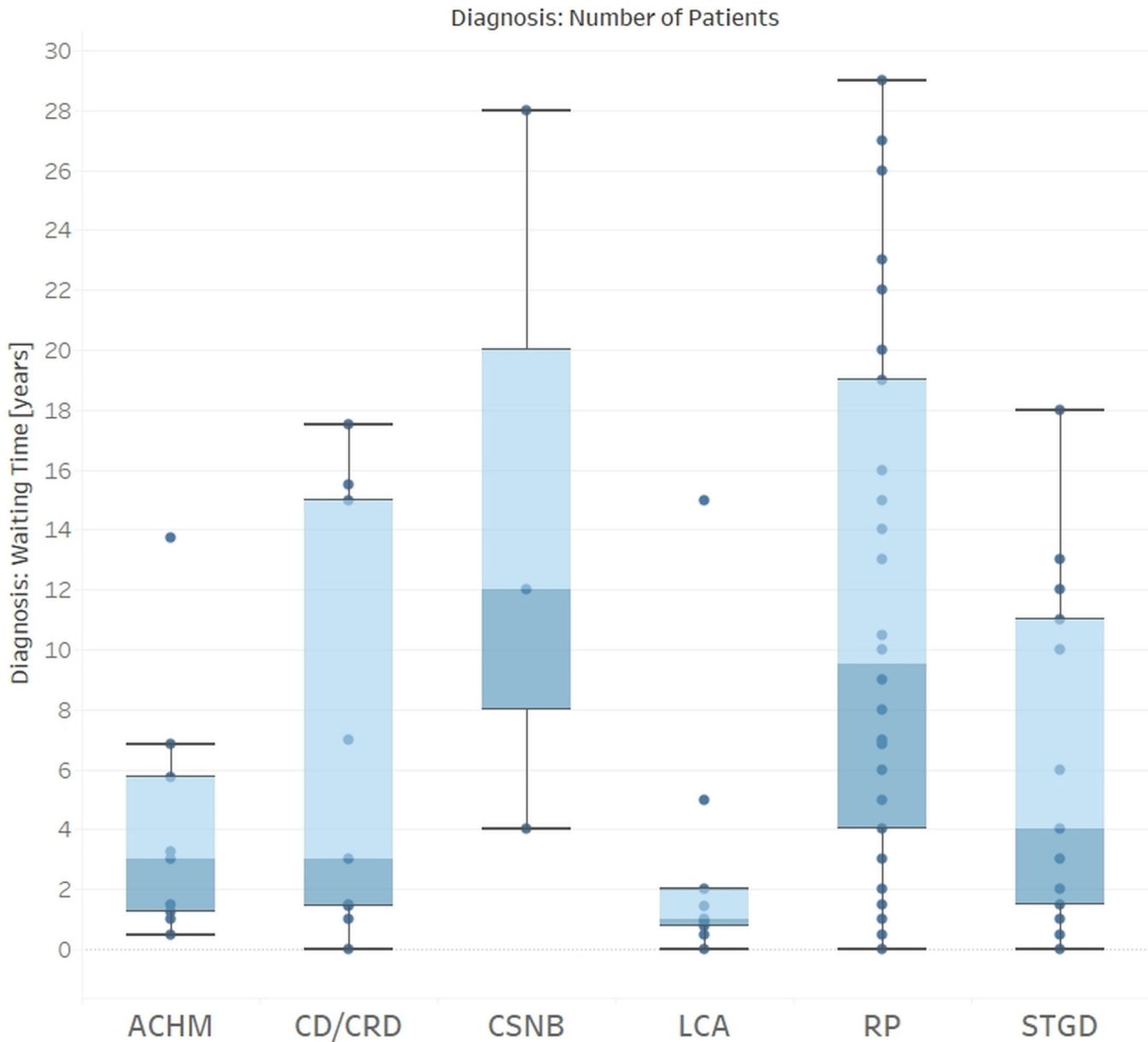


Figure 2. The number of individuals diagnosed with a specific entity and the wait time for the diagnosis. The box plot for each phenotype is divided into two differently shaded parts with the median value between them (in years). Each data point represents a patient. ACHM, achromatopsia; CD/CRD, cone or cone-rod dystrophy; CSNB, congenital stationary night blindness; LCA, Leber congenital amaurosis; RP, retinitis pigmentosa; STGD, Stargardt disease.



The patient with the variant in *RP2* (X-linked), c.768+2T>C, p.(?), which was not present in his mother, was not hemizygous, but represented a mosaic of normal and mutated cells. This was confirmed in epithelial cells from a buccal swab and leukocytes from peripheral blood. Therefore, this alteration represents a de novo change that might have appeared in the early stages of the patient’s embryonic development.

We identified pathogenic alterations in *ABCA4* as the primary cause of IRDs in Poland: solved or probably solved cases accounted for 31% of the whole group (59/190). Patients with STGD had their mutation recognized in 47 out of 50 cases; in two negative cases, ES was performed, but to no avail. There was a visible sex imbalance in the subgroup carrying c.5882G>A, p.(Gly1961Glu). The women to men ratio was 7:2, a phenomenon we described in a large study [12] showing that, especially with *ABCA4* hypomorphic alleles, STGD1 should probably be considered a multifactorial rather than monogenic disorder.

Additional sequencing of the whole *ABCA4* gene in single-allele cases revealed a missed mutation in exon 46 in individual 302 (Appendix 3 and Appendix 4), but not in individuals 383 and 385, in whom these mutations were most probably a chance finding [13]. There is a high probability that these probands carry deep intronic mutations or infrequent copy number variants (CNVs) in the *ABCA4* gene [14]. However, this hypothesis could not be tested in our setting.

Some genes commonly mutated in other populations were rarely mutated in this cohort. The *EYS* gene has been described multiple times as the leading cause of recessive RP in many populations, for example, 12% in France [15], 15.9% in Spain [16], and 40% in Japan. In comparison, in Polish individuals with recessive and isolated RP this proportion was 2/80 (2.5%). We found an additional single variant in individual 419 (c.9380\_9399del, p.(Thr3127fs\*13)), bearing two other, most probably non-causative mutations in *ABCA4* (c.5882G>A, p.(Gly1961Glu) and c.1411G>A, p.(Glu471Lys)). In this case, the search for a deletion or a duplication in the *EYS* gene, which is prone to large rearrangements, is the best

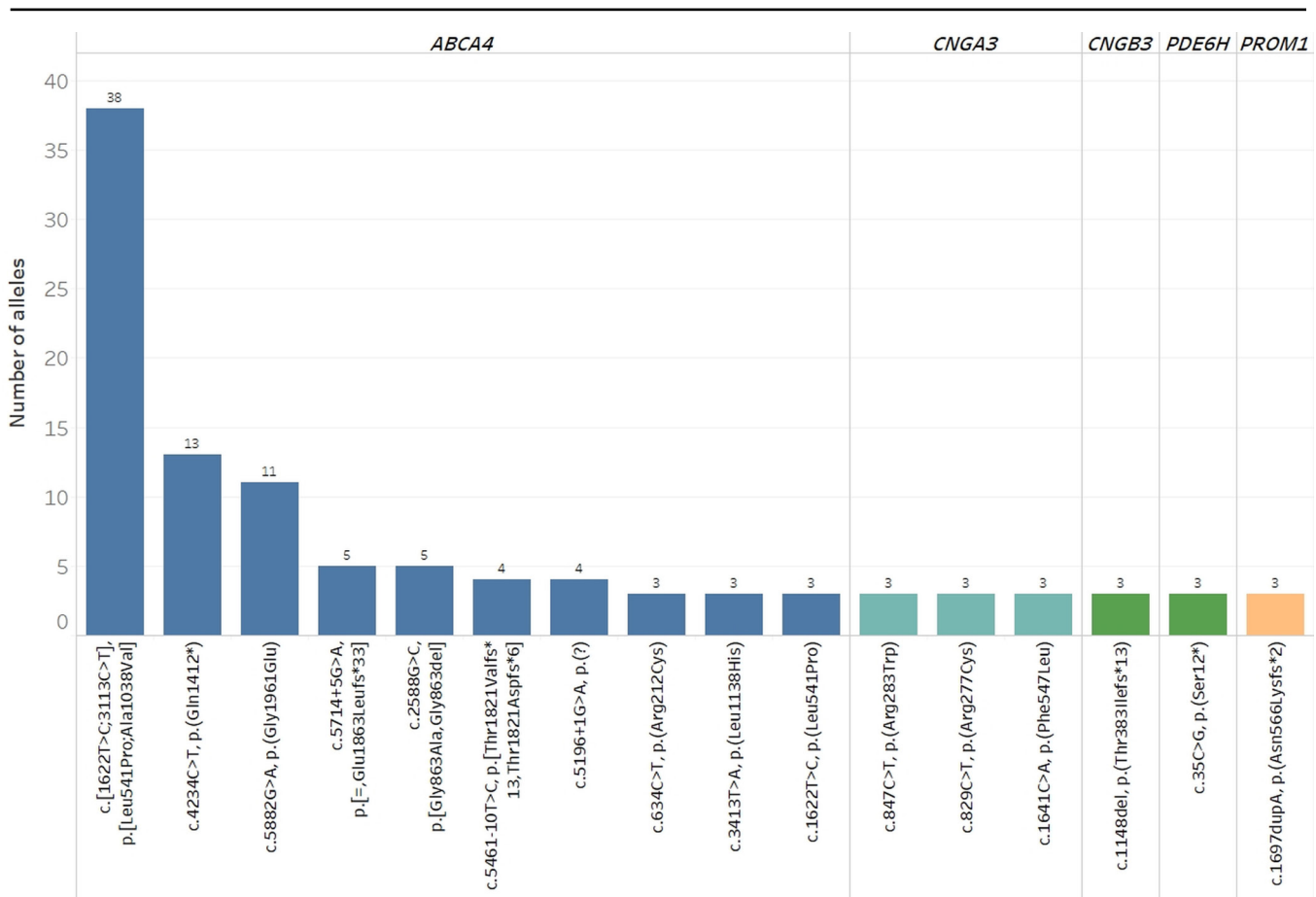


Figure 3. Most common mutations (allele count).

follow-up option. The relatively low depth did not allow us to use the internal CNV module of SeqNext in this case. However, in two other cases we discovered large rearrangements in *EYS* (c.(7723+1\_7724-1)\_(8071+1\_8072-1)dup and *ABCA4* (c.(2653+1\_2654-1)(\*1\_?)del) [13]; regrettably, the actual breakpoints could not be determined. Because Zampaglione et al. reported that almost 9% of IRD cases are due to CNVs [17], this number (two in 190 individuals) is probably highly underestimated. Thus, the next most logical step in probands with identified single allele variants in *ABCA4*, *CEP290*, *CNGA3*, *CNGB3* (Gene ID: 54714), *EYS*, *IMPG2*, *MERTK* (Gene ID: 10461), *PROM1* (Gene ID: 8842), and *USH2A* (Gene ID: 7399) would be using a technique which enables detection of such deletions and duplications, for example, microarrays (for larger rearrangements) or multiplex ligation-dependent probe amplification (MLPA, a tool for detecting deletions or duplications at the exon level), or deep targeted resequencing. In many cases, however, even with confirmed pathogenicity and matching phenotype, single allele variants may be chance findings. Null mutations in IRDs were shown to be carried by approximately 20% of all individuals examined with ES [18]. Moreover, in a recent publication, Hanany et al. estimated the number of pathogenic IRD variant carriers to be half of the entire human population [19]. Therefore, if no pathogenic variants are identified with deep targeted sequencing, the method of choice should be whole genome sequencing. This method provides comprehensive CNV screening and allows breakpoint sequencing. Whole genome sequencing also enables the identification of deep intronic variants, which remain elusive with the use of other techniques. Ultimately, it may serve to prove that the single variant was an incidental finding, and the true cause of the disease lies elsewhere.

Previously, there were no comprehensive reports of IRD causes within the Polish population. We analyzed 108 genes out of approximately 130 genes involved in non-syndromic IRDs (there are more than 250 including all IRD forms, and the numbers are still growing). However, we did not find additional variants in the genes outside the panel using whole exome sequencing (WES) in unsolved patients for whom we had DNA of family members, except *DYNC2HI* (Gene ID: 79659), which resulted in publication of a novel non-syndromic RP gene [20]. Only two additional families were solved in this manner, and they carried mutations in genes which were present in the panel but insufficiently covered in the MIP samples.

We indicated that apart from patients with STGD, patients with CD/CRD and RP should undergo the initial *ABCA4* test in the absence of funding for next-generation sequencing of

a large gene panel or exome sequencing. Other genes were unremarkable: six mutations in *RPGR* make it the second most frequently altered gene in this cohort (Figure 1, Appendix 4). The most frequent pathogenic allele was the complex *ABCA4* c.[1622T>C;3113C>T, p.[Leu541Pro;Ala1038Val], present on 38 alleles (Figure 3). Because the actual inheritance was, in many cases, different from the inheritance pattern derived from family history, we suggest that the diagnostics should not involve dividing affected individuals by inheritance patterns and testing only genes within a certain pattern, as typically performed currently. A new diagnostic algorithm should be implemented, involving initial targeted NGS testing for the whole *ABCA4* gene. All isolated and familial male-only families should then be tested for *RPGR*, including ORF15, which could not be entirely covered in this study.

As one of the post-communist countries in the European Union, Poland is still lagging behind the rest of Europe, in terms of diagnostics, application of new techniques, and developments in some of the medical fields in the country's largely underfunded public healthcare system. The lack of specialists able to properly recognize rare eye disorders is obvious, with many adult patients describing their "odyssey" from doctor's office to office before they receive a diagnosis. This trend was not visible in pediatric patients diagnosed at CMHI, because they were examined at the country's reference center, where many genetic disorders are diagnosed, and there is available equipment for more advanced procedures, such as ERG. As IRDs are uncommon, the average ophthalmologist can see only a few cases during his or her entire career. Therefore, the discrepancy between the age of onset and the age at diagnosis may be a result of lack of experience with such disorders and limited access to proper diagnostic procedures.

The mean difference between the age of onset and the age at diagnosis was almost 5 years, and it was largest in the CSNB/RP group (Figure 2), which may be partially explained by the long follow-up time to monitor disease progression. The mean wait time for genetic diagnosis since onset was 17.8 years, which emphasizes the pressing need for increasing availability of genetic tests for IRDs. In some cases, genetic diagnosis is helpful in determination of the final diagnosis. However, many patients lack the financial means to perform genetic tests, as they are not fully reimbursed by insurance, especially because these diseases are not lethal, and for most forms, there is no available treatment.

The data presented here expose the underestimation of rare disorders, a phenomenon happening in many areas of medical care in Poland. It is absolutely necessary to raise awareness of rare retinal disorders among Polish

ophthalmologists. Additionally, it must be stressed that in some cases genetic analysis of the patient is necessary to achieve accurate diagnosis.

#### APPENDIX 1. PEDIGREES OF ALL RECRUITED PATIENTS AND FAMILIAL VARIANT SEGREGATION DATA.

To access the data, click or select the words “[Appendix 1.](#)” [added as a PDF file to the submission]

#### APPENDIX 2. IRD PHENOTYPES OF AFFECTED INDIVIDUALS IN THIS STUDY.

To access the data, click or select the words “[Appendix 2.](#)”

#### APPENDIX 3. PHENOTYPE, GENOTYPE AND DEMOGRAPHIC DATA OF THE COHORT.

To access the data, click or select the words “[Appendix 3.](#)” + - wild type, ACHM - achromatopsia, AD - autosomal dominant, AR - autosomal recessive, BEST - bestrophinopathy, CD - cone dystrophy, CHM - choroideremia, CRD - cone-rod dystrophy, CSNB - congenital stationary night blindness, F - female, LCA - Leber congenital amaurosis, M - male, RP - retinitis pigmentosa, STGD - Stargardt disease, VUS - variant of unknown significance, XR - X-linked recessive. 1An additional heterozygous mutation in PROM1 - c.1697dup, p.(Asn566Lysfs\*2), was detected in this patient, which may contribute to the phenotype. 2A single heterozygous alteration was detected in this patient, which is most probably a chance finding.

#### APPENDIX 4. INVESTIGATED VARIANTS IDENTIFIED IN THE COHORT.

To access the data, click or select the words “[Appendix 4.](#)” ADA - a splicing prediction score derived from database of all potential human SNVs within splicing consensus regions and their functional annotations, alt - alteration, CADD - Combined Annotation Dependent Depletion, chr - chromosome, clinvar - Genomic variation as it relates to human health, DANN - deleterious annotation of genetic variants using neural networks, DM - disease-causing mutations, DFP - disease-associated polymorphism with additional supporting functional evidence, HGMD - Human Gene Mutation Database, LASSIE - Linear Allele-Specific Selection Inference, ref - reference, 1Compound heterozygous alterations were detected in ABCA4 gene, which is most probably a chance finding due to high frequency of the variants.

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