II. Anatomy and physiology of retina

The retina is a thin, multilayered sheet of neural tissue that lines the inner aspect of the posterior wall of the eye globe. This layer is vital for the vision process since it contains neurons that are capable to sense, amplify, integrate and transmit the visual signals to the brain. The photoreceptors respond to the light particles and convert them into electrical impulses. Subsequently, these impulses travel down the optic nerve to the visual cortex for further processing and visual perception.

The retina is a highly organized structure that comprises two distinct layers: the outer pigmented layer called the retinal pigment epithelium (RPE) and the adjacent inner layer of nerve tissue called the neurosensory retina (14;15).

Figure 1. The layers of the retina (16).
II. 1. 1. The retinal pigment epithelium

The RPE is a monolayer of hexagonal cells that is located between the light-sensitive outer segments of the photoreceptors and the choroid. RPE is attached to the Bruch’s membrane and forms the choroid border. This layer plays an essential role in the retinal physiology by forming the outer high-resistance basolateral blood-retinal barrier and supporting the function of the photoreceptors (17).

II. 1. 2. The neurosensory retina

The complex of neurosensory retina formed by the neural extension of the brain is responsible for the detection and transmission of the signals from the photoreceptors to the brain cortex. This inner layer of retina comprises of six major types of cells: the amacrine cells, bipolar cells, ganglion cells, horizontal cells, Müller cells and the photoreceptor cells. The neurosensory part of retina is divided into three distinct layers. The outermost layer is formed by the photoreceptor cells which convert the light signal into an electrical impulse. These impulses are then transferred to the second layer consisting of nuclei of interneurons (bipolar, horizontal and amacrine cells). The third layer comprises of ganglion cells, which collect the signals of from the bipolar cells, and their axons will subsequently transmit this information to the visual cortex of the brain (14;18-20).
II. 1. 3. The photoreceptor cells

Photoreceptors are photosensitive neurons in the outer part of the retina. Morphologically, two types can be distinguished: rod and cone cells. The retina contains approximately 120 million rods and 6 million cones (21;22).

Rods are particularly sensitive to light, being able to detect even a single photon (23). This photoreceptor type allows vision in dim light. In contrast, cones are less (30-100 fold) sensitive to the light, playing an important role in mediating bright-light and color vision (16,24). Rods are predominantly located in the peripheral area of retina whereas cones are predominantly located in the center of retina, called the fovea (14).

Figure 2. The photoreceptor cells: rod and cone
The photoreceptors are two types of cells: rod and cone, which can be distinguish by their shape. Both can be divided into 4 parts: an outer segment, an inner segment, perikaryal region containing the cell nucleus and an axon (16).
All photoreceptors consist of four main components – an outer segment, which contains the visual pigments, an inner segment, a perikaryal region containing the cell nucleus, and an axon. The molecular components in the outer segment are needed for light absorption and conversion to electrical signals, whereas the inner segment harbors the machinery for protein synthesis and production of energy. The electrical light response is then transmitted to the horizontal and bipolar cells (24-26).

The blood supply of retina provided by two main sources: the choriocapillaris immediately outside Bruch's membrane, which supplies the outer third part of the retina, including the outer plexiform and outer nuclear layers, the photoreceptors, and the retinal pigment epithelium; and branches of the central retinal artery, which supply the inner two-thirds. The fovea is supplied entirely by the choriocapillaris (14).

II. 2. Visual process

Two main processes are essential for the mechanism of vision in the retina, phototransduction - the process in which absorbed photons are converted into electrical responses - and visual cycle. Studies of genes linked to human hereditary blindness have been crucial for improving the understanding about this pathway.
II. 2. 1. Phototransduction

Phototransduction is the process of absorbing light stimulus and creating a response by photoreceptor cells. This biochemical cascade takes place in the outer segment of a photoreceptor (27). The membranous discs of outer segment are loaded with photosensitive pigments, rhodopsin in rods and cone opsins in cones. These pigments covalently linked to 11-cis-retinaldehyde (11-cis-RAL) by a Schiff-base linkage. Upon absorption of a light photon, the opsin activates by 11-cis-RAL isomerization into all-trans-retinal. The activated form of the rhodopsin photopigment, metarhodopsin II, triggers the activation of a trimeric G-protein called transducin by allowing the change of the bound GDP nucleotide to GTP. The alpha subunit of transducin then activates the phosphodiesterase (PDE) alpha and beta subunits which subsequently hydrolize cGMP into 5'-GMP. The reducing of the cytoplasmic cGMP concentration modifies the conductance of the cGMP-gated channels leading to their closure and thereby blocking the influx of sodium cations towards the cells. As a result, positively charged potassium flows out of cell more rapidly than sodium and calcium is transported to the cell. The cell becomes depolarized and suppresses its release of glutamate neurotransmitter enabling the signal to travel down the optic nerve neurotransmitter (26-28). This cascade provides enormous signal amplification, as a single activated rhodopsin molecule is able to activate
500 transducin molecules, leading to hydrolyzation hydrolysis of about $10^5$ cGMP (29;31;32).

In the termination phase of this cascade, rhodopsin and other molecules are rapidly deactivated and recycled. The activated rhodopsin will be phosphorylated by rhodopsin kinase which then allows the protein arrestin to bind to rhodopsin. The binding of arrestin blocks the access of G protein to the activated rhodopsin, thus ‘arresting’ its further activity. Finally, rhodopsin will be recycled back to a form that can absorb light (33-35).

II. 2. 2. The retinoid cycle

The retinoid cycle is a complex recycling system that supplies the 11-cis-retinal chromophore of rod and cone visual pigments after isomerized to all-trans-retinal by light. This cycle takes place in retinal rod and cone photoreceptor outer segments and the retinal pigment epithelium. After inactivation of metarhodopsin II, all-trans-RAL will dissociate from the opsin protein and transported into the bilayer of outer segment disc membranes. ATP-binding cassette transporter called ABCR has been implicated to facilitate reduction of all-trans-RAL by transport it from disc membranes to the cytoplasmic space to become all-trans-retinol (all-trans-ROL). Retinal dehydrogenase will subsequently catalyze all-trans-RAL to all-trans-ROL. All-trans-ROL will be released to the interphotoreceptor matrix (IPM) space by the photoreceptor cells.
Lecithin retinol acyl transferase (LRAT) is the major retinyl ester synthase in RPE cells, which catalyzes the transfer of a fatty acyl group from phosphatidylcholine to all-trans-ROL. The isomerases in RPE cells will catalyze the conversion from all-trans-retinoid to the sterically constrained 11-cis-ROL. Eventually, 11-cis-ROL will be oxidated to formed 11-cis-RAL(33-34).

II. 3.  Retinitis pigmentosa

II. 3. 1. Definition and clinical manifestations

RP is a clinically and genetically diverse group of hereditary retinal dystrophies affecting primarily rods, with subsequent cone photoreceptor cell degeneration. The onset and progression vary between individuals with RP (2;4;36). The first symptom of RP is night blindness at an early stage, followed by visual field constriction and central severe visual impairment due to photoreceptor degeneration as the disease progresses (37). These clinical symptoms correlate with a predominantly affected rod photoreceptor cells followed by the cone photoreceptors degeneration causing complete blindness at the final stage (38). This disease can be subdivided into non syndromic retinitis pigmentosa, in which there is no systemic abnormalities, and syndromic retinitis pigmentosa where the retinal degeneration is associated with other disorders. The most frequent syndromic form of RP is Usher Syndrome, followed by Bardet Biedl syndrome (BBS) and other syndrome
RP patients displays a classic triad of RP in the fundus: bone-spicule pigmentation, arteriole attenuation and pale, waxy of the disc optic (Figure 3) (39;40).

![Fundus appearance of normal eye and RP](image)

**Figure 3. Fundus appearance of normal eye and RP**
(a) Normal fundus appearance; (b) Fundus appearance of RP patients which shows bone-spicule like pigmentation, attenuation of arterioles and pallor of the optic disc (16).

**II. 3. 2. Pathophysiology of retinitis pigmentosa**

RP is a group of retinal dystrophies which primarily affect rod photoreceptor cells followed by cone degenerations. The degeneration of photoreceptor cells is subsequently followed by retinal outer layers degenerations, whereas the inner layer of retina is still well preserved until the later stage of the disease. In the previous study in mice model implicated that this degeneration are due to the disability to form rod outer segment (OS) and consecutively lead to rod photoreceptor cells loss. Cone degenerations which triggered by toxic byproducts of rod cells degeneration or due to the loss of trophic factors that are normally produced by rod, will subsequently occur and lead to a successive loss of outer retinal layers. The loss of photoreceptors will reduce the retinal thickness and alters retinal
integrity. In the normal retina, photoreceptor layer is located between RPE and retinal vessels. The loss of photoreceptor layer will result in approximation of RPE to retinal vessels. This contact stimulates the detachments of RPE cells from Bruch’s membrane and migrate to along the adjacent retinal vessels to perivascular sites in the retina. The nuclei of RPE cells are translucent, whereas the cell body is dark due to melanin. The RPE cells will form three-dimensional pigmented cell clusters around retinal vessels which are known as bone-spicule pigmentations.

Attenuation of retinal blood vessels is a funduscopic hallmark of RP which caused by the occlusion of vessels lumina by extracellular matrix deposits. Thickening of the blood vessels wall and the occlusion of lumina correlates with the retinal vasculature atrophy and sclerosis. RPE cells will also reestablished the blood-retina barrier by forming tight junctions thereby reducing leakage from vascular endothelial fenestrations (39).

Another typical appearance of RP fundus is disc optic atrophy. It is suspected that the ganglion cells axon are compressed by the retinal blood vessel and lead to the loss of ganglion cells. At the later stage, more ganglion cells will be lost and cause the pale, waxy, atrophic optic disc appearance (39).
II. 3. 3. Histopathology appearance of retina with RP

The rod photoreceptor cells outer segments (OS) shortening is one of the first histopathology finding in the retina at the early stage. At the later stage, RPE cells will be migrate upwards along the retinal vessels (39). The pigmented cells will cluster around retinal vessels and forms bone spicule pigmentation (Figure 4). Extracellular matrix (ECM) is accumulated and causes the occlusion of retinal vessels (Figure 5).
II. 3. 4. Molecular genetics of retinitis pigmentosa

RP can be inherited in an autosomal dominant (adRP, 30 - 40%), autosomal recessive (arRP, 50 - 60%) X-linked (XLRP, 5 - 20%) and digenic fashion (1;3;5). A number of cases (termed “isolated” or “sporadic” RP) cannot be classified since proband is the only affected person in the family. Hence, both autosomal recessive and dominant inheritance, arising from de novo alterations as well as X-linked inheritance in isolated male cases, are possible. About 50 genes are currently known to be causative for RP, several displaying allelic heterogeneity; some have been reported to cause both dominant and recessive modes of inheritance (1;2). Although many genes have been described as causative for RP, 50% of the genetic defects remain unsolved. Several kinds of approach are needed to discover new
mutations and new genes that are responsible for the remaining cases (Figure 6).

Figure 6. All identified RP genes
Currently, 39 genes has been reported responsible for arRP (courtesy of R.W.J Collin)

Based on the molecular pathway and the retinal structure, RP-associated genes can be classified as involved in: the rod phototransduction cascade, the retinoid cycle, and ciliary transport (illustrated in Figure 7). Thus, knowledge about molecular mechanisms of vision is important to understand the involvement of the genes in the visual process (6).
There are three major processes that are essential in human rod photoreceptor cells and the RPE: retinoid cycle, phototransduction cascade and ciliary transport (6).

II. 4. Molecular genetic analysis

II. 4. 1. Homozygosity mapping

Homozygosity mapping has been proven to be an effective approach in the search for genes and the discovery of mutations in known
arRP genes (41-45). A homozygous mutation is likely to reside within homozygous region detectable with a high resolution genome-wide single nucleotide polymorphism (SNP) genotyping array. The application of homozygosity mapping leads to the identification of the genetic lesions both in consanguineous and non-consanguineous cases, as shown in patients with autosomal recessive kidney diseases (44). This technique has been proven to be a powerful method for detection of new mutations in known genes implicated in retinal dystrophies and mutations in new disease genes.

In autosomal recessive mode of inheritance, alterations on both alleles are required for the disease manifestation. Homozygosity mapping is a method that utilizes homozygous regions shared by the affected individuals of the same family. In consanguineous families, affected individuals are more likely to inherit two copies of the same disease allele from their common ancestor. Therefore, the region surrounding disease causing mutations will be homozygous. A closer relative marriage will present a larger homozygous region (46-48).

In non-consanguineous families, homozygous regions can be found as well, due to a common ancestor who cannot be traced from the available family history. The size of homozygous region is related to the number of generations between the parents of the patient and their common ancestor. The closer the generation of the founder, the bigger size homozygous region will be found.
II. 4. 2. Pathogenecity prediction of missense mutations

In a missense mutations, in silico web based prediction programs, including Polymorphism Phenotype (PolyPhen), Sorting Intolerant from Tolerant (SIFT) and Align GVGD were used to predict the pathogenecity of missense mutation. These programs are able to calculate the potential functional and structural impacts based on Grantham scores (comparison of the differences in physical properties of the amino acids side chains) and PhyloP scores (evolutionary conservation of the nucleotide). Project hope will be also used to visualized the protein’s crystal structure changes caused by mutation.

II.4.2.1 SIFT

This web based program uses sequence homology to predict whether an amino acid substitution effect to the protein function and contribute to a disease phenotype. The idea of this approach is that important amino acids tend to be highly conserved across species. SIFT, which assigns scores from 0 to 1, predicts substitutions with scores less than 0.05 as deleterious, whereas those greater than or equal to 0.05 are considered to be tolerated.

II.4.2.2 Polyphen

PolyPhen includes the evolutionary conservation of the amino acid subjected to the mutation and the physicochemical characteristics of
the wild-type and mutated amino acid residue and the consequence of the amino acid change for the structural properties of the protein to predict the effect of mutation.

II.4.2.3 Align GVGD

Align GVGD combine Grantham variation (GV) and Grantham deviation (GD) to predict the pathogeneicity of mutations. GV measures the degree of biochemical variation among amino acids found at a given position in the multiple sequence alignment, whereas GD reflects the 'biochemical distance' of the mutant amino acid from the observed amino acid at a particular position. Align-GVGD can be used to predict the transactivation activity of each missense substitution. A score of GV=0 consider as a residue that is invariant in the alignment, a value of GV of 60–65 is the upper limit of conservative variation across species, and a value of GV>100 is indicative of positions that are under little functional constraint. A value of GD=0 corresponds to a missense substitution that is within the cross-species range of variation at its position in the protein; at invariant positions (GV=0); GD=60–65 is the upper limit of a conservative missense substitution.

II.4.2.4 Project hope

This web-based program was used to analyze the effect of a point mutation on the 3D-structure of a protein. This program collects
structural information from a several of sources, including calculations on the 3D protein structure, sequence annotations and protein predictions from other prediction tools. This informations were combined to analyze the effect of a certain mutation on the protein crystal structure. Hence, we can predict and visualized the missense mutation effect on the mutant protein structure and function.

II. 4. 3 Novel mutations confirmation

Mutation analysis of candidate genes residing within the homozygous regions may revealed either known mutations or novel mutations. Pathogeneicity of the known mutations can be determined from the available mutations database, such as Human Genome Mutation Database (HGMD). This website provide informations and link of other databases which have a complete informations regarding the mutations. The available informations are including the pathogenecity prediction, molecular mechanism prediction, the protein expression, knock-out murine phenotype and human phenotype with this specific mutation.

Conversely, in a novel mutation, those data are not available since the functional studies has not been performed for these novel mutations. Therefore, supporting data to predict the pathogeneicity of mutations are needed. Several in silico web based programs such as SIFT, align GVGD and Polyphen are useful for the mutations pathogeneicity prediction. However, these programs can only predict the
effect of the mutations but cannot confirm whether this gene defects are really the disease-causing mutations for probands or not. Thus, segregation analysis and frequency analysis in unaffected ethnically matched controls are needed. Segregation analysis is performed using direct Sanger sequencing method in the patient’s parents and siblings. In the segregation analysis, mutations should exclusively found in the affected individual, whereas in the unaffected individual, mutations will be either absence or found in a heterozygous state. By performing this method, the mode of inheritance can also be determined and risk of the next generation can be calculated.

Frequency analysis in the unaffected ethnically matched controls is performed to prove that the variants found is a mutation not a common variants. RFLP PCR and ARMS PCR are the effective techniques to perform frequency analysis. A mutation should be absent in all of the control samples. If the same amino acid changes found in many of the control samples, it means that this is a common variant, and not a mutation. The technique of RFLP PCR and ARMS PCR explained below.

II.4.3.1 RFLP PCR

Restriction Fragment Length Polymorphism (RFLP) is is used to identify a base pair change in the DNA sequence that occurs at a site where a restriction enzyme cuts. Restriction enzymes are isolated from
bacteria that recognize specific short sequences of DNA and cut the DNA at those sites. The restriction enzyme is added to the DNA being analyzed and incubated for several hours, the restriction enzyme will cut at its recognition sites. By running the DNA in the gel electrophoresis, the fragments of DNA will separate according to size. DNA fragments can be visualized and assessed whether or not the DNA was cut by the enzyme by comparing the band size using a certain marker.

In this study, RFLP was used to confirm whether the base pair changes in the DNA are mutations or a common variant by performing this method in the ethnically matched control samples. This technique was also performed in the screening of all identified mutations in Indonesian population that were found from the previous study.

Volume, incubation temperature and time of the restriction enzymes are different for each enzyme. This information provided by the enzyme manufacturer which can be found in the enzyme manual kit.

In some mutations, RFLP PCR can not be performed due to unavailability of the restriction enzymes that cut in the mutation sites. Therefore, ARMS PCR was used as the next approach in this situation.

II.4.3.2 *ARMS PCR*

ARMS PCR method is a technique for point mutation or small deletion analysis. ARMS PCR consists of two complementary reactions:
one containing an ARMS primer specific for the normal DNA sequence and cannot amplify mutant DNA at a given locus and the other one containing a mutant-specific primer and does not amplify normal DNA.

Figure 8. An illustration of RFLP and ARMS PCR.

(a) RFLP PCR, a change in the DNA sequence can create or abolish recognition site of the enzyme, thus affecting the quantities and length of the DNA fragments resulting from enzyme digestion which can be visualized by gel electrophoresis.

(b) ARMS PCR, the figure above display an interpretation of ARMS PCR, Lane 1 shows the DNA size ladder. Lane 2 presents the results from a normal individual using the ARMS primer with the normal or wild-type sequence, resulting in the target DNA product. Lane 3 also presents the results from a normal individual, but the ARMS primer now corresponds to the mutant sequence; thus, PCR amplification only occurs with the control reaction. Lanes 4 and 5 reveal the results from a patient with the mutation on both alleles (homozygote). Lane 4 represents the results with an ARMS primer that corresponds to wild-type sequence, with PCR amplification therefore only occurring in the control reaction; lane 5 uses an ARMS primer that corresponds to the mutant sequence, with amplification of both target and control DNA. Lanes 6 and 7 reveals the results with a heterozygous individual. In lane 6, the ARMS primer corresponds to wild-type sequence with PCR amplification of target DNA (from one allele) plus the control DNA, and in lane 7, the ARMS primer corresponds to mutant sequence with PCR amplification of target DNA (from the other allele) plus the control DNA (52).

The genotype of an individual can be determined by analysis of the amplified products: for homozygote individual PCR, products were obtained in only one reaction (either the one with the “wild type” primer
for the normal homozygous probands, either the one with “mutant” primer for probands with homozygous mutations) and for a heterozygote genotype PCR products were obtained in both reactions. ARMS PCR will be used in this study to confirm whether the amino acid changes are mutations or a common variant by performing this method in the ethnically matched control samples. This technique will be also performed in the screening of all identified mutations in Indonesian population that were found from the previous study.

II. 5. The important role of molecular diagnostics

Accurate molecular genetic diagnosis has been proven to be essential to determine the prognostic and therapeutic approach for individuals with inherited eye disorders. Knowledge of the underlying molecular mechanism of the disease is critical in providing information about its nature, course, and prognosis. The development of gene therapy for retinal dystrophies is making molecular diagnosis increasingly important.

Some syndromic diseases involve retinal dystrophy as the first sign preceding the other organ involvement, as in Senior–Loken syndrome. This syndrome involves retinal dystrophy followed by nephrolithiasis at the later stage (49). Molecular diagnosis at an early stage may allow physicians to monitor patients carrying mutations in
“syndromic genes” more closely for the involvement of other organs and slow the progression of the disease.

Genetic diagnosis is also important to provide the patient and the family with genetic counseling about the inheritance manner, the recurrence risk of the diseases and the possibility to perform an early intervention to slow the disease progression.

II. 6 Early intervention for RP

Gene therapy is a very promising approach to treat RP patients. Nonetheless, this approach still not applicable for the patients. Several studies need to be done to prove the safety and efficacy of this therapy. Thus far, therapy for RP patients are very limited. Physicians should emphasize the therapies that are available to help patients. The aim of the patient management is to slow the disease progression and help patients retain their vision to maintain the normal daily function.

Vitamin A (beta-carotene) is an antioxidants that has been implicated to slow RP progression. A recent comprehensive epidemiologic study concluded that very high daily doses of vitamin A palmitate (15,000 U/d) slow the progress of RP by about 2% per year. The effects are modest and there is a risk of hepatotoxicity and teratogenicity caused by this regiment. Liver enzymes and vitamin A level tests are need to be performed annually for the patients whose consumed high dose vitamin A in a routine basis.
Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid and antioxidant. Studies have shown a correlation of ERG amplitudes with patients' erythrocyte-DHA concentration. Others studies reported trends of less ERG change in patients with higher levels of DHA. However, a recent study compared DHA plus vitamin A to vitamin A alone in patients with RP over 4 years. In this study, the benefit of DHA was not seen. Further clinical trials must be done to determine DHA benefit (50).

Ascorbic acid consumption (1000 mg/d) has been recommended, but there are no evidence exists that ascorbic acid is helpful. Although several agents has been suggested to slow the disease progression, there were still no strong evidence that prove the effectivity of these therapy to improve or retain patient’s vision (50). Therefore, these agents has to be consumed wisely with also considering the side effects to the other organ and the effectivity of the agents. Meanwhile, avoidance to oxidants agents may slow the disease progression, such as UV light avoidance, stop smoking and alcohol consumption.

II. 7. Genetic Counseling

Genetic counseling of the affected individual with RP or their relatives will improve their understanding about the disease. The aim of genetic counseling is to inform patients about the hereditary nature of their RP disease and mode of inheritance based on pedigree analysis,
genotype and also the risk of the next generations. Counseling modalities consist of genetic counseling, psychological counseling, and low vision rehabilitation counseling. The risk of passing the disease to the next generations can be calculated based on the mode of inheritance. Informations gained from counseling will help the affected individuals in the decision making regarding the future strategy, such as pregnancy, vocational choices and medical interventions. Counseling about prognosis should be included with providing informations regarding the great variation among and within inheritance groups, families, and individuals with respect to age of onset and natural history of the disorder. Because no treatment is currently available for most RP patients, genetic counseling and supportive follow-up should be viewed as an essential service for this common group of genetic disorders, and co-operation with the ophthalmologists should be actively sought. The availability of support groups are very useful for the RP patients. Patients can shared their experience, knowledge and latest information about RP. Furthermore, patients will also get a psychological supports from other RP patients
II. 8 Theoretical Framework

Epithelial growth factor

Phototransduction

Vit.A metabolism

Specific gene for Photoreceptor

Trafficking of intercellular

Ph Regulation

Gene mutation

Inherited

Mutagen:
- Free radicals
- Nutrition
- Viral infection
- Chemicals
- Unknown

Autosomal recessive (homozygous/compound heterozygous)

Autosomal Dominant

X-Linked

Non Syndromic Retinitis Pigmentosa

Homozygosity mapping
II.9. Conceptual Framework

Gene Mutation

Inherited

Autosomal recessive (homozygous/compound heterozygous)

Autosomal Dominant

X-Linked

Non Syndromic Retinitis Pigmentosa

Homozygosity mapping