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# Reassessing Inherited Retinal Degeneration Families Clinically and Genetically After Initial Negative Results from Panel-Based Next-Generation Sequencing

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## Abstract

Inherited retinal degenerations (IRDs), although uncommon, are the primary cause of blindness among adults of working age. These disorders are genetically complex, involving over 300 different loci, and establishing a molecular diagnosis is crucial for access to emerging therapies and clinical trials. Standard panel-based next-generation sequencing (pNGS) identifies causative variants in approximately 70–80% of cases, leaving a significant subset unresolved. This study examines patients with negative first-tier pNGS results, highlighting the role of detailed clinical reassessment and the application of targeted second-tier genetic testing. By excluding individuals without IRDs and applying appropriate follow-up genetic analyses, we were able to determine a molecular cause in 56% of previously undiagnosed families, increasing the overall diagnostic yield to 92% (388/423). Our findings indicate that while pNGS remains the most cost-efficient initial strategy for diverse IRD populations, further testing should be guided by refined clinical evaluation—such as multimodal imaging and electrophysiology—and genetic indicators, including the presence of single alleles in recessive conditions, to achieve accurate and cost-effective diagnoses.

**Keywords:** Genetic testing, Next-generation sequencing, Whole-exome sequencing, Single-gene sequencing, Inherited retinal degenerations, Retinal dystrophy

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## Introduction

Inherited retinal degenerations (IRDs) are rare genetic conditions caused by pathogenic variations in more than 300 loci, leading to progressive and variable vision loss [1]. In many Western countries, IRDs represent the leading cause of visual impairment among adults of working age [2, 3]. The broad clinical and genetic diversity of these disorders makes precise molecular diagnosis challenging. Panel-based next-generation sequencing (pNGS) provides first-line genetic analysis with a detection rate of roughly 70–80% [4–6]. Expanding testing to whole-exome or whole-genome sequencing (WES/WGS) can further resolve up to 79% of previously undiagnosed cases [7, 8]; however, this comes with higher costs, increased data management requirements, and the need to handle incidental findings [9, 10].

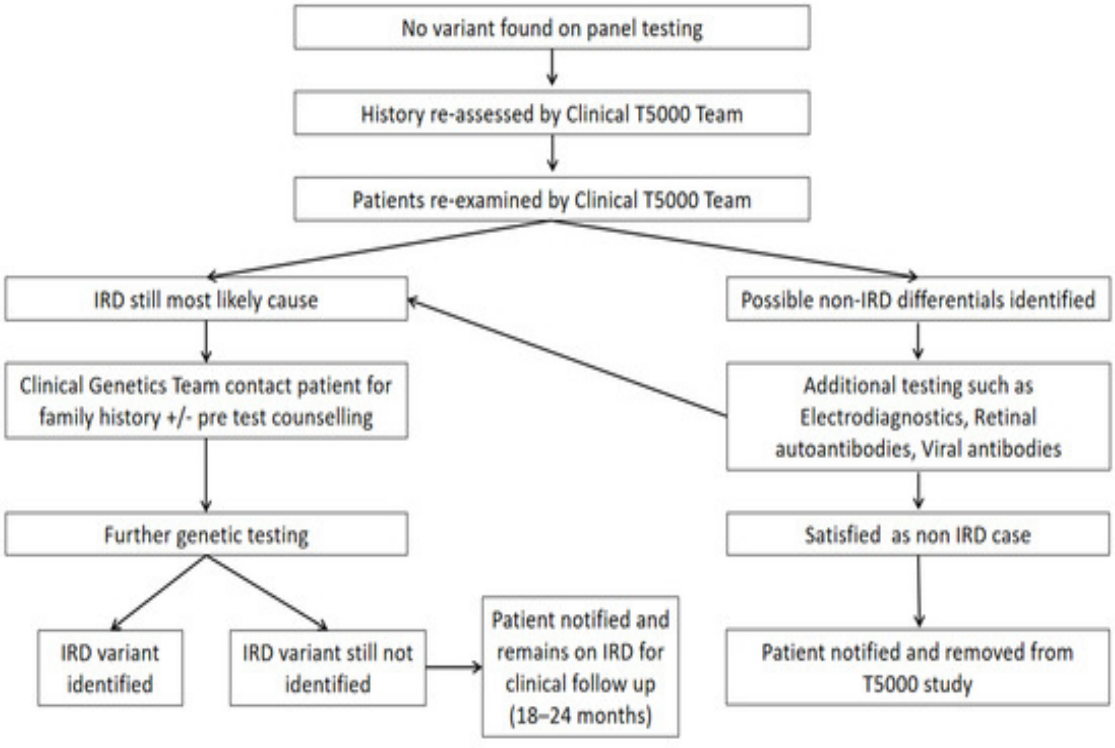
Careful clinical characterization can guide the selection of the most suitable genetic testing strategy [11]. Accurate genotyping has become increasingly important with the rise of gene- and stem cell-based therapies, as it determines eligibility for clinical trials and approved interventions [12–15]. A confirmed molecular diagnosis also enables families to understand inheritance patterns, assess reproductive risk, and consider options such as prenatal or pre-implantation genetic testing. Maximizing the diagnostic yield in IRDs is therefore essential.

This study outlines the approach for re-evaluating patients with negative first-tier pNGS results, detailing the integration of clinical reassessment and subsequent targeted genetic testing to improve the overall resolution of IRD cases.

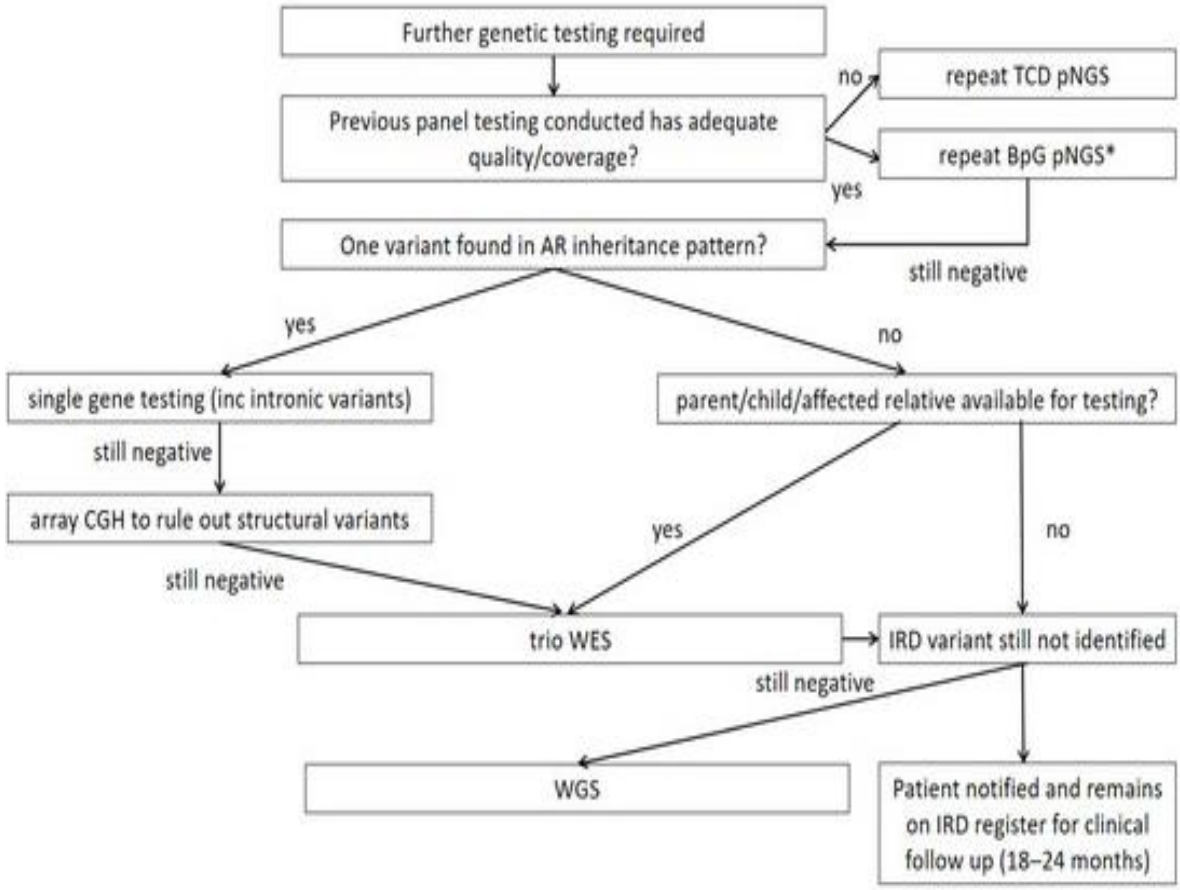
## Materials and Methods

Participants were recruited through the Mater Clinical Ophthalmic Genetics program as part of the Irish national IRD registry (Target 5000) and underwent evaluation for potential genetic causes of ophthalmic and syndromic features. Comprehensive clinical assessment included visual acuity (LogMAR, Optos plc, Scotland, UK), formal visual fields (Humphrey Field Analyzer, Carl Zeiss Meditec, CA, USA), color vision testing (Lanthony D15, Gulden Ophthalmics, PA, USA), ocular motility and nystagmus assessment, and slit-lamp biomicroscopy with Goldmann applanation tonometry (Haag-Streit UK Ltd, UK). Multimodal imaging comprised color fundus photography, fundus autofluorescence (Optos 'California,' Optos plc, Scotland, UK), and spectral-domain optical coherence tomography (OCT, Cirrus 5000, Carl Zeiss Meditec, CA, USA). Visual electrophysiology (ERG, Metrovision, France) was performed as indicated. All participants had previously undergone research-grade pNGS of 250 IRD-associated genes at the Ocular Genetics Unit, Trinity College Dublin [4, 5, 16].

For patients with negative initial pNGS findings, existing clinical records—including imaging, electrophysiology, and visual fields—were reviewed by three clinicians independently and in a blinded manner (KS, TB, DK). A fourth investigator (JZ) resolved any disagreements, with concordant cases presented to the clinical genetics multidisciplinary team (MDT) and discordant cases recalled for in-person reassessment. On-site reassessment focused on functional testing (VA, VF, electrophysiology) and structural evaluation (multimodal imaging) to clarify the diagnosis **(Figure 1a)**.




a)




b)

**Figure 1.** Workflow for Clinical and Genetic Re-Evaluation of 'Gene-Negative' Cases. (a) Clinical reassessment workflow for patients with negative first-tier genetic results. (b) Decision pathway for selecting appropriate second-tier genetic testing strategies. *https://blueprintgenetics.com/tests/panels/ophthalmology/retinal-dystrophy-panel (accessed 8 November 2021).

Families in which the phenotype remained consistent with an inherited retinal degeneration (IRD) after initial evaluation were referred to the clinical genetics multidisciplinary team (MDT) to determine the most suitable subsequent genetic testing strategy. Decisions were based on clinical presentation, family history, and any findings from first-tier pNGS, such as the presence of a single pathogenic allele in recessive disorders.

Second-tier genetic testing in this study included several approaches:

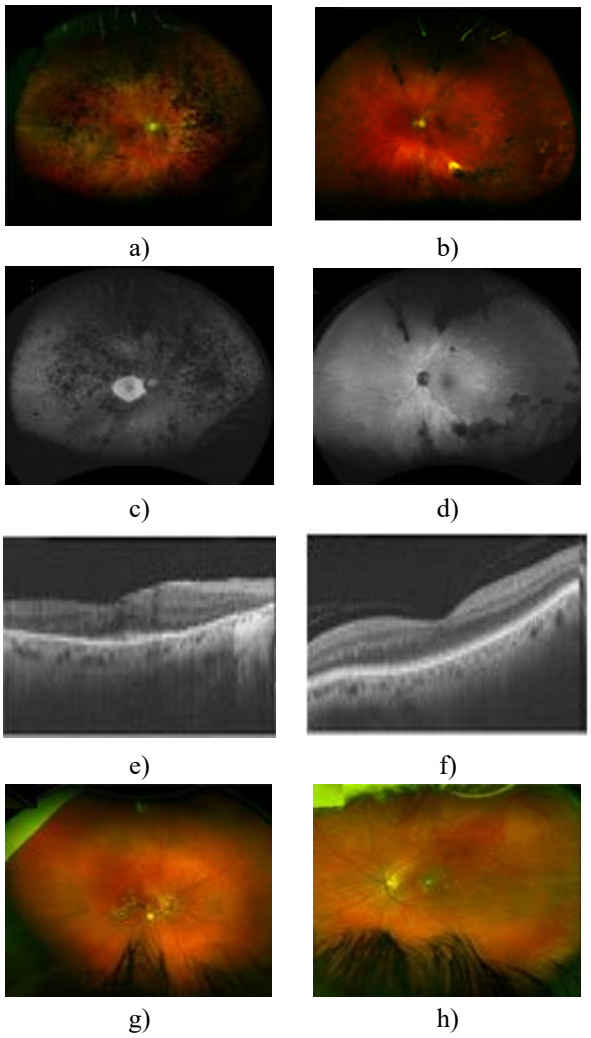
1. Repeat or manual review of pNGS data: For cases where initial gene coverage was considered insufficient, either a repeat sequencing run of the 250-gene panel or direct inspection of BAM files was performed (pedigrees 19, 20, 23).
2. Expanded gene panel testing: Where first-tier coverage was adequate, a larger panel of 351 IRD-associated genes was applied by a commercial laboratory (Blueprint Genetics, Helsinki, Finland) (pedigrees 24, 26) [17].
3. Targeted single-gene sequencing: Applied in cases with one candidate variant identified previously or classic phenotypes such as Stargardt Disease (OMIM#248200). This included sequencing exons, introns, and flanking regions for genes such as ABCA4 (pedigrees 21, 22), ADGRV1 (pedigree 31), BBS1 (pedigree 32), CDH23 (pedigree 29), CNNM4 (pedigree 28), EYS (pedigree 25), PEX7 (pedigree 18), and TRIM32 (pedigree 30).
4. Trio whole-exome sequencing (WES): For four pedigrees without candidate variants on first-tier pNGS, sequencing was performed on the proband and both parents (Blueprint Genetics). Three of these pedigrees presented with non-syndromic retinitis pigmentosa (RP) with X-linked, autosomal dominant, and autosomal recessive inheritance (pedigrees 27, 33, 34), and one pedigree exhibited autosomal dominant vitreoretinopathy (pedigree 35).

All identified variants were reported using HGNC nomenclature, validated via bidirectional Sanger sequencing, and compared to the GRCh37/HG19 reference genome **(Figure 1b)**.

## Results and Discussion

Among 441 patients (331 pedigrees) evaluated at the Mater Clinical Ophthalmic Genetics Unit prior to 2019, 69 individuals (52 pedigrees, 16%) remained genetically unresolved after initial pNGS testing. Phenotypic reassessment revealed that 51 patients (74%) continued to display features consistent with IRD, whereas 18 patients (26%) were reclassified as having acquired, non-IRD conditions **(Table 1 and Figure 2)**. The mean age was 58.06 ± 16.97 years in the non-IRD group and 50.57 ± 16.12 years in the IRD group, with females comprising 61% and 53% of the respective cohorts.

Of the 51 patients (35 pedigrees) retaining an IRD-consistent phenotype, 34 individuals from 18 pedigrees proceeded to further genetic testing **(Table 2)**. Seventeen patients were unavailable for additional testing due to death (n = 2), absence of suitable family members for trio WES (n = 4), or personal choice during the SARS-CoV-2 pandemic (n = 11).



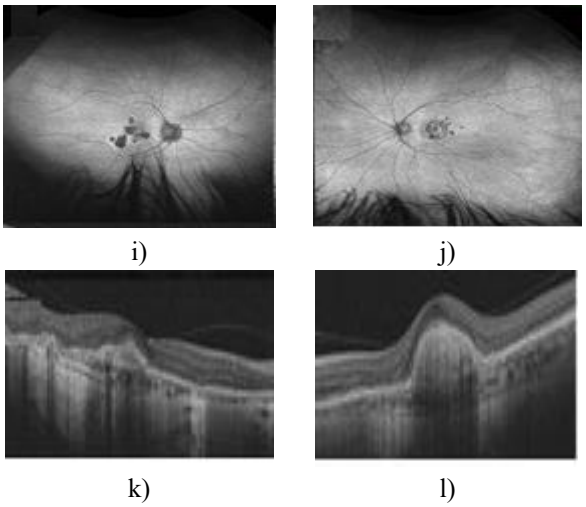




**Figure 2.** Illustrative Non-IRD ('Gene-Negative') Cases Exhibiting Strongly Asymmetric Retinal Changes. Pedigree #2: A 70-year-old male presented with highly uneven retinal pigmentation between eyes. The right eye (a) showed features typically associated with retinitis pigmentosa, whereas the left eye (b) displayed only mild paraarteriolar pigment migration. Fundus autofluorescence revealed a small central area of preserved retinal pigment epithelium in the right eye (c), while the left eye (d) demonstrated minor paravascular hyperautofluorescence inconsistent with RP. OCT imaging confirmed the asymmetry: the right eye retained much of the central outer retina (e), while the left eye maintained normal retinal layering (f). No pathogenic variants were identified through pNGS. The patient's ocular history included childhood meningitis, with no familial retinal disease, stable vision (6/12 right, 6/6 left), and no symptom progression. Clinical review concluded these findings represented asymmetric post-inflammatory pigmentary changes, and no further genetic testing was recommended. Pedigree #3: A 47-year-old female exhibited asymmetric macular degeneration. Fundus photography (g,h) showed localized macular atrophy, and autofluorescence imaging (i,j) demonstrated hypoautofluorescent lesions corresponding to atrophy interspersed with hyperautofluorescent regions, with otherwise normal peripheral retina and vessels. OCT (k,l) identified outer retinal loss, focal subretinal fibrosis, and areas of choroidal thinning. After thorough review, the condition was classified as punctate inner choroidopathy. Genetic testing was not pursued, and the patient was referred to a uveitis specialist for further management.

**Table 1.** Categories and Demographic Characteristics of Non-IRD Cases

| Diagnosis Group | Female (%) | Mean Age (Years ± SD) | n (%) |
|---|---|---|---|
| Posterior uveitis | 57% | 51 ± 16.39 | 7 (39%) |

| | | | |
|---|---|---|---|
| Age-related macular degeneration (AMD) | 25% | 76 ± 8.39 | 4 (22%) |
| Myopic / pachychoroid-related degeneration | 50% | 45.5 ± 3.54 | 2 (11%) |
| Inherited optic neuropathy (ION) | 100% | 50 | 1 (6%) |
| Clinically normal | 75% | 49 ± 12.92 | 4 (22%) |

SD= standard deviation.

**Table 2.** Summary of Outcomes Following Additional Genetic Testing. Pedigrees 18–27 achieved a molecular diagnosis, while pedigrees 28–36 remained unresolved despite further testing. Variants listed in plain text were detected during the initial pNGS run, whereas bolded variants were identified through subsequent testing approaches specified in the 'Method' column. All variants reported are classified as ACMG class 5 (pathogenic).

| Pedigree | N | Phenotype | Inheritance | Gene | Variant 1 | Variant 2 | Method | Issue in 1st-Tier pNGS |
|---|---|---|---|---|---|---|---|---|
| 1–17 | 18 | Non-IRD | - | - | - | - | - | - |
| 18 | 1 | Refsum disease | AR | PEX7 | c.875T>A, p.Leu292* | c.40A>C, p.Thr14Pro | Single-gene testing | Limited coverage |
| 19 | 2 | EOSRD | AR | CFAP410 | c.218G>C, p.Arg73Pro | c.218G>C, p.Arg73Pro | Repeat pNGS (R) | Misaligned reads, index hopping |
| 20 | 1 | Syndromic RP | AR | FLVCR1 | c.1022A>G, p.Tyr341Cys | c.1307+5G>T † | Repeat pNGS (R) | Additional phenotype information |
| 21 | 1 | Stargardt disease | AR | ABCA4 | c.752del, p.Phe251Serfs*11 † | c.5461−10T>C, p.Thr1821Aspfs*6, Thr1821Valfs*13 | Single-gene testing | Intronic variant |
| 22 | 1 | Stargardt disease | AR | ABCA4 | c.4363T>C, p.Cys1455Arg | c.4253+43G>A, p.Ile1377Hisfs*3 | Single-gene testing | Intronic variant |
| 23 | 1 | Bardet–Biedl Syndrome | AR | BBS10 | c.2119_2120del, p.Val707* | c.687del, p.Val230Phefs*7 | Repeat pNGS (R) | Poor coverage |
| 24 | 3 | Non-syndromic RP | AD | RP1 | c.2321_2322insAlu | - | Repeat pNGS (A) | Complex structural variant |
| 25 | 1 | Non-syndromic RP | AR | EYS | c.2620C>T, p.Gln874* | c.(?-*538-1*)(2023+1_2024-1)del † | Single-gene testing | Copy number variants |
| 26 | 3 | Non-syndromic RP | XL | RPGR | c.2777_2778del, p.Glu926Glyfs*152 † | - | Repeat pNGS (A) | Low complexity ORF15 region |
| 27 | 2 | Non-syndromic RP | XL | RPGR | c.2571_2572del, p.Glu859Glyfs*219 | - | Trio WES | Low complexity ORF15 region |
| 28 | 1 | Syndromic macular dystrophy | AR | CNNM4 | c.1660G>T, p.Ala554Ser | Unresolved | Single-gene testing | - |
| 29 | 1 | Usher Syndrome | AR | CDH23 | c.289-1G>A, p.Arg964Gln | Unresolved | Single-gene testing | - |
| 30 | 1 | Non-syndromic RP | AR | TRIM32 | c.691del, p.Ala231Glnfs*21 | Unresolved | Single-gene testing | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 31 | 1 | Usher Syndrome | AR | ADGRV1 | c.18025C>T, p.Arg6009* | Unresolved | Single-gene testing | - |
| 32 | 1 | Bardet–Biedl Syndrome | AR | BBS1 | c.478C>T, p.Arg160Trp | Unresolved | Single-gene testing | - |
| 33 | 3 | Non-syndromic RP | AD* | - | - | Unresolved | Trio WES | - |
| 34 | 1 | Non-syndromic RP | AR* | - | - | Unresolved | Trio WES | - |
| 35 | 9 | Vitreoretinopathy | AD* | - | - | Unresolved | Trio WES | - |
| 36–52 | 16 | Clinically consistent with IRDs | - | - | Unresolved | Unresolved | - | Retesting postponed due to SARS-CoV-2 pandemic |

*Abbreviations:* AD, autosomal dominant; AR, autosomal recessive; XL, X-linked; BBS, Bardet–Biedl syndrome; EOSRD, early-onset severe retinal dystrophy; sRP, syndromic retinitis pigmentosa; nsRP, non-syndromic retinitis pigmentosa; sMD, syndromic macular dystrophy; STGD, Stargardt disease; USH, Usher syndrome; VRO, vitreoretinopathy.
*Notes:* * Presumed inheritance pattern based on available family history. (A)—repeat pNGS at accredited laboratory. (R)—repeat pNGS at research laboratory. † novel variant.

Re-evaluation of patients allowed revision of the clinical diagnosis in 18 individuals, identifying them as non-IRD cases. Using the additional genetic testing strategies summarized in **Table 2**, 16 further patients from 10 IRD pedigrees were successfully resolved, raising the overall genetic resolution rate in this cohort to 92 percent (388/423).

All patients undergoing repeated pNGS achieved a genetic diagnosis (n = 5; pedigrees 19, 20, 23, 24, 26). For cases where first-tier pNGS coverage (250-gene panel) was sufficient, a larger 351-gene panel was applied for second-tier testing. Conversely, if coverage of clinically relevant genes was inadequate, such as BBS10, the initial 250-gene panel was repeated.

Targeted single-gene sequencing, including exons and introns, was used for autosomal recessive IRDs where one pathogenic allele had already been identified, applied in nine cases; 44% of these yielded a second pathogenic variant (pedigrees 18, 21, 22, 25).

Whole-exome sequencing (WES) was performed for 15 patients from four pedigrees lacking candidate variants from first-tier pNGS; however, three pedigrees remained unresolved (34, 35, 36). Across all second-tier testing, four novel variants were identified in ABCA4, EYS, FLVCR1, and RPGR **(Table 2)**. Pedigrees that remain unresolved after second-tier testing are planned for further evaluation using array comparative genomic hybridization and/or whole-genome sequencing to detect structural variants or copy number changes.

The 18 patients (17 pedigrees) reclassified as non-IRD were referred to relevant ophthalmic subspecialties, including uveitis, neuro-ophthalmology, and medical retina clinics, and subsequently discharged from the IRD service.

Among the 69 patients (16%) not resolved by first-tier pNGS, clinical reassessment guided management: 74% were directed toward additional genetic testing, and 26% were reclassified as acquired disease. After further genetic investigation, 47 percent of patients (16 of 34) or 56 percent of pedigrees (10 of 18) available for second-tier testing were resolved, including identification of four novel variants **(Table 2)**. Consequently, 92% of the total IRD cohort (388/423) received a molecular diagnosis.

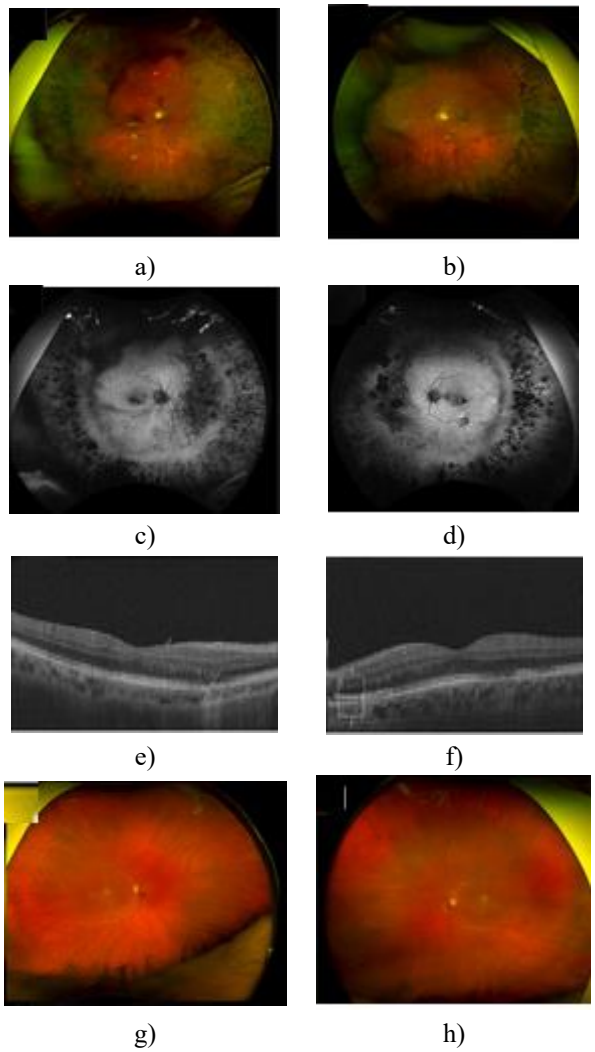
### *Clinical reassessment*

Factors supporting a diagnosis of IRD included early onset (<40 years), symmetrical disease, a positive family history, evidence of progression, and associated ocular or systemic features (e.g., juvenile posterior subcapsular cataract, sensorineural hearing loss, post-axial polydactyly) [18, 19]. Classic retinal phenotypes indicative of IRD are shown in **Figure 3**. In contrast, non-IRD cases were often unilateral or asymmetrical **(Figure 2)** or had late onset (e.g., age-related macular degeneration after 60 years). While no single feature is diagnostic, consideration of multiple indicators strengthens the suspicion of a genetic etiology.

Acquired retinal diseases may mimic IRDs in advanced stages, exhibiting arteriolar attenuation (e.g., retinal vasculitis, arteriolar occlusion), optic disc pallor (e.g., anterior ischemic optic neuropathy, glaucoma), or intraretinal RPE migration (e.g., retinal pigment epitheliitis, late-stage multifocal choroiditis) [20]. In this cohort, interocular asymmetry was absent in all resolved IRD cases (0/16) but present in 33% (6/18) of non-inherited cases, highlighting the importance of evaluating for acquired causes before proceeding to genetic testing.

Both subjective (history, family history, symptom progression) and objective (clinical findings, multimodal imaging, electrophysiology) information contribute to assessing the likelihood of a genetic etiology. Conditions commonly misdiagnosed as IRDs include autoimmune retinopathy, infectious or non-infectious posterior uveitides, and drug-induced retinal toxicity (e.g., hydroxychloroquine mimicking bullseye maculopathy/STGD1, deferoxamine toxicity).

Early in this study, challenging cases with partial IRD features were advanced to genetic testing in hopes of identifying causative variants. Experience from the multidisciplinary team (MDT) has since demonstrated that unrelated genetic findings can complicate diagnosis, delaying accurate conclusions and causing patient anxiety. Negative results from appropriate first-tier IRD genetic testing (e.g., pNGS) should prompt thorough clinical reassessment to exclude acquired causes. This approach maximizes the likelihood of resolving pedigrees and prevents overestimation of unresolved inherited retinal degeneration cases in the cohort.



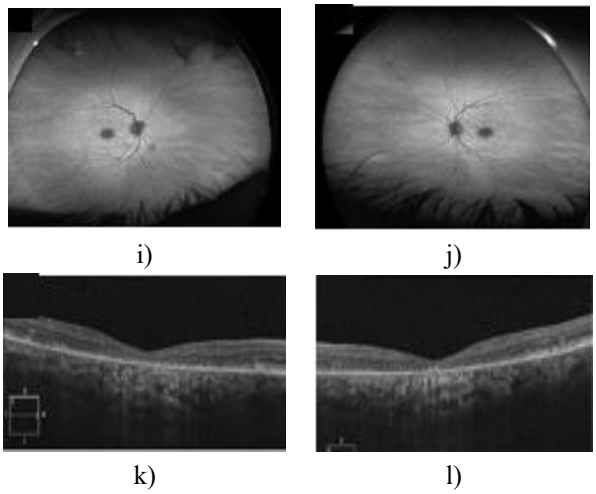




**Figure 3.** Multimodal Imaging of Genetically Confirmed IRD Cases. Pedigree #18: Colour fundus images (A,B) reveal symmetric, primarily midperipheral pigmentary changes. Fundus autofluorescence (C,D) shows patchy hypoautofluorescence in the midperiphery and focal areas at the posterior pole, consistent with RPE atrophy. OCT scans (E,F) demonstrate overall preservation of the photoreceptor and RPE layers, with a localized nasal defect of the photoreceptor inner segments in E and multiple inner segment/RPE defects nasally and temporally in F. Single-gene sequencing identified a second pathogenic PEX7 variant (OMIM*601757, c.40A>C, p.Thr14Pro), confirming autosomal recessive Refsum disease (OMIM#614879). The patient also exhibited systemic features including ataxia. Pedigree #22: Colour fundus photographs (G,H) show macular atrophy with surrounding subretinal flecks, sparing the areas outside the vascular arcades. Autofluorescence imaging (I,J) confirms foveal hypoautofluorescence bordered by hyperautofluorescent flecks primarily confined to the macula, with additional flecks nasal to the optic disc in J. OCT (K,L) demonstrates foveal outer retinal atrophy. These multimodal imaging characteristics align with autosomal recessive Stargardt disease (OMIM#248200). Single-gene testing of ABCA4 revealed a second pathogenic allele (c.4253+43G>A, p.[=, Ile1377Hisfs*3]), providing molecular confirmation for this case.

### *Second-tier genetic testing strategies and cost considerations*

The research-based panel NGS (pNGS) workflow, validated in an accredited laboratory as implemented in the Target 5000 program, provides substantial cost efficiency **(Table 3)** [5, 16]. Compared with whole-exome sequencing as an initial screening tool, pNGS offers lower bioinformatic burden, identifies the causative variant in the majority of IRD patients, and reserves resources for second-tier testing of more complex cases **(Figure 1b)**.

This tiered strategy has been adopted in other centers as well, resolving roughly one-third of partially characterized cases on second-tier testing, particularly where a single pathogenic allele is detected in autosomal recessive IRDs [6, 21].

**Table 3.** Estimated Total Costs and Resolution Rates for Genetic Testing Modalities Used in This Study (Trinity College Dublin, Ireland; Blueprint Genetics, Finland)

| Test Type | Number of Tests (N) | Cost per Test (€) | Resolution Rate (%) | Total Cost (€) |
|---|---|---|---|---|
| pNGS (initial negative in research lab + expanded panel at accredited lab + validation for other affected relatives) | 5 + 3 variant confirmations | 1,120 (€250 research + €870 ± €350 accredited) | 100% (5/5) | 6,350 |
| pNGS (resolved via research lab + accredited lab validation) | 441 | 600 (€250 research + €350 accredited) | 84.4% (372/441) | 240,450* |
| WES / trio WES (initial negative in research lab + accredited trio WES + validation for other affected family members) | 4 + 1 variant confirmation | 2,550 (€250 research + €2,300 ± €350 accredited) | 25% (1/4)** | 10,550 |
| Single-gene testing (initial negative in research lab + accredited single-gene test + validation for relatives) | 9 | 700 (€250 research + €450 ± €350 accredited) | 44% (4/9) | 6,300 |

*Note:* The 69 unresolved cases in this study underwent only initial research-grade pNGS prior to this investigation. Therefore, the total cost calculation includes (372 × €600) + (69 × €250).

**The single WES-resolved case could have been identified using the expanded 351-gene pNGS panel.

The costs and diagnostic yield of the various genetic testing strategies applied in this study are summarized in **Table 3**. Among re-tested patients, single-gene sequencing provided a molecular diagnosis in 44 percent (n = 9), while WES resolved 25 percent(n = 4). These findings underscore that pNGS remains the most cost-effective first-line approach, with more expensive and computationally intensive methods reserved for cases unresolved after clinical and phenotypic reassessment. The relatively modest success rate of single-gene testing may reflect instances in which first-tier pNGS identified a spurious variant, focusing analysis on one gene when a broader screening approach, such as WGS or array comparative genomic hybridization, might have yielded a higher probability of resolution [22, 23]. Careful evaluation of variants identified by pNGS—including ACMG classification and in silico functional predictions—will inform the selection of appropriate second-tier testing. Similarly, the low resolution rate with WES likely reflects prior comprehensive exon coverage via pNGS, suggesting that WGS may be more suitable to detect deep intronic or structural variants. Applying these broader techniques directly to previously untested IRD populations may increase the diagnostic yield, albeit at a higher relative cost compared with pNGS [21, 24].

Ongoing updates to NGS panel design allow reapplication to existing DNA samples, offering an efficient and cost-effective approach since the primary expenses involve sample preparation, sequencing, and panel design [25]. The total cost to reassess 34 patients was €23,200 (approximately €1,450 per resolved case), in addition to the original pNGS expenditure and an estimated €10,600 for clinician and genetic counselor time.

### *Resolution challenges and solutions*

Maximizing patient throughput per sequencing run is critical to optimize costs in research-based NGS, but aggressive pooling can result in some samples receiving suboptimal coverage. In specific phenotypes, targeted manual review of sequencing data (e.g., BAM files) can resolve candidate variants. This was demonstrated in pedigree #23, where re-phenotyping revealed systemic features, including diabetes and polydactyly, alongside teenage-onset RP consistent with Bardet–Biedl syndrome (BBS, OMIM#209900). This refinement reduced the gene search from >57 genes associated with autosomal recessive RP to 16 BBS-associated genes, of which BBS1 and BBS10 account for ~45% of cases [26]. Targeted manual inspection, despite suboptimal coverage, identified two pathogenic frameshift variants in BBS10: c.2119_2120del, p.(Val707*) and c.687del, p.(Val230Phefs*7), subsequently confirmed by direct sequencing.

Panel-based NGS relies heavily on PCR-based amplification to capture genes and incorporate indexing for multiplexed sequencing, which can lead to poor coverage in challenging regions. A notable example is the ORF15 region of RPGR (OMIM*312610), characterized by repetitive, low-diversity sequences. Cloning and bidirectional sequencing of this region have proven to be a cost-effective solution [27, 28]. Accurate assessment of RPGR ORF15 is critical, as approximately 60% of variants causing X-linked RP are located in this hotspot

[29]. Pedigrees #26 and #27 were resolved through improved coverage strategies: pedigree #26 via an expanded 351-gene pNGS panel and pedigree #27 using trio WES [30]. In hindsight, the variant in pedigree #27 could have been detected with the expanded commercial pNGS panel, which likely employs proprietary capture or hybridization methods superior to the research laboratory approach, further supporting pNGS as a first-tier investigation for IRDs.

On rare occasions, the zygosity of a variant may be incorrectly determined by sequence alignment algorithms, causing a patient to appear heterozygous for a variant in a recessive gene when they are, in fact, homozygous. This phenomenon was observed in pedigree #19 (CFAP410, OMIM*603191) and attributed to index hopping during the sequencing run, a known artifact in multiplexed sample sequencing [31]. Index hopping occurs when the sequencing platform misassigns reads to the wrong sample, resulting in erroneous genotype calls—in this case, a true homozygous variant being classified as heterozygous.

Panel-based NGS predominantly targets exonic regions. While canonical splice site variants adjacent to exons are generally captured, deep intronic or near-exon variants may be inconsistently detected, depending on factors such as capture probe design, hybridization efficiency, sequencing depth, and the analysis pipeline. To standardize variant interpretation, bioinformatic thresholds often focus on purely exonic variants and canonical splice sites (±1–2 nucleotides), which can inadvertently filter out pathogenic intronic variants. Coverage drop-off outside exon targets further reduces the likelihood of detecting near-exon aberrant RNA (NEAR) or deep-intronic variants [32]. For example, in pedigree #22, a single ABCA4 variant (c.4363T>C, p.Cys1455Arg) was identified on initial pNGS, while single-gene sequencing later revealed a second pathogenic variant (c.4253+43G>A, p.Ile1377Hisfs*3), confirming the diagnosis.

In recessive IRD cases, detection of one likely pathogenic allele provides additional support for a clinical/genetic diagnosis and justifies single-gene sequencing of the candidate locus. Using this approach, 44 percent (n = 4/9) of patients had a second pathogenic variant identified in intronic regions **(Table 2)**. Deep intronic variants activating cryptic splice sites account for up to 5 percent of pathogenic ABCA4 alleles [33, 34], which aligns with our findings: pedigree #21 harbored a novel non-canonical splice site variant, and pedigree #22 carried a NEAR intronic variant.

For the remaining 56 percent of partially resolved cases where second-tier single-gene sequencing did not yield a diagnosis, the initially detected heterozygous variants from pNGS were likely spurious, potentially directing attention to the wrong gene. Notably, in pedigrees resolved via second-tier single-gene testing, the first-tier variants were ACMG class 5 (pathogenic), whereas the unresolved pedigrees contained class 3 (variant of unknown significance) or class 4 (likely pathogenic) variants. Moving forward, only class 5 variants will automatically progress to single-gene sequencing, while variants of lower classification will undergo rigorous in silico analyses to determine whether focused gene re-evaluation or broader testing of IRD-associated genes is warranted. If the initial variant cannot be upgraded to class 5, broader approaches such as WES or WGS are considered more appropriate. Although WGS is highly effective for resolving such cases, its high financial and computational demands currently limit its use to situations where first- or second-tier approaches are insufficient.

Whole exome sequencing (WES) is increasingly being adopted as a primary tool for genetic evaluation of IRDs [29]. One advantage of WES is that it allows the storage of complete exomes, enabling the creation of a ‘virtual panel’ of genes relevant to a specific clinical presentation [35]. This facilitates retrospective re-analysis when new genotype–phenotype correlations are discovered, without requiring re-sequencing. Additionally, WES data can later be interrogated with alternate gene panels for other organ systems (e.g., cardiac, neurological) if new clinical features emerge, and it can support determination of population-specific variant frequencies. While sequencing costs are incurred upfront, the data can be securely archived for future clinical or research use. Published WES studies report a 49–63% diagnostic yield in IRD cohorts [22, 29, 36, 37], highlighting that the cost-efficiency advantage of pNGS remains relevant for first-tier testing, as in our workflow [4, 5]. Although panel-based methods limit re-analysis with expanded virtual panels, this is less of a concern for IRDs, given that most associated genes have already been identified, with relatively few novel gene discoveries in recent years [1].

Exon-focused analysis, such as pNGS, resolves approximately 70–80% of IRDs, whereas deep intronic variants account for 1.4–25% and copy number variants (CNVs) up to 9%, which are generally missed by short-read sequencing [5, 33, 38, 39]. Implementing WGS as a first-line diagnostic tool is currently impractical due to high cost, extensive bioinformatic requirements, and only marginal gains in diagnostic yield over pNGS [28]. WGS may also detect numerous rare non-pathogenic polymorphisms, potentially complicating the determination of the causative genotype and delaying accurate molecular/clinical diagnosis. However, as with WES, storing WGS data digitally allows for future virtual panel analyses and incorporation of newly discovered gene associations. First-tier WGS also demands substantial data-processing infrastructure, potentially

requiring artificial intelligence pipelines for timely clinical interpretation [40], and may reveal significant secondary findings unrelated to IRD (e.g., cancer predisposition genes) [10, 41]. Nevertheless, results from the UK 100,000 Genomes Project indicate that second-tier WGS can achieve >40% diagnostic yield in previously unresolved heritable ophthalmic cases, addressing non-coding, structural, mitochondrial variants, and regions with insufficient exon coverage [8, 42].

When considering cost-effectiveness in IRD genetic testing, it is important to account for not only sequencing expenses but also supporting resources, including genetic counsellors, MDT time, molecular genetics staff, and clinic usage. Broader techniques like WGS increase the likelihood of non-diagnostic or potentially misleading findings, which require reporting and interpretation [10, 16, 41]. Utilizing first-tier WGS in smaller health services may divert limited resources and reduce the number of patients that can be assessed. Consequently, pNGS remains the most efficient approach for maximizing diagnostic yield in small- to medium-sized countries, with broader approaches reserved for unresolved cases. Large-scale collaborative WGS initiatives are likely to develop optimized condition-specific algorithms and standardized strategies for managing secondary findings. WGS may eventually replace pNGS as the first-tier sequencing method for IRDs globally once costs decrease and infrastructure and interpretive guidelines improve.

### *Implications for gene therapy*

Accurate molecular diagnosis is increasingly crucial as gene therapy trials expand for a growing number of IRD etiologies **(Table 4)**. In this cohort, only a small fraction of patients (15.6%, n = 5) were eligible for forthcoming RPGR-targeted gene therapy trials (NCT03316560, NCT03252847, NCT03116113), which require specific genotypes for enrollment. Establishing a validated genetic diagnosis also facilitates precise genetic counselling and family planning, which is particularly relevant for IRD patients of reproductive age, including options for prenatal and pre-implantation genetic testing [2, 3].

**Table 4.** List of ongoing IRD gene therapy clinical trials.

| Gene | NCT Number | Technique | Phase | Status |
|---|---|---|---|---|
| **Rod-Cone Dystrophies** | | | | |
| MERTK | NCT01482195 | AAV | 1/2 | Completed |
| PDE6B | NCT03328130 | AAV | 1/2 | Recruiting |
| RHO | NCT04123626 | AON | 1/2 | Recruiting |
| RPGR | NCT03252847 | AAV | 1/2 | Completed |
| | NCT03116113 | AAV | 1/2 | Completed |
| | NCT03316560 | AAV | 1/2 | Recruiting |
| RLBP1 | NCT03374657 | AAV | 1/2 | Recruiting |
| USH2A | NCT03780257 | AON | 1/2 | Not recruiting |
| MYO7A | NCT01505062 | LV | 1/2 | Terminated |
| **Macular/Cone Dystrophies or Cone Dysfunction Syndromes** | | | | |
| RS1 | NCT02416622 | AAV | 1/2 | Terminated |
| | NCT02317887 | AAV | 1/2 | Recruiting |
| ABCA4 | NCT01367444 | LV | 1/2 | Terminated |
| CNGB3 | NCT03001310 | AAV | 1/2 | Completed |
| | NCT02599922 | AAV | 1/2 | Recruiting |
| CNGA3 | NCT03758404 | AAV | 1/2 | Completed |
| | NCT02935517 | AAV | 1/2 | Recruiting |
| | NCT02610582 | AAV | 1/2 | Recruiting |
| **Leber Congenital Amaurosis** | | | | |
| RPE65 | NCT02781480<br>NCT02946879 | AAV | 1/2 | Recruiting |

| | | | | |
|---|---|---|---|---|
| | NCT00643747 | AAV | 1/2 | Completed |
| | NCT01496040 | AAV | 1/2 | Completed |
| | NCT00821340 | AAV | 1 | Completed |
| | NCT00749957 | AAV | 1/2 | Completed |
| | NCT00481546 | AAV | 1 | Completed |
| GUCY2D | NCT03920007 | AAV | 1/2 | Recruiting |
| CEP290 | NCT03913143 | AON | 3 | Not recruiting |
| | NCT03872479 | Gene editing | 1/2 | Recruiting |
| | **Choroidal Dystrophies** | | | |
| | NCT02341807 | AAV | 1/2 | Completed |
| | NCT02671539 | AAV | 2 | Completed |
| | NCT01461213 | AAV | 1/2 | Completed |
| | NCT02077361 | AAV | 1/2 | Not recruiting |
| CHM | NCT02553135 | AAV | 1/2 | Completed |
| | NCT03507686 | AAV | 2 | Completed |
| | NCT03496012 | | 3 | Completed |
| | NCT02407678 | AAV | 2 | Completed |
| | NCT04483440 | AAV | 1 | Recruiting |

AAV = adeno-associated virus. AON = antisense oligonucleotide. LV = lentiviral vector NCT = Reference number for study on clinicaltrials.gov.

### *Limitations*

The current study was conducted during the global SARS-CoV-2 pandemic, which limited some patients' ability or willingness to attend in-person clinical assessments, potentially affecting the overall power of the genetic investigations and the final resolution rate. Two patients had passed away prior to reassessment, precluding further genetic analysis. Trio WES could not be performed for five patients due to the unavailability of relevant family members (e.g., deceased, residing abroad, or unwilling to participate during the pandemic). Given the rarity of IRDs, even a national cohort exceeding 1,000 patients does not provide sufficient statistical power to draw definitive conclusions regarding the optimal genetic testing approach for individual genes or variants. The strongest support for initial pNGS remains its cost-effectiveness and high diagnostic yield, allowing resources to be strategically allocated to more expensive 2nd-tier genomic techniques in cases where phenotypic reassessment has refined the clinical focus.

## Conclusion

Second-tier genetic testing resolved 56 percent of previously unresolved pedigrees with pathogenic variants in IRD-associated genes, raising the overall genetic resolution rate to 92% (388/423). The application of 2nd-tier testing should be informed by comprehensive clinical reassessment—including multimodal imaging, electrophysiology, and detailed pedigree analysis—as well as prior genetic findings such as single alleles in autosomal recessive disease, to achieve molecular diagnoses efficiently and cost-effectively. Thorough phenotyping of pedigrees also allows non-IRD cases (e.g., AMD, uveitis) to be redirected to appropriate care pathways, preventing unnecessary genetic investigations. The apparent plateau in sequencing resolution observed internationally may reflect the inclusion of patients with non-inherited conditions; refining diagnostic accuracy has the potential to improve resolution rates for truly inherited retinal degenerations.

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Informed consent was obtained from all subjects involved in the study.

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