Characterization of macular structure and function in two Swedish families with genetically identified autosomal dominant retinitis pigmentosa

Wissam Abdulridha-Aboud, Ulrika Kjellström, Sten Andréasson, Vesna Ponjavic

Department of Ophthalmology, University of Lund, Lund, Sweden

Purpose: To study the phenotype in two families with genetically identified autosomal dominant retinitis pigmentosa (adRP) focusing on macular structure and function.

Methods: Clinical data were collected at the Department of Ophthalmology, Lund University, Sweden, for affected and unaffected family members from two pedigrees with adRP. Examinations included optical coherence tomography (OCT), full-field electroretinography (ffERG), and multifocal electroretinography (mfERG). Molecular genetic screening was performed for known mutations associated with adRP.

Results: The mode of inheritance was autosomal dominant in both families. The members of the family with a mutation in the PRPF31 (p.IVS6+1G>T) gene had clinical features characteristic of RP, with severely reduced retinal rod and cone function. The degree of deterioration correlated well with increasing age. The mfERG showed only centrally preserved macular function that correlated well with retinal thinning on OCT. The family with a mutation in the RHO (p.R135W) gene had an extreme intrafamilial variability of the phenotype, with more severe disease in the younger generations. OCT showed pathology, but the degree of morphological changes was not correlated with age or with the mfERG results. The mother, with a de novo mutation in the RHO (p.R135W) gene, had a normal ffERG, and her retinal degeneration was detected merely with the reduced mfERG.

Conclusions: These two families demonstrate the extreme inter- and intrafamilial variability in the clinical phenotype of adRP. This is the first Swedish report of the clinical phenotype associated with a mutation in the PRPF31 (p.IVS6+1G>T) gene. Our results indicate that methods for assessment of the central retinal structure and function may improve the detection and characterization of the RP phenotype.

Of the 23 known genes that play a major role in the pathogenesis of non-syndromic autosomal dominant retinitis pigmentosa (adRP) [1], three have been reported to be the most prevalent: the rhodopsin gene (RHO; Gene ID: 10692, OMIM: 605224), which causes approximately 26%; RP1 (Gene ID: 6101, OMIM: 603937), which causes 6%; and the gene for pre-mRNA processing factor 31 (PRPF31; Gene ID: 26121, OMIM: 606419), which causes 5% of adRP in the United States [2]. In Europe, the prevalence is lower for mutations in RHO (16.5%) [3] and higher for mutations in PRPF31 (6.7%), compared to the American population [4]. In the Swedish population, RHO mutations have been found previously [5-13], but no PRPF31 mutations have been reported. The only previous report concerning PRPF in Sweden [7] describes a large genomic deletion with almost entire loss of the PRPF31 gene, and no characterization of the associated clinical phenotype has been presented.

Variations in the clinical phenotype are frequent in adRP, particularly in the case of PRPF31 mutations [14-19] and in RHO mutations [3,15,20,21]. Genetic modifiers have been discussed as a cause of the phenotypical variability. CNOT3 (Gene ID: 4849, OMIM: 604910) belongs to the Ccr4-Not complex, a conserved multiprotein structure involved in the regulation of gene expression, and has recently been found as a modifier in PRPF31-associated adRP [17], thus explaining the incomplete penetrance observed in families with this genotype. In addition, in families with RHO mutations, a modifier (ROM1; Gene ID: 6094, OMIM: 180721) has been suspected to cause the extreme intrafamilial variability in the clinical phenotype, as previously reported in a Swedish family with adRP [22]; however, this could not be verified as it is known that ROM1 is non-pathogenic in the single heterozygous state.

Mutations in GUCY2D (Gene ID: 3000, OMIM: 600179) are most frequently associated with autosomal recessive retinal degeneration, such as Leber congenital amaurosis (LCA) [23,24]. However, cone dystrophy [25-28] and cone-rod dystrophy [28,29] have been reported previously in association with this genotype. Recently, mutations in GUCY2D have also been associated with central areolar choroidal dystrophy (CACD) [30]. It has been shown that in one family
with RPE65 (Gene ID: 6121, OMIM: 180069)-related LCA, a missense mutation in GUCY2D acts as a genetic modifier, causing a phenotypic variability in two siblings [31]. Thus, mutations in GUCY2D may either cause hereditary retinal disease or modify the expression of other disease-causing genes. However, other studies have shown that this single nucleotide polymorphism (SNP) is highly prevalent in a normal population as well [32], and therefore, the presence of this SNP does not automatically imply a modifying quality.

Standardized full-field electroretinography (ffERG) is still the conventional method in RP diagnostics. It is highly sensitive in detecting early photoreceptor disease and in selectively assessing the total response from rods and cones. However, during the past decade, assessment of central retinal function and diagnostic imaging methods have been used more frequently in RP diagnostics [33-36]. Multifocal electroretinography (mfERG) is used for the assessment of central retinal function, and optical coherence tomography (OCT) is used to evaluate macular structure, preferably combining both for accurate estimation of macular function [33]. These methods are non-invasive, are not time-consuming, and are usually easily performed in most patients with RP, including adolescents. As diagnostic methods to supplement the ffERG, they offer detailed and objective information about central retinal structure and function, which may improve detection and characterization of the clinical phenotype in RP.

The aim of the present study was to characterize the clinical phenotype in two Swedish families with a similar (ad) inheritance pattern but with two different disease-causing mutations: PRPF31 (p.IVS6+1G>T) and RHO (p.R135W), by assessing total retinal function and macular structure and function.

METHODS

The study was approved by the Ethics Committee of Lund University, Sweden. The research procedures were performed in accordance with institutional guidelines and according to the tenets of the Declaration of Helsinki. Informed consent was obtained from the participants.

Subjects: Twelve subjects from two families were studied. Family A, with a mutation in the PRPF31 (p.IVS6+1G>T) gene, included affected family members from four generations. Five family members were examined at our department. Family B, with mutations in the RHO (p.R135W) gene, included seven family members from four generations, and all were examined at our department.

Methods:

Ophthalmological examination—The patients underwent a thorough ophthalmological examination, including assessment of best corrected visual acuity (VA) using a Snellen chart, slit-lamp biomicroscopy, and ophthalmoscopy. Fundus photographs were obtained with a Topcon 3D OCT-1000 (Topcon, Inc., Paramus, NJ). Kinetic perimetry was performed with a Goldmann perimeter using standardized objects I4e and V4c.

Full-field electroretinography: Standardized ffERGs were recorded in a Nicolet analysis system (Nicolet Biomedical Instruments, Madison, WI), as described previously [37]. The examinations were conducted according to the ERG standards of the International Society for Clinical Electrophysiology of Vision (ISCEV) [38]. In one subject (I-2, Family B) the ffERG was recorded with an Espion E2 analysis system (Diagnosys; LLC, Lowell, MA).

Multifocal electroretinography: MfERGs were recorded with a Visual Evoked Response Imaging System (VERIS Science 6; EDI, San Mateo, CA) using settings that adhere to the ISCEV guidelines [39]. The stimulus matrix consisted of 103 hexagonal elements, scaled with eccentricity to elicit approximately equal amplitude responses at all locations. Each hexagon independently alternated between black and white according to a pseudorandom binary m-sequence at 75 Hz. The pupils were maximally dilated with cyclopentolate 1% and 10% phenylephrine. Retinal activity was registered using a Burian-Allen bipolar ERG contact lens electrode that was placed on the anesthetized (oxybuprocaine) cornea. Fixation was monitored with an IR eye camera. The first order component of the mfERG was analyzed regarding amplitudes (A) and implicit times (IT) of the first positive peak (P1) within the five concentric rings around the fovea (Rings 1–5). Ring 1 is composed of the summed responses from the central and surrounding five hexagons.

Optical coherence tomography: Retinal thickness was measured with a Topcon 3D OCT-1000 (Topcon, Inc., Paramus, NJ) as described previously [40]. The retinal thickness map contains three circles with a diameter of 1, 3, and 6 mm. The inner ring (1) of the OCT corresponds to the central zone of the mfERG (Ring 1 including the central and surrounding five hexagons) [41]. The second ring of the OCT (2) corresponds to the second full ring of the mfERG, and the third ring of the OCT (3) corresponds to the third full ring of the mfERG. To measure the retinal nerve fiber layer (RNFL) thickness of the optic disc, scan mode “2D circle scan” was used as formerly described [37].
DNA analyses: Blood samples for molecular genetic analysis were drawn from as many family members as possible, including those not affected with RP. The blood samples were screened for known mutations in adRP. Screening for mutations was performed at Asper Biotech using the genotyping microarray (Asper Ophthalmics, Tartu, Estonia). The following genes were screened: \textit{CA4} (Gene ID: 762, OMIM: 114760), \textit{FSCN2} (Gene ID: 6121, OMIM: 25794), \textit{IMPDH1} (Gene ID: 3614, OMIM: 146690), \textit{NRL} (Gene ID: 4901, OMIM: 162080), \textit{PRPF3} (Gene ID: 9129, OMIM: 607301), \textit{PRPF8} (Gene ID: 10594, OMIM: 607300), \textit{PRPF31}, \textit{RDS} (Gene ID: 5961, OMIM: 179605), \textit{RHO}, \textit{ROM1}, \textit{RPI}, \textit{RP9} (Gene ID: 6100, OMIM: 607331), \textit{CRX} (Gene ID: 1406, OMIM: 602225), \textit{TOPORS} (Gene ID: 10210, OMIM: 609507), \textit{NPR/NR2E3} (Gene ID: 10002, OMIM: 604485), \textit{KLHL7} (Gene ID: 55975, OMIM: 611119), and \textit{CUGY2D}. The identified mutations were verified with sequencing.

RESULTS

Genotype: The pedigrees of the two families are presented in Figure 1. The genotype is shown in parentheses after the pedigree ID, in all subjects with a known genotype.

Family A included eight family members affected with RP from four generations. Five of these patients were examined at our department, and they all had the same mutation in \textit{PRPF31} (p.IVS6+1G>T). The proband (III-4) is indicated in the pedigree with a red arrow. The proband’s youngest child (IV-3), a 3-year-old boy, was diagnosed with RP at our department through genetic screening and had a history of night blindness from birth, but he was too young to participate in the clinical examinations.

Family B included seven family members from four generations (Figure 1). The proband (III-1) is indicated in the pedigree with a red arrow. The grandmother (I-1) had no clinical signs of RP and a normal genotype. The grandfather (I-2) had a mutation in \textit{GUCY2D} (p.P701S) but not in \textit{RHO} (p.R135W). The mother (II-1) and all of her three children (III-1, III-2, and III-3) had mutations in \textit{RHO} (p.R135W) and in \textit{GUCY2D} (p.P701S). These children had three different fathers who were not available for inclusion in the study. The proband’s daughter (IV-1) had a mutation in \textit{RHO} (p.R135W) but no mutation in \textit{GUCY2D} (p.P701S).

 Phenotype:

Visual fields—The results from visual field testing of both families are presented in Figure 2. In Family A, all affected individuals had residual visual fields; however, they were severely constricted in older generations. Further, the visual field defect was characteristic for the RP phenotype with ring scotoma (remaining peripheral islands and a preserved central visual field). In patient IV-5 (21 years of age at examination), there was severe constriction of the visual field when tested with I4e, which is frequently associated with early stages of RP. The youngest affected patient (IV-3) was not examined with kinetic perimetry because of the patient’s young age at the time of examination (3 years).

In Family B, the visual field testing showed the opposite result with a completely normal result in the affected mother (II-1) while two of her children (III-1 and III-3) had small remaining visual fields with preserved central function. Her younger daughter had severe constriction of the visual field.

![Figure 1. Pedigree and genotype. A: Schematic pedigree for Family A with the PRPF31 (p.IVS6+1G>T) mutation. B: Schematic pedigree for Family B with the RHO135 (p.R135W) mutation. Circles indicate females. Squares indicate men. Filled symbols indicate affected individuals. Red arrow = proband. () Genotype is shown in parentheses after pedigree ID: (+) = mutation identified, (-) = no mutation identified, * Assessed with ERG, ● Mutation in GUCY2D (p.P701S).](image-url)
when tested with I4e. The youngest affected patient (IV-1) in Family B was not examined with kinetic perimetry because of the patient’s young age at the time of examination (1 year).

**Fundus appearance:** The fundus photographs from both families are presented in Figure 3. Family A showed a fundus appearance characteristic of the RP phenotype with thinning of retinal vessels, pigmentary degeneration in the periphery, and a relatively normal macula, at least in the young individuals.

Family B showed the opposite, with normal fundi in the older generation, while the young patients had degenerative changes at an early age. Further, the pathology in the fundus appearance was mainly located in the central parts of the fundus, with a loss of pigmentation around the fovea and normal pigmentation in the periphery.

**Multifocal electoretinography:** Results of the mfERG examinations for Family A are presented in Figure 3, and the amplitudes and implicit times for Ring 1 of the mfERG registrations are presented in Table 1. A small remaining foveal peak was identified (most distinct in the younger individuals, III-4 and IV-5), but severe perifoveal deterioration was demonstrated in all subjects. There was a good correlation between the mfERG results and the OCT measurements. The deterioration of the macular structure and function were correlated with increasing age and duration of disease.

Results of the mfERG examinations for Family B are presented in Figure 3 and Figure 4, and the amplitudes and implicit times for Ring 1 of the mfERG registrations are presented in Table 1. The three affected children (III-1, III-2, and III-3) showed a small remaining foveal peak, but severe perifoveal deterioration, in contrast to their mother (II-1), who had a relatively preserved mfERG response from the central fundus and a slight parafoveal reduction. For Family

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**Figure 2. Phenotype.** A: Visual fields in Family A with the **PRPF31 (p.IVS6+1G>T)** mutation, demonstrating an age-correlated loss of visual field. B: Visual fields in Family B with the **RHO135 (p.R135W)** mutation, showing normal visual fields in patient II-1 and variable loss in the visual field in her three children.
Figure 3. Phenotype. The top figure shows fundus photos, optical coherence tomography (OCT) and multifocal electroretinography (mfERG) results for the family with the *PRPF31* (p.IVS6+1G>T) mutation, demonstrating affected patients from three generations and increasing retinal pathology with increasing age. The bottom figure shows fundus photos, OCT, and mfERG results for the family with *RHO135* (p.R135W) mutations, demonstrating marked intrafamilial variability in the clinical phenotype, regarding retinal structure and function. For patient III-2, the mfERG is from her left eye.
B, no significant correlations were found between the mfERG parameters and the OCT measurements.

**Optical coherence tomography:** The results of the OCT macular examinations for Family A are presented in Figure 3. All three affected individuals showed a similar pattern of the OCT retinal thickness map with severe retinal attenuation in the two outer circles (2 and 3) but retinal thickness within normal limits in the central circle (Ring 1). Concerning the RNFL thickness around the optic nerve all individuals in the family showed normal results for the temporal segment, corresponding to the macular region. For the other segments, the pattern was variable with normal thickness in the youngest generation (IV-5), slight attenuation (superior segment) in the middle generation (II-4), and more widespread attenuation (superior, nasal, and inferior segment) in the oldest generation (II-1).

The results of the OCT macular examinations for Family B are presented in Figure 3 and Figure 4, and they show a more variable pattern. Patient I-2, who carried only the GUCY2D (p.P701S) mutation, had normal retinal thickness in all segments of the macular map. The subjects with mutations in both RHO (p.R135W) and GUCY2D (p.P701S) showed normal retinal thickness for the inner circle (Ring 1), in all subjects but III-2, who instead showed increased central retinal thickness compared to the normal material of the OCT. For the outer circles (2 and 3), no consistent pattern concerning retinal thickness was found. The proband (III-1) showed attenuated retina in all but one of the outer segments. Only the nasal segment of the second circle was within normal limits. Patient III-2 instead showed increased retinal thickness in one segment of circle 3 (inferior) and normal retinal thickness in the rest of the segments. In patient II-1, two segments were attenuated, and in patient III-3, one segment showed attenuation. The peripapillar RNFL was generally thickened in all family members with mutations in RHO (p.R135W) and GUCY2D (p.P701S).

**Full-field electroretinography:** The results of the ffERG examinations are shown in Figure 5. In Family A, all subjects affected with RP had a severely reduced response to stimulation with bright white light. The isolated cone response was also severely reduced in amplitude, and the implicit time was prolonged. The two unaffected children (IV-1 and IV-2) had a normal ffERG. The proband’s youngest child (IV-3) had a mutation in PRPF31 (p.IVS6+1G>T) but was not examined with ffERG.

In Family B, the grandfather’s (I-2) and the mother’s (II-1) ERG responses were normal, while all three children (III-1, III-2, III-3) and the grandchild (IV-1) had severely reduced rod and cone responses. In the case of the mother (II-1), the diagnosis of RP was obtained only through functional analysis of the macula (mfERG) and structural analysis with OCT (macular scan). The genotype was affirmed retrospectively.

### Table 1. Family characteristics.

| Family | Subject (Pedigree ID) | Age (years) | Visual acuity OD/OS | Mutation PRPF31 | Mutation RHO | Mutation GUCY2D | mfERG ring 1 ampl.(mV/deg2) | mfERG ring 1 IT (ms) |
|--------|-----------------------|-------------|---------------------|----------------|--------------|----------------|----------------------------|-------------------|
| A      | II-1                  | 86          | 0.3/0.4             | +              |              |                | 2.5                       | 34.2              |
| A      | III-4                 | 50          | 0.8/0.7             | +              |              |                | 7.5                       | 28.3              |
| A      | IV-5                  | 21          | 1.0/1.0             | +              |              |                | 13.5                      | 24.2              |
| A      | IV-1                  | 8           | 0.9/0.9             | -              |              |                | -                         | -                 |
| A      | IV-2                  | 6           | 0.8/0.8             | -              |              |                | -                         | -                 |
| A      | IV-3                  | 3           | -                   | +              |              |                | -                         | -                 |
| B      | I-1                   | 65          | -                   | -              |              |                | 51.3                      | 29.2              |
| B      | I-2                   | 66          | -                   | -              | +            |                | 25.5                      | 29.2              |
| B      | II-1                  | 45          | 0.7/0.9             | +              | +            |                | 24.1                      | 28.3              |
| B      | III-1                 | 19          | 0.7/0.8             | +              | +            |                | 3.5                       | 30                |
| B      | III-2                 | 17          | 0.6/0.5             | +              | +            |                | 16.1                      | 29.2              |
| B      | III-3                 | 11          | 0.7/0.7             | +              | +            |                | 17.2                      | 29.2              |
| B      | IV-1                  | 1           | -                   | +              | -            |                | -                         | -                 |

Family characteristics of family A with a mutation in PRPF31 (p.IVS6+1G>T), and of family B with a mutation in RHO (p.R135W), showing pedigree ID, age, visual acuity, genotype and results of mfERG, ring 1.
DISCUSSION

To date, more than 50 adRP-associated mutations in PRPF31 have been identified, including missense and nonsense mutations, splicing mutations, deletions and insertions, small indels, and complex mutations [42]. However, to our knowledge, there is only one previous report of the specific mutation PRPF31 (p.IVS6+1G>T) associated with adRP [18]. This novel heterozygous splice-site mutation was associated with a heterogeneous clinical phenotype where only three of nine affected individuals were symptomatic. Our results from Family A show that this specific genotype may cause a more homogeneous clinical phenotype with symptomatic disease in all affected individuals.

This is the first report of the clinical phenotype in a Swedish family with a mutation in PRPF31 causing adRP. Mutations in PRPF31 have been found in up to 10% of patients with adRP throughout the world [1,4] but have not been characterized in a Swedish family previously. It has been shown that some families with this mutation are homogeneous in phenotype [43], while others are exceptionally heterogeneous in phenotype [17,18,44]. In the present study, Family A showed a homogeneous phenotype with a progressive disease course, and there was a positive correlation between the degree of visual loss and age. The homogenous phenotype may indicate an absence of genetic modifiers in this particular family. Previously, CNOT3 has been suggested to be a genetic modifier of clinical expression in PRPF31 mutations [17,44], but this modifying gene was not included.

Figure 4. Phenotype. Patient II-1 with autosomal dominant retinitis pigmentosa (adRP) and a de novo mutations in RHO135 (p.R135W), showing normal fundi, a slightly reduced optical coherence tomography (OCT) scan and a reduced multifocal electroretinography (mFERG). This patients with normal full-field electroretinography (fFERG) and visual fields shows the importance of assessing central retinal structure and function in the diagnostics of RP.
Figure 5. Phenotype. Results of full-field electroretinography. Family A with the PRPF31 (p.IVS6+1G>T) mutation showed a severely reduced rod and cone response in the affected individuals (II-1, III-4, and IV-5), while the two unaffected children (IV-1 and IV-2) had normal full-field electroretinography (ffERG). Family B with the RHO135 (p.R135W) mutation showed in the unaffected grandparent (I-2) and in the mother (II-1) normal rod and cone responses, while all three affected children (III-1, III-2, and III-3) and the grandchild (IV-1) had a severely reduced rod and cone response.
in the genetic analyses in our study. We conclude that the homogeneity of the phenotype supports a monogenic pathogenesis and is therefore sufficient in the screening procedure, eliminating the need for further and more extensive genetic evaluations in search of genetic modifiers.

Mutations in RHO are well-known and are frequently found in Sweden. Phenotypic heterogeneity has been reported in RHO (p.R135W)-associated adRP [3,12,15,20,21]. The p.R135W of RHO is known as a “Class A” rhodopsin mutation that shows severe degeneration. In contrast, our study shows that an individual with this mutation can be only slightly affected, with a normal ffERG and with normal visual fields, which has not been reported previously (Family B).

The p.P701S of GUCY2D has been studied previously, and an association has been suggested with LCA, CORD, and CACD [31,45]. Furthermore, one research group has reported a missense mutation in GUCY2D that acts as a genetic modifier in RPE65-related LCA [31]. However, some studies have shown that this SNP is highly prevalent in a normal population as well [32]. The role and significance of GUCY2D (p.P701S) in this family are not clear. We initially suspected that a mutated GUCY2D could be a genetic modifier of the clinical expression, causing a mild RP phenotype in the affected mother (II-1). However, the mother and her three children had the same genotype, despite the heterogeneity of the phenotype; therefore, we cannot conclude that this mutation has a modifying effect in this family. There may be additional modifying mutations that we have not found. We conclude that whenever heterogeneity of the phenotype is obvious, in a family with a known genotype, further and more extensive genetic evaluation should be performed to try to find a possible genetic modifier.

An important finding in this study is the obvious diversity of the clinical phenotype in adRP. These two families, both affected with adRP, showed extreme inter- and intrafamilial variability in the clinical phenotype regarding pedigree, visual acuity, visual fields, macular structure, and macular function. Determination of genetic pathogenesis using DNA analysis (mutation screening methods) is important; however, this study indicates that even a profound knowledge of genotype does not fully explain the clinical phenotype regarding the patient’s visual dysfunction. Assessment of macular function may detect RP in patients who present without any symptoms of retinal degeneration and who appear completely unaffected on clinical examination. In Family B, the mother (II-1), who had a de novo mutation in the RHO (p.R135W) gene, had normal fundi, visual fields, and fERG. However, the mFERG was reduced, and the OCT slightly attenuated, indicating a retinal disease that was later confirmed with genetic analysis. What initially appeared to present as autosomal recessive retinal pigmentosa (arRP) was found to be adRP. What seemed to be an unaffected subject (the mother, II-1) was actually a patient with RP and genetically identical to her three children. The diagnosis of RP could easily have been missed in this mildly affected mother of three severely affected children. The extensive investigation including genetic and phenotypic analyses (with methods assessing total and central structure and function) was essential for the detection of her eye disease.

A possible explanation for the mild phenotype in the mother (II-1) in Family B may be mosaicism, a characteristic we did not test for specifically, as it was not the primary purpose of the study. Not testing for mosaicism is however a limitation of our study. If present, mosaicism would indicate that this de novo mutation has occurred post-zygotically. In particular, it could have occurred during the early embryonic development of II-1 and affect progenitor cells for large areas of her adult body, including the ovaries and the bone marrow, but not the retinas. Mosaicism as a cause of phenotypical heterogeneity has been reported previously in families with RP [46-51], and it may have caused the extremely mild retinal disease in our patient. Another limitation of the study is the fact that all family members were not included in the study. In Family A, patient III-6, the affected mother of patient IV-5, declined to participate, and the proband’s son, patient IV-3 (3 years), was too young perform visual field testing and ERG. In Family B, the three different fathers did not wish to participate, and therefore, we could not exclude contribution of different modifying genes from each father. The youngest child in Family B (patient IV-1) was only 1 year old and was tested for mutations and with ffERG but not with mfERG and OCT. We had no access to longitudinal data for either family. Last, we could not examine wide-field autofluorescence, which is another diagnostic method that may be of value in the assessment of hereditary retinal diseases.

In summary, adRP may show extreme inter- and intrafamilial variability in the clinical phenotype in the central visual structure and function, suggesting that OCT and mfERG are valuable additional methods of investigation in RP diagnostics and detection, supplementing conventional ffERG and visual field testing. The clinical phenotype cannot always be sufficiently explained by what we know about the genotype and may be evaluated further using these methods.
ACKNOWLEDGMENTS

We thank Ing-Marie Holst and Boel Nilsson for skillful technical assistance. The study was supported by grants from Stiftelsen Synfrämjandets Forskningsfond, Cronquists Stiftelse, The Swedish Society of Medicine, Kronprinsessan Margaretas Arbetsnämnd, Torsten och Ragnar Söderbergs Stiftelse, the Swedish Medical Research Council (project no. 2007–3385), Stiftelsen för synskadade i före detta Malmöhus län and the Faculty of Medicine at the University of Lund, Sweden. The funding organizations had no role in the design or conduct of this research.

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