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Perspective

Genetic testing for retinal dystrophies and dysfunctions: benefits, dilemmas and solutions

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Abstract

Human retinal dystrophies have unparalleled genetic and clinical diversity and are currently linked to more than 185 genetic loci. Genotyping is a crucial exercise, as human gene-specific clinical trials to study photoreceptor rescue are on their way. Testing confirms the diagnosis at the molecular level and allows for a more precise prognosis of the possible future clinical evolution. As treatments are gene-specific and the 'window of opportunity' is time-sensitive; accurate, rapid and cost-effective genetic testing will play an ever-increasing crucial role. The gold standard is sequencing but is fraught with excessive costs, time, manpower issues and finding non-pathogenic variants. Therefore, no centre offers testing of all currently 132 known genes. Several new micro-array technologies have emerged recently, that offer rapid, cost-effective and accurate genotyping. The new disease chips from Asper Ophthalmics (for Stargardt dystrophy, Leber congenital amaurosis [LCA], Usher syndromes and retinitis pigmentosa) offer an excellent first pass opportunity. All known mutations are placed on the chip and in 4 h a patient's DNA is screened. Identification rates (identifying at least one disease-associated mutation) are currently ~70% (Stargardt), ~60–70% (LCA) and ~45% (Usher syndrome subtype 1). This may be combined with genotype–phenotype correlations that suggest the causal gene from the clinical appearance (e.g. preserved parafoveolar retinal pigment epithelium suggests the involvement of the *CRB1* gene in LCA). As ~50% of the retinal dystrophy genes still await discovery, these technologies will improve dramatically as additional novel mutations are added. Genetic testing will then become standard practice to complement the ophthalmic evaluation.

Key words: CEP290, gene therapy, Leber congenital amaurosis, retinal dystrophies, Retinitis pigmentosa, RPE65.

Introduction

Human retinal dystrophies and dysfunctions are a common group of inherited retinal diseases that are genetically complex, and exhibit significant clinical overlap between the different types. Retinal dystrophies lead to photoreceptor death or dysfunction and blindness with a profound impact on the individual and society, as the blindness is lifelong and currently irreversible. Over the past decade, our understanding of the causes and consequently the potential therapies for retinal dystrophies has dramatically improved. The purpose of this review is to highlight these new insights, by illustrating the importance of diagnosing retinal dystrophies through genetic testing. We will also discuss the daunting task of this endeavour, provide several possible solutions to this problem with the aid of new diagnostic technology and discuss several spectacular treatment successes in animal models with retinal dystrophies.

> With this profound new knowledge, humankind is on the verge of gaining immense new power to heal. Genome science will have a real impact on all our lives, and even more on the lives of our children. It will revolutionize the diagnosis, prevention, and treatment of most, if not all, human diseases (June 26, 2000, President Bill Clinton, announcing the completion of the first draft of the human genome).

Despite the unparalleled advances made by and as a result of the human genome project, sadly, the practical impact of new retinal gene discoveries have had little impact on the general ophthalmic community, thus far. Is it possible that

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expectations have been raised unreasonably? Are we to expect a real practical impact of molecular diagnostics in our practices in the near future? Or are there unforeseen or underestimated problems in bringing genetic diagnostic data from the bench to the bedside?[1] We will identify some of the problems in bringing genotype information from the bench to the bedside, and suggest several solutions that may facilitate this important process. First we will show with four concrete examples, the utility of genetic testing in the management of blind patients, as it improves the diagnostic and visual prognostic capabilities for the practising ophthalmologist, while at the same time identifies disease pathways for the vision scientist. Second, we will discuss the state of the art micro-array technology including the latest disease chips by Asper Ophthalmics for Stargardt macular dystrophy, Leber congenital amaurosis (LCA), autosomal recessive retinitis pigmentosa (ARRP), autosomal dominant retinitis pigmentosa (ADRP) and Usher syndrome. We will then discuss the gene specific nature of the successful gene replacement.

Genetic testing and determining a molecular diagnosis of the complex group of diseases such as the retinal dystrophies allows for a more *specific characterization of the disease* than the clinical phenotyping can provide. At the same time it provides the ophthalmologist and involved families an estimate of the *probable clinical course of the disease*. We will now discuss seven purposes for doing genetic testing as it aids ophthalmologists in the clinical management of retinal dystrophy patients and facilitates vision scientists in their quest to identify novel disease genes.

1. Improve diagnostic accuracy
2. Provide prognostic information
3. Establish a genotype–phenotype correlation system, in order to suggest the causal gene from the retinal phenotype
4. Identify new retinal pathways
5. Provide prenatal screening
6. Identify new genes
7. Guide therapy

Background information

Inherited retinal degenerations, such as adult onset retinitis pigmentosa (RP), congenital onset Leber congenital amaurosis (LCA), complete achromatopsia and congenital stationary night blindness (CSNB), are a highly heterogeneous, currently untreatable group of human diseases of the photoreceptors that lead to blindness. The genetic and clinical diversity is unparalleled in Mendelian human diseases as more than 185 chromosomal loci have now been identified, harbouring retinal degeneration genes and over 132 of these genes have now been cloned.[2] It is speculated that these 185 loci harbour approximately 50% of the genetic defects in patients, so that the remaining 50% of genes still remain to be identified. The retinal gene discoveries have led to new insights into disease mechanisms, which in turn has led to cautious optimism regarding retinal cell rescue. Therapies with gene replacements, neurotrophic factor administration and several pharmacological agents have been shown to rescue photoreceptors in animal models of retinal dystrophies, predicting human clinical trials in the near future.[3–5] This enthusiasm has been tempered somewhat by the realization of the magnitude of the genotyping endeavour and its tribulations. The current genotyping dilemma can be divided into genetic and clinical problems. Genetically, the problems are the unparalleled genetic diversity (>132 retinal genes and counting), the multiple inheritance patterns (autosomal dominant, autosomal recessive, X-linked, digenic and mitochondrial), the number of mutations per gene (e.g. >100 rhodopsin mutations), the possible existence of modifier alleles, and the finding of non-disease causing genetic variants (polymorphisms). Clinically, the difficulties are the overlapping symptoms and signs of the genetically distinct entities and the intra- and inter-familial variability, even in patients with the same mutation and gene. To illustrate the complexity of genetic testing: a simplex male patient with RP could carry one autosomal dominant, one X-linked mutation, or two autosomal recessive mutations, in any of the currently known 37 RP genes, which would require screening (sequencing) of almost 500 exons (DNA regions that are coded into the protein), representing ~120.000 base pairs, an almost impossible task.

In a study by Weiss and Biersdorf,[6] the authors found that patients with retinal dystrophies and congenital blindness visit on average seven ophthalmologists before the final diagnosis is made. This final diagnosis is usually made in the teenage years. However, with carefully chosen genetic testing (as we will illustrate), retinal dystrophies can be diagnosed before the age of 1 year.

Clinical and genetic overview of LCA, achromatopsia, CSNB and RP

Leber congenital amaurosis, complete achromatopsia and CSNB are three types of congenital retinal dystrophies (dysfunctions) that overlap clinically, as all patients present in early childhood with visual impairment and nystagmus. LCA is predominantly an autosomal recessive entity and can be caused by mutations in nine genes, which encode proteins that participate in a wide variety of retinal functional pathways. LCA-associated proteins participate in phototransduction (GUCY2D), vitamin A metabolism (RPE65, RDH12), photoreceptor development (CRX), photoreceptor morphology and retinal architecture (CRB1), biosynthesis and farnesylation of cGMP phosphodiesterase (PDE) (AIPL1), GTP biosynthesis (IMPDH1), and protein trafficking (RPGRIP1, CEP290). LCA genes at chromosomal loci 6q11, 14q24 and 1p36 remain to be identified. The nine currently known LCA genes account for ~60% of the cases, while genes underlying the remaining 40% of cases await

discovery. LCA represents the most severe entity in the retinal dystrophy spectrum and the visual prognosis is poor. Patients present early in life (at around 6 weeks of age) with nystagmus, poor fixation and vision, amaurotic pupils, oculodigital behaviour, a non-detectable ERG and a spectrum of retinal appearances, ranging from essentially normal to a diversity of pigmentary changes.[7,8]

In contrast, complete achromatopsia is a rare autosomal recessive disease of the cone system, and three causal genes (*CNGB3*, *CNGA3* and *GNAT2*) have been identified. All three genes encode proteins that have important functions in the phototransduction cascade. Symptoms and signs of this disease overlap substantially with LCA and CSNB. Symptomatically, patients with complete achromatopsia typically present with striking photoaversion and blepharospasm in the light; while in the dark their eyes open and vision seems to improve. ERG cone responses are non-detectable or severely impaired in complete and incomplete achromatopsia, and the rod-mediated ERG is often normal. In some patients with complete achromatopsia, however, residual cone function can be demonstrated psychophysically. Retinal appearance is essentially normal and acuities are usually 6/60. The most characteristic aspect of the disease and the reason that genetic testing and confirmation of the diagnosis is so essential is that the disease is stationary.

Congenital stationary night blindness is a group of congenital retinal dystrophies with two X-linked genes (*NYX*, *CACNA1F*) and two autosomal recessive genes (*GRM6* and *CABP4*) identified thus far. Mutations in *GRM6* cause autosomal recessive CSNB with a distinctive scotopic 15-Hz flicker electroretinogram,[55] while mutations in *CABP4*, the gene encoding the Ca^{2+}-binding protein 4,[56] also cause autosomal recessive night blindness. CSNB overlaps significantly with LCA and achromatopsia. The X-linked genes encode proteins that are involved in Ca^{2+} ion exchange (CACNA1F) and in cell adhesion, differentiation and migration (Nyctalopin). The *GRM6*-encoded protein, metabotropic glutamate receptor 6, is involved in the signal transmission from the photoreceptors to ON-bipolar cells. CABP4, a member of the calcium-binding protein (CABP) family, is located in photoreceptor synaptic terminals and is directly associated with the C-terminal domain of the Cav1.4 alpha protein. Patients suffer from complete night blindness and may have visual acuity loss (up to 6/60). Retinal appearance is not normal as many patients have significant refractive errors including myopic astigmatism. Again, this group is stationary. The ERG in the X-linked form is also characteristic and consists of a Schubert-Bornschein waveform, which is recognized as an electronegative ERG. This appearance may not be obvious in the first year of life.

In addition to the above challenges with congenital retinal dystrophies, there are clinical and genetic challenges for the group of adult onset retinal dystrophies, namely RP. RP phenotypes are very similar, as overlaps exist for disease onset, severity, progression and retinal appearances. It is therefore still nearly impossible to distinguish the different types of RP from the clinical appearance alone. This may change as more detailed and sophisticated technology is appearing to probe the phenotype (autofluorescence, optical coherence tomography and others). Also this may be easier for LCA as several genotype–phenotype associations have been published and confirmed that allow for the prediction of the gene from phenotypic characteristics. The fact that LCA appears to have genotype–phenotype correlations may be due to the fact that LCA represents a developmental disorder. Coupled with the fact that RP has significant clinical overlap with other retinal diseases (cone and cone-rod dystrophy [CRD], trauma, posterior uveitis and others), and with a variety of syndromes (Bardet–Biedl syndrome, Joubert syndrome, Alström syndrome, Usher syndromes and others), one can see that a new genotyping methodology is necessary. This editorial will start with a discussion of the reasons to perform genetic testing, their benefits and illustrations with actual cases.

The benefits of genetic testing

To secure the diagnosis at the molecular level

Case 1

A baby with a remote family history of LCA presented with poor vision and nystagmus at 4 months. There was a history of tachypnea, acidosis and mild chronic pulmonary disease, psychomotor developmental delay and increased CSF spaces. Eye examinations showed vertical and horizontal nystagmus, inability to fix and follow on faces and small objects and −6.00 D myopia at 4 months. An ERG showed markedly decreased rod and cone responses, compatible with LCA. The retinal appearance was significant for mild optic pallor, vessel narrowing and RPE mottling. LCA mutation screening was negative. At age 2 years the child was protesting in the dark. Repeat ERG at age 2 years was unchanged. Because of the nyctalopia, nystagmus high myopia and the negative LCA gene screening, the diagnosis of CSNB was entertained and the *CACNA1F* gene was screened, which revealed a c.2488C > T which is predicted to lead to a stop mutation p.R830X. At age 3 years the scotopic ERG was electronegative (with the a-wave larger than the b-wave) and the cone ERG was non-detectable. The electronegative ERG was not present on the first and second ERG.

This case illustrates the ability of genetic testing to distinguish between two overlapping retinal dystrophies. In summary, this child was initially diagnosed with LCA, based on the absent visual responses, nystagmus, family history and markedly reduced ERG recordings. Genetic testing strongly suggested CSNB and this was confirmed a year later by the third ERG and the clinical phenotype. This case illustrates the difficulties in making the exact diagnosis of the retinal dystrophy, especially early, at the first time the parents bring the visually impaired child, usually at around age 6 weeks. Implication for recurrence risk calculation is as follows; autosomal recessive LCA has a 25% recurrence rate while X-linked CSNB has a recurrence of 50% in men and 0% in women. LCA is usually progressive, while CSNB is a stationary disease.

Case 2

A 3-month-old boy presented with nystagmus by a paediatric ophthalmologist and was found to fix intermittently, but did not follow, cycloplegic refraction was +4.50 both eyes, the retinal appearance was found to be within normal limits, and the presumptive diagnosis was congenital motor nystagmus, with differential diagnosis LCA, achromatopsia and CSNB. ERG testing revealed a non-detectable rod and cone ERG and LCA was discussed with the parents. A second paediatric ophthalmologist added that the child seemed to like the lights and objected and cried in the dark, and noted narrowing of the retinal arterioles. LCA gene screening was negative. At 6 months improved fixation and following in both the light and dark was reported, and no photoaversion was documented. ERG testing at 9 months was very difficult, but a large mixed rod-cone b-wave (153 μV/121 μV, while >200 V is normal) but essentially a non-detectable cone b-wave could be recorded as the child vehemently protested during the procedure. Examination showed oculodigital sign with mild enophthalmos, and marked arteriolar narrowing with no pigment degeneration was found. In view of the striking mixed rod and cone ERG findings the diagnosis of LCA was not maintained, but in view of the unreliable cone ERG results, a new diagnosis was not yet given. At age 1 year, the child was found to have head bobbing, and striking photoaversion (with shutting of the eyes to slits in the light and opening of the eyes and fixing in the dark). ERG was less difficult, and showed a robust mixed rod-cone signal and a non-detectable cone signals, and the diagnosis of complete achromatopsia was given. Molecular testing identified compound heterozygous *CNGB3* mutations c.1148delC (p.T383fsX) and c.888C > A (p.R283Q) in the β-subunit of the cGMP gated cone channel, confirming the diagnosis of complete achromatopsia.

In this case the ERG was technically difficult and appeared to be non-detectable at age 3 months. At age 1 year, it was found that some development of the ERG had taken place as an essentially normal mixed rod-cone ERG was recorded. This illustrates that the ERG, although extremely helpful at age 1 year, was not diagnostic at age 3 months. The clinical sign of liking the lights at 6 months, and developing photoaversion at 12 months illustrates the fact that clinical symptoms can be confusing and non-contributory. The clinical diagnosis of complete achromatopsia became obvious at age 1 year when the child became extremely photophobic and preferred the dark, and the ERG revealed a non-detectable cone response. Genetic testing before the age of 1 year was extremely helpful in making the correct diagnosis.

Case 3

A 23-month-old patient presented with light perception vision, nystagmus and a normal retinal appearance. The mother had been diagnosed with stage V cervical cancer during pregnancy and required emergency *cis*-platinum chemotherapy at the 28th week of pregnancy and was induced at 31. The mother passed away from disseminated cancer. The specific question on consultation was whether the chemotherapeutic agents might have caused the blindness. VEP and ERG were non-detectable while the brain CT was normal. LCA gene screening revealed a homozygous c.2074-81del8bp in *GUCY2D* (p.E692FsX), which predicts a truncation of the GUCY2D protein. Our conclusion was that this patient had LCA with a *GUCY2D* defect, and that the *cis*-platinum was not the cause of the visual loss.

These cases illustrate the difficulties in diagnosing retinal dystrophies, because of overlapping symptoms and signs of CSNB, complete achromatopsia and LCA, technical difficulties with ERGs in this age group and the usefulness of genetic testing in distinguishing between the three entities.

In a study by Lambert *et al.*[9] a diagnostic reappraisal of 75 patients diagnosed with LCA was performed and in 30 (40%) of the cases, the diagnosis was revised to CSNB (in five cases), to complete achromatopsia (in four cases), juvenile RP (in four cases) and to a systemic disorder (in 17 cases). Weleber *et al.*[10] documented two children with congenital blindness, nystagmus, poor fixation, slightly abnormal retinal appearances, and markedly abnormal photopic and scotopic ERG responses, which were diagnosed with LCA. On follow up, these two children started to fix and follow and developed a measurable ERG, which at age 1 year became electronegative, suggesting the diagnosis CSNB. Fulton *et al.*[11] documented that normal infants have significant immaturities of retinal processes, and showed that the cone and rod responses of children are significantly smaller than those of adults. ERG amplitudes increase with age and are similar to adults only at approximately age 1 year. In the largest cohort of normal term infants, Fulton *et al.* showed that ERG analysis revealed that the youngest group of subjects, from 1 to 5 weeks old, had no detectable ISCEV rod response. A normal developmental increase in ERG responses was subsequently documented in these normals. Also, a developmental increase in the ERG parameters is noted in patients with retinal dystrophy, as in Weleber *et al.*[10] and in our examples above.

To provide a visual prognosis

Four small longitudinal studies of LCA cohorts have identified three categories of visual prognosis.[7] Irrespective of how visual function was measured and followed, Snellen visual acuity, grating acuities, dark adapted visual thresholds or flash visual evoked potentials, most LCA patients were found to have stable visual function (75%), followed by deterioration in 15%, and improvement in 10% of the cases.[9,12–14] How the natural history of visual function corresponds to the specific LCA gene defect is now being studied and reported, but much work remains to be done in this important area. Lorenz *et al.* found that retinal dystrophy patients with *RPE65* defects have measurable vision, transient improvements in function, followed by eventual deterioration,[15] while Koenekoop *et al.* found improvements in visual acuity, visual field and cone ERG b-wave amplitudes in an LCA patient with a *CRX* gene

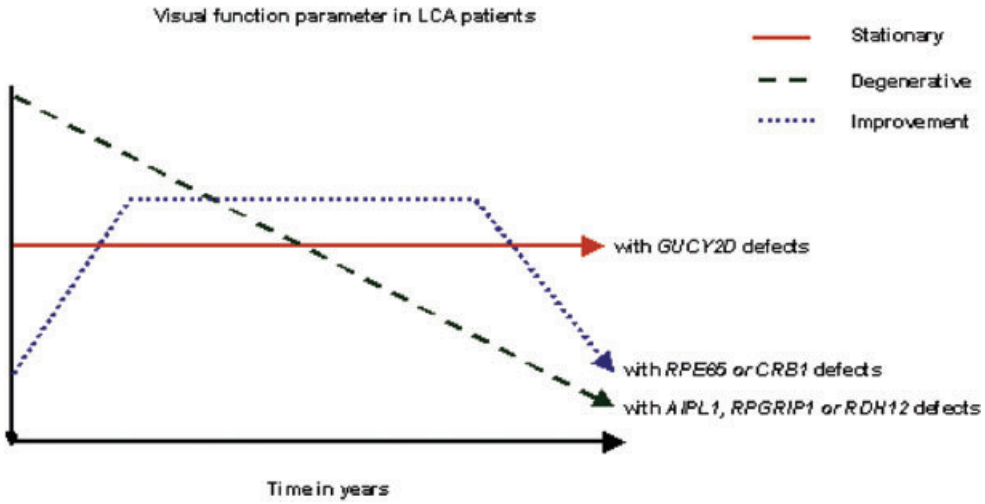

Figure 1. Shows the visual evolution of three types of LCA. Patients with *AIPL1*, *RPGRIP1* and *RDH12* mutations have the degenerative type of evolution with steadily declining vision. Patients with *GUCY2D* (and *CEP290*) mutations exhibit the stationary type of evolution, with stable vision, while patients with *CRB1* and *RPE65* mutations exhibit the improvement type of visual function, which improves, remains stable for a period of time, and then declines.

defect that was followed by the authors for 12 years.[16] Patients with mutations in the *AIPL1* gene have deteriorating visual function.[17] Patients with *GUCY2D* mutations have essentially poor but stable visual function (small series).[7]

Case 4

A male baby presented at 2 months with nystagmus and visual impairement. At 14 months we found vertical nystagmus, the oculo-digital sign, paradoxical and amaurotic pupils, a cycloplegic refraction of $-1.00 + 1.00 \times 90°$ both eyes, and we noted that the child could follow large bright objects, he protested in the dark. ERG testing revealed that 10% remained of the cone b-waves and the mixed rod-cone ERG was non-detectable. Retinal exam revealed narrowing of vessels, optic disc pallor, absence of a pigmentary retinopathy and a very striking translucency of the RPE layer, so that the choroid and choriocapillaris were easily visible. We diagnosed the child with LCA. LCA gene screening revealed a heterozygous c.700C > T (p.R234X) nonsense mutation and a heterozygous c.272G > A (p.R91Q) missense mutation in *RPE65*. At age 3 years we found acuities of 6/30 (Allen), and 6/15 at near.

Figure 1 illustrates the correlations between LCA gene defects and the natural history of visual function in LCA patients. The 'degenerative' group shows a decline of visual function and may be found in patients with *AIPL1*, *RDH12* and *RPGRIP1* mutations.[7] The 'improvement' group, shows a transient improvement in visual function and may be documented in patients with *RPE65*, *CRB1* and possibly *CRX* mutations.[16] A 'stationary' group may be documented in patients with *GUCY2D* and *CEP290* mutations.

A genotype–phenotype correlation system (to suggest the causal gene from the phenotypic appearance)

Despite the facts that the multiple RP and LCA gene defects converge on a limited number of apoptotic death pathways,

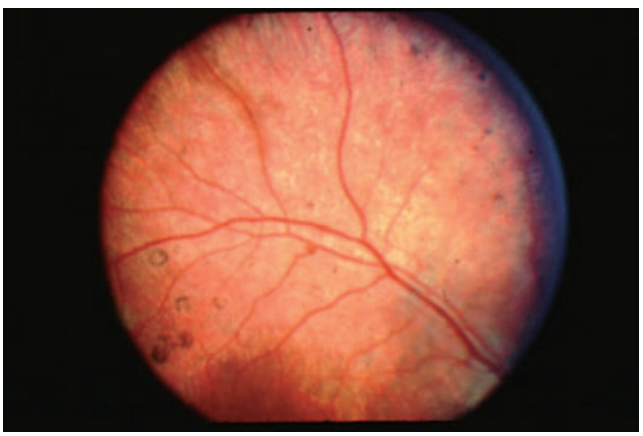

Figure 2. Colour retinal photo of the right eye of a 13-year-old girl with autosomal recessive retinitis pigmentosa who has preserved para-arteriolar retinal pigment epithelium along the top left arteriole branched off the superior arcade. Also obvious are the nummular pigment lesions.

and that the highly specialized retina only has a limited number of injury responses, it appears possible to identify the causal gene based on retinal appearance in selected cases. These types of genotype–phenotype studies have only just recently started, and must be viewed with caution as the sequence from gene defect to resulting phenotype is not straightforward. Environmental, genetic background, modifier alleles and overlap between gene defects affect this relationship. Nonetheless, several important patterns have emerged.[53,54]

Preserved para-arteriolar retinal pigment epithelium (PPRPE) was first documented in autosomal recessive RP (ARRP) patients with hyperopic refractive errors, and a relatively severe phenotype.[18] Subsequently, RP and LCA patients with PPRPE were found to have *CRB1* mutations.[19,20] We have been able to predict *CRB1* mutations in several consecutive LCA patients with PPRPE (see Fig. 2), in which the retinal appearance and the mfERG regional pattern of

dysfunction in the carrier parents strongly suggests the causal gene (*CRB1*).[21] Also, a thickened retina on optical coherence tomography suggests involvement of the *CRB1* gene.[22] These clinical parameters in *CRB1* patients provide powerful genotype–phenotype correlations. Patients with *GUCY2D* mutations can be clinically distinguished from patients with *RPE65* patients,[23,24] as patients with severe visual loss (count fingers, hand motions and light perception), high hyperopia (>+5.00) and essentially normal retinal appearance would suggest *GUCY2D*, while measurable vision and transient improvement in vision with a translucent RPE would suggest *RPE65*. In a large series of LCA patients with *AIPL1* defects,[17] we documented that most patients developed a striking maculopathy and optic disc pallor, while we have not found this to be the case for those with *RPGRIP1*, *RPE65* and *GUCY2D* mutations. In patients with adult RP, hearing loss suggests involvement of the *RPGR* [25–27] or Usher syndrome type 2 genes, *USH2A* [28] and *VLGR1*. [29] Severe RP or CRD with an early maculopathy and/or macular colobomas may suggest *ABCA4* involvement,[30] nummular pigment may indicate defects in *NR2E3*,[31] sectoral pigment degeneration may indicate *Rhodopsin* involvement,[32] while non-penetrance in the RP pedigree may indicate a defect in the pre mRNA splicing factor gene *PRPF31*.[33] Ophthalmologists can make important contributions to this evolving field, and add genotype–phenotype correlations so that detailed eye examinations may suggest the underlying gene defect.

To identify new retinal pathways

Identification of *CRB1*, the crumbs homolog 1 gene, is an example of how genetic analysis can discover a previously unknown retinal pathway and function.[34] The outer limiting membrane (OLM) is an adhesion belt of the outer retina that separates the subretinal space from the photoreceptors. CRB1 is part of a multiprotein complex that maintains the zonula adherence junctions that is formed between photoreceptors and Müller cells at the OLM. The identification of CRB1 therefore has shed light on a new aspect of photoreceptor morphogenesis. CRB1 is involved in the maintenance of cell polarity and alignment of the photoreceptors, and without it (as shown in two CRB1-deficient mouse models) the retina develops folds and pseudorosettes, a disrupted OLM, two photoreceptor layers, and eventual photoreceptor death.[35,36]

To identify carriers and support prenatal screening

Once both the maternal and the paternal mutations have been identified in a child with a severe retinal dystrophy like LCA, and the mutations have been shown to cosegregate in the family (two mutations in the affecteds, one or none in the unaffected sibs and one in each parent) prenatal screening can be offered to parents who want another child, but who do not want to accept the 25% recurrence risk of LCA. We identified a 4-year-old LCA child with light perception vision and *AIPL1* mutations. We found a paternal c.834G > A (p.W278X) mutation and a maternal c.487C > T (p.Q163X) mutation in the affected child that cosegregated in the family, and expression studies had convincingly shown that both the p.W278X and the Q163X mutations were deleterious. The parents requested prenatal screening, and for the first pregnancy, chorionic villous sampling of the unborn sib at 14 weeks gestation we identified both *AIPL1* mutations and the parents elected to terminate the pregnancy. For the second prenatal screening for this couple a year later again by chorionic villous sampling we identified only the paternal p.W278X mutation, and we predicted a normal child with normal vision. The parents elected to continue the pregnancy and the baby was born normally with normal fixation. Genetic testing can also be utilized for preimplantation diagnosis in selected cases, approved by the ethical review committee. Here oocytes and sperm are harvested, embryos are created *in vitro*. At the eight cell stage, one cell is genetically tested for the known mutations. Embryos with one or none of the mutations are selected for implantation.[37]

To identify new retinal genes

All those LCA and RP patients who have undergone screening for the currently known LCA and RP genes and are negative, which represents approximately ~60–70% of the patients, potentially harbour mutations in novel, currently unknown LCA and RP genes. These LCA and RP patients and their families are extremely valuable for further genetic studies, which can now be more focused on finding the new chromosomal locus and subsequently the new LCA or RP gene. Much time, effort and finances will be saved concentrating on these individuals and families. For example, in a consanguinous LCA pedigree with four affected sibs from Quebec, we excluded seven LCA genes by comprehensive gene screening. Homozygosity mapping by single nucleotide polymorphism microarray (Affymetrix 10K chip) revealed a homozygous region on chromosome 12q21, which represented a novel locus for LCA. We screened a new gene, that is, centrosomal protein 290 (*CEP290*) in this interval and found a homozygous mutation in this family.[38]

To guide therapeutic strategies

The most exciting reason to perform genetic testing on all retinal dystrophy patients is to prepare them for future therapeutic trials, many of which will be gene-specific.

Replacement of the rod cGMP phosphodiesterase *gene*

One of the first gene therapy attempts in retinal dystrophy models was with the *rd* mouse, which lacks the phototransduction enzyme rod cGMP phosphodiesterase (*PDE6B*). Lem

et al.[39] showed that it was possible to rescue the photoreceptors by a transgenic introduction of the normal gene by subretinal injection in 1-day-old mouse embryos. It is important to know that at the day 1 stage, the photoreceptors are still dividing and have not yet undergone complete degeneration.

Replacement of the Peripherin/RDS *gene before the retinal degeneration is established*

Travis *et al*.[40] then showed similar rescue results in the *rds* mouse, which lacks the structural photoreceptor protein peripherin/RDS. Both genes (*βPDE* and *RDS*) were re-introduced into the mouse models at a time when the degenerative disease process had not yet fully taken place, and when cell division was still active. The question emerged whether it would be possible to replace genes later in the disease process, when outer segments have been lost owing to retinal degeneration.

Replacement of the Peripherin/RDS *gene after the retinal degeneration is well established*

Ali *et al*.[41] performed subretinal injections of recombinant adeno-associated virus (AAV) containing the normal peripherin/RDS gene in the *rds* mice that had an established retinal degeneration. Post-injection immunostaining revealed normal peripherin protein and rhodopsin localization to the outer segments of the photoreceptors, indicating successful expression of the introduced gene in the correct layer in the retina. Photoreceptor outer segments and the characteristic stacked discs were very similar to the wild type mice and very unlike the diseased *rds* mice. Functional studies showed significantly improved b-waves in the treated animals.

Replacement of a RPE gene that encodes a retinoid cycle enzyme (RPE65)

To determine whether gene replacement would work in a larger animal model, Acland *et al*.[3] studied the effects of *RPE65* replacement in the Briard dog. This dog model harbours a naturally occurring, homozygous 4-bp deletion in the *RPE65* gene (which predicts a lack of functional RPE65 protein in the RPE) and is essentially blind at birth. ERG function is non-detectable, despite the normal appearance of the retinas, including the essentially normal histological appearance of the photoreceptor layer in this dog model. Subretinal injections in one eye of three dogs containing the AAV virus with cDNA of dog *RPE65*, with a CMV promotor; β-actin enhancer and internal ribosome entry sequence were performed at age 4 months. Rod and cone mediated ERGs, visual evoked potentials, pupillometry and behavioural testing all showed dramatic improvements in visual function at about 8 months of age. Wild type *RPE65* was found in the retina and in RPE cells. These experiments were repeated in other laboratories[42] and found to be accurate, while follow-up visual function testing revealed stable function for at least 3 years, after one subretinal injection of the gene.

Photoreceptor gene RPGRIP1 *replacement rescues blind mice with established retinal degeneration*

Three important aspects of the successful *RPE65* gene replacement may have biased a favourable outcome; (i) *RPE65* is a gene expressed in the RPE layer and in cone photoreceptors, (ii) *RPE65* encodes an enzyme (not a structural protein), and (iii) the retinal architecture of the *RPE65* dog model was not severely degenerated. The question now is; does rescue work for *photoreceptor* genes that encode *structural proteins*, when the *degenerative process* is well established? Pawlyk *et al*.[43] using the well-known *RPGRIP1* knockout mouse answered this question. *RPGRIP1* is expressed in the ciliary axoneme that connects the inner to the outer photoreceptor segment and *RPGRIP1* mutations cause abnormalities in protein trafficking from the inner segment to the outer segment (especially rhodopsin), aberrant disc morphogenesis of the outer segment discs and a rapid early degeneration of the entire retina. Five months post injection of *RPGRIP1* cDNA by an AAV vector, they showed that RPGRIP1 (and its partner RPGR) correctly colocalized to the connecting cilia and documented preservation of photoreceptor nuclei in the outer nuclear layer. ERGs showed a marked improvement of the rod photoreceptor b-wave amplitudes and the rates of ERG decline were also significantly smaller in the treated eyes.

Replacement of a retinal gene that encodes a protein that functions as glue between the inner and outer retina

Can retinal gene therapy rescue other cell types in the retina, when the gene product is an extra-cellular molecule involved in keeping the retina together as natural glue? Retinoschisin (RS) gene replacement rescues the RS null mice as shown by Min *et al*.[44] and Zeng *et al*.[45] Viral constructs containing the normal human RS cDNA, injected subretinally in the RS deficient mouse led to RS expression in retinal extracts and led to marked improvements in both the rod-mediated and cone-mediated signals (including the reversal of the electronegative ERG, which is the hallmark of the human and mouse disease phenotype) all of which indicates a rescue effect on the retinal architecture.[44] The effects on the cone ERG, especially the flicker ERG were the most dramatic. Also apparent were the retinal changes seen by scanning laser ophthalmoscopy (SLO), as the untreated eyes revealed the characteristic cystic lesions, while the transfected retinas were devoid of these pathological structures and resembled wild type retinas. Photoreceptor inner and outer nuclear layers, outer plexiform layer and bipolar cells of the inner nuclear layer in addition to the inner plexiform layers all revealed RS staining, which illustrates the important concept that retinal gene replacement injected subretinally may lead to protein localization longitudinally and laterally into the retina.

Dilemmas of genetic testing

When a patient with a retinal degeneration presents to an Ophthalmologist and requests molecular testing for his/her condition, theoretically a maximum of 132 retinal degeneration genes would need to be screened.[2] If a patient is found to have RP, 37 RP genes would need to be screened. Comprehensive genetic testing of RP patients is a problematic and challenging endeavour because it is extremely time-consuming, expensive and labour intensive, as RP patients with unknown inheritance would need to be screened for almost 500 exons, which represents almost 120 000 base pairs. There are also other problems: the current technology is not 100% sensitive, and the screening results will be full of false positive results (polymorphic variants). Once a sequence variant is identified, it must be determined whether it is disease causing or may be a polymorphism. Short of performing functional assays on mutations in *in vitro* cell culture, mutations can be compared with polymorphisms for the following five attributes which improves the probability that the mutation causes a defect in the resulting protein: (i) the predicted effect of the base pair change on the RNA and/or protein product, (ii) the relative frequency of the variation in LCA patients versus normal ethnically matched controls (>1% in the controls will be assigned as a polymorphism), (iii) cosegregation of the mutant allele(s) in the affected members of the families, (iv) homozygosity or compound heterozygosity in recessive disease, and (v) conservation of the mutant codon across other species. Several web-based programs exist that are helpful in distinguishing benign from deleterious effects (polyphen, SIFT, Blossum). Therefore, comprehensive RP screening is currently not available in the world in a single laboratory.

The endeavour is slightly better for RP patients with known inheritance patterns. RP patients with a clear X-linked inheritance pattern (XLRP) would only need to be screened for two genes (*RPGR* and *RP2*), which are divided into 24 exons and include almost 7000 base pairs. *RPGR* mutations may account for 70–90% of all XLRP patients, and may be the most common RP gene, overall. For ARRP patients, which represents the most common RP inheritance subtype, 20 RP genes are required for screening, divided over 337 exons, which include almost 75 000 base pairs. Finally, if the RP patient has clear dominant inheritance, 15 genes need to be screened, divided over 135 exons, including almost 38 000 base pairs.

In addition to this insurmountable task, conventional mutation screening methods, such as SSCP and dHPLC, can miss mutations, and are not 100% sensitive. Automated sequencing of almost 500 exons for one retinal dystrophy patient would cost about $10 000.00 (CDN) and evaluation of all sequence data and repeat analysis may add another $2–3000. Another problem would be the enormous number of DNA variations that would be found which have nothing to do with the disease. Each variation would have to be tested in the family to verify whether it cosegregates with the disease phenotype. Deciding which of the variations in which gene is the actual mutation responsible for the disease would be extremely difficult, especially in the most common type of RP which is sporadic (also called isolated RP, in which there is only one affected patient). Fortunately, there are several possible solutions to this problem, some of these solutions are being actively tested and evaluated.

Possible solutions for genetic testing

We will discuss three possible solutions: (i) utilize the retinal phenotype (including retinal aspect and natural history of vision) to suggest the genetic defect; (ii) start with the most frequently mutated gene or with the most frequent mutation(s); and (iii) utilize new high throughput microarray technology.

Solution 1: Utilize genotype–phenotype correlations to suggest the causal gene

Ideally, an ophthalmologist could predict the causal gene in a RP patient by documenting the clinical phenotype, including age of onset, rate of progression, optical status, retinal appearance and associated features, to name a few. This is not possible in all cases, because of many reasons, including the fact that there is no straightforward and direct relation between the gene and the phenotype, the action of interfering factors from the environment and genetic background, the influence of modifier alleles and the fact that RP genes may have similar functions in the retina or participate in similar retinal cycles. Also, this area of investigation is in its infancy, and there are more genes known than robust genotype–phenotype correlations. Despite these shortcomings, there are several good examples of retinal dystrophy genotype–phenotype correlations (see *Possible solutions for genetic testing* and Table 1). This exciting area of investigation is also in its infancy and needs to be expanded and tested.

Solution 2: Focus on the most frequent mutation(s) and/or mutated gene(s) first

Another solution to screening may be to start with the most commonly mutated RP genes, that is, *RPGR* screening may provide a molecular diagnosis in ~10% of all RP patients, 70–90% of patients with XLRP, and up to 25% of males with sporadic RP. Another common gene mutated in 15–20% of all ADRP is the rhodopsin gene, especially the Pro23His mutation, which is common in North America but not elsewhere. *USH2A* screening is recommended for patients with Usher (USH) syndrome as 40–50% have mutations in *USH2A*, but also for patients with ARRP, as up to 14% may harbour mutations in *USH2A*. For LCA, it appears that the most frequently mutated gene is *CEP290*. In more than 20% of new LCA patients, a single mutation p.C998X can be found. With an inexpensive and simple blood test this mutation can be found, establishing the genotype.[38]

Table 1. Phenotype-Genotype correlations for retinal dystrophies, linking a clinical characteristic to a specific retinal gene defect.

Phenotypic parameter	Suggested retinal gene
RP patients	
Hearing loss	*RPGR/USH2A/VLGR1*
Sectoral retinal changes	*Rhodopsin, RP1*
Non-penetrance (skipped generations)	*PRPF31*
Severe RP with maculopathy	*ABCA4*
Nummular pigment formation	*NR2E3*
Families with RP and maculopathy	*ABCA4/RDS*
Clumped pigment deposits	*NRL*
LCA and juvenile RP patients	
PPRPE	*CRB1*
Thickened retina on OCT	*CRB1*
Nummular pigment	*CRB1*
Coats reaction	*CRB1*
Severe, with maculopathy and optic disc pallor	*AIPL1*
Severe, with relatively normal retinal aspect	*GUCY2D, CEP290*
Rapid peripheral visual loss and maculopathy	*RDH12*
Transient visual improvement, followed by decline, and translucent RPE	*RPE65*
Relatively stable visual loss, blond fundus	*LRAT*
Severe, progressive, retinal pigmentation	*RPGRIP1*
Severe central visual loss, relative preservation of visual field, and perifoveal yellow annulus	*TULP1*
Moderate visual loss, maculopathy	*CRX*

LCA, leber congenital amaurosis; OCT, optical coherence tomography for in vivo microscopy of the retina; PPRPE, preserved para-arteriolar retinal pigment epithelium; RP, retinitis pigmentosa.

Solution 3: High throughput mutation microarrays

Disease chips (asper ophthalmics)

The first microarray ('disease chip') was designed for Stargardt macular dystrophy (STGD1) and contains all currently known disease-associated alleles of the *ABCA4* gene. Other retinal diseases may be caused by or are associated with *ABCA4* mutations, namely fundus flavimaculatus, CRD, RP and age-related macular degeneration, and can be screened on this chip as well. Other disease chips are now available for LCA, Usher syndrome ARRP, Bardet Biedl Syndrome, ADRP, XLRP and age-related macular degeneration (see http://www.asperbio.com). The screening by the disease chips is rapid (~4 h per patient), reliable and affordable (~150–200 USD), and can be updated, as new genes and new mutations become available.

Microarrays are designed and manufactured with the arrayed primer extension (APEX) method, also known as solid-phase minisequencing and details can be found in Kurg *et al.*[46] and Tonisson *et al.*[47] There are basically six steps (see Fig. 3): in Step 1 Oligonucleotides (oligos) are designed for each known mutation, with the 5′ end immobilized on the glass slide and the 3′ end immediately adjacent to the variable (queried) site. Step 2 is polymerase chain reaction (PCR) amplification of each DNA segment (which harbours the known mutations) of the new (to be tested) patient's genome. In Step 3, all the patient's PCR fragments are spread over the slide containing the oligos and annealing is allowed to take place, between the patient's DNA fragments and the immobilized oligos. In Step 4, DNA polymerase is added, with dye labelled dideoxynucleotides (C, T, G and A) so that a sequence specific extension of one nucleotide only takes place at the 3′ end of the oligo, using the patient DNA as a template. In Step 5, the patient's DNA not annealed to a homologous oligo is washed off, to reduce noise, and in Step 6 signal detection takes place by laser and computer. Advantages are the low cost (~150–200 USD), rapid results (4 h per sample), ability to update the chip and the ability to enter only known pathogenic mutations to the platform. A disadvantage is that APEX technology only detects the known mutations, although a new mutation in a nucleotide already included on the chip will be detected. In patients with autosomal recessive diseases, sequence analysis may be required to identify a second allele once APEX technology had detected the first.

The ABCA4 disease chip. The first disease chip designed was the ABCR400 chip specifically for the *ABCA4* gene, which has 50 exons. Unlike, for example, cystic fibrosis, which is also caused by a member of the ABC super family and in which the p.F508del is a common mutation found on ~70% of cystic fibrosis alleles, the commonest STGD1 mutation is only found in ~10% of patients. The array now contains ~500 ABCA4 mutations, and was >98% effective in detecting a patient's variations. Jaakson *et al.*[48] showed that the disease chip was 54–78% effective in detecting at least one of the two STGD1 patients mutations, depending on the geographical cohorts. In a second study, Klevering *et al.*[49] showed that the *ABCA4* chip is also effective in 33% of ARCRD patients, and 6% effective in severe ARRP patients in locating at least one of the two mutant alleles.

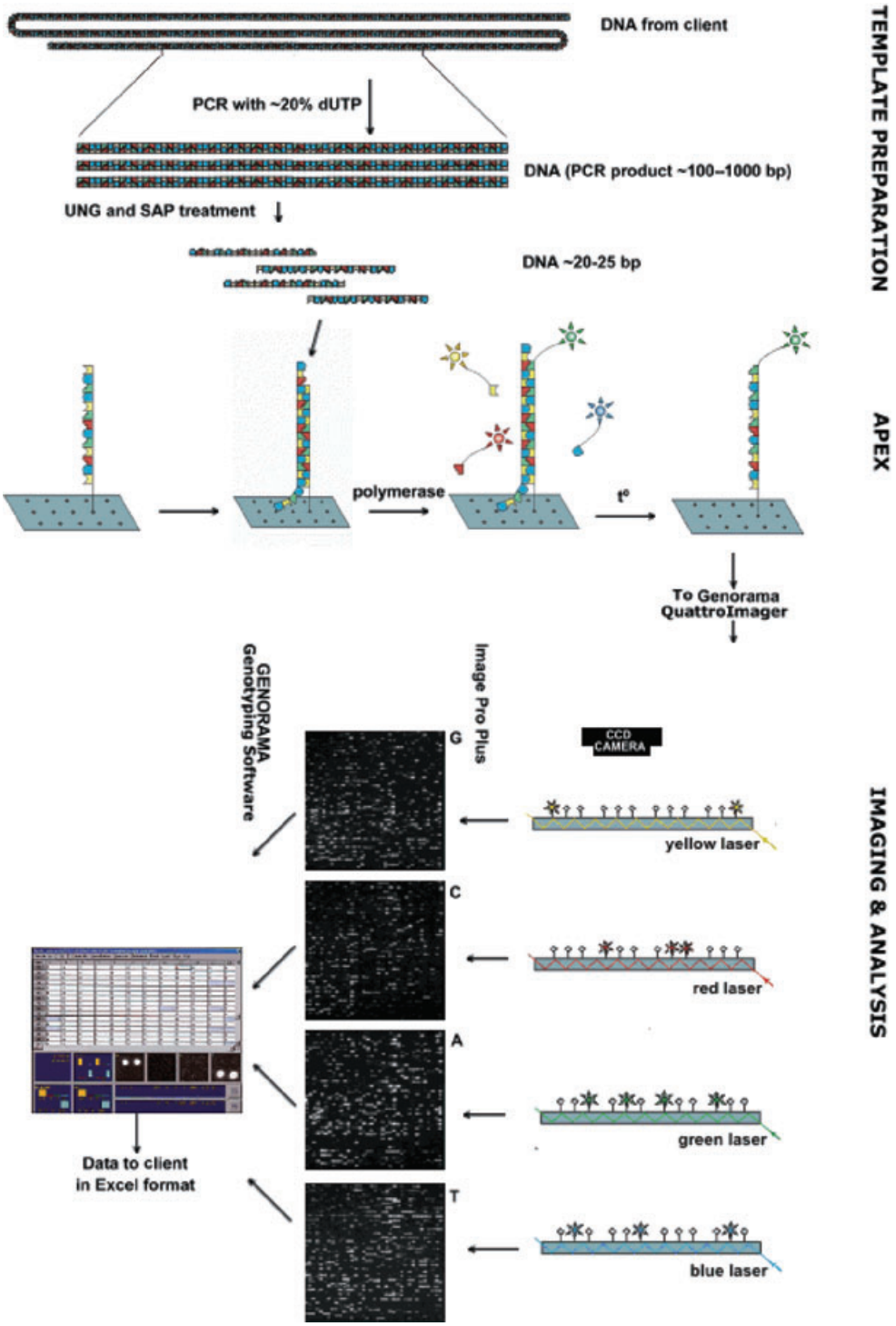

Figure 3. Arrayed primer extension (APEX) technology. PCR, polymerase chain reaction.

The LCA disease chip. In the design of the second disease chip, for LCA, we wanted to test whether the technology was valid for a complex retinal dystrophy with multiple causal genes and again extensive allelic heterogeneity. LCA can be caused currently by mutations in nine known retinal genes (*GUCY2D*, *RPE65*, *AIPL1*, *CRX*, *RPGRIP1*, *CRB1*, *CEP290*, *IMPDH* and *RDH12*). In addition, *TULP1*, *PROML1*, *MERTK* and *LRAT* are associated with juvenile RP, which has significant clinical overlap with LCA. We designed the LCA chip with >300 known mutations in six LCA genes (*CEP290*, *RDH12* and *IMPDH* were not discovered yet, but are now on the platform) and three juvenile RP genes, and found that the array was able to detect at least one of the two mutant alleles in ~35% of the newly screened patients (Zernant *et al.*[50]). In 22/300 (7%) of LCA patients we found three mutant alleles, suggesting the possibility of modifier alleles. In several sib

pairs we were able to demonstrate altered phenotypes in the sib with three alleles versus those with two alleles. Several recent studies have confirmed the utility and efficiency of the new LCA chip. Pathogenic mutations were identified in 19 out of 58 LCA patients for an efficiency rate of 33% by Yzer *et al*.[51] These results are encouraging for at least two reasons. In comprehensive screening studies (by sequencing all the exons of all the LCA genes) an 47.5% efficiency is reached (Hanein *et al*.[24]). While the LCA disease chip will identify mostly the known mutations entered onto the platform, a 33–35% detection rate is surprising and will increase proportionately to the addition of new mutations added to the chip. Recently, with the addition of several new LCA genes and mutations, the success rate of the LCA chip has increased to ~60–70%. As the chip is fast, relatively cheap and accurate, we propose to use the LCA chip as a first pass screening tool for all patients with LCA and juvenile RP.

The usher disease chip. Three clinical Usher syndrome subtypes have been described (USH1-3) and eight USH genes have currently been reported (USH1 genes: *CDH23, MYO7A, PCDH15, Harmonin* and *SANS;* USH2 genes: *USH2A* and *VLGR1;* and USH3 gene: *USH3A*). Identification of the causal Usher mutations is important for all the above reasons, but also to identify the patient with congenital hearing loss, in order to implant cochlear devices as early as possible. As is the case for RP and allied retinal degenerations, testing for Usher disease is also hampered by genetic heterogeneity (eight genes), many exons (179 coding units) and the great number of known mutations. In a recent study, Cremers *et al*.[52] designed a new Usher disease chip containing the currently known 298 Usher mutations (from the eight currently known Usher genes), and the efficiency of the array was tested using DNA from 370 untested Usher patients. Mutations were found in 45% of USH1, 23% of USH2 patients and 28% of the USH3 patients, which represents a very encouraging detection rate, considering that not all Usher genes nor all mutations have been identified.

Conclusions. Now more than ever, the genotyping of retinal dystrophy patients is a crucial exercise, as human gene-specific clinical trials to study rescue of photoreceptors are on their way. Genetic testing confirms the diagnosis at the molecular level and allows for a more precise prognosis of the possible future clinical evolution of the disease based on the gene defect. Also, as human treatments are gene specific and the treatment 'window of opportunity' is likely time sensitive; accurate, rapid and cost-effective genetic testing will play an ever increasing crucial role. Currently there is no single 'perfect' technology to quickly and affordably determine the genotype in a particular retinal dystrophy patient. The gold standard technique of automated sequencing is fraught with excessive costs, time and manpower issues and finding non-pathogenic variants (which are not a problem if strict criteria are used with regard to their pathogenicity, they do however, add much time and work to the genotyping effort). Therefore, no current RP laboratory or centre offers testing of all known RP genes (37 genes and counting). In the past few years several new and exciting (micro array) technologies have emerged that offer the possibility to genotype retinal dystrophy patients rapidly, cost-effectively and accurately. Each of these new technologies has advantages and disadvantages and each one needs to be tested in large genotyping efforts. We suggest that the new disease chips from Asper Ophthalmics offer an excellent first pass opportunity. This may be combined with genotype–phenotype correlations that suggest the causal gene from the clinical appearance (e.g. PPRPE suggests the involvement of the *CRB1* gene in LCA and juvenile RP) and regular sequencing of the suggestive gene. Also starting with the most frequently mutated genes (*RPGR, Rhodopsin, USH2A*) may be prudent.

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