School of Medicine, University of Coimbra

6th Medical Year – Master's Degree

Review Article

GENE THERAPY FOR STARGARDT AND OTHER *ABCA4*-RELATED DISEASES: LESSONS FROM THE *RPE65*-LCA TRIALS

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March, 2010

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Gene Therapy for Stargardt and other *ABCA4*-Related Diseases: Lessons from *RPE65*-LCA Trials.

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Abstract

Stargardt disease is the most common among recessively inherited macular dystrophies, with 600 new disease cases diagnosed every year. It is caused by mutations in *ABCA4*, a photoreceptor-bound gene involved in normal visual cycle kinetics. The resulting disease phenotype, with accumulation of lipofuscin and other by-products in the retinal pigmented epithelium, is also recognized in the *abca4* mouse model of the disease. Currently, Stargardt remains an incurable condition and there is little that can be done to improve the visual function of these patients. However, recent developments in ocular gene therapy will likely change this scenario. In the past decade, extensive research has been done in gene therapy for another form of retinal degeneration, Leber Congenital Amaurosis (LCA) associated with mutations in the *RPE65* gene. Successful and sustainable rescue of vision in several animal models and human patients with *RPE65*-LCA, using AAV-mediated *RPE65* gene replacement therapy, has led to the natural and rather easy application of the concept to Stargardt disease, and in fact, an *ABCA4*-based gene replacement trial is expected to enter Phase I/II development during 2010. If proven successful, this trial will provide unprecedented treatment possibilities for Stargardt disease and spectrum of retinal phenotypes associated with *ABCA4* variation.

Keywords: ABCA4, Stargardt, retinal degeneration, Leber Congenital Amaurosis, LCA, RPE65, gene therapy.

INTRODUCTION

Stargardt disease (STGD1, OMIM entry #248200) is an autosomal recessive macular dystrophy, first described by German ophthalmologist Karl Stargardt (Stargardt 1909), and accounts for about 7% of all retinal degenerations. It is a prevalent disorder, grossly affecting 1 in 10.000 individuals (Maia-Lopes et al. 2009), and it is also the most common among recessively inherited macular dystrophies (Tombran-Tink et al. 2007). Until now, there were limited treatment options available and the possibility of cure for this visually debilitating condition remains scarce. However, in the past decade, extensive research has been done in the field of gene therapy, with particular applications to ocular diseases. By far, most research has been done in animal models of Leber Congenital Amaurosis (LCA) and its results are overwhelming, paving the way for human clinical trials, which are already underway and whose first published data is becoming available. With this review, we intended to summarize all the work that has been done in the past decade regarding gene therapy for

LCA in order to analyze the possible applicability of the concept to Stargardt and other ABC*A*4-related diseases.

STARGARDT DISEASE: GENERAL CONSIDERATIONS AND GENETICS

Stargardt disease is a genetic disorder of the retina caused by mutations in the *ABCA4* gene (Allikmets et al. 1997), which encodes for a homonymous retinal specific protein involved in the process of phototransduction, the phenomenon by which light can be converted into an electrical signal, which is then conducted, via the optic nerves, to the occipital cortex for central processing. Recessive inheritance is most common, but dominant forms of Stargardt-like disease have been reported, involving mutations in structural genes from chromosomes 6 and 4 (STGD3, OMIM entry #600110 and STGD4, OMIM entry #603786).

In general, the disease has no gender or race predilection and is typically characterized by its wide variation in age of onset, visual impairment, clinical appearance and severity (Holz et al. 2007). The *ABCA4* gene maps to the short arm of chromosome 1 and presents extraordinary allelic heterogeneity, with over 490 disease-associated variants discovered thus far, most of which are missense mutations (Rozet et al. 1998 and 1999, Maugeri et al. 1999, Allikmets 2000a, Maia-Lopes et al. 2008). Immediately, this leads to a common problem often present when one studies Stargardt disease from a molecular standpoint: the most frequent *ABCA4* disease-associated alleles (e.g. G1961E, G863A/delG863 and A1038V) account for only about 10% of patients, making the *ABCA4* gene a rather difficult diagnostic target (Tombran-Tink et al. 2007). Also, some have alerted for the presence of “ethnic group-specific” *ABCA4* alleles responsible for founder phenomena in different areas of the globe (Kuroiwa et al. 1999, Maugeri et al. 1999, Allikmets 2000b, Rivera et al. 2000). Examples include the T1428M allele which, although extremely rare in populations of European descent, is rather frequent in the Japanese, with an estimated frequency of approximately 8% (Kuroiwa et al. 1999), and the G863A/delG863 allele, which is currently considered a founder mutation in Northern Europe (Maugeri et al. 1999). To overcome the genetic screening challenge of *ABCA4*, the ABCR400 microarray was developed, and contains all currently known disease-associated genetic variants and many common *ABCA4* polymorphisms. The ABCR400 microarray is more than 99% effective, making it a first line option for systematic screening of *ABCA4* mutations (Jaakson et al. 2003).

One aspect of critical relevance is the significant carrier frequency for *ABCA4* alleles, reaching as much as 5 to 10% in the general population, which means that at least 1 in every 20 people carries a disease-associated variant of the gene (Maugeri et al. 1999, Jaakson et al. 2003). This finding raised serious implications regarding the way we face *ABCA4*-related disease, suggesting that the amount of retinal pathology associated with *ABCA4* variation may be more than previously thought (Tombran-Tink et al. 2007). That said, it has in fact been noted that different combinations of *ABCA4* alleles are predicted to result in distinct phenotypes, in a continuum of retinal disease manifestations. It appears the severity of disease would be inversely proportional to residual ABC*A*4 activity. Likewise, there is up-to-date evidence indicating that *ABCA4* is also implicated in the pathogenesis of various other retinal diseases beyond Stargardt, and we now consider age-related macular degeneration (AMD), some cone-rod dystrophies and forms of retinitis pigmentosa, *ABCA4*-related disorders as well (Fig. 1, Rozet et al. 1998 and 1999, Allikmets 2000a, Rivera et al. 2000, Dean 2003). Accordingly, Stargardt disease would result from partial but not complete inactivation of the gene, allowing for residual low level ABCA4 expression (Lewis et al. 1999), whereas retinitis pigmentosa, the most severe of *ABCA4*-related conditions, would derive from the presence of two null alleles of the *ABCA4* gene, fully compromising its pattern of genetic expression (Martinez-Mir et al. 1998, Rozet et al. 1999). These assumptions are further supported by the fact that, today, *ABCA4* mutations are thought to be involved in 30 to 60% of autosomal recessive cone-rod dystrophies (Maugeri et al. 2000, Briggs et al. 2001, Ducroq et al. 2002, Fishman et al. 2003). Even within the same family, different *ABCA4* allelic combinations can be responsible for distinct phenotypes as was demonstrated by Cremers and colleagues who discovered that in a consanguineous family with retinitis pigmentosa in one branch and a cone-rod dystrophy in the other, a variety of recessive *ABCA4* splice site mutations were co-segregated (Cremers et al. 1998).

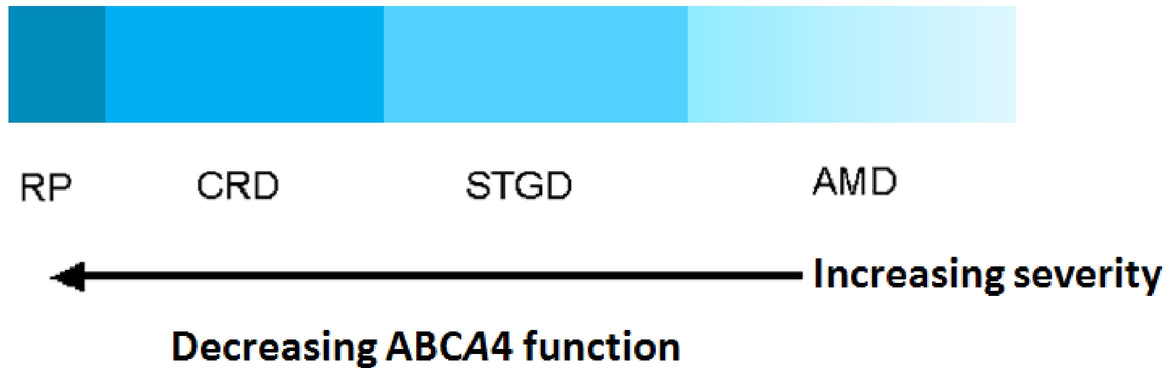


Fig. 1 Spectrum of *ABCA4*-related phenotypes. Decreasing function of *ABCA4* is thought to be associated with age-related macular degeneration (AMD) in some mutation heterozygotes. Stargardt (STGD) patients have at least one missense allele; in cone-rod dystrophy (CRD) and retinitis pigmentosa (RP) patients have two severe mutations. *Adapted from Dean 2003.*

Even if one considers Stargardt disease alone, different *ABCA4* allelic combinations can explain the high clinical heterogeneity of the disease, leading some authors to suggest three major clinical phenotypes for Stargardt disease based on fundus examination at time of presentation: phenotype I, characterized by an atrophic-appearing macula, sometimes surrounded by localized perifoveal yellowish-white flecks, absence of a dark choroid and normal electroretinographic (ERG) amplitudes; phenotype II, with a dark choroid, more diffuse flecks in the fundus and inconsistent ERG amplitudes; and phenotype III, with a more extensive and diffuse atrophy of the retinal pigmented epithelium (RPE) and reduced ERG amplitudes of both types of photoreceptors (Tombran-Tink et al. 2007). Others have suggested further phenotypic classifications, but none has been conventionally established. It was further demonstrated by Yatsenko and co-workers that late-onset Stargardt disease is associated with missense mutations that map outside known functional domains of ABCA4, thereby resulting in milder mutant alleles (Yatsenko et al. 2001), and suggesting that some *ABCA4* variants and combinations lead to less severe and later-onset subsets of the disease, like fundus flavimaculatus. Nonetheless, it is important to note that the observed clinical phenotype of Stargardt patients is also significantly influenced by age at time of diagnosis and, accordingly, progression of the disease (Cideciyan et al. 2009a). Moreover, other genes and/or environmental factors may contribute to *ABCA4* expression, influencing the resulting phenotype (Zhang et al. 1999, Klevering et al. 2005). Likewise, although there is evidence that ABC*A*4 is implicated in development of retinitis pigmentosa and some forms of cone-rod dystrophies, it is important to state that these conditions are genetically heterogeneous and

their clinical phenotypes may originate from a number of mutations in distinct genes (Klevering et al. 2005).

There is substantial evidence pointing to the fact that heterozygotes for *ABCA4* mutations may carry an increased risk of developing AMD (Allikmets 2000a, Mata et al. 2001, Shroyer et al. 2001, Yatsenko et al. 2001). In fact, it was shown, on a multicenter international study carried out by Allikmets and colleagues, that heterozygotes for the G1961E *ABCA4* allele had a fivefold risk of developing AMD, and that carriers of the D2177N variant had a threefold risk of developing the same condition (Allikmets 2000a). The results obtained were considered statistically significant. (Klevering et al. 2005) However, it has also been stated before that, although *ABCA4* mutations might have an influence on the development of AMD, they are only a minor cause of the disease (Stone et al. 1998). Indeed, several smaller-sized mutation screening studies, and most of the co-segregation studies in AMD families, failed to establish a direct correlation between the disease and *ABCA4* (De La Paz et al. 1999, Souied et al. 2000, Guymer et al. 2001, Schmidt et al. 2003). Accordingly, although the relationship between AMD and *ABCA4* remains speculative, we can assume that *ABCA4* possibly relates to some forms of the condition and agree that very unlikely is it the only factor involved in its development.

Quite interestingly, Maia-Lopes and co-workers state that morphologically non-affected Stargardt relatives who carry *ABCA4* mutations could have a possible predisposition to AMD. More importantly, however, they found that these same individuals, at least for some mutations, could have insufficient ABCA4 expression despite normal visual acuity, leading to subnormal visual function, as revealed by both psychophysical and electrophysiological approaches (Fig. 2a and 2b). In that way, normal carrier Stargardt relatives were found to have intermediate visual performance, between age-matched control subjects and Stargardt patients. The authors further advance that relatives of Stargardt patients should be periodically followed-up, because their visual function as a group is subnormal (Maia-Lopes et al. 2008). This is a pioneer and innovative study which clearly brings further insights into the involvement of *ABCA4* in retinal pathology.

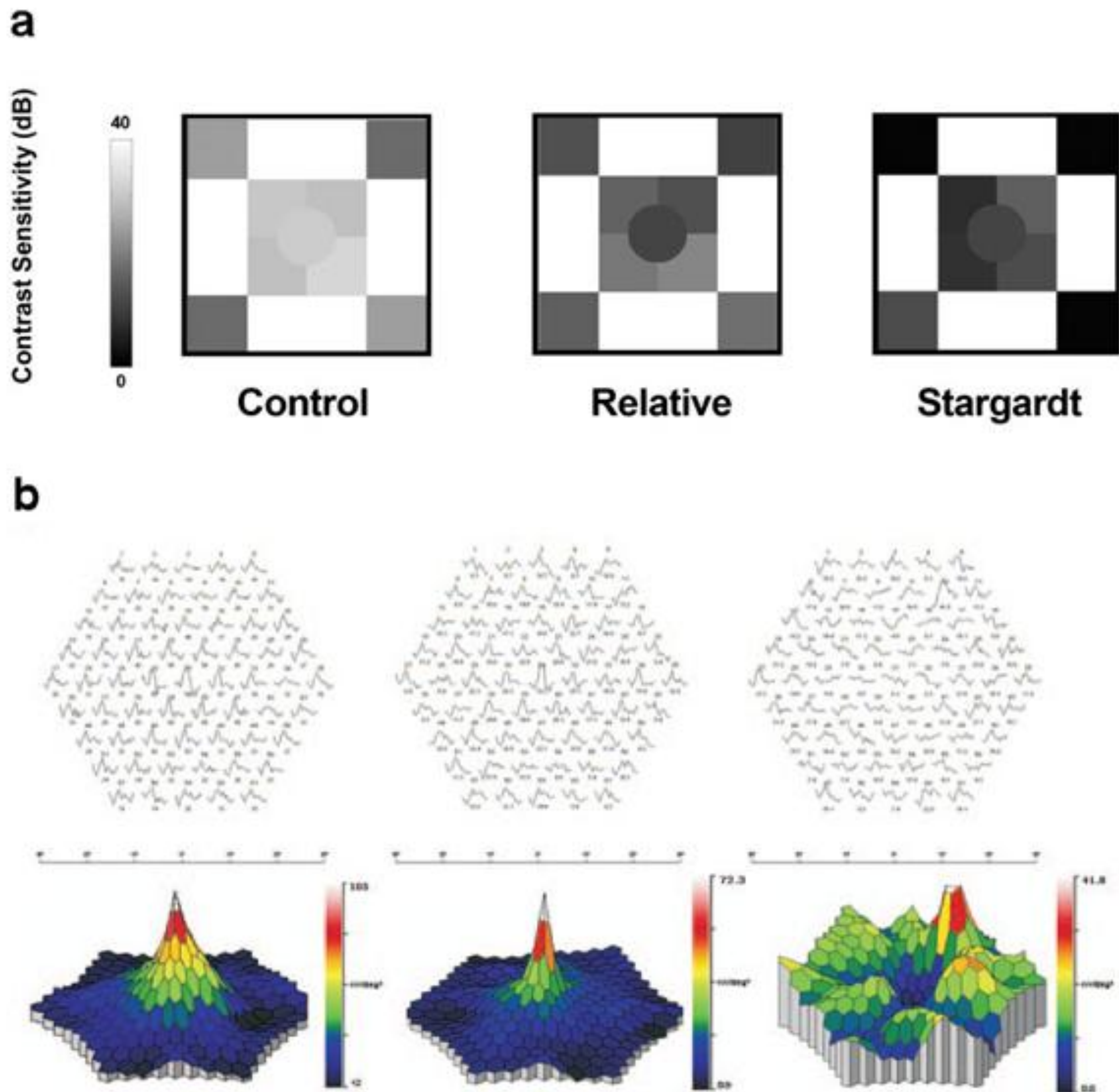


Fig. 2a Representative maps of contrast sensitivity of normal control subjects, Stargardt relatives and patients obtained by an intermediate spatial frequency method. Note the intermediate results in the Stargardt relatives subgroup. **2b Multifocal ERG results obtained from representative normal control subjects, Stargardt relatives and patients.** Decreased responses were observed for Stargardt patients and their relatives. *Adapted from Maia-Lopes et al. 2008.*

Genotype-phenotype correlations are becoming increasingly more important as gene therapy starts to emerge as a treatment possibility for diseases such as Stargardt. Indeed, screening patients or their siblings for *ABCA4* variants may allow for prediction of disease progression, significantly influencing the efficacy of gene therapy, which is essentially determined by stage of disease at the time of treatment.

PATHOPHYSIOLOGY OF STARGARDT DISEASE

The ATP-binding cassette (ABC) superfamily comprises a broad and heterogeneous group of proteins specialized in the active transport of various substrates across cellular membranes, against a concentration gradient (Higgins 1992). Substances transported include amino acids and other peptides, ions, metals, lipids and fatty acid derivatives, steroids, organic anions, vitamins and drugs, among others. ABC proteins exist in virtually every living organism and are involved in various human diseases. Grossly, their molecular structure consists of two transmembrane domains that provide a pathway for substrate translocation and two ATP-binding domains that bind and hydrolyze ATP, thereby supplying the energy required for substrate transport (Molday 2007). At least 48 genes are known to encode ABC transporters across the genome (Dean et al. 2001, Dean et al. 2005). Moreover, the ABC superfamily is further organized in seven subfamilies, ranging from ABCA to ABCG. In particular, the ABCA subfamily has been recently implicated in severe inherited diseases involving defects in lipid transport (Borst et al. 2000, Kaminski et al. 2006). That is the case of *ABCA4*, the gene linked to Stargardt disease.

Although ABC transporters are spread across the entire human organism, it is interesting to observe that ABC*A*4 localizes specifically to the retina and is present virtually nowhere else. Northern blot analysis and hybridization studies confirm that and sub-localize ABC*A*4 to the photoreceptor cell layer of the retina (Allikmets et al. 1997). With additional immunofluorescence labeling (Sun et al. 1997) and immunoelectron microscopy (Illing et al. 1997) it is possible to further precise ABC*A*4 location to the disc membranes present in the outer segments of rod and cone photoreceptors, where phototransduction initiates. This anatomical localization, together with the fact that mutations in *ABCA4* are associated with Stargardt disease, suggests that ABC*A*4 detents an important function in photoreceptor physiology and that it is required for long-term cell survival.

In the human retina, two types of photoreceptor cells are generally considered: rod and cone photoreceptors. Although both share basic structural anatomy (Fig. 3), their individual functions are quite different. Conventionally, rod cells are linked to vision under dimly lit environments and monochromatic vision, whereas cone cells ensure vision under bright light conditions, color and high definition vision. The general distribution of photoreceptors throughout the retina also differs. Whereas rods are somewhat

homogenously distributed throughout the retina, cone photoreceptors are predominant in its central area, with highest density at the fovea.

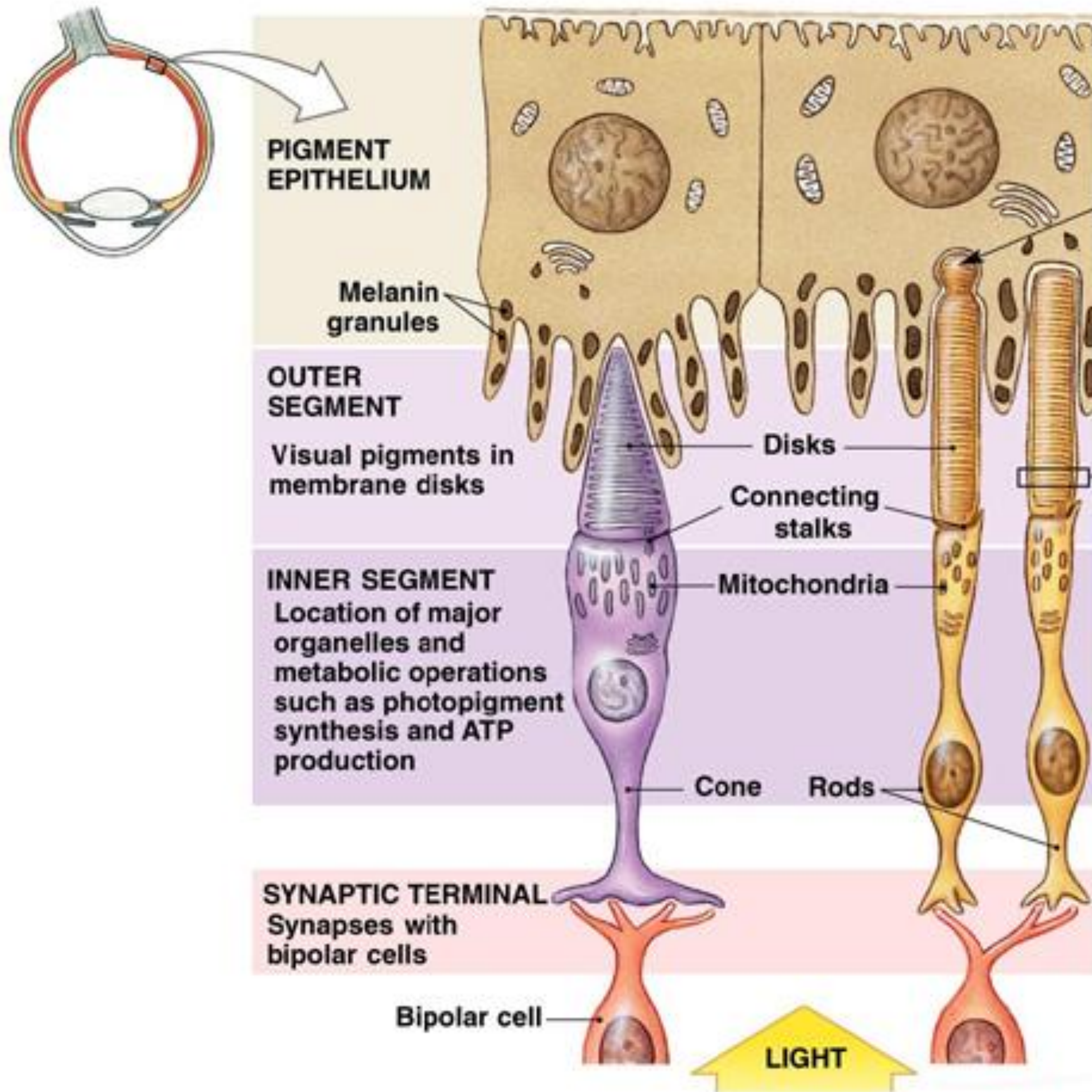


Fig. 3 General photoreceptor anatomy. Both cone and rod photoreceptors share a basic structural anatomy. Externally they consist of an outer segment where the visual chromophore, rhodopsin, is located, specifically in the so called disc membranes. Cone photoreceptors have a conic outer segment whereas rods have a more elongated, rod-like one. The outer segment is structurally supported by the retinal pigmented epithelium (RPE). The inner segment comprises the middle part of the photoreceptor cell and contains its nucleus, and other major organelles, performing the cell's main metabolic operations. A connecting stalk unites the outer and inner photoreceptor segments. The inner most part of the photoreceptor is the synaptic terminal, where the photoreceptor connects with neighboring bipolar cells. Rod photoreceptors tend to more elongated than cones. *Image copyright ©Pearson Education Inc. 2007.*

A critical step of the phototransduction process is the so called visual or retinoid cycle (Fig. 4), in which ABC*A*4 is thought to develop an essential role. Briefly, it consists of a number of

enzymatic reactions, which ultimate goal is the recycling of rhodopsin, or 11-*cis* retinal, the visual chromophore, so that it can be re-stimulated by a different photon, thus returning the photoreceptor to its dark adapted state. These reactions take place in a sequential manner on the two outermost cellular layers of the human retina, both the photoreceptor cell layer, where ABC*A*4 is expressed, and the RPE (Molday 2007).

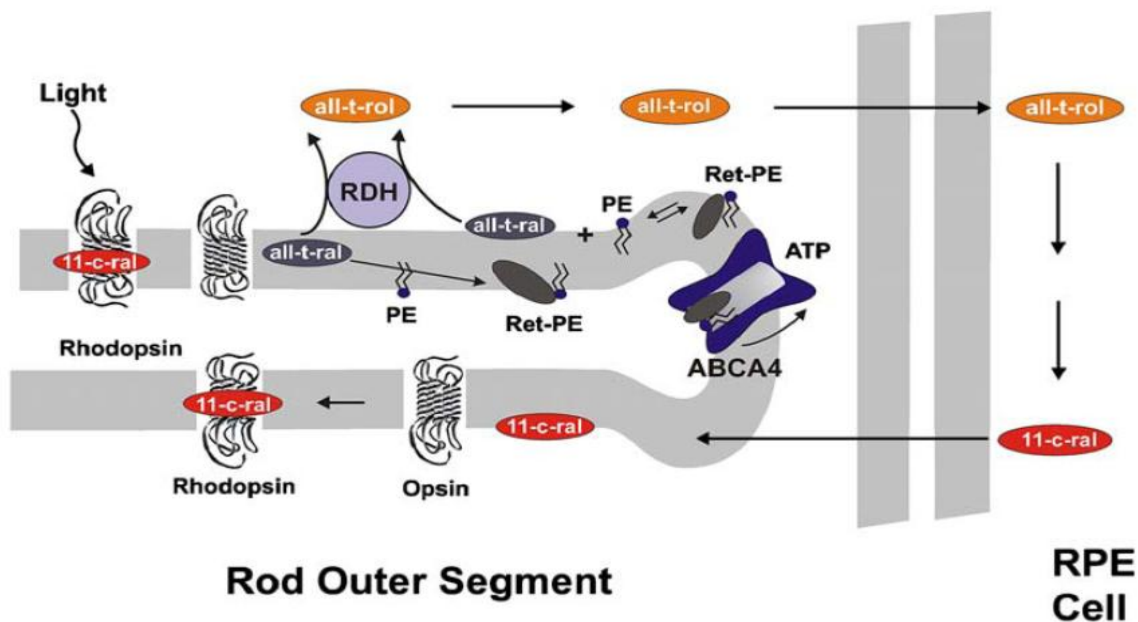


Fig. 4 General schematics of the retinoid cycle. When rhodopsin, or 11-*cis* retinal (11-c-ral), is stimulated by a photon it suffers a molecular conversion into all-*trans*-retinal (all-t-ral), a process which occurs in the disc membrane of photoreceptors' outer segments. Then, newly formed all-t-ral needs to be transported across the disk membrane into its cytoplasmic side. Some of the all-t-ral diffuses directly to that subcellular space but a significant fraction of it diffuses into the lumen aspect of the disc membrane where it reacts with phosphatidylethanolamine (PE) to form N-retinylidene-PE (ret-PE) which then becomes trapped, because of its protonated state. ABC*A*4 actively transports ret-PE across the disc membrane. When on the cytoplasmic side, ret-PE dissociates into all-t-ral and PE. Then, all-t-ral is reduced to all-*trans* retinol (all-t-rol) by all-*trans* retinol dehydrogenase (RDH). Afterwards, all-t-rol is transported to retinal pigmented epithelium (RPE) where the cycle continues, as depicted. *Adapted from Molday 2007.*

It is theorized that ABC*A*4 works as a “flippase” actively transporting N-retinylidene-phosphatidylethanolamine (N-retinylidene-PE), a product from the photobleaching of rhodopsin, across the disc membranes of photoreceptors but, although this is widely accepted, it remains to be experimentally confirmed (Molday 2007).

To further characterize ABC*A*4 function and study the effects of its deficiency, *abca4* knockout mice have been genetically engineered by Travis and colleagues (Weng et al. 1999, Mata et al. 2000 and 2001). In this animal model, it was possible to observe that mice, either homozygous or heterozygous for *ABCA4* blockade, maintained normal retinal appearance

and photoreceptor structure, as well as normally organized outer segments, in contrast to what is often observed in degenerative retinal conditions (Weng et al. 1999, Mata el al. 2000 and 2001). Their electrophysiological studies also remained normal, but a delayed rod dark adaptation was detected (Weng et al. 1999). However, the most striking finding was observed when the same mice were studied at a molecular basis. Abnormally high levels of protonated N-retinylidene-PE, all-*trans*-retinal and phosphatidylethanolamine (PE) were found to be trapped inside their photoreceptor outer segments. Similarly, in the RPE of *abca4* knockout mice exposed to continuous or cycling lighting conditions, equally abnormally high levels of another compound, Di-retinoid-pyridinium-ethanolamine (A2E), were found (Weng et al. 1999, Mata et al. 2001, Radu et al. 2004). A2E is known to be a major component of lipofuscin (Koenekoop 2003). Already these studies indicate that ABC*A*4 is not required for normal photoreceptor structure or morphogenesis. On the other hand, ABC*A*4 seems to play a metabolic role, being responsible for the removal of all-*trans*-retinal and N-retinylidene-PE from disc membranes after the photobleaching of rhodopsin, thereby preventing retinoid accumulation in this sub-cellular space (Weng et al. 1999, Sun et al. 1999). Although most studies refer to rod photokinetics, it is likely that ABC*A*4 plays a similar role in cone photoreceptors, since subsequent studies also localized the protein to the outer segments of foveal and peripheral cone photoreceptors (Molday et al. 2000).

Studies in *abca4* knockout mice support the proposed pathophysiology model for human disease. Accordingly, mutant ABC*A*4 would become unable to transport N-retinylidene-PE across the disc membranes of photoreceptors' outer segments leading to accumulation of this compound inside their disc lumina. Equally, all-*trans*-retinal would be excessively accumulated. When in excess, all-*trans*-retinal can re-associate with opsin to form a complex that activates the visual cascade, although less efficiently than photoactivated rhodopsin (Buczylko et al. 1996, Surya et al. 1998). This low level of activity can explain the prolonged dark adaptation usually found in Stargardt patients and in *abca4* knockout mice, and explains the residual vision often observed in Stargardt patients (Weng et al. 1999, Mata et al. 2000 and 2001). However, the most important consequence of N-retinylidene-PE accumulation comes from the fact that it can also react with all-*trans*-retinal to form Di-retinoid-pyridinium-phosphatidylethanolamine (A2PE), through chemical condensation, rearrangement and oxidation (Fig. 5b; Mata et al. 2000, Ben-Shabat et al. 2002). Every 10 days or so, aged photoreceptor outer segments undergo renewal through

shedding and phagocytosis by adjacent RPE cells. New membrane components are then added at the base of the photoreceptors' outer segments (Molday 2007, Molday et al. 2009). Upon outer segment phagocytosis into the RPE cells, phagosomes, containing A2PE, all-*trans*-retinal dimers and other retinoid compounds, fuse with lysosomes to form phagolysosomes. Further hydrolysis of A2PE leads to the formation of A2E, which cannot be further hydrolyzed, resulting in its progressive accumulation as lipofuscin deposits in the RPE cell (Fig 5a; Mata et al. 2000, Ben-Shabat et al. 2002). Indeed, measurements of lipofuscin accumulation in the RPE of Stargardt patients can reach up to five times the normal values (Delori et al. 1995, Koenekoop 2003). Excess A2E in the RPE has its consequences since this compound exerts a negative effect on the epithelium's function and survival. A2E can act as a detergent, compromising normal cellular membrane architecture and inhibiting normal RPE metabolic functions (Eldred et al. 1993). Additionally, in the presence of oxygen and blue-wavelength light it forms free radical epoxides, which induce RPE cell death (Fig 5a; Sparrow et al. 2000 and 2005).

Loss of RPE has negative consequences over the photoreceptor cell layer. Beyond giving structural support and promoting photoreceptor renewal, RPE also provides nutritional support to photoreceptors and participates in the regeneration of rhodopsin. Likewise, death of RPE inevitably leads to irreversible secondary photoreceptor degeneration and, consequently, loss of vision (Molday 2007). Recently, Gomes and co-workers, using autofluorescence imaging and optical coherence tomography, postulated that photoreceptor loss might actually precede RPE cell death (Gomes et al. 2009).

This pathophysiologic model is supported by the characteristic features found in *abca4* knockout mice and Stargardt patients, in whom accumulation of byproducts derived from the photobleaching of rhodospin plays a central role. In fact, evidence of lipofuscin deposition in the RPE has been acquired in histological examination of human Stargardt retinas (Fig. 6, Yannoff and Duker's Ophthalmology, 3rd edition, 2008).

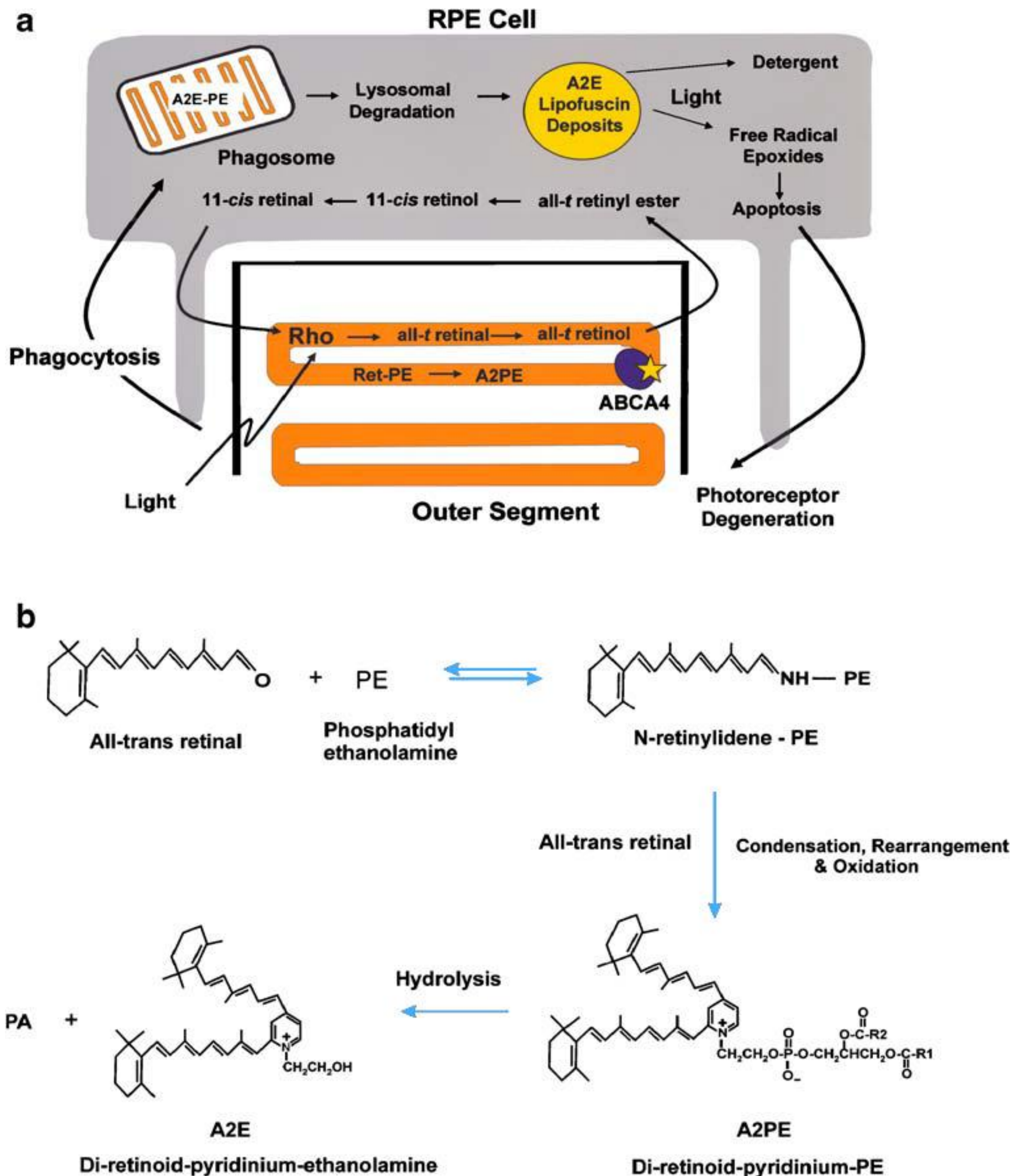


Fig. 5a General pathophysiology of Stargardt disease. When ABC*A*4 is defective, N-retinylidene-phosphatidylethanolamine (Ret-PE) and all-*trans*-retinal (all-t-retinal) accumulate in the outer segment of photoreceptors and both compounds react to form Di-retinoid-pyridinium-phosphatidylethanolamine (A2PE). Every 10 days, photoreceptors undergo renewal by shedding and phagocytosis by neighboring retinal pigmented epithelium cells (RPE cell). Once inside the RPE, A2PE incorporated into phagolysosomes is further hydrolyzed to A2E, which cannot undergo any further degradation. In this way, A2E accumulates inside RPE cells as lipofuscin deposits exerting negative effects on RPE function and survival. **5b. Chemical reactions leading to A2E formation.** *Adapted from Molday 2007.*

Although this pathophysiological model is widely accepted, some issues remain to be elucidated. It still remains to be experimentally demonstrated that ABC*A*4 works directly as a transporter for N-retinylidene-PE across disc membranes, and its ATP-dependent function is still not fully understood. Likewise, the direction of transport, from the lumen to the cytoplasmic side of disc membranes, turns out to be opposite to the direction of substrate transport of other well-studied mammalian ABC transporters, and this issue still requires further clarification. To date, there are still to be identified regulators of ABC*A*4 activity (Molday 2009).

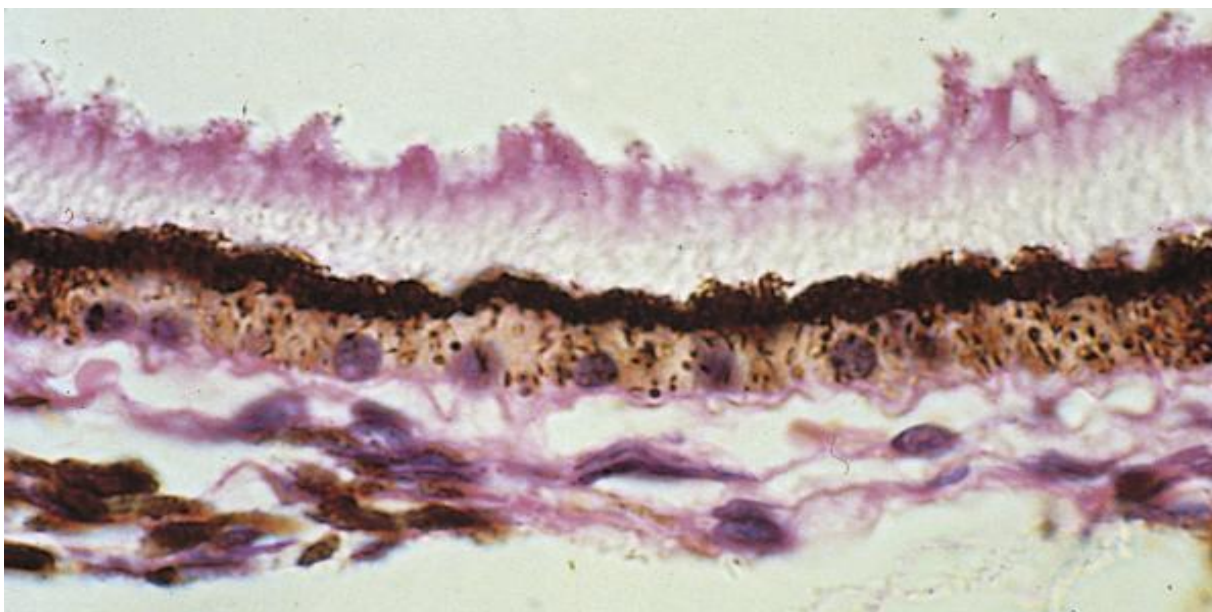


Fig. 6 *Post-mortem* histological specimen from the retina of a patient with Stargardt disease. Histological examination confirms lipofuscin deposition on the retinal pigmented epithelium. This compound can be accumulated up to 5 times normal values. *Adapted from Yannof and Duke's Ophthalmology, 3rd edition, Mosby-Elsevier © 2008.*

Like in other diseases, not necessarily eye conditions, learning the pathophysiology for Stargardt disease represents the basis for understanding its clinical expression and designing logical and intuitive therapeutic interventions.

STARGARDT DISEASE: CLINICAL FEATURES AND DIAGNOSIS

Diagnostic evaluation of Stargardt disease is based on family history, visual acuity, fundus examination, visual field testing, fluorescein angiography, fundus autofluorescence, ERG, and cross-sectional imaging using state-of-the-art techniques like optical-coherence

tomography (OCT). Genetic testing is not currently performed on a routine basis (Berisha et al. 2009).

Stargardt patients may be asymptomatic but most commonly present with bilateral central visual loss, photophobia and color vision abnormalities, parafoveal scotomata and slow dark adaptation. Vision deterioration is rapidly progressive and its age of onset is highly variable, although it most often occurs between childhood and adolescence or early adulthood (Stargardt 1909, Hadden et al 1976, Lois et al. 2001, Westerfeld et al. 2008). Presenting visual acuity may range between 10/10 and 0.5/10, with prior visual acuity being frequently normal, and very few patients further deteriorate their vision to counting finger or hand motion level (Hadden et al. 1976, Rotenstreich et al. 2003, Westerfeld et al. 2008). Interestingly, it seems the prognosis for visual outcome is highly dependent of age of disease onset. Rotenstreich and co-workers estimate that patients presenting with a visual acuity of 5/10 at an early age (20 years old or below) would develop a more rapid course of disease achieving a visual acuity of 2/10 or worse at a mean time of 7 years (Rotenstreich et al. 2003). Likewise, patients who presented with a visual acuity of 5/10 between 21 and 40 or 41 and 60 years would experience slower visual deterioration over a mean time of 22 and 29 years, respectively (Rotenstreich et al. 2003).

A positive family history of Stargardt disease is an invariable finding, with an autosomal recessive pattern of inheritance being the rule. Accordingly, both genders are equally affected and the disease is equally transmitted by them. Most affected individuals have normal progenitors, heterozygotes have a normal phenotype, affected parents will always have carrier offspring, and close relatives are more likely to have affected children.

Color vision in Stargardt patients is typically compromised but it is not frequently evaluated on a clinical basis. Using Hardy-Rand-Rittler or Ishihara color plates (Fig. 7a) it is possible to detect a mild red-green dyschromatopsia in patients with Stargardt disease. Moreover, when these patients are submitted to a Farnsworth-Munsell Hue Test (Fig. 7b), it is possible to note a tritan axis (or short wavelength) deviation (Mantyjarvi et al. 1992).

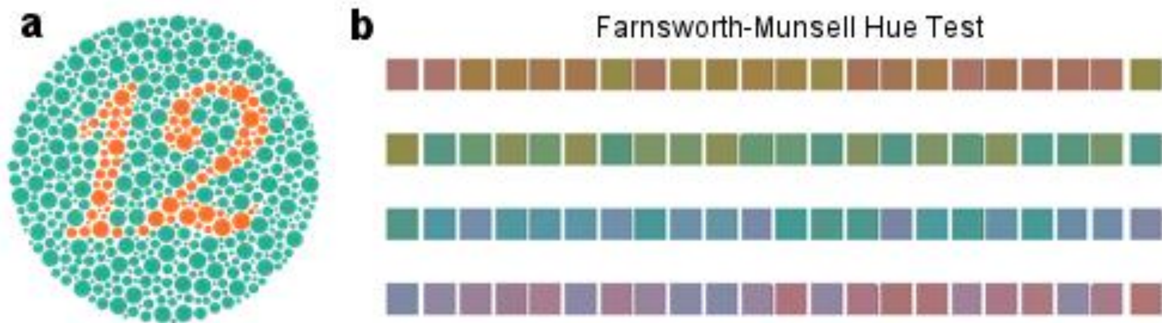


Fig. 7a Ishihara color plate. Used to evaluate type and degree of color deficiency. Briefly, the patient is asked to identify colored shapes or numbers that lie within a jumble of dots which vary in color and intensity. The physician detects and categorizes the deficiency based on the patient's responses. **7b Farnsworth-Munsell Hue Test.** Evaluates the patient's ability to identify gradations of color by asking him to place different hues in order. *Images adapted from Wikipedia © and X-rite ©, respectively*.

The first diagnostic approach for Stargardt disease can be simply made by direct ophthalmoscopy. Stargardt disease affects the macula with variable centrifugal expansion, but fundus examination is frequently normal early on the course of the disease, even if patients already complain of visual loss (Stargardt 1909). At this stage, the clinical diagnosis of Stargardt might be missed and patients' complaints can be easily interpreted as functional visual loss, especially if the patient is a child or a teenager (Holz et al. 2007). Later on, typical fundus manifestations arise, including pigment mottling, frank macular atrophy, a bull's eye appearance and fundus flecks (Fig. 8; Hadden et al. 1976). However, it should be underscored that Stargardt disease presents with highly variable phenotypes, influenced by a combination of several factors (Zhang et al. 1999, Klevering et al. 2005).

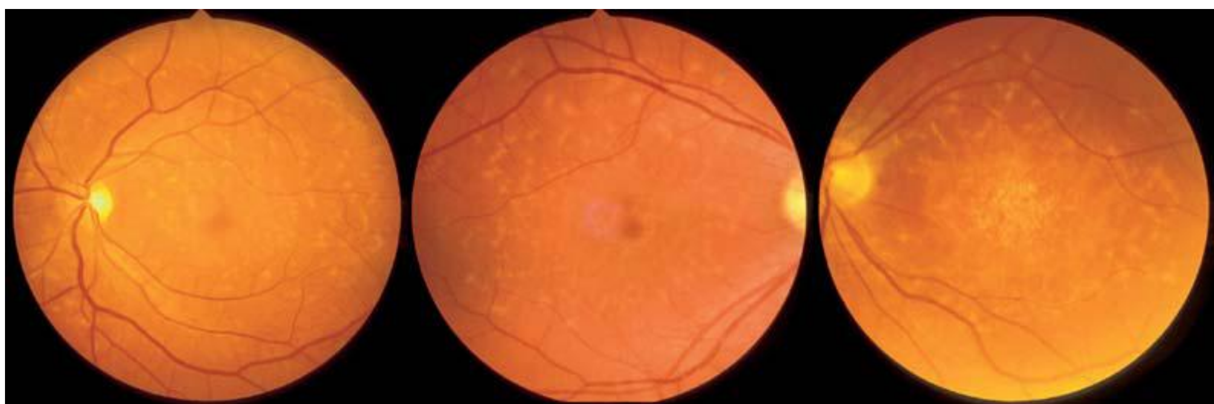


Fig. 8 Color fundus photographs of three patients with Stargardt disease. One patient presents a normal-looking fovea (left) whereas the other two show a bull's eye maculopathy (middle) and foveal atrophy (right), respectively. *Adapted from Holz et al. 2007.*

Fundus flecks (Fig. 9) are pisciform, round or dot-like yellow-white lesions typically found in Stargardt patients and should be seen at a given point in order to consider the clinical diagnosis of the disease (Stargardt 1909). Flecks translate the accumulation of lipofuscin in the RPE but may also represent areas of regional depigmentation and atrophy (Holz et al. 2007). Accordingly, some have suggested the term “active flecks” to define the former and “reabsorbed flecks” for the latter (Fishman 1976, Aaberg 1986). However, these classifications have not been thoroughly accepted possibly because fleck distribution may change over time, does not correlate well with the visual loss, and there seems to be no intra-familiar concordance regarding their presence (Hadden et al. 1976, Lois et al. 1999 and 2001).

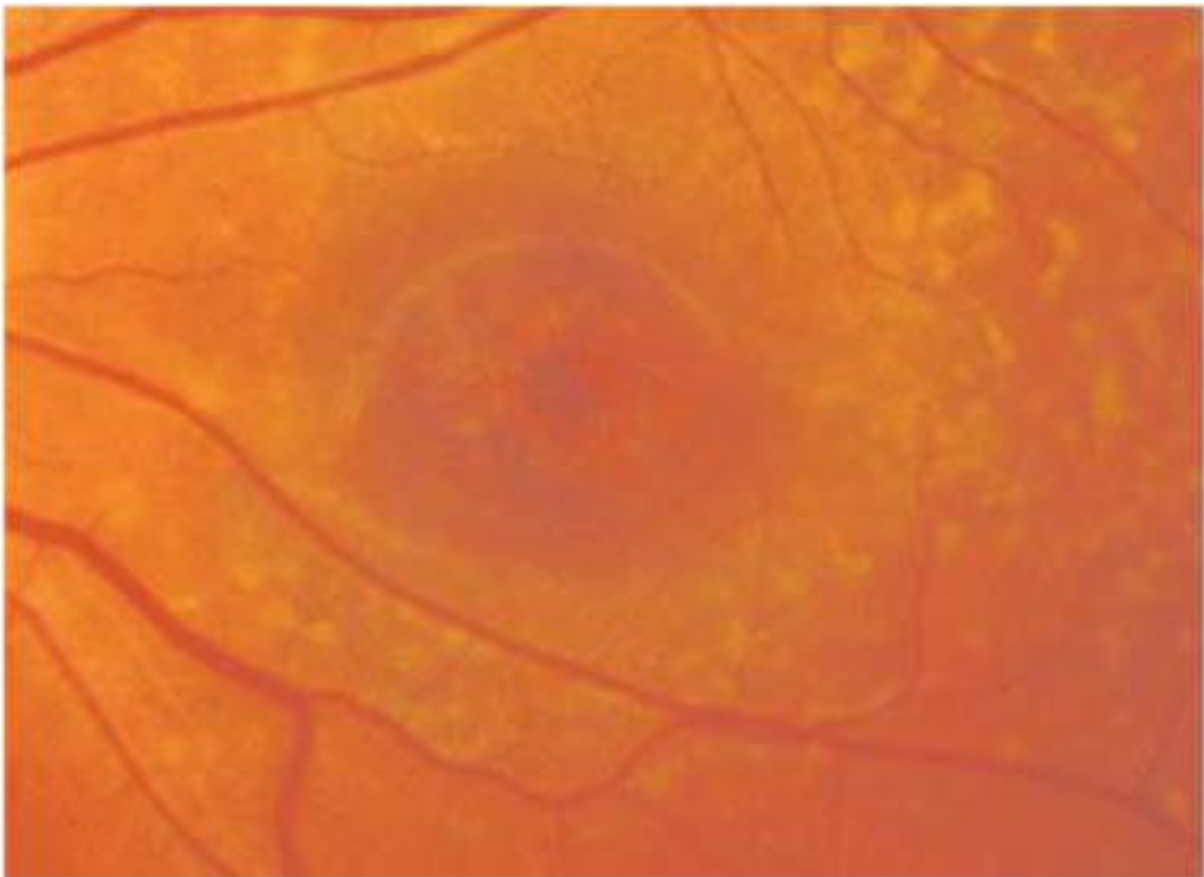


Fig. 9 Typical morphology of fundus flecks in a patient diagnosed with Stargardt disease. Flecks are observed as pisciform, round or dot-like yellow-white aggregates, forming individual or confluent lesions. They relate to lipofuscin accumulation in the retinal pigmented epithelium and may also represent regional depigmentation and atrophy. Distribution of flecks may change during evolution of the disease. Note the macular involvement, with atrophy. *Adapted from Holz et al. 2007.*

Flecks may form individual or confluent patterns and have a typical central distribution in Stargardt patients, with variable mid-periphery involvement (Franceschetti 1965). In fact, they are the main feature of the once-thought-distinct clinical entity termed fundus flavimaculatus (Franceschetti 1963). This condition shares obvious phenotypic similarities with Stargardt disease. Indeed, it is now accepted that fundus flavimaculatus and Stargardt disease are genetically linked and that the former represents a subset of Stargardt manifestations, with intermediate forms between the two equally considered (Hadden et al. 1976, Aaberg 1986, Armstrong et al. 1998, Fishman et al. 1999, Holz et al. 2007). It should be pointed that Stargardt and fundus flavimaculatus differ in important aspects. In contrast to Stargardt disease, patients with fundus flavimaculatus often have a later disease onset and slower visual deterioration, making fundus flavimaculatus a milder condition than typical Stargardt disease (Klevering et al. 2005). Surprisingly, if one looks at fundus photographs of both conditions, one might get the opposite impression because in fundus flavimaculatus there appears to be a more widespread retinal involvement (Fig. 10). However, although in fundus flavimaculatus flecks are diffusively scattered throughout the posterior pole ,extending out to the midperiphery, in this condition the macula is typically less involved, therefore enabling better visual performance.

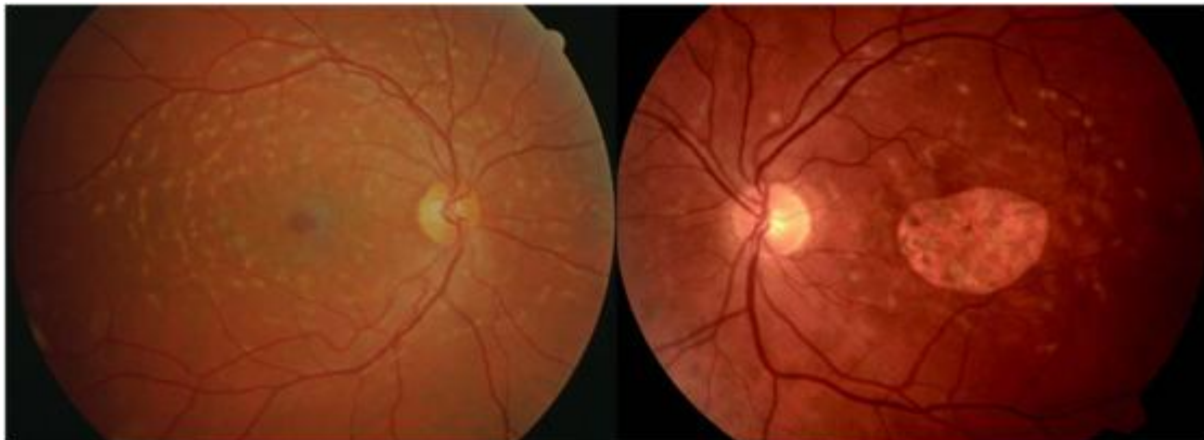
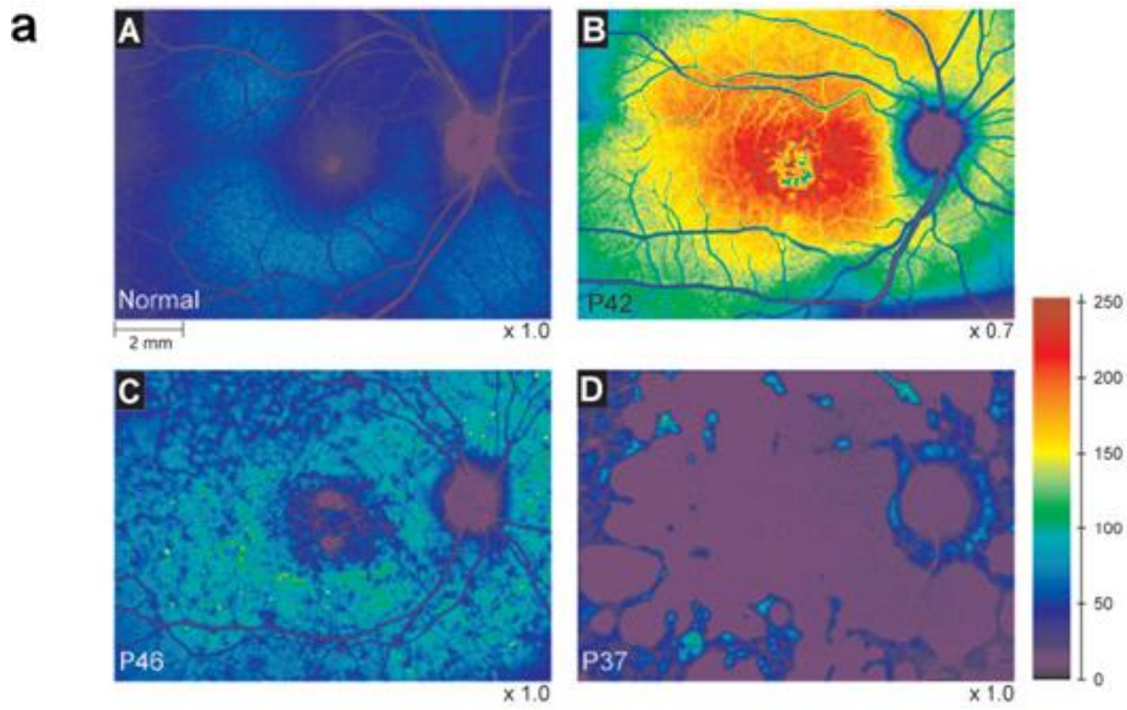


Fig. 10 Comparison between fundus flavimaculatus and Stargardt disease fundus photographs. Fundus photograph of a patient with fundus flavimaculatus (left) shows more widespread retinal involvement with scattered yellow-white flecks in the mid-periphery; the macula shows a bulls-eye appearance, but is relatively preserved. Fundus photograph of a patient with Stargardt disease (right) denotes obvious macular involvement with some surrounding flecks. *Images adapted from Le Syndicat National des Ophthalmologistes de France (left) and from Tombran-Tink et al. 2007 (right).*

Visual field testing in Stargardt patients is often normal in early stages of the disease. Over time, relative central scotomata develop, further progressing to absolute central scotomata, in a variable fashion. Typical Stargardt patients usually preserve their peripheral visual fields. However, in severe cases, with widespread retinal atrophy, visual constriction can occur (Armstrong et al. 1998). Another particularly important finding is the change in preferred retinal locus of fixation. Early in Stargardt's disease natural evolution, most patients maintain a foveal fixation, but as disease progresses absolute central scotomas develop and the preferred retinal fixation becomes eccentric, as demonstrated by several microperimetry studies (Rohrschneider et al. 1997, Messias et al. 2007, Reinhard et al. 2007). In most cases, this new eccentric fixation point localizes above the foveal lesion (Sunness et al. 1996, Rohrschneider et al. 1997, Reinhard et al. 2007, Berisha et al. 2009). This finding might have a normal physiological explanation. Indeed, Silva and colleagues recently demonstrated, through psychophysical studies, increased contrast sensitivity to low spatial and high temporal frequency stimuli in the superior retina compared to its inferior division (Silva et al. 2008). This is also consistent with the fact that the superior retina has higher densities of ganglion cells (Curcio et al. 1990). Further astonishing data came from Cideciyan and co-workers who, in a 41-patient study, found that *ABCA4*-related disorders, including Stargardt disease, spare structure and function of the parapapillary retina (the area that normally surrounds the optic nerve head) as determined by fundus autofluorescence (Cideciyan et al. 2005). Indeed, a parapapillary ring of normal-appearing fundus autofluorescence was identifiable in all disease stages (Fig. 11a). Additional histological examination confirmed that the structural abnormalities increased as a function of distance from the center of the optic nerve head (Fig. 11b). Remarkably, this area served as a preferred retinal locus of fixation in up to 30% of patients (Cideciyan et al. 2005).

Fluorescein angiography has a limited role in the diagnostic evaluation of Stargardt disease and is not performed on a routine basis as other diagnostic techniques, like fundus autofluorescence, are less invasive and provide similar data. However, it can be useful at initial presentation whenever fundus changes are not so evident (Westerfeld et al. 2008).



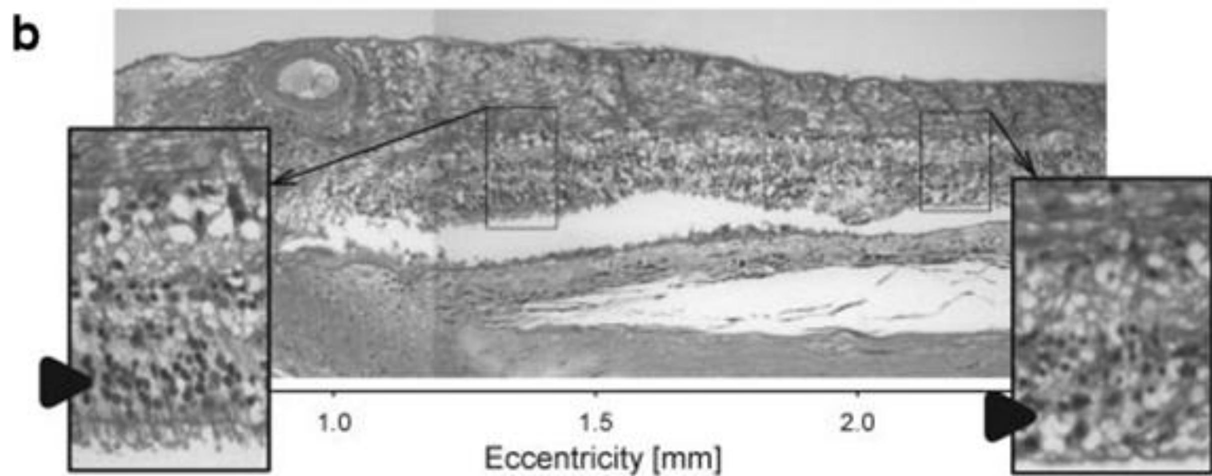


Fig. 11a Standardized fundus autofluorescence images of a representative normal subject (A) and three Stargardt patients (B-D; P42, P46, P37). Patients present in different disease stages. Note the preserved ring of parapapillary retina in all patients. **11b. Histopathology of a donor eye with Stargardt disease, picturing the parapapillary region nasal to the optical nerve head.** Structural abnormalities of photoreceptors and retinal pigmented epithelium increase and become more evident as a function of eccentricity relative to the center of the optical nerve head. *Adapted from Cideciyan et al. 2005*.

In Stargardt patients, fluorescein angiography reveals a "dark-choroid" sign (Fig. 12) in up to 62% of patients (Rotenstreich et al. 2003). This sign, unexclusive of Stargardt disease, derives from a lack of early choroidal hyperfluorescence, which is blocked by high-grade lipofuscin accumulation in the RPE, thus enabling visualization of the small retinal capillaries that

become easily evident over the dark, non-fluorescent and high-contrast choroid. Fundus flecks are seen as small irregular hyperfluorescent lesions (Fig. 12). Their presence over a "dark-choroid" background further suggests the diagnosis of Stargardt disease (Holz et al. 2007).

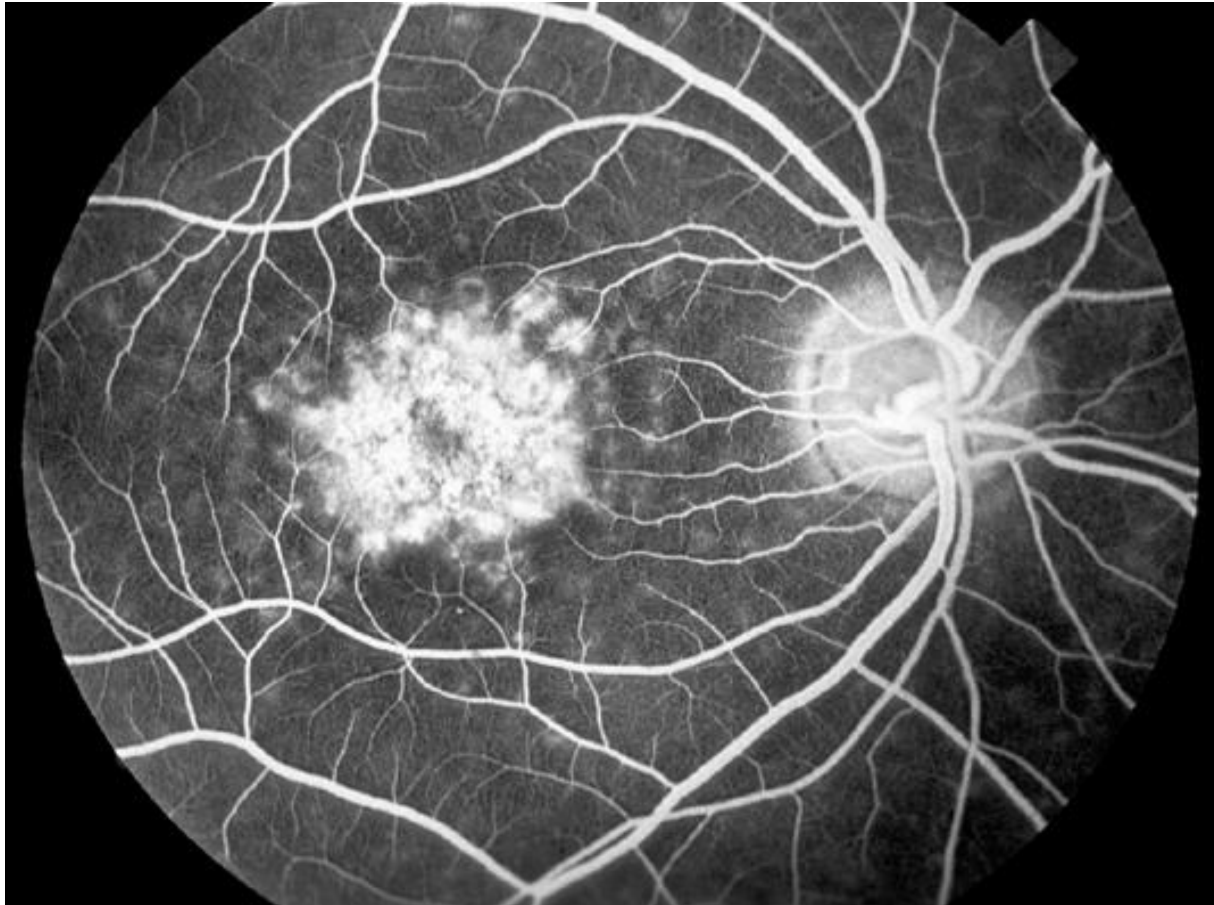


Fig. 12 "Dark-choroid" sign. The "dark-choroid" sign is unexclusive to Stargardt disease and relates to high-grade lipofuscin accumulation in the retinal pigmented epithelium, which blocks early choroidal fluorescence, thus creating a dark, non-fluorescent and high-contrast background, enabling easy visualization of retinal capillaries, otherwise less visible. The presence of small irregular hyperfluorescent fundus flecks further suggests the angiographic diagnosis of Stargardt disease. *Adapted from Holz et al. 2007.*

Fundus autofluorescence imaging, a relatively novel diagnostic procedure, provides a fast non-invasive way to study the health and viability of the RPE. In brief, it uses the natural fluorescent properties of lipofuscin to quantify its distribution throughout the RPE. Technically, fundus autofluorescence involves excitation of the eyes with a monochromatic short-wavelength light after which the pattern of fluorescence is detected and recorded by a Confocal Scanning Laser Ophthalmoscope (cSLO), allowing the spatial distribution of fundus autofluorescence over large retinal areas to be documented. Likewise, correlations between

the pattern of fundus autofluorescence and pathologic features observed on fundus examination can be performed (Audo et al. 2005). Normal fundus autofluorescene parallels histological data concerning lipofuscin distribution (von Rückmann et al. 1995). Higher levels of fluorescence are achieved in the parafoveal area, peaking on a ring located at about 10° eccentric to the fovea, gradually decreasing towards the peripheral retina, closely correlating with the relative photoreceptor densities and underlying RPE metabolic loads. The optic disc and the retinal vessels typically lack fluorescence, because there is no RPE in the former, and because it is masked by the overlying blood column in the latter (Audo et al. 2005). Abnormally increased fundus autofluorescence represents excessive lipofuscin accumulation in the RPE. Inversely, decreased areas of fundus autofluorescence relate to low level of RPE metabolic activity which normally underlies local atrophy with secondary photoreceptor loss (Fig. 13a and 13b; von Rückmann et al. 1995, Katz et al. 2001). Therefore, fundus autofluorescence is a perfectly adequate exam to study and diagnose Stargardt disease and it proved to be one of the most efficient in determining the stage of disease especially if combined with ultrasctructural data derived from OCT (Gomes et al. 2009). Indeed, abnormalities of autofluorescence intensity are an early sign of *ABCA4*-related disease and correlate well with local severity (Cideciyan et al. 2005). This supports the work of Cideciyan and colleagues who further advance that abnormally high autofluorescence intensity with all other normal parameters suggests that RPE lipofuscin deposition may be the first pathophysiological event in *ABCA4*-related disease (Cidecyian et al. 2004).

Electrophysiological studies in Stargardt patients reveal that they typically maintain normal or subnormal full-field electroretinographic scotopic (rods) and photopic (cones) responses. However, patients with more widespread disease can present with notably abnormal scotopic and photopic responses on full-field ERG (Lois et al. 2001). Given that there is no reliable way to predict the type of functional visual loss based on the fundus examination alone, electrophysiological testing is essential to evaluate patients with Stargardt disease. Specifically, these tests have a prognostic value as patients that present with early peripheral photoreceptor dysfunction have a higher chance of developing greater functional losses (Lois et al. 2001). Curiously, there seems to be intra-familial homogeneity in the qualitative pattern of functional loss (Aaberg 1986, Lois et al. 1999). Electroretinography can further demonstrate the dark adaptation typical of Stargardt patients, correlating with underlying slow rod kinetics. Indeed, Cideciyan and co-workers found that delay of dark

adaptation is strongly correlated with the absolute dark-adapted rod sensitivity at the same retinal locus, suggesting a direct relationship between the extent of local rod photoreceptor degeneration and abnormality of retinoid cycle kinetics (Cideciyan et al. 2004). More central retinal locations showed slower kinetics and lower sensitivities than more peripheral loci. Cone dark adaptation kinetics showed similar results. Likewise, both rod and cone thresholds presented comparable loss. Interestingly, it was also noted that retinoid cycle slowing down tends to progress like the underlying retinal degeneration, meaning that younger individuals with less severe disease had faster photoreceptor responses than older individual with more advanced disease (Cideciyan et al. 2004).

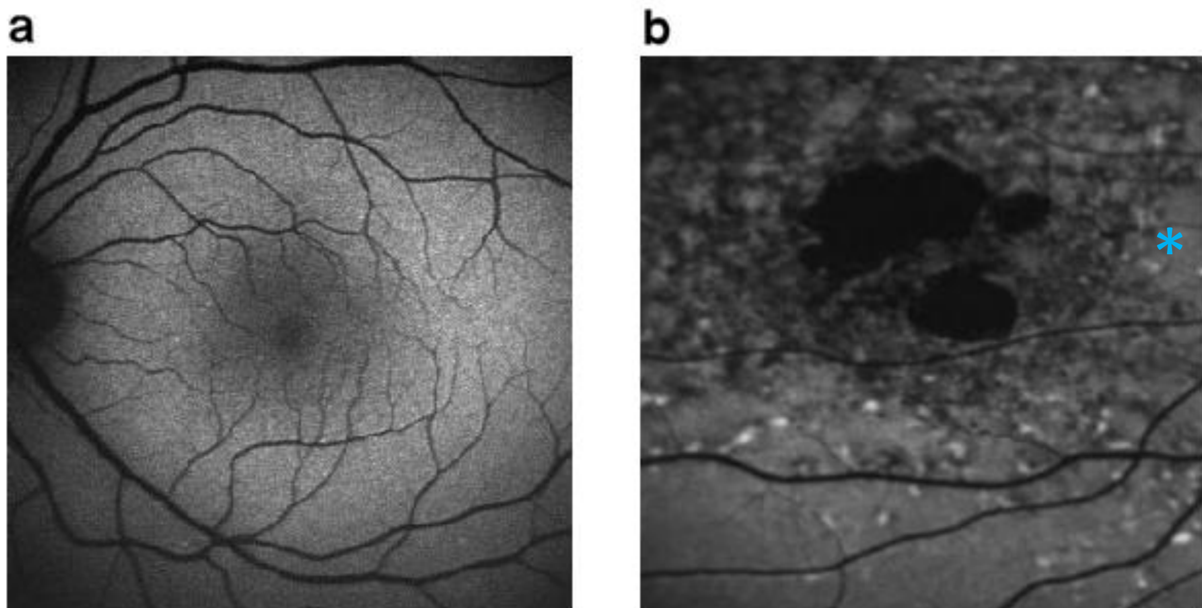


Fig. 13a Normal pattern of fundus autofluorescence. Note the high fluorescence in the parafoveal area and the progressive decrease towards the periphery. The optic nerve head and blood vessels typically lack autofluorescence. **13b Central retinal fundus autofluorescence in the right eye of a 31-year old Stargardt patient.** A sharply demarcated central area lacks fluorescence, denoting foveal atrophy. Irregular hyper and hypofluorescent lesions are dispersed across the posterior pole. Note the sparing of the parapapillary retina (blue asterisk*). *Adapted from Audo et al. 2005*.

Ultrastructural imaging like the one provided by optical coherence tomography (OCT) is a fast evolving tool, which has been applied to the real-time, non-invasive, *in vivo* study of retinal diseases. Basically, employing near-infrared light, OCT captures micrometer-resolution, three-dimensional images of the retina allowing for easy visualization of its structural organization and accurate detection of early changes in its different layers. Newer high-resolution tools using superluminescent diodes and ultrashort pulsed lasers, allow sub-

micrometer resolution, further improving image detail. OCT has been increasingly used in the study of Stargardt disease. It allows early detection of lipofuscin accumulation in the RPE and photoreceptor layer disorganization. Combined with autofluorescence data, OCT can provide valuable information regarding disease staging. Recently, Gomes et al studied 22 patients combining data obtained from autofluorescence and OCT; they concluded that OCT provides a more precise evaluation of local disease severity than autofluorescence, a relationship that is further accentuated as autofluorescence intensity values decrease. Indeed, these investigators showed that when areas of absent fluorescence were analyzed using OCT, the degree of photoreceptor loss was greater than expected. Areas of reduced fluorescence showed a better correlation with OCT findings. These findings lead to the assumption that photoreceptor loss might actually precede RPE cell death, bringing new insights into the yet-to-be-fully-understood pathophysiology of Stargardt disease (Gomes et al. 2009).

THERAPEUTIC OPTIONS FOR STARGARDT DISEASE

At present, no definite cure is known for Stargardt disease. Current therapeutic options include photoprotection and pharmacological slowdown of the visual cycle.

There is evidence showing that all-*trans*-retinal induces photo-oxidative damage to the unusually sensitive ABC*A*4, further compromising its function (Sun et al. 2001, Cideciyan et al. 2004). Given that Stargardt patients already have impaired ABC*A*4 function and increased levels of all-*trans*-retinal trapped inside their photoreceptors, it is logical to think that these patients would be extremely sensitive to the effects of light exposure (Sun et al. 2001). Furthermore, Sparrow and colleagues have demonstrated that di-retinoid-pyridinium-ethanolamine (A2E) does not accumulate in the RPE of *abca4* knockout mice kept in total darkness (Sparrow et al. 2000). Accordingly, Stargardt patients are usually advised to avoid direct ocular exposure to sunlight. In that way, ultraviolet-blocking sunglasses are useful.

Given that A2E cannot be further hydrolyzed in the RPE, there is considerable interest in preventing its synthesis. Isotretinoin is reportedly capable of dampening A2E deposition in the RPE *abca* knockout mice (Radu et al. 2003). However, considerable side effects associated with chronic intake of isotretinoin prevent its use in humans. Thus, further research is needed in order to develop better-targeted compounds or forms of

administration that will enable effective treatment with minimal and/or acceptable side effects (Tombran-Tink et al. 2007).

In recent years, the field of gene therapy has suffered remarkable advances. Of interest, gene therapy for ocular diseases has gained considerable attention among the ophthalmology community. Much of that attention is due to successful preliminary results of gene therapy trials performed in animal models of degenerative retinal diseases such as Leber Congenital Amaurosis (LCA). At the same time, these results encouraged other investigators to study gene therapy possibilities for other ocular conditions, including Stargardt disease. Gene therapy holds the promise of cure for most Stargardt patients who have to deal daily with their visual disability.

GENE THERAPY

Gene therapy can be simply defined as “the use of genes as medicine”. In detail, the concept of gene therapy involves the transfer of genetic material into a cell, tissue or whole organ in order to cure a disease or at least improve the patient’s clinical status (Verma et al. 2005). The primary goal is to replace defective, non-functioning genes with new fully-functional ones so that normal levels of genetic expression can be achieved. Alternatively, gene therapy can also produce gene silencing, through the inhibition of the expression of a gene or gene product, and genetic addition, by delivery of a gene whose product provides beneficial effects regardless of the primary defective gene’s condition (Colella et al. 2009). Thus, gene therapy offers a wide therapeutic potential for inherited and acquired diseases. However, some prerequisites need to be met. The condition for which gene therapy is to be performed requires a good understanding of its pathophysiology and the underlying faulty gene.

The process of gene delivery and expression is known as transduction and invariably requires vectors to mediate gene transfer. Vector development remains one of the most challenging obstacles to widespread application of gene therapy (Verma et al. 2005). An ideal vector would be one that could be produced in a highly concentrated form, through convenient and reproducible production schemes, and one that could be injected as many times as desired. Additionally, it should be able to target a specific cell type and achieve stable, sustained gene expression through integration of the vector DNA in the host genome or maintenance as an episome. Finally, the ideal vector should not elicit any undesirable

immune response or adverse effect (Kumar-Singh 2008, Smith et al. 2009). Unfortunately, such a vector does not yet exist.

Vectors can be of two different natures: viral and non-viral. Non-viral vectors consist of naked DNA, cationic lipids, DNA nanoparticles and small interfering RNA (siRNA). They offer several advantages over viral vectors. In general, non-viral approaches are less toxic and immunogenic, lack a pathogenic effect, present a lower risk of insertional mutagenesis and are more easily manipulated and manufactured. However, non-viral vectors lack the efficacy of viral vectors regarding gene delivery and duration of subsequent genetic expression (Liu et al. 2007). As a consequence, viral approaches continue to be the preferred vector system for gene delivery.

Viral approaches for gene transfer use the natural ability of viruses to deliver genetic material into the cell they are infecting. The basic process of viral vector production relies on the ability to individualize the components needed for replication of the virus from those capable of causing disease (Fig. 14). Viral vectors are generated after RNA or DNA viruses and can have integrating or non-integrating properties. Integrating vectors favor lifelong gene expression but carry the risk of insertional mutagenesis, the random integration into the host cell genome, which can have potential negative consequences (Verma et al. 2005, Ralph et al. 2006). Non-integrating viral vectors are usually kept as an episomal structure and can promote efficient gene expression, particularly in non-diving cells (Verma et al. 2005). In reality, non-integrating vectors are not suitable for dividing cells due to dilutional loss of vector genomes during cellular replication, resulting in transient transgene expression. Fortunately, this is a minor issue for the retina since it is entirely composed of post-mitotic cells, therefore enabling efficient, sustained transgene expression after a single non-integrating vector administration (Alloca et al. 2006, Kumar-Singh 2008).

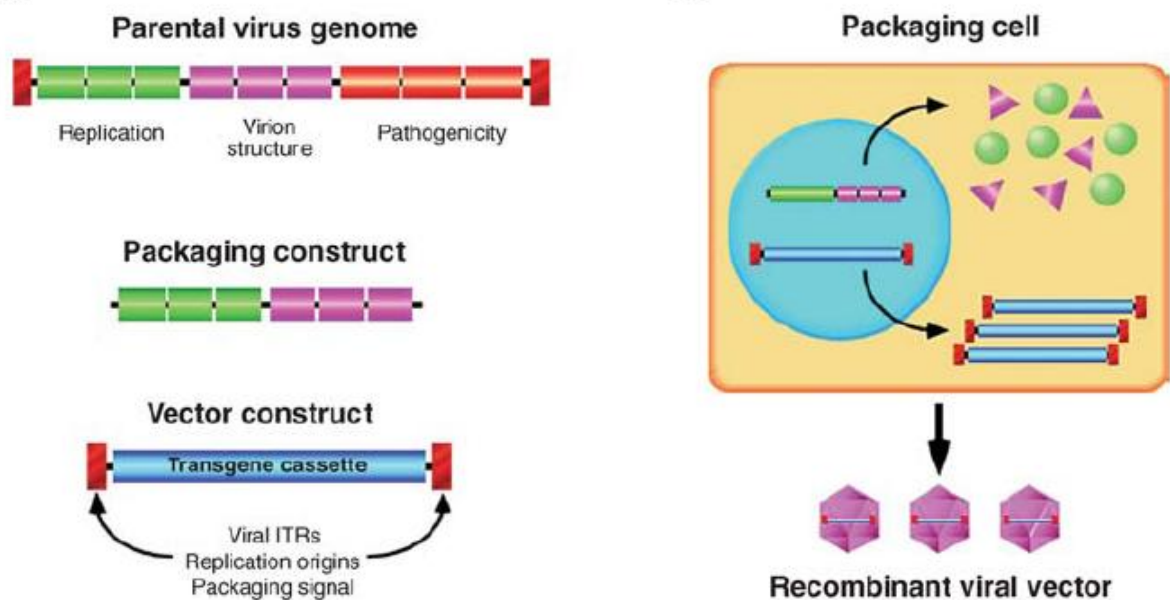


Fig. 14 Generating a viral vector for gene therapy. The parental virus genome contains sequences involved in the replication, production of the virion and pathogenicity of the virus. The genome is flanked by *cis*-acting sequences (red rectangles) that provide the viral origin for replication and the signal for encapsidation. A packaging construct is built after the parental virus genome, through removal of its pathogenic sequences. Further, the *cis*-acting sequences are incorporated in the vector construct, which contains the transgene cassette that holds the gene of interest and the required regulatory elements for its transcription. Both the packaging and the vector constructs are introduced inside a packaging cell where recombinant viral vector production will take place. *Adapted from Verma et al. 2005*.

The human eye gathers several specific characteristics that render it as an ideal target for gene delivery. Indeed, the eye is an immediately accessible organ with well-defined anatomy. Its small size and enclosed structure allow the use of low vector doses to achieve a therapeutic effect. Furthermore, it contains transparent media, which allow direct visualization of intraocular structures, enabling easy, non-invasive, *in vivo* monitoring of the effects of gene therapy through objective tests such as OCT, ERG, visual-evoked potentials (VEPs) and measurement of afferent pupillary light responses. Transparent media of the eye also allow easy detection of potential harmful local adverse reactions (Ralph et al. 2006, Liu et al. 2007, Colella et al. 2009). Importantly, the great majority of eye conditions theoretically suitable for gene therapy detain a well understood molecular and genetic basis. In addition, several small and large animal models are available and correlate well with human pathology. Concerns over a possible host immune response against the viral vector have long blunted the application of gene therapy in human subjects. The presence of both tight junctions, between the RPE cells and the blood-retina barrier, limits vector and/or gene leakage into the circulation, thus confering an immune-privileged status to the eye.

However, inflammatory reactions relating to the viral vectors or to the inoculation procedures have been reported (Liu et al. 2007, Colella et al. 2009).

Viral vectors capable of efficiently transducing different eye tissues are available and this makes them the preferred vector system for ocular gene delivery. The administration route is highly dependent on the targeted cell type. Thus, subretinal injections are more suitable for gene delivery to the outer retina (photoreceptors and RPE), whereas intravitreal gene delivery primarily targets the inner retina (retinal ganglion cells). Vector injections in the anterior chamber and in the subconjunctival space are also possible (Fig. 15; Colella et al. 2009). The use of tissue-specific promoters enables further restriction of transgene expression to the desired cell subtype. Accordingly, combining these promoters with appropriate vectors and route of administration ideally allows highly selective transduction of specific target cells in the eye (Alloca et al. 2007, Lebherz et al. 2008).

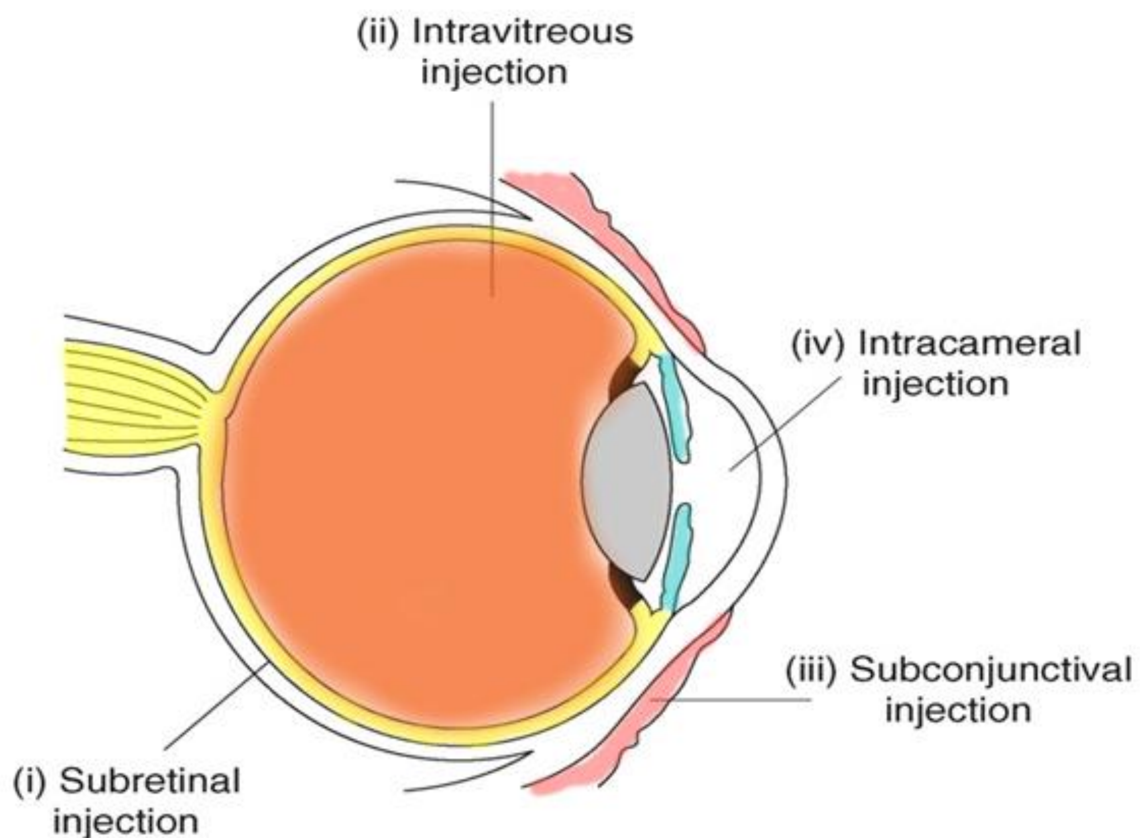


Fig. 15 Vector injection routes for ocular gene therapy. Subretinal injection (i) exposes the retinal pigmented epithelium and photoreceptors in the outer retina to the inoculated vectors, whereas intravitreal injection (ii) targets primarily the inner retina (retinal ganglion cells). Periocular vector delivery can be achieved through subconjunctival injection (iii). Intracameral injection (iv) directly delivers the vectors to the anterior chamber of the eye. *Adapted from Colella et al. 2009*.

Viral vectors most commonly used for ocular gene transfer are adeno-associated, adenoviral, and lentiviral vectors. The first two are non-integrating vectors whereas the third fully integrates the host genome.

Adeno-associated viral (AAV) vectors seem to be the most promising vectors for ocular gene transfer and are currently the most commonly used for retinal gene therapy (Colella et al. 2009, Smith et al. 2009). They have proved to effectively transduce both types of photoreceptors and the RPE. As a matter of fact, they seem to be the only virus-derived vector capable of doing so (Bainbridge et al. 2006, Surace et al. 2008). Generated from small, non-pathogenic, single-stranded DNA viruses, AAV vectors exist in over 100 distinct variants, defined as serotypes or genomovars. Indeed, this extreme serotype diversity is responsible for their ability to transduce various ocular cell types. Initial AAV vectors were based on AAV serotype 2, the most common serotype among humans. Today, through a process called pseudotyping it is possible to combine different serotypes in one unique vector, further increasing target-cell specificity (Colella et al. 2009, Smith et al. 2009). In brief, pseudotyping consists of packaging AAV serotype 2 (AAV2) genomes inside capsid proteins of another AAV serotype, therefore creating chimeric vectors (Smith et al. 2009). Likewise, it has been demonstrated that combining AAV serotype 2 genomes with serotype 5 capsids resulted in a 400-fold increase in the number of efficiently transduced photoreceptor and RPE cells after subretinal injection when compared to the use of the same genomes inside serotype 2 capsids (Yang et al. 2002). Recently, Alloca and colleagues postulated that using serotype 2 genomes combined with capsids from serotypes 7, 8 and 9 resulted in a six to eightfold increase of specific photoreceptor transduction after subretinal injection (Alloca et al. 2007). On the other hand, it has also been shown that using serotype 2 genomes combined with serotype 1 and 4 capsids results in exclusive transduction of RPE (Rabinowitz et al. 2002). AAV vectors present one potential disadvantage which is their cargo capacity, known to be restricted to 4.7 kb (Colella et al. 2009, Molday et al. 2009). However, a major breakthrough was recently presented by Alloca and co-workers who managed to demonstrate, in a murine model, that AAV 2/5 chimeras can efficiently transduce both photoreceptors and RPE cells while carrying genomes of up to 8.9 kb (Alloca et al. 2008). In fact, among all vectors studied, this particular vector was found to have the highest packaging capacity (Alloca et al. 2008). These results have significant implications regarding the possibility of gene therapy for Stargardt disease, which would require high capacity vectors since *ABCA4*, with 6.8 kb, is a

large gene (Molday et al. 2009). Alternatively, lentiviruses have been shown to be a plausible alternative vector system for Stargardt disease (Kong et al. 2008).

Lentiviruses are double-stranded RNA viruses, surrounded by a glycoprotein-rich lipid envelope. The glycoproteins influence the tropism of the virus for both its native and recombinant forms. Lentiviral vectors either derive from the type 1 human immunodeficiency virus (HIV-1) or from non-primate lentiviruses such as the equine infectious anemia virus (EIAV). It is possible to create hybrid lentiviral vectors using heterologous envelope glycoproteins, thereby influencing the virus tropism. For recombinant lentiviral vectors, the most frequently used protein is the G glycoprotein of the vesicular stomatitis virus (VSV-G), which enables a broad vector tropism. When compared to AAV vectors, lentiviruses present one major advantage which relates to their high cargo capacity, allowing for accommodation of genomes of up to 8.0 kb. However, as integrating vectors, the carried genome then randomly integrates the host chromosomes, which can cause insertional mutagenesis (Colella et al. 2009). Recently engineered, non-integrating human immunodeficiency viruses promote long-term transgene expression with reduced risk of insertional mutagenesis (Yanez-Munoz et al. 2006). Lentiviruses have been widely used for ocular gene delivery with good results. These vectors have been found to efficiently transduce RPE cells resulting in long-term transgene expression, upon subretinal injection (Bainbridge et al. 2006). Evidence for photoreceptor transduction is less convincing. Efficient transduction of photoreceptors has been achieved in neonatal and embryonic retinas, but results in adult animal retinas are more variable (Bainbridge et al. 2006, Kong et al. 2008). Even so, lentivirus-derived vectors based on the equine infectious anemia virus (EIAV) seem to transduce photoreceptors better than the HIV-1 (Kong et al. 2008).

Adenoviral vectors are non-enveloped double-stranded DNA viruses which figure as the least suitable viral vectors for long-term ocular gene therapy, since they elicit strong immune responses that blunt the potential for long-term transgene expression (Reichel et al. 1998). In contrast, they can be useful in situations that require transient gene expression, like the treatment of cancer through expression of toxic transgenic products. Indeed, adenoviral vectors have recently been successfully tested in clinical trials of patients with retinoblastoma, demonstrating good safety and efficiency (Chevez-Barrios et al. 2005). One additional advantage of adenoviral vectors is their very high genomic packaging capacity, of up to 36 kb. To overcome the problem of adenoviral-mediated immune responses, helper-

dependent adenoviral vectors (Hd-Ad) have recently been developed, which lack all viral genetic sequences, thereby preventing the development of vector-targeted immune responses that would otherwise compromise transgene expression. Recent animal studies proved that Hd-Ad vectors' advantages are a reality, demonstrating that they can achieve up to lifelong transgene expression following their injection (Kim et al. 2001, Kreppel et al. 2002, Lamartina et al. 2007). Importantly, Hd-Ad vectors were found capable of transducing retinal cells, primarily RPE cells. Photoreceptor transduction by these vectors is still not satisfactory. However, it is possible to further engineer these vectors to improve their target-cell specificity and future developments will certainly enable photoreceptor transduction. Additional advantages of Hd-Ad vectors include their high cargo capacity and the possibility of re-administration (Kumar-Singh 2008). Thus far, Hd-Ad vectors are looking very promising for future employment as gene delivery systems for gene therapy, including that applied to ocular conditions.

The field of gene therapy has suffered major advancements in recent years and the most impressive breakthroughs were observed in retinal conditions, particularly Leber Congenital Amaurosis (LCA), which dominates the current panorama of research.

LEBER CONGENITAL AMAUROSIS

Leber Congenital Amaurosis (LCA) is the most severe form of inherited retinal dystrophy. Although rare (population frequency between 1/30.000 and 1/81.000), LCA accounts for at least 5% of all inherited retinopathies and about 20% of children attending schools for the blind (Koenekoop 2004, Stone 2007). More than 400 mutations in 14 different genes are known to be involved in the development of LCA and together they account for 70% of cases. The three most frequently mutated genes are *CEP290* (15%), *GUCY2D* (11.7%) and *CRB1* (10%). Similarly to *ABCA4*, founder mutations have also been identified, explaining differences in the relative prevalence of LCA mutations in distinct areas of the globe (den Hollander et al. 2008). Clinically, LCA patients present with blindness or severe visual impairment at an early age, usually within the first six months of life, which represents one of the essential clinical diagnostic criteria for LCA. Visual function and acuity range widely, but only rarely exceed 1/10. Three longitudinal studies involving a total of 90 LCA patients have been performed. Globally, they have demonstrated that vision tends to remain stable in up to 75% of patients, while 15% experienced further deterioration, and 10% actually

registered some degree of visual improvement (Heher et al. 1992, Fulton et al. 1996, Brecelj et al. 1999). More recently, it has further been advanced that visual function outcome might be dependent of the underlying genetic mutation. Koenekoop and colleagues observed improvements in visual acuity, visual field and cone ERG responses in a patient with documented LCA linked to *CRX* mutations (Koenekoop et al. 2001). Similarly, LCA patients with mutations *CRB1, LCA5* and *RPE65* mutations may experience subtle vision improvements but decline after a variable period of stability (Lorenz et al. 2000, Yzer et al. 2003). *CEP290* and *GUCY2D* mutations have been found to be associated with very significant loss of vision with long-term stability, whereas LCA patients who harbor *AIPL1* and *RPGRIP1* mutations have progressive visual deterioration (Dharmaraj et al. 2000, Koenekoop et al. 2007).

Additional clinical features include a positive family history of LCA typically associated with an autosomal recessive pattern of inheritance, although some dominant forms of LCA have been reported (Weleber et al. 2006, den Hollander et al. 2008). The oculo-digital sign is a non-pathognomonic feature of LCA and consists of poking, rubbing or pressing of the eyes (Franceschetti et al. 1954, Fazzi et al. 2003), resulting in progressive enophthalmos. It still remains a mystery why this phenomenon occurs but it is thought to relate to the production of phosphenes, which produce sparks of light that may please patients, or to some kind of stereotypic behavior derived from the blinding state (den Hollander et al. 2008). Repetitive trauma to the cornea may implicate the oculo-digital sign in the pathogenesis of the keratoconus, a condition frequently observed in LCA patients (Weleber et al. 2006). It has also been postulated that keratoconus in LCA patients might have other etiologies, including genetic and retinal degeneration-related causes (den Hollander et al. 2008). Other clinical findings associated with LCA include a near-absent pupillary light response, which reflects severe retinal dysfunction, a pendular or roving nystagmus, photophobia, and high hyperopia (Weleber et al. 2006). Patients with LCA also appear to have a higher predisposition to the development of cataracts (Koenekoop 2004).

Fundus appearance in LCA is as diverse as the genetic heterogeneity of the disease (Dharmaraj et al. 2004, Koenekoop et al. 2007). Still, the full phenotypic spectrum of retinal changes in LCA remains to be correlated with the underlying genotype. Unlike Stargardt disease, LCA frequently presents with a more widespread retinal involvement. Currently, LCA fundus presentation ranges from essentially a normal-appearing retina to mild retinal vessel

attenuation, pseudopapilledema, maculopathy, macular coloboma, bone spicule-like pigmentation, nummular pigmentation, salt and pepper pigmentation, yellow confluent peripheral spots, white retinal spots, marbleized retinal changes, preserved para-arteriolar RPE, and Coats reaction (Fig. 16; den Hollander et al. 2008).

A flat ERG (non-detectable cone and rod responses) is diagnostic in LCA patients, reflecting the underlying widespread defective retinal function (Weleber et al. 2006, den Hollander et al. 2008). Other procedures like fundus autofluorescence and OCT provide highly variable findings further supporting the fact that LCA shelters a wide range of gene-specific pathologic changes. Additionally, OCT studies suggest that in most cases, and despite retinal remodeling, photoreceptors remain viable even in late disease stages (Milam et al. 2003, Cideciyan et al. 2007, Jacobson et al. 2007).

Given the highly diverse genetic nature of LCA, its molecular diagnosis is not always an easy task. However, a microarray containing all known mutations linked to LCA is currently available, enabling a relatively cost-effective detection of LCA-related genetic defects with an efficiency of up to 55%, and facilitating genetic counseling of affected families. Furthermore, as visual performance in LCA seems to partially correlate with the underlying causative gene mutation, a more accurate prognosis can be established if that gene mutation is known (den Hollander et al. 2008). Knowledge of the underlying genetic cause is also a prerequisite for the design of therapies aimed at replacing defective genes, such as in gene therapy. LCA, particularly *RPE65*-associated disease, has attracted much attention over the last ten years.

The *RPE65* gene is responsible for about 6% of all LCA cases (den Hollander et al. 2008). In normal retinoid cycle physiology, *RPE65* codifies for an important isomerase involved in the conversion of all-*trans*-retinyl esters to 11-*cis*-retinol, a critical step in the process of rhodopsin regeneration after its photobleaching (Cai et al. 2009). Given its monogenic nature, relatively simple pathophysiology and the existence of several natural and genetically engineered animal models, including a naturally occurring large-animal model, LCA linked to *RPE65* mutations gained the spotlight and became a preferred target for gene replacement therapy. Indeed, *RPE65*-LCA is today one of the most extensively described retinal degenerations and the amount of medical literature available regarding its molecular basis, pathogenesis, clinical features, and early gene therapy trials is extraordinarily vast.

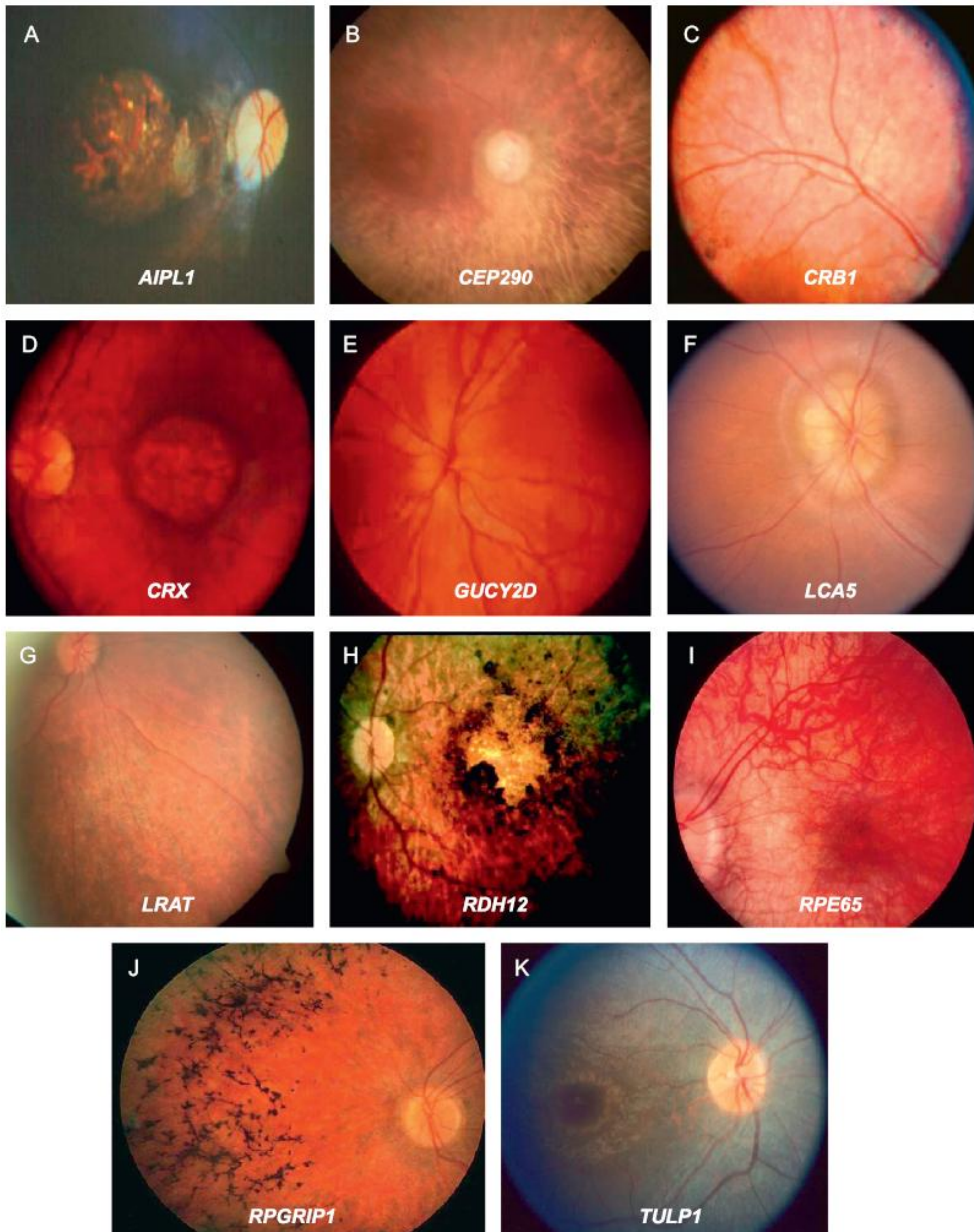


Fig. 16 Spectrum of fundus appearance in LCA. (A) Fundus photograph of an 8-year old black female LCA patient, who carries an *AIPL1* compound heterozygous missense mutation, presents with a prominent macular coloboma, retinal arteriolar narrowing and optic disc pallor. (B) Fourty-year old French-Canadian male LCA patient with a *CEP290* homozygous nonsense mutation has marked choroidal sclerosis, pale optic discs, barely visible retinal vessels, and relative preservation of the posterior pole. (C) A 10-year old female shows preserved para-arteriolar retinal pigmented epithelium and nummular pigmentation relating to a homozygous *CRB1* missense mutation. (D) A prominent maculopathy and relatively normal-appearing vessels and optic discs are the result of a heterozygous *CRX* frameshift mutation in this 10-year old female. (E) Compound heterozygous *GUCY2D* missense and frameshift mutations result in a relatively normal retinal appearance in this

25-year-old female LCA patient. (F) Prominent optic disc drusen, relatively well preserved retinal aspects and mild vessel narrowing are seen in this 25-year old female with a large homozygous deletion in the *LCA5* gene. (G) A 10-year old male presenting with a normaç optic disc, mild vessel narrowing and a salt and pepper fundus pattern was found to carry a homozygous *LRAT* frameshift mutation. (H) This 8-year old male has a homozygous *RDH12* missense mutation responsible for a prominent maculopathy and severely impaired visual performance. (I) A characteristic retinal pigmented epithelium translucency and mild vessel narrowing are seen in the retina of this 20-year old male with a homozygous mutation in *RPE65*. (J) A homozygous *RPGRIP1* nonsense mutation is responsible for a bone spicule-like retinal degeneration in this 10-year old female with LCA. (K) Fundus photograph of a 15-year old female shows a yellow perifoveal ring and mild pigmentary changes related to a homozygous *TULP1* splice-site mutation. *Adapted from den Hollander et al. 2008.*

ANIMAL MODELS OF RPE65-LCA

Availability of animal models that sufficiently translate human disease sequence is an essential prerequisite for any gene therapy research trial. LCA related to mutations in the *RPE65* gene enjoys the existence of several of those models. There are currently three available animal models for LCA linked to *RPE65* mutations, two rodent and one canine models (Table 1; Song et al. 2007).

Name	Nature of Mutation	Species	Features	Reference
***Rpe65**$^{-/-}$*	Knockout	Mouse	Slowly progressive retinal degeneration, residual scotopic ERG responses, absent rhodopsin and 11-cis-retinal, retinyl ester accumulation in the RPE.	Redmond et al. 1998
rd12	Naturally occurring	Mouse	Slowly progressive retinal degeneration, decreased scotopic ERG responses, absent rhodopsin and 11-cis-retinal, retinyl ester accumulation in the RPE, white dots on retina.	Pang et al. 2005
Briard dog	Naturally occuring	Swedish Briard dog	Slowly progressive retinal degeneration, severely diminished ERG responses, centripetal photoreceptor loss, lipid-like inclusions in the RPE.	Veske et al. 1999

Table 1 Animal models of *RPE65*-linked Leber Congenital Amaurosis.

Soon after *RPE65* was cloned and implicated as causative gene for LCA, the *Rpe65* knockout mouse was genetically engineered in order to better understand the disease and plan logical therapeutic interventions (Redmond et al. 1998). These mice were found to develop a slow retinal degeneration, depicting normal retinal anatomy at 7 weeks of age, with normal photoreceptor structure and retinal outer nuclear layer thickness (Redmond et al. 1998, Rohrer et al. 2003). However, starting at the age of 15 weeks, their photoreceptor outer segments start to shorten and inclusions develop inside the RPE cells (Redmond et al. 1998). By 12 months of age, *Rpe65* knockout mice retain about 70% of their photoreceptor nuclei,

but further photoreceptor loss continues through the ages of 18 and 24 months (Rohrer et al. 2003). In this model, absence of functioning RPE65 results in lack of 11-*cis*-retinal and 11-*cis*-retinyl esters leading to excessive accumulation of all-*trans*-retynil esters in the RPE, thus suggesting a block in normal visual cycle kinetics. This further supports the idea that RPE65 functions as an isomerase converting the all-*trans*-retinyl compounds to their *cis* isoforms (Redmond et al. 1998, Ablonczy et al. 2002). Like in humans, dark-adapted ERG responses obtained from *Rpe65* knockout mice are nearly undetectable (Redmond et al. 1998).

There has long been some controversy regarding the retinal location of RPE65 expression. It is known that RPE65 is particularly abundant on and almost specific of the RPE (Cai et al. 2009). However, some authors have demonstrated additional RPE65 expression in cone photoreceptors (Znoiko et al. 2002), but others have failed to confirm it (Hemati et al. 2005). RPE65 expression in rod photoreceptors has not been reported. Nonetheless, despite this controversy, it seems now clear that RPE65 is important for cone photoreceptor function, since cone degeneration in *Rpe65* knockout mice can reportedly begin as early as from 2 weeks after birth (Znoiko et al. 2005). Indeed, it has been postulated that mammalian cones might utilize a different retinoid processing cycle in addition to the one traditionally used by rods (Znoiko et al. 2002, Jacobson et al. 2005). Accordingly, Wenzel and colleagues found that in mice with cone only retinas RPE65 was expressed in high levels and ablation of its expression led to the absence of 11-*cis*-retinal (Wenzel et al. 2007). Furthermore, Seeliger and co-workers had previously demonstrated, through selective impairment of rod or cone function in RPE65-deficient mice, that no ERG responses were detected on the rod-defective mice, meaning that the remaining functional rod photoreceptors are responsible for the residual visual function observed in RPE65-deficient mice, and that rods might depend less on RPE65-mediated retinoid kinetics than cones (Seeliger et al. 2001). The authors state that the dramatic decrease in functional chromophore in the RPE65-deficient retina enables rods to respond under photopic conditions, which in otherwise normal conditions would quickly saturate rod function (Seeliger et al. 2001). Evidence pointing to early loss of cone photoreceptor in LCA patients with *RPE65* mutations further supports these assumptions (Znoiko et al. 2005, Chen et al. 2006, Cottet et al. 2006). However, human findings of residual retained cone structure and function after years of the disease suggest that cone survival might be co-influenced by alternative pathways (den Hollander et al. 2008).

The naturally occurring *rd12* mutant model is caused by a nonsense mutation in exon 3 of the *RPE65* gene involving a cytosine to thymine transition which creates a stop codon, resulting in non-functional protein due to truncation and mRNA degradation (Pang et al. 2005). The *rd12* mouse model shares its phenotypical features with the *Rpe65* knockout mouse. Likewise, small deposits of retinyl esters are observable in the RPE cells as soon as the age of 3 weeks and scotopic ERG responses are profoundly diminished. Similarly to the *Rpe65* knockout mouse, the retinal degeneration in the *rd12* model further progresses at slow velocity, with normal retinal structure still observable at the age of 6 weeks. With 7 months of age, *rd12* mice present with an up to 30% loss in the number of photoreceptor nuclei (Pang et al. 2005, Pang et al. 2006). The only notable difference between the two mouse models is that fundus examination of *rd12* mice discloses small white dots evenly dispersed throughout the retina at the age of 5 to 9 months (Pang et al. 2005).

The Swedish Briard dog comprises the second naturally occurring model of *RPE65*-associated LCA. It is also the only available large-animal model of *RPE65*-LCA. Molecular analysis revealed a 4-bp (AAGA) deletion in the *RPE65* gene leading to a frameshift and premature stop codon, which ultimately results in the formation of a truncated non-functional protein (Veske et al. 1999). Affected dogs maintain normal fundus appearance until up to the age of 3. However, abnormal ERG responses, with severely diminished scotopic responses and very low photopic amplitudes, can be recorded from the age of 5 weeks. Like in the mouse models, the canine disease is slowly progressive. Over time, lipid-like inclusions become visible inside RPE cells and the outer segments of both types of photoreceptors suffer a progressive disarrangement with subsequent loss of cones and rods, usually with a centripetal pattern (Narfström et al. 2003a). From all the *RPE65*-LCA disease models, the Briard dog is considered to resemble the most to the human sequence of pathological events, with a strikingly similar retinal phenotype (Wrigstad 1996, Cai et al. 2009).

The fact that *RPE65* animal models correlate well with human disease, combined with findings of viable photoreceptors in adult LCA patients (Jacobson et al. 2005, Aguirre et al. 2007), has paved the way for the development of gene therapy clinical trials, first in animal and then in human subjects.

GENE THERAPY IN ANIMAL MODELS OF RPE65-LCA

Back in 2001, in Philadelphia, Acland and co-workers took advantage of the at-the-time recently characterized Swedish Briard dog to accomplish what turned out to be the first successful gene replacement therapy for an ocular condition (Acland et al. 2001). In that study, each of the three treated RPE65-defective dogs received a unilateral subretinal injection of AAV2/2 carrying a chicken β-actin-promoter/CMV enhancer-driven normal canine *RPE65* gene. As a result, significant restoration of vision in these previously blind dogs was achieved. Objectively, treated eyes showed improvements in blue light stimulated dark-adapted and cone flicker ERG responses of up to 16%, when compared to uninjected eyes. Additionally, improvements in pupillometry-assessed pupillary constriction and VEPs, both of which target higher order visual function, were also reported. However, the most notorious effect of therapy was observed when the treated dogs were subject to qualitative behavioral assessments, by simply putting them in a maze before and after gene therapy, under dark conditions. Treated dogs were found to have indistinguishable photopic visual performance when compared to normal sighted controls, avoiding obstacles in a similar manner and much more efficiently than untreated dogs (Acland et al. 2001). For the first time, this investigation demonstrated proof-of-principle of gene therapy for ocular conditions, at least for those affecting retinal cells, and thanks to it, LCA attracted considerable amounts of attention from the scientific community. In fact, the positive results obtained by Acland and colleagues served as a first stimulus for the quite astonishing amount of subsequently developed gene replacement trials involving *RPE65* (Kaplan 2008).

Proof-of-principle for the use of gene therapy to eye diseases was further confirmed elsewhere by Narfström and colleagues, and Ford and co-workers, who also managed to successfully treat visually-impaired RPE65-defective dogs using gene replacement therapy (Ford et al. 2003, Narfström et al. 2003b). More importantly, these studies brought new insights into the subject. Narfström confirmed the proof-of-principle of ocular gene therapy using similar methods as those used by Acland, but to a wider extent, since they treated a larger cohort of RPE65-deffective dogs. In their experiments, eleven dogs, aged 4 to 30 months old, each received a subretinal injection of an AAV vector containing the normal *RPE65* gene, in one eye, and a subretinal injection of a control vector, in the contralateral eye. ERG assessment, 3 months after therapy, showed greatly improved visual function, with a dark-adapted b-wave amplitude recovery averaging 28% of normal and light-adapted b-

wave amplitudes reaching mean values of 32% of normal, in both young and older dogs. Incredibly, these findings remained stable at a 6 to 9-month follow-up period, meaning that prospects of a long-lasting positive effect after a single administration of the viral vector were made aware. In addition, these researchers performed immunocytochemical and ultrastructural studies in two of the treated eyes of dogs that were sacrificed at 3 and 6 months post-treatment, respectively. Accordingly, RPE65 was found to be expressed in the RPE of the treated eyes and a reversal of RPE lipid accumulation was observed at the transgene injection site, a phenomenon that did not occur in retinal locations that were not exposed to the vector (Narfström et al. 2003b). Of note, Narfström and colleagues had previously reported in a similar study, involving five RPE65-defective dogs, that the changes in visual function are measurable as early as 4 weeks after treatment (Narfström et al. 2003a). Interestingly, in that study they also found that the contralateral untreated control eye also demonstrated improvements in ERG responses, similar to those observed in the treated eye. Although this phenomenon remains unexplained, the researchers advanced that it might be due to transfer of the RPE65 protein from the treated to the untreated eye (Narfström et al. 2003b). This assumption is based on previous findings by Dudus et al who found that mice and dogs given intraocular injections of AAV vector carrying a fluorescent genetic marker demonstrated the presence of fluorescence beyond the retina, namely in brain tissues known to be innervated by retinal ganglion cells (Dudus et al. 1999). However, it was found that transgene expression did not surpass the first synapses of the transduced cells, indicating that it was the fluorescent protein that was exchanged between cells, and not the transgene (Dudus et al. 1999). An alternative explanation could be the existence of a low-level release into the bloodstream of 11-*cis*-retinoids from the treated eye that would then be taken up by the contralateral eye (Narfström et al. 2003b). This hypothesis is supported by the work of van Hooser et al who partially reversed the electrophysiological phenotype of *Rpe65* knockout mice through oral gavage with 9-*cis*-retinal (van Hooser et al. 2000). Recent data, derived from human studies, further advance that contralateral improvement in vision after gene transfer might be due to dampening of nystagmus or improved visual signal processing related to plasticity of visual cortex (Maguire et al. 2009).

To assess the possible dose-dependent effects of gene therapy, Ford and co-workers demonstrated that the improvements of vision after gene therapy in RPE65-defective dogs are volume-dependent for the injected vector (Ford et al. 2003). In this study, 11 dogs

received a unilateral subretinal injection of a either a high (70-100 microliters) or a low volume (30-60 microliters) of an AAV vector carrying the normal *RPE65* gene. Gene transfer resulted in significant improvements in visual function in both groups. However, in the high vector-volume group, vision in daylight conditions was found to be significantly better than in dim light, whereas, in the low vector-volume canine group, no significant differences were observed between both light conditions. Overall visual performance was found to be better in the high-volume group of dogs, which actually demonstrated no differences in daylight visual function when compared to normal controls (Ford et al. 2003). Observations that gene therapy seems to better restore vision in daylight than in dim light conditions suggest that primary cone rescue might preferentially occur (Narfström et al. 2003a).

Embryonic studies demonstrating retinal histological abnormalities in human fetuses with *RPE65* mutations led Dejneka et al to suggest that the success of gene therapy for *RPE65*-LCA might be dependent on time of intervention, and thus an early intervention might be necessary to achieve full disease phenotype correction (Dejneka et al. 2004). Likewise, Dejneka tested the efficacy and viability of gene therapy at the earliest possible intervention, conducting AAV2/2-mediated *RPE65* subretinal gene transfer to the eyes of fifty-two *Rpe65* knockout mice fetuses. Thirty young adult mice, aged 1 to 2.5 months old, also received unilateral subretinal injections of the same vector. Immunohistochemical examination of *in utero*-treated animals revealed that RPE65 expression was detectable at birth and persisted throughout 6 months of postnatal follow-up. From the 13 *in utero*-treated animals that survived to adulthood, it was possible to observe that no micro or macroscopic abnormalities were found in their eyes, supporting that *in utero* gene therapy does not compromise normal fetal retinal development. Furthermore, *in utero* treated mice demonstrated measurable rescue of vision 1 to 2 months after gene transfer, including two animals with near normal ERG responses. One of two animals that were used for visual pigment measurements was found to have a near normal rhodopsin complement, which suggests that gene therapy restores the ability of photoreceptors to generate this compound. Postnatal treated animals were also found to have major vision improvements, with an overall 80% treatment success rate (Dejneka et al. 2004).

The work of Dejneka et al seems to demonstrate no significant differences in visual outcome whether one uses *in utero* or postnatal interventional approaches. However, Jacobson and co-workers alerted for the fact that the success of gene therapy was strongly

dependent of the number of viable photoreceptors at time of intervention and that the preliminary successful animal results were certainly influenced by the unusual feature of dissociation between structure and function that characterizes *Rpe65* knockout mice and RPE65-defective Briard dogs (Jacobson et al. 2005). In these animal models, photoreceptor structure remains relatively unchanged despite severe functional impairment and, indeed, preliminary gene therapy successes were obtained at disease stages in which photoreceptors were still viable. Thus, Jacobson further investigated the relationship between gene therapy success and time of intervention, maintaining *Rpe65* knockout mice (n = 25) until older ages, between 17-24 months old, and treating them with subretinal gene injections. In addition, another group of mice (n = 24), aged 17-26 months old, was given 9-*cis*-retinal by oral gavage. It was found that older mice could be successfully treated with *RPE65* gene transfer, but in a significant smaller percentage of eyes (Jacobson et al. 2005), when compared to younger counterparts (Dejneka et al. 2004). Overall, ERG responses from older-treated mice were far less improved than the ones obtained from younger-treated animals (Fig. 17a). Statistically significant improvements in ERG responses were seen in only 4 of 25 mice, resulting in a modest treatment success rate of just 16%. Likewise, retinal biochemical analysis showed improvement in only 5 of 17 mice after gene therapy, which corresponds to a 29% success rate. Mice treated with oral 9-*cis*-retinal showed similar results, with success rates not significantly different from the ones obtained with gene transfer, suggesting that the lower success rates of the latter in the older group of mice are not due to the surgical procedures (Fig. 17b; Jacobson et al. 2005). Outer nuclear layer analysis, using high-definition OCT demonstrated significant variability in the amount of photoreceptor degeneration. Accordingly, in the older age group some mice were found to have three to four layers of photoreceptor nuclei whereas other mice were reduced to a single row of nuclei (Fig. 17c). Furthermore, there was a linear correlation between successfully treated eyes and outer nuclear layer thickness, such that mice with less impaired outer nuclear layers registered significantly better improvements than mice with severely reduced outer nuclear layer thickness (Fig. 17d). These findings were similar for both gene therapy and treatment with oral retinoids (Jacobson et al. 2005). Consequently, this confirms the general idea that younger animals present with better preserved photoreceptors and that older mice tend to suffer greater degrees of photoreceptor loss (Rohrer et al. 2003). However, given the significant variability in the amount of

degeneration, found particularly in the older animals, it is feasible to say that prediction of gene transfer success should not be based exclusively on the age at treatment but primarily on photoreceptor viability, as defined by outer nuclear thickness values. Of note, it is acceptable to consider the age of the patient as a suggestion of the degree of photoreceptor degeneration, but only if the underlying disease is sufficiently characterized. Indeed, Jacobson and colleagues advance that the identification of viable photoreceptors is a prerequisite for the success of human gene therapy (Jacobson et al. 2005). Likewise, these investigators have studied several LCA patients, aged 11 to 53 years old, and have demonstrated that a range of outer nuclear thickness topographies, suggesting distinct severities of retinal degeneration, exists among adults with *RPE65* mutations and a simple relationship between age and retinal thickness could not be elicited (Jacobson et al. 2005). Importantly, the work of Jacobson has also demonstrated the presence of a definable photoreceptor layer in adult patients with *RPE65* mutations (Jacobson et al. 2005). As a surprise, some *RPE65*-mutant retinas were found to have greater photoreceptor layer thickness than what could be predicted from the degree of functional disability. Interestingly, graphs of outer nuclear layer thickness as a function of retinal distance suggest greater preservation of the photoreceptor lamina at the region of highest rod density, precisely at 3-5mm eccentricity (Jacobson et al. 2005). Furthermore, Jacobson and colleagues indicate that there seems to be no strong relationship between age and retinal structure or function until after the fourth decade of life (Jacobson et al. 2007 and 2008). These investigators have found that in human LCA, central cone photoreceptor layer abnormalities can occur from as early as age 3 (Jacobson et al. 2007), suggesting that there is a human counterpart to the early cone degeneration noted in the first month of life for *Rpe65* knockout mice (Znoiko et al. 2005, Bemelmans et al. 2006, Chen et al. 2006, Cottet et al. 2006). However, for reasons yet to be confirmed, a subset of human macular cone photoreceptors seem to survive this initial insult and could persist for decades, demonstrating slowly progressive degeneration (Jacobson et al. 2007). This has serious implications regarding the possibility of gene therapy for human patients, further supporting the necessity of pre-treatment photoreceptor layer assessment in order to identify patients who can benefit the most from gene replacement therapy. Importantly, the effects of genetic background and environmental influence on disease severity still remain to be

elucidated (Jacobson et al. 2008), but it is feasible to think that they might also play an important role.

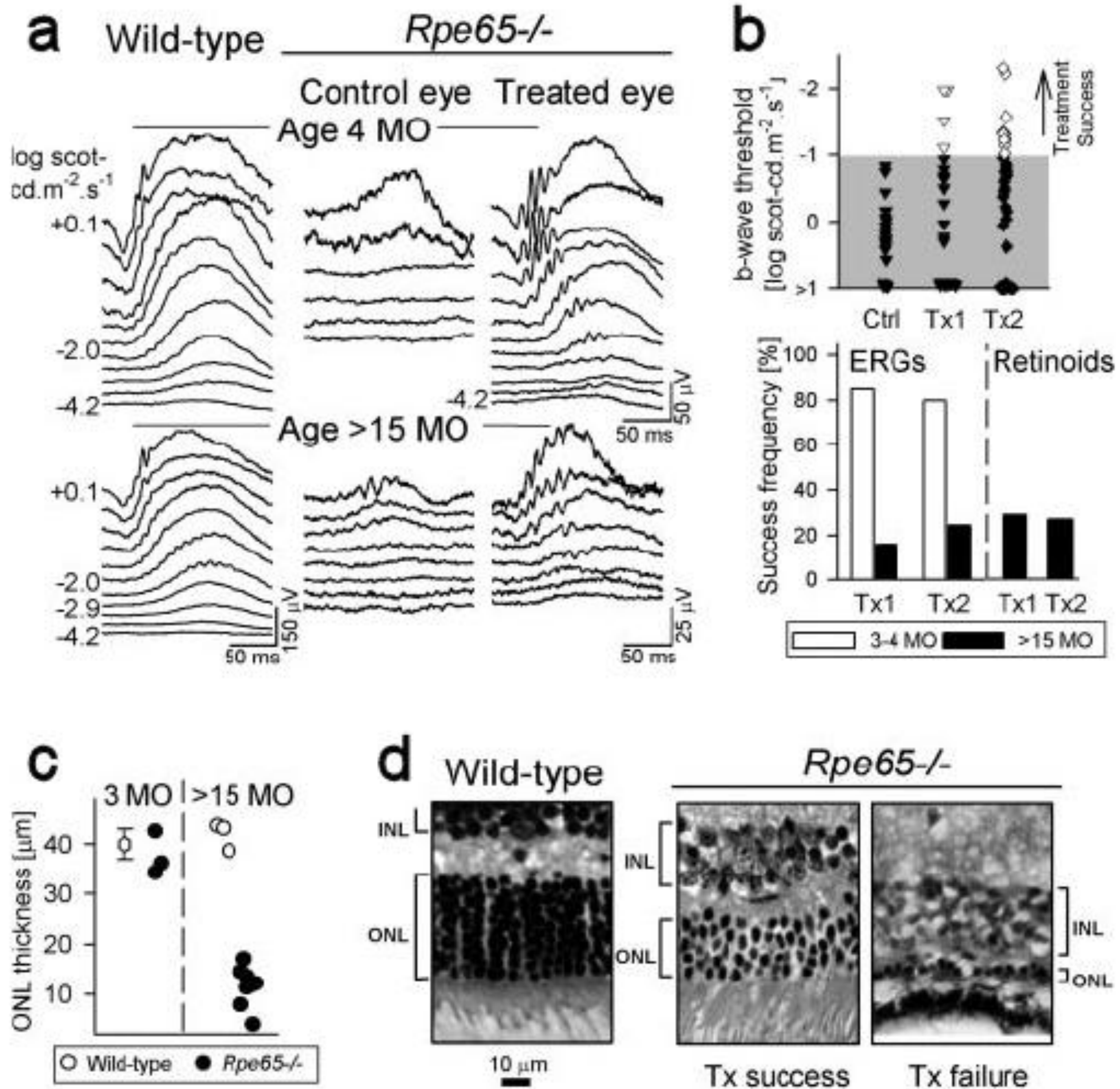


Fig. 17a ERG comparison between wild-type and *Rpe65* knockout mice treated at different ages. Gene therapy successfully restores vision at both age groups, but less efficiently in the group of mice treated after 15 months of age. **17b Treatment success for mice treated with gene therapy (Tx1) or oral retinoids (Tx2) at different ages.** In both groups, treatment success was greater in animals treated at 4 months of age, as measured by ERG testing and retinoid biochemistry analysis. **17c Comparison of outer nuclear thickness in wild-type and *Rpe65* knockout mice at the ages of 3 and more than 15 months old.** At 3 months old, outer nuclear thickness is similar in both wild-type and *Rpe65* knockout mice, depicting preserved photoreceptor anatomy. At more than 15 months old, mutated mice present with significantly lower outer nuclear layer thickness, when compared to their wild-type counterparts. **17d Ultrastructural comparison between wild-type and successfully and unsuccessfully treated *Rpe65* knockout mice.** Failure of therapy is correlated with less preserved outer nuclear layer, where significant reduction of photoreceptor nuclei is observed (far-right picture). Treatment success is seen in animals presenting with relative preservation of outer nuclear layer thickness (middle image). *Adapted from Jacobson et al. 2005.*

Further canine research was reported by Acland and co-workers, providing new data on unresolved questions such as the real magnitude and predictability of a single subretinal vector administration, types of photoreceptors treated, long-term stability of visual improvement after a single treatment, and morphological and biochemical recovery (Acland et al. 2005). They found that RPE65-defective dogs that were injected with a single subretinal dose of an AAV-*RPE65* vector demonstrated normal cone and rod ERG waveforms, although with subnormal amplitudes. The same did not happen in dogs receiving intravitreal injections of the same vector, confirming this as an ineffective approach for targeting photoreceptor cells and the RPE. To explain the range of cone and rod ERG amplitudes obtained from the successfully treated eyes, Acland and colleagues tried to establish a possible correlation with the location of the subretinal vector injection. On average, injection areas in the superior retina were about half as large as those in the inferior retina but, surprisingly, both cone and rod ERG amplitudes in the eyes with superior retinal injection locations were significantly higher than those obtained from eyes that received vector injections in the inferior retina. Furthermore, rod ERG amplitudes as a function of the subretinal injection area were much larger in the superior compared to the inferior retina (Acland et al. 2005). These regional differences between treatment responses can be related to distinct photoreceptor densities in these retinal areas and suggest a predictable behavior of photoreceptors upon gene therapy (Kemp et al. 1992). Importantly, Acland and colleagues concluded that only the areas treated directly by subretinal injection regain functional RPE65 expression and visual pigment production. Of note, recovery of retinal function was found to be strictly limited to treated eye (Acland et al. 2005), a finding that opposes previous studies reporting bilateral functional improvements after unilateral vector administration (Narfström et al. 2003b). Two successfully treated dogs previously enrolled in the proof-of-principle study (Acland et al. 2001) had yearly ERG recordings in order to evaluate long-term functional consequences of single subretinal injections. Remarkably, post-treatment improvements in vision remained stable during the 3 years of follow-up (Acland et al. 2005), further supporting the idea that long-term visual restoration is possible after a single vector administration (Narfström et al. 2003b).

In 2006, Pang et al were able to reproduce in *rd12* mice the previous achievements reported for the Swedish Briard dogs and the *Rpe65* knockout mice. Using recombinant AAV2 vectors pseudotyped with serotype 5 capsids, the investigators were able to

successfully treat 100 newly-born mice, resulting in improved rhodopsin levels and nearly normalized ERG responses. Importantly, RPE65 expression was found to be exclusively localized to the RPE (Fig. 18a-d) and persisted, with stability, throughout the 7 months of follow-up. Significant reduction in RPE lipid accumulation (Fig. 18e-f), and maintenance of normal photoreceptor structure and number, were also observed. Perhaps the most important effect of gene therapy in this study was the restoration of normal vision-dependent behavior, in mice who received injections of the *RPE65*-containing vectors. Curiously, visual improvements and RPE65 expression were found to be strictly limited to the treated eyes (Pang et al. 2006).

Further suggestion that early intervention might provide better treatment results was advanced by Nusinowitz and colleagues (Nusinowitz et al. 2006). In their study, *rd12* retinas treated with a subretinal injection of a serotype 5 recombinant AAV2 vector carrying a normal human *RPE65* gene, demonstrated successful functional recovery of vision that was better for mice treated at early ages. Interestingly, gene therapy appeared to better rescue cone-mediated vision, but only when treatment occurred at the early age of 18 days (Nusinowitz et al. 2006). The observation of better cone rescue seems to support prior findings of better visual performance under daylight than under dim light conditions after gene therapy in the Swedish Briard dog (Ford et al. 2003, Narfström et al. 2003a). Jacobson and colleagues suggest that better cone preservation seems also to be the case for humans, rendering them more amenable to gene replacement therapy (Jacobson et al. 2005). In a rather innovative way, Nusinowitz also studied *rd12* cortical visual function after gene therapy, comparing post-treatment ERG results with VEPs obtained with identical stimuli. This represents an important issue since there are considerable amounts of evidence suggesting that limiting sensory input to the visual system ultimately leads to severe and often irreversible visual deficits later in life, both in animals and humans (Ellemberg et al. 2000, Lewis et al. 2002, Tian et al. 2003, Lickey et al. 2004). Moreover, the timing and type of deprivation affect the character and severity of alteration of cortical function (Hensch 2004). VEPs, captured through electrodes placed at the surface of the occipital bone, indirectly test visual pathway integrity, meaning that a normal VEP requires an intact visual pathway from the retina to the primary visual cortex.

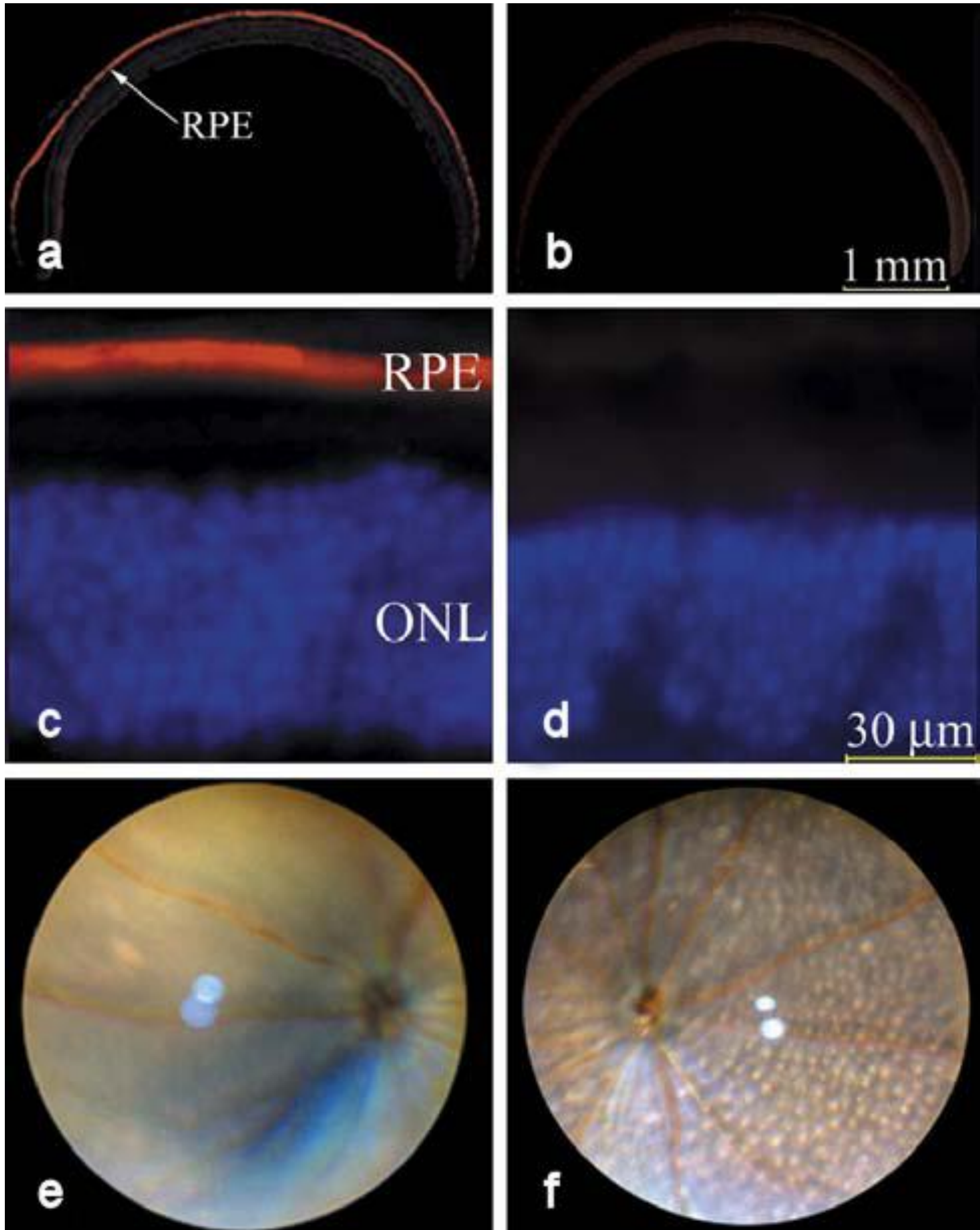


Fig. 18a-d RPE65 immunohistochemistry in the treated (a and c) and contralateral untreated (b and d) eyes of a 7 month old *rd12* mouse treated at postnatal age of 14 days. In the treated eye, gene therapy successfully restores RPE65 expression, which localizes exclusively to the retinal pigmented epithelium (RPE). **18e-f Representative fundus photographs of the treated (e) and untreated (f) eyes of the same mouse.** After treatment there is significant and notable reduction in the amount of lipid-droplets accumulated in the RPE. ONL - outer nuclear layer. *Adapted from Pang et al. 2006.*

Correlating ERG responses with VEP signals accurately evaluates the functional integrity of cells throughout the visual pathway, and in normal animals both parameters correlate linearly. Likewise, in the Nusinowitz study, treated *rd12* mice with departures from this relationship were implied to have dysfunction of the post-retinal visual pathway. Overall, cortical visual function, as measured by VEPs, corresponded well to the ERG-determined degree of retinal rescue for luminance information modulated at low temporal frequencies, except at the oldest age of treatment, where visual pathway disruption was suggested. However, in all ages of treatment, VEPs for high temporal and spatial frequencies were found to be lower than could be predicted from the ERG outcome, suggesting distinct and partially overlapping timing of development of visual pathways (Nusinowitz et al. 2006). These results allow us to conclude that visual deprivation in the *rd12* mice leads to disruption of visual pathway that is not completely recovered by late-employed gene therapy.

On a different approach, Aguirre and co-workers further studied the effects of gene replacement therapy in the cortical function of RPE65-mutant dogs using blood oxygenation level dependent functional magnetic resonance imaging (BOLD fMRI) (Aguirre et al. 2007). In brief, BOLD fMRI uses the focal uncoupling of cerebral blood flow and metabolism to detect focal brain activation. Neuronal activity involves both a local increase in blood flow and a disproportionate consumption of oxygen by the active cortex, leading to a focal net decrease in deoxyhemoglobin concentration near the activation site. Deoxyhemoglobin is paramagnetic, effectively reducing the magnetic resonance signal emanating from its surroundings. Thus, a decrease in the concentration of deoxyhemoglobin related to cortex activation will locally increase the magnetic resonance signal. In the Aguirre study, it was found that the visual cortex of RPE65-mutant dogs remains responsive to gene therapy, even in dogs deprived from any kind of visual input for over 4 years. Moreover, results pointed to a rapid recovery of visual cortical function, since it was observed as soon as 1 month after therapy (Fig. 19; Aguirre et al. 2007). If we take in account that the onset of retinal transgene expression with AAV2 vectors occurs 2-4 weeks after injection (Auricchio et al. 2001), cortical neurons seem to have recovered quite rapidly following restoration of retinal function. The effects of gene therapy on cortical function were also persistent, since restored cortical responses were observed in animals treated 18-30 months before BOLD fMRI evaluation (Fig. 19; Aguirre et al. 2007). It was noted that some dogs additionally

demonstrated recovery of responses in cortical areas usually linked to higher-order visual function (Fig. 19). After treatment, pupillary light reflexes were also restored to normal values, proving that brainstem visual pathways are functional after retinal gene therapy (Fig. 19; Aguirre et al. 2007). Importantly, Aguirre also studied the cortex of *RPE65*-mutant humans and demonstrated that it preserves visual pathway anatomy and detectable cortical activation despite the limited visual experience imposed by LCA (Aguirre et al. 2007).

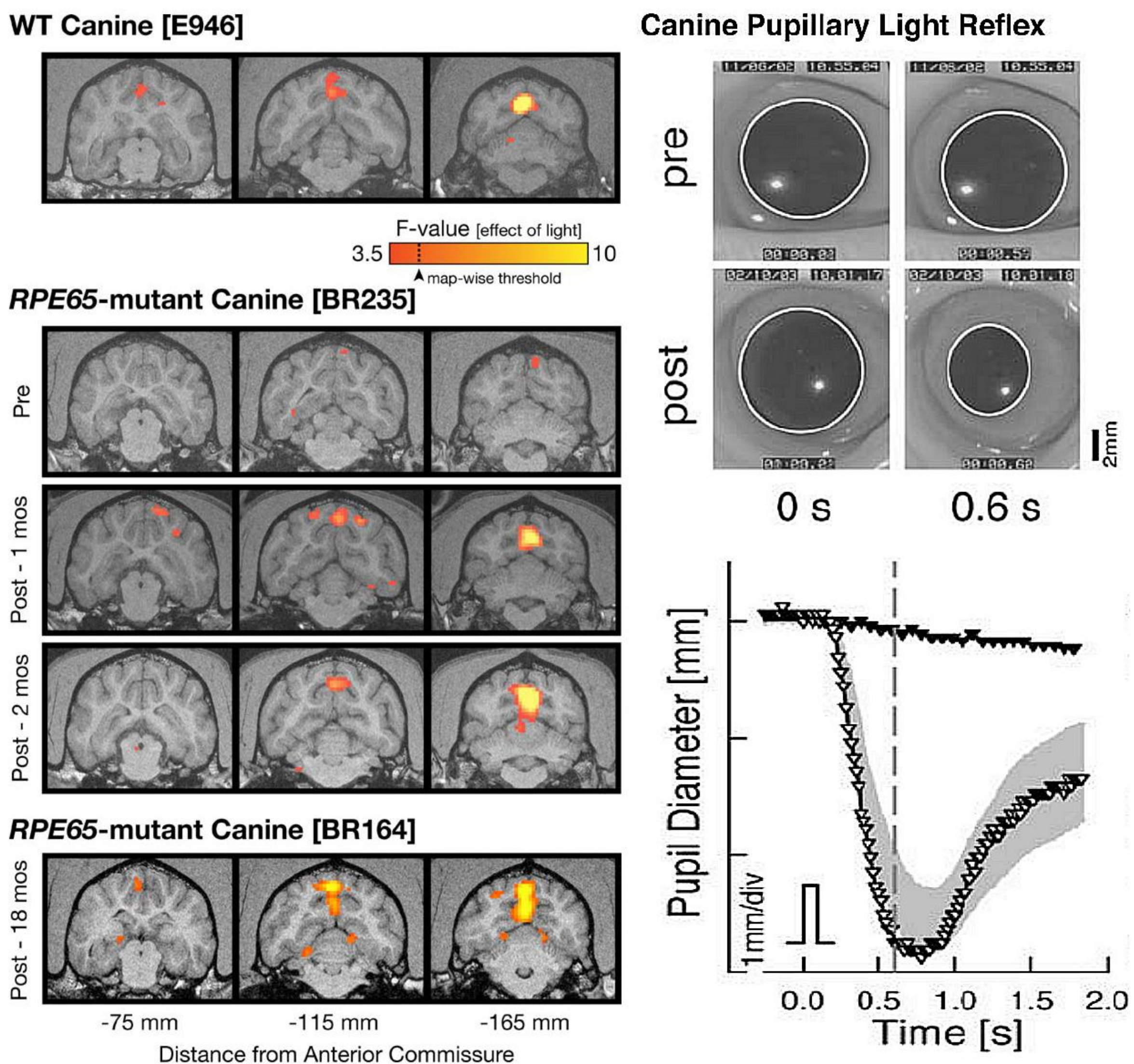


Fig. 19 Functional cortex responses before and after gene therapy in RPE65-mutant dogs, as measured by fMRI and pupillary light responses. Gene therapy successfully restores visual cortex activity that is measurable as soon as 1 month after treatment (left three middle rows) and persists for at least 18 months (left lower row). Note that prior to therapy, cortical responses to light were minimalistic. Improvement of pupillary light reflexes to normal values following gene therapy (right images and graphic), indicate functioning brainstem visual pathways. *Adapted from Aguirre et al. 2007.*

The vast majority of gene therapy studies for *RPE65*-LCA used AAV vectors as the transgene delivery system. Additionally, Bemelmans and co-workers have demonstrated that lentiviral vectors carrying the normal *RPE65* gene can also promote successful restoration of retinal function in *Rpe65* knockout mice (Bemelmans et al. 2006). As a primary objective, the authors intended to better characterize cone rescue after gene therapy, since preservation of cone-mediated vision is of crucial importance and the ultimate goal for human therapies. For that, they used the immunofluorescent labeling of specific cone photoreceptor markers, including the GNAT2 transducin, S-opsin (Short-wavelength opsin, blue cones) and M/L-opsin (Medium/Long wavelength opsin, red/green cones), after gene therapy was performed. Likewise, Bemelmans et al found that lentiviral-mediated *RPE65* gene transfer prolongs cone survival until at least 4 months of follow-up, but only when treatment is initiated at the early age of 5 days after birth. Mice treated at 4-5 weeks of age failed to demonstrate the same degree of recovery (Bemelmans et al. 2006). This comes to term with previous reports suggesting that, despite the slow photoreceptor degeneration that characterizes the *Rpe65* knockout mouse, cone degeneration in these animals can occur as early as from 2 weeks after birth (Znoiko et al. 2005). Additionally, these findings support prior results obtained by Nusinowitz et al who observed better rescue of cone function after gene therapy in the *rd12* mouse, but only in animals early-treated at the postnatal age of 18 days (Nusinowitz et al. 2005). Importantly, the Bemelmans study also demonstrated a linear correlation between the size of the transduced area and the extent of cone survival, which was found to exceed the area of therapeutic transgene expression. A possible explanation for this phenomenon could be the heterogeneity in transduction efficiency within the injected area, such that its center would be highly transduced whereas its periphery would express the transgene at a functionally relevant, but lower level. Alternatively, lateral diffusion of 11-*cis*-retinal could influence cone survival in the vicinity of the transduced area (Bemelmans et al. 2006). This strong linear correlation between the actual sizes of the transduced area and of the cone survival area is of critical significance regarding treatment of human patients, since it will certainly help to target precise retinal locations in order to achieve a therapeutic effect of 11-*cis*-retinal at the fovea. Thus, it might be sufficient to treat the vicinity of the fovea with a limited dose of the vector, avoiding the surgical risk inherent to foveal manipulation. Moreover, the existence of this transitional area, where cones are

rescued but transgene is not expressed in the RPE, further enhances safety features of the procedure and optimizes restoration of vision (Bemelmans et al. 2006).

Further data concerning possible vector dose-related efficacy came from the work of Jacobson et al who demonstrated significant differences in ERG outcome between Briard dogs treated with low AAV2/2-*RPE65* doses and others with high doses of the same vector, through unilateral subretinal injection performed between 2.7 and 7.4 months of age. Indeed, ERG rescue in the high-dosage group was found to have a success rate of 94% for dark-adapted and 72% for flicker responses, whereas in the low-dosage group overall therapy success was only about 7% (Jacobson et al. 2006). Furthermore, ERG rescue showed good correlation with post-treatment immunocytochemical staining of RPE65. Accordingly, dogs submitted to the highest doses of vector were all found to have positive RPE65 immunolabeling, whereas dogs receiving lower doses of the vector had little or no RPE65 staining, although for some dosage values ERG responses could still be detected (Jacobson et al. 2006). Later, Roman and co-workers were able to reproduce these findings in *rd12* mice. Like in the canine model, *rd12* mice were also found to have dose-dependent ERG improvements, after unilateral AAV2-mediated *RPE65* gene transfer was performed at the average age of 3.3 weeks (Roman et al. 2007). Indeed, it was noted that increasing doses accentuated differences in visual recovery between treated and untreated eyes. Likewise, eyes submitted to the lowest vector dose were found to have b-wave and photoreceptor waveforms not different from untreated eyes, whereas at higher doses, treated eyes showed greater b-wave amplitude and a faster photoresponse with larger amplitudes (Fig. 20a-c; Roman et al. 2006).

When one envisions the possibility of extrapolating animal methodologies to human patients, safety issues are a matter of top priority and need to be systematically analyzed. Remarkably, AAV-mediated gene transfer for *RPE65*-LCA has proven safe in the various animal studies, motivating its long-awaited application to humans. Adverse reactions reported so far seem to be mostly related to surgical procedures and to the biological nature of the injected material. The latter, however, are the most worrying since, unlike surgical complications, they are independent of human behavior and, consequently, less controllable. Recently, Jacobson et al demonstrated localized retinal toxicity after AAV-mediated gene therapy in RPE65-mutant dogs, despite no systemic or retina-wide toxicity could be detected (Jacobson et al. 2006). It was advanced that local toxicity could be

mediated through both vector-independent and vector-dose-dependent components. Indeed, traumatic lesions, leading to photoreceptor loss and RPE changes, were common in the injection site, but outer nuclear layer thinning occurred only in dogs exposed to the two highest vector doses. Curiously, however, despite evidence of toxicity, treatment efficacy was obtained. Self-limited mild uveitis developed in the treated eyes and has been reported elsewhere (Narfström et al. 2003, Jacobson et al. 2006).

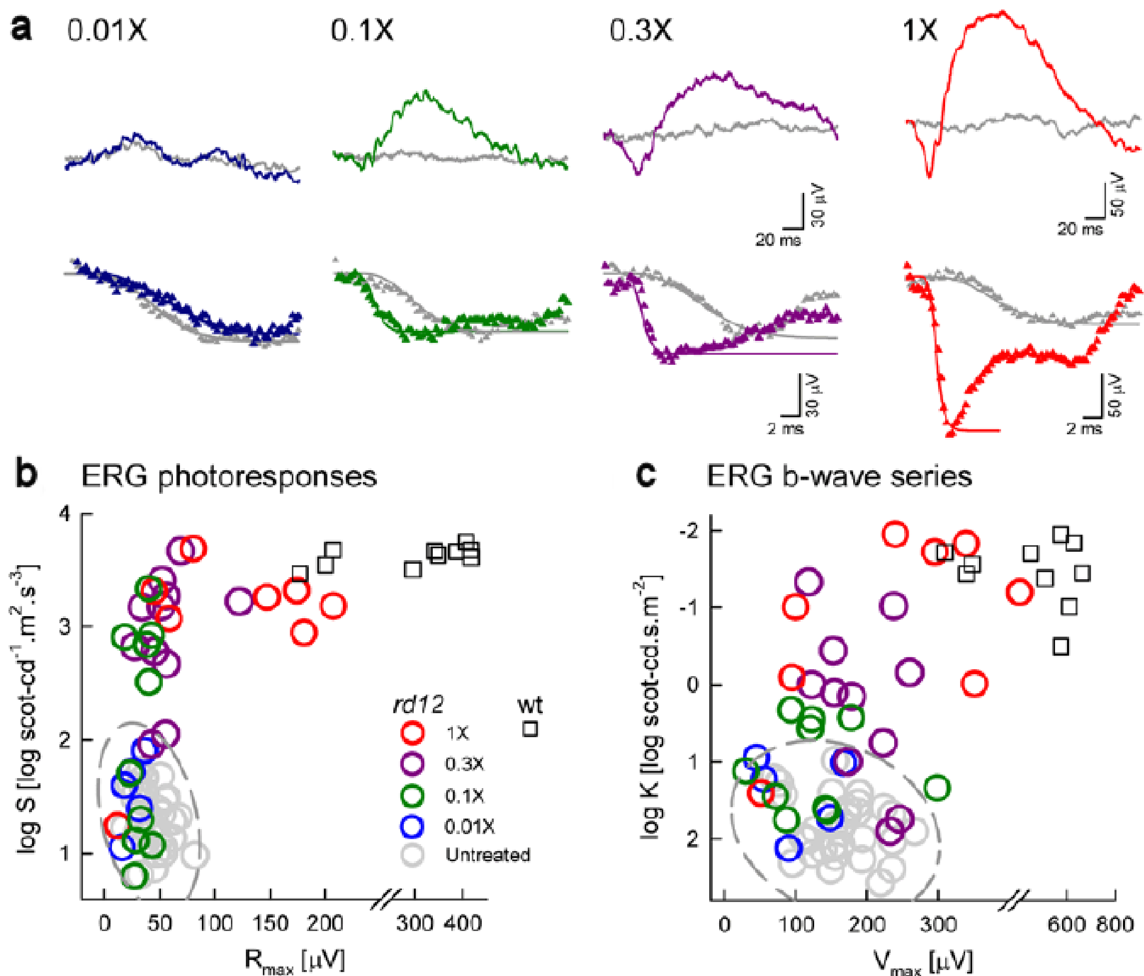


Fig. 20a-c Electroretinographic parameters of *rd12* mice treated with different doses of subretinal AAV2-RPE65. 20a ERG evoked by 0.1 log scot-cd.s.m^{-2} flashes (upper row) and by 3.6 log scot-cd.s.m^{-2} flashes (lower row) in treated (colors) and untreated (gray) eyes demonstrate that, with increasing vector dose, responses become asymmetric with treated retinas showing increasing b-wave amplitude and faster photoresponses. **20b** As vector dose increases, photoresponse parameters of treated eyes drift outside of the 99% confidence interval (dashed ellipse) defined by the untreated eyes of *rd12* mice and start to approach wild-type levels. **20c** Luminance response parameters in treated *rd12* eyes show a similar dose-related progression. *Adapted from Roman et al. 2006*.

Ocular inflammation following gene therapy could relate to an immunopathogenic reaction to the RPE65 molecule (Narfström et al. 2003), but inadequate vector purification has also been suggested (Acland et al. 2005). Importantly, Jacobson did not detect a humoral immune response to the AAV vector or to any of its components (Jacobson et al. 2006). Biodistribution of the transgene product is also an important safety issue and, accordingly, many authors have addressed this subject. It has been found that intravitreal injection of AAV2/2 vectors results in persistent detection of vector sequences in canine brain and visual pathways (Dudus et al. 1999, Provost et al. 2005). Similarly, AAV sequences have also been identified in canine optic nerve following subretinal injection (Provost et al. 2005). More recently, however, Jacobson et al reported little or no detectable vector sequences outside the injected eyes of both RPE65-mutant dogs and normal mice, following intravitreal or subretinal injection routes (Jacobson et al. 2006).

Several factors served as a starting point for the extrapolation of animal methodologies to human patients, leading to the development of Phase I human clinical trials. Among those factors we should point out some that were absolutely essential. Perhaps the most important is the fact that gene therapy in animal models of LCA has proven to be successful and, above all, safe. Equally important were the findings of viable photoreceptors in human LCA patients, rendering them amenable to gene replacement therapy.

GENE THERAPY RESULTS FOR HUMAN LCA

The first two human clinical trials using gene therapy for LCA associated with RPE65 mutations took place in London (UK) and in Philadelphia (USA), in 2008.

In the Philadelphian study (ClinicalTrials.gov NCT00516477), Maguire and colleagues injected AAV2 containing the wildtype human *RPE65* carrying a chicken β actin promoter into the subretinal space of three LCA patients, ranging from 19-26 years (Maguire et al. 2008). The injection created a dome-shaped retinal detachment starting in the superior nasal retina and further extending into the macula. Visual function was assessed before and after gene transfer through pupillometry, nystagmus frequency, ETDRS visual acuity, Goldman peripheral visual fields, and ability to navigate an obstacle course. ERG assessment was not reported. Two weeks post-surgery, all patients self-reported vision improvement in dimly lit environments, but dark-adapted functional assessments were not reported.

Clinically significant improvements in visual acuities, ranging from 3 to 4.5 ETDRS lines, were also observed and visual field width improved from 80° before to 200° after therapy, in all patients. Perhaps the most notorious effect of therapy was seen when patients were tested in the obstacle course. Video recordings show truly astonishing improvements in confidence and time taken to complete this task, for all patients, when compared to their pre-treatment performance. Objective measures of nystagmus frequency and pupillary constriction were much improved after treatment, which reflects enhanced retinal sensitivity, and better transmission of visual input signals to the brainstem. Of note, clinical benefits remained stable during the 6 months of follow-up. Importantly, no serious systemic adverse events were reported in any of the patients (Maguire et al. 2008). However, a full-thickness macular hole developed in one of the patients. This can possibly be explained by the proximity between the site of the retinotomy and the foveal region (Hauswirth et al. 2008).

In the London trial (ClinicalTrials.gov NCT00643747), Bainbridge and colleagues included three patients, ages 17 to 23 years, who underwent subretinal injections of an AAV2/2 vector containing the normal human *RPE65* gene and promoter (Bainbridge et al. 2008). Resulting injection blebs included the fovea and extended over about one third of the total retina. Prior to treatment, structural integrity of the retina was analyzed using fundus photography, fundus autofluorescence and OCT. Additionally, visual acuity, contrast sensitivity, color vision, and cone flicker sensitivities were performed and perimetric procedures enabled the assessment of visual fields, before and after treatment was conducted. Electrophysiological evaluation included full field, flicker, pattern and multifocal ERG. Ultimately, patients were submitted to obstacle course testing, at different illumination conditions, to evaluate their real-world visual performance. At baseline, all three patients were found to have slightly better visual acuities than their counterparts from the Maguire trial. However, only one of the patients showed visual improvement after gene therapy was performed. Of note, this patient was not the youngest-treated, but he probably had less advanced retinal disease at time of intervention, which may explain the lack of visual improvement in the other patients. Visual acuities in his study eye improved over a follow-up period of 12 months, but because his control eye also showed a similar improvement, the gain was deemed non-significant (Bainbridge et al. 2008). It is possible that the improvements in the control eye relate to nystagmus dampening (Koenekoop 2008) or to transfer of RPE65 protein to the untreated eye (Narfström et al. 2003). In the Bainbridge

study, the patient with visual improvement was also found to have a dramatic improvement in performance through the obstacle course, reducing the walk time from 77 to a mere 14 seconds. In this study, no serious adverse effects were reported (Bainbridge et al. 2008).

Improved visual acuities in both studies are likely due to photoreceptor rescue but, since no detectable improvements in ERG responses were reported in either study, it is impossible to confirm this assumption (Hauswirth et al. 2008, Koenekoop 2008).

Both the Philadelphia and London trials demonstrate that, like in non-human animal models, gene therapy successfully restores vision in human LCA patients. Differences in visual outcome between the two trials may be due to several factors, including differences in patient genotypes, or differences in the promoters that were use to enhance RPE65 expression. Furthermore, better visual acuities at baseline in the Bainbridge study might be another contributing factor. Common to both studies were the facts that low doses of the viral vector were used, and patients were at an age considered old, in which photoreceptor loss is likely to already have commenced (Koenekoop 2008). Thus, given that previous studies in *Rpe65* mice have shown superior treatment success in younger animals (Dejneka et al. 2004), it seems logical to think that functional improvements might be better in younger patients (Bainbridge et al. 2008, Hauswirth et al. 2008, Maguire et al. 2008) and with higher doses of the vectors (Hauswirth et al. 2008). However, we should remember that a strong relation between age and retinal structure or function has been said not to exist before the fourth decade of life (Jacobson et al. 2007 and 2008).

In a parallel study, Cideciyan and colleagues performed gene therapy to three human LCA patients, ranging from 21-24 years old, restoring normal RPE65 function through AAV2-mediated gene transfer (Cideciyan et al. 2008). After treatment, rod-dependent night vision increased in sensitivity up to 63000 fold in the three patients, an improvement observed exclusively in the retinal areas exposed to therapy. Moreover, the magnitude of improvement differed among patients, and those with a better preserved photoreceptor layer in the treated area were found to have greater rod rescue. Cone-mediated vision was also found to have robustly improved, with up to 50 fold increased sensitivities, in two of the patients, throughout the 3 months of follow-up. Of note, none of the patients showed a decrease in nystagmus frequency. It was suggested that the increase in rod sensitivity probably relates to an increased 11-*cis*-retinal synthesis mediated by the *RPE65* transgene. Surprisingly, Cideciyan et al found that although therapy appeared to successfully restore

normal retinoid cycle activity, it did so but with abnormally slow rod kinetics. Indeed, after a desensitizing light flash, rod recovery in two of the patients progressed slowly, lasting at least 8 hours. The investigators suggest that the prolonged rod recovery could relate to slowed delivery of 11-*cis*-retinal from the RPE to the rod photoreceptors either due to a reduced synthesis rate, possibly relating to limited transgene expression, or to an increased obstruction to its inter or intracellular transport, eventually related to the underlying retinal degeneration (Cideciyan et al. 2008). The finding of slow post-treatment rod kinetics has practical implications since it will surely influence the design of future clinical trials. Cideciyan et al highlight this issue alerting for the fact that maximum increase in vision after gene therapy can only be judged after patients have undergone a long period of dark adaptation, in order to provide enough time for full rod photoreceptor recovery. Likewise, comparisons of visual function between patients within or between trials cannot be made unless rigorous attention is given to previous light exposure and length of dark adaptation (Cideciyan et al. 2008).

One year after gene therapy was performed in the Cideciyan and colleagues trial, patients remained healthy and free of vector-related serious adverse events (Cideciyan et al. 2009b). Additionally, functional and structural assessment results were found to remarkably resemble those measured at the 3-month time point, suggesting stability in the improvements obtained after gene therapy was conducted. The unexpected finding of slowed rod kinetics in the treated retina (Cideciyan et al. 2008) was also present at the 12-month time point (Cideciyan et al. 2009b). The durability of the human visual improvements after gene therapy seems to be consistent with the electrophysiological data from the long-term studies of treated *RPE65*-mutant dogs (Acland et al. 2005, Aguirre et al. 2007, Cideciyan et al. 2009b).

At the 12-month follow-up, a noteworthy observation of one patient of the Cideciyan trial prompted further investigation (Cideciyan et al. 2009b). For the first time in her life, this patient self-reported the ability to read the illuminated numerical clock display on the dashboard of her family vehicle, while sitting in the front seat. The numerals implied a visual angle equivalent to a visual acuity of 1/10, which was not different from her formally measured visual acuities at baseline or at 1 year after treatment. When quantifying fixation of the patient's gaze to dim targets over a range of luminances, it was primarily found that the patient could perceive the lowest luminance target, for the first time. Surprinsigly, this

new perception was accompanied by a shift in fixation into the previously treated superotemporal retina. This retinal location, then termed as “pseudo-fovea”, was found to have remarkably increased sensitivities, greatly different from its counterpart in the control eye. It is thought that the change in fixation was driven by the treatment-created extrafoveal cone vision with better sensitivity and greater expanse than the untreated foveal region, and it might correlate to experience-dependent plasticity of the human visual system (Cideciyan et al. 2009b). This finding was not reported by the two other patients included in the trial (Cideciyan et al. 2009b).

Suggestion that gene therapy might produce better vision rescue in younger patients has significantly influenced the approval of human clinical trials involving children with LCA. Likewise, Maguire et al were the first to include children in human clinical trials of gene therapy for the disease (ClinicalTrials.gov NCT00516477; Maguire et al. 2009). In that trial, 12 patients were treated, ranging from 8-44 years old, using AAV-*RPE65* vectors. Of note, this was the first trial to use a vector dose-escalation scheme, therefore enabling analysis of possible vector dose-dependent effects. Starting 2 weeks after unilateral subretinal injection of AAV-*RPE65*, all patients reported improved vision, an effect that remained unchanged throughout a 2 year follow-up period. Importantly, no serious local or systemic adverse effects were reported by any of the patients, but the presence of detectable vector in blood samples after injection in two patients with widespread retinal degeneration, suggests that transient systemic exposure to the vector may occur after injection. Visual acuities were found to significantly improve in three of the low dose patients, three of the middle dose, and one receiving the high dose. Importantly, however, there was no obvious dose-dependent effect with respect to improvements in visual acuity. Moreover, the improvement was not associated with age, meaning that mean visual acuity gain was similar in young and older patients. However, since children had better acuities at baseline, correlating with a far less advanced degenerative process, the qualitative effect of therapy in this patient group was more perceivable. Visual fields were found to widen in all the patients and improvement was reportedly age-dependent. Indeed, even patients, aged 19 years or older, receiving large volumes of vector could not achieve visual field improvements like the ones obtained by their younger counterparts, a difference certainly related to greater photoreceptor loss in older LCA patients. Of note, visual field recovery was found to roughly correlate with the injected area. Full-field retinal sensitivity to light was markedly improved

in all treated eyes and demonstrated age and baseline sensitivity dependence, being especially noteworthy in younger patients. This correlated well with improved pupillary light responses in the treated eyes. Curiously, although full-field sensitivity and pupillary light response improvements were limited to the vector-treated retinas, a mild bilateral improvement of visual function, like the one obtained in visual acuity, was noted in many patients (Maguire et al. 2009), a phenomenon possibly related to nystagmus dampening (Maguire et al. 2008, Simonelli et al. 2010) or to improved signal processing in the visual cortex, which has been found to remain responsive despite early degeneration in LCA patients (Aguirre et al. 2007). On light of these assumptions, Maguire et al further advance that bilateral simultaneous or immediately consecutive treatment of both eyes might show a synergistic effect (Maguire et al. 2009), a tempting concept surely to be explored in future trials. Ultimately, Maguire and co-workers enrolled four of the treated children through an obstacle course, before and after gene transfer was performed. Amazingly, after therapy, all children were found to navigate the course more accurately and rapidly than before, proving that gene therapy was successful in the restoration of real-world visual performance (Maguire et al. 2009).

Preliminary results of gene therapy for human LCA are indeed overwhelming and seem to extend the successful achievements obtained during animal experimentation. In a total of 21 patients, all but two managed to report significant improvements in their visual function. Moreover, in some of the patients, improvements were as great as to allow reconsideration of their legally blind status (Maguire et al. 2009). Importantly, all human trials proved safe, a major contributing factor to their success. Altogether, these favorable results will certainly allow for the development of future human clinical trials, in which new, previously unexplored premises will surely be addressed, bringing further insights into the emerging field of ocular gene therapy, particularly that related to LCA. Additionally, gene therapy's success in human LCA patients has nurtured hope regarding the possibility of its applicability to other visually debilitating diseases, such as Stargardt disease.

GENE THERAPY FOR STARGARDT DISEASE: LESSONS FROM LCA TRIALS

The possibility of using gene therapy to treat patients with Stargardt disease is tempting and much can be learned from its application to LCA.

First of all, the monogenic nature of Stargardt disease and its relatively although-not-yet-fully-understood simple pathophysiology, render it as a preferred target for gene replacement therapy. Indeed, replacement of the mutant *ABCA4* gene by its wildtype counterpart is theorized to produce successful results as the ones obtained from *RPE65* gene replacement in LCA. However, prospects of gene therapy for Stargardt disease will require good animal supporting studies and careful analysis of several other factors, including vector-related issues and clinical assessment of patients potentially amenable to gene transfer. A better understanding of the pathophysiology of Stargardt disease is also a pre-requisite for successful gene replacement therapy.

Animal testing using gene therapy for Stargardt disease remains yet very limited, and its feasibility has only recently been explored. Kong and co-workers were able to successfully treat the Stargardt phenotype in *abca4* knockout mice using lentiviral gene therapy (Kong et al. 2008). Each mouse received a single unilateral subretinal injection of ABC*A*4-carrying equine infectious anemia viral vectors, which resulted in significant rescue of the retinal phenotype. Indeed, treated eyes showed marked reduction in the accumulation of disease-associated A2E compared to untreated and mock-treated eyes. Moreover, 1 year after gene transfer, A2E accumulation in the treated eyes were found to match A2E levels in normal wildtype controls (Kong et al. 2008). This was only a morphological study and functional effects of therapy have therefore not been reported by it. However, it confirms that the primary, although indirect, target of *ABCA4* replacement is the RPE. In other words, by directly correcting the photoreceptor-bound defective *ABCA4*, gene therapy allows reduction of A2E accumulation and, therefore, secondary RPE rescue. Likewise, rescue of RPE is deemed essential for photoreceptor survival.

Discussion regarding the type of vector to use for *ABCA4*-related gene transfer must certainly not be overlooked. The relatively large *ABCA4* gene presents a unique challenge regarding packaging capacities of available viral vectors, owing to its significant genomic size of 6.8 kb. Lentiviral vectors, due to their 8.0 kb packaging capacity and satisfactory photoreceptor transduction ability, are seen as the most suitable for *ABCA4* gene transfer and the work of Kong et al proves that they can do so in a very successful way (Kong et al. 2008). However, based on clinical trials of ocular gene therapy, experimental data is largely more detailed for AAV vectors, since these have been the preferred vector system so far. Yet, since AAV vectors are very limited in terms of their packaging capacity (4.7 kb), they

have been used mostly in gene therapy studies involving smaller-sized transgenes. Recent evidence, however, suggests that AAV2/5 chimeras are suitable for photoreceptor transduction while providing a packaging capacity of up to 8.9 kb (Alloca et al. 2008). In the future, new vector systems, such as recently developed Hd-Ad vectors, or the overgrowing development of non-viral vectors, will surely provide innovative alternatives for the transfer of large genes such as *ABCA4.* Moreover, continuous improvement of currently available vector systems will certainly accelerate the development of new animal studies of gene therapy not only for Stargardt disease but also for other ocular genetic conditions (Cideciyan et al. 2009a).

Given that Stargardt, like LCA, is a retinal degeneration, identification of viable photoreceptors will also play a central role in the selection of patients amenable to gene replacement therapy. Thus, the use of imaging resources will surely be essential to photoreceptor viability assessment, when selecting patients who may benefit the most from gene therapy. Among these resources, ultrastuctural retinal imaging, like the one provided by high-definition OCT, must be highlighted, since it provides the most accurate assessment of outer nuclear layer structural integrity, therefore identifying early photoreceptor degeneration. More recently, the incorporation of adaptive optics technology into ocular imaging is looking to allow more accurate and detailed photoreceptor viability assessment. Adaptive optics promotes reduction of the effects of rapidly changing optical distortion in order to improve the performance of optical systems. It has long been used in astronomical telescopes and laser communication systems, but only recently have its applications to ocular imaging been made aware. Adaptive optics technology enhances lateral resolution in retinal images up to 3-4µm, allowing the visualization of individual photoreceptors, and literally enabling their individual count (Fig. 21). One of its key advantages is the possibility of combining it with almost any other imaging technology including charge-coupled device (CCD) cameras, scanning laser ophthalmoscopy (SLO), and OCT, among many other existing systems. Indeed, adaptive optics technology is destined to revolutionize ocular imaging systems, and prototypal testing is already taking place in Paris. Widespread application of this technology to ophthalmology can surely be regarded as eminent.

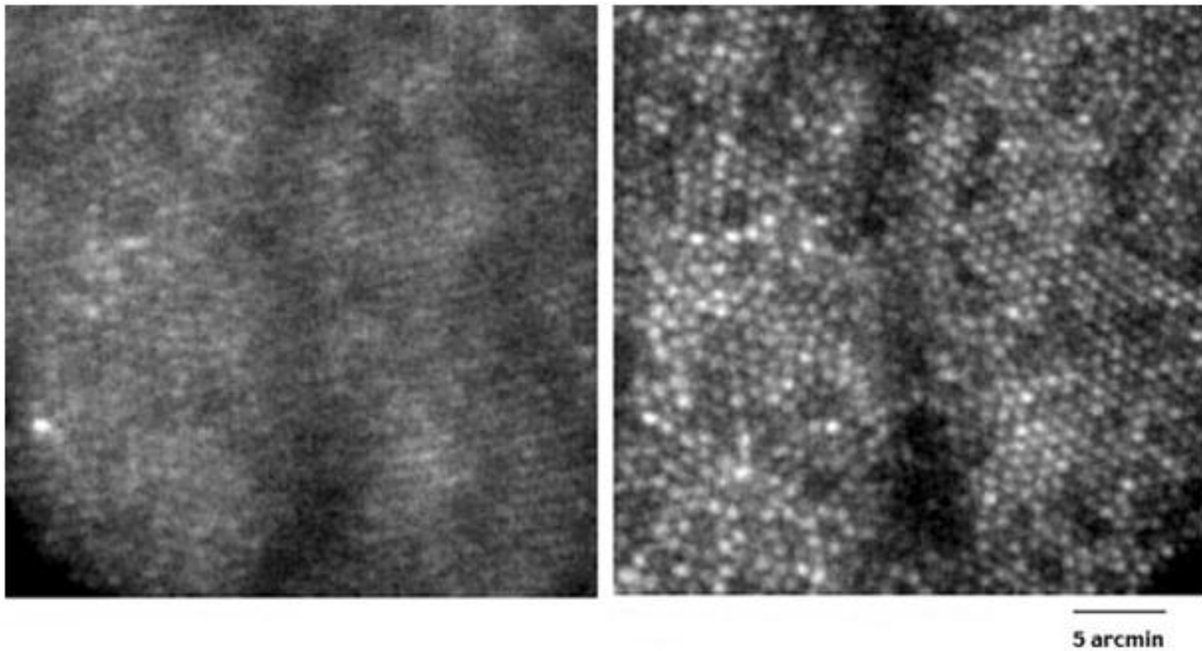


Fig. 21 Image of the retina without (left) and with (right) adaptive optics compensation. Adaptive optics technology remarkably enhances image resolution, allowing individual photoreceptor assessment. *Image by Austin Roorda*.

Unlike LCA, the onset of Stargardt disease has been known to occur later, usually between childhood and adolescence (Stargardt 1909, Hadden et al 1976, Lois et al. 2001, Westerfeld et al. 2008), but that can only come as an advantage since it will allow for early recognition of the disease and implementation of therapy before the condition develops or at least at a very early disease stage, ultimately treating Stargardt patients before any significant vision loss occurs. For that matter, the identification of *ABCA4* mutations in target individuals is deemed to be a crucial element in this sequence, since it will allow for close follow-up of patients and intervention at the most suitable time point. Still, the most realistic scenario is envisioned to be clinical trials that will enroll individuals already with overt macular degeneration, in whom gene therapy will mostly have a limiting role regarding disease progression. Therefore, reliable prediction of disease evolution will surely be an essential determinant. However, there is currently no way of predicting whether or when an individual will progress from localized macular involvement to retina-wide degeneration, and we yet remain unaware of the true contribute of environmental conditions or possible genetic modifiers to the phenotypic variation among Stargardt patients (Cideciyan et al. 2009). Surely, future studies will bring new insights into these yet unresolved issues.

CONCLUDING REMARKS

Years of gene therapy research for LCA and underlying RPE65 mutations, have produced incomparable breakthroughs, which will invariably serve as a foundation for further research involving many other ocular conditions. Indeed, the impact of this success has certainly been the main motivating factor leading some biomedical research centers to explore the commercial benefits of gene therapy. Accordingly, in December 2009, Oxford Biomedica™ announced that StarGen™, a gene-based therapy that uses the Company's LentiVector® technology for the treatment of Stargardt disease, has received orphan designation from the Committee for Orphan Medicinal Products of the European Medicines Agency (EMEA). In collaboration with sanofi-aventis, both companies aim to advance StarGen™ into PhaseI/II development during 2010. The US charity, Foundation Fighting Blindness, is also supporting the programme and previously funded preclinical development. With this initiative, Oxford Biomedica™ is deemed to bring considerable hope for the 600 new cases of Stargardt disease diagnosed every year and for many other Stargardt patients that currently await treatment for their visually-debilitating condition. If successful, StarGen™ will elevate the topic of ocular gene therapy to unprecedented levels.

ACKNOWLEDGEMENTS

The author would personally like to thank his master and friend, professor Eduardo José Gil Duarte Silva, for his irreplaceable support and supervision, without which this work would have not been possible.

ACRONYMS

A2E - Di-retinoid-pyridinium-ethanolamine; **A2PE** - Di-retinoid-pyridinium-phosphatidylethanolamine; **AAV** - Adeno-Associated Vectors; **ABC** - ATP-biding cassette; **AMD** - Age-related Macular Degeneration; **CRD** - Cone-Rod Dystophies; **ERG** - Electroretinogram/Electroretinography; **Hd-Ad** - Helper-dependent Adenoviral Vectors; **LCA** - Leber Congenital Amaurosis; **PE** - Phosphatidylethanolamine; **RP** - Retinitis Pigmentosa; **RPE** - Retinal Pigmented Epithelium; **STGD** - Stargardt Disease; **VEPs** - Visual-Evoked Potentials.

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