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Association of single nucleotide polymorphisms in the CFH, ARMS2 and HTRA1 genes with risk of developing age related macular degeneration in Egyptian patients

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Association of Single Nucleotide Polymorphisms in the *CFH*, *ARMS2* and *HTRA1* Genes with Risk of Developing Age Related Macular Degeneration in Egyptian patients

A thesis submitted to

The Biotechnology Graduate Program

In partial fulfillment of the requirements for

The degree of Master of Science in Biotechnology

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Bachelor of Pharmaceutical Sciences, Cairo University

Under the supervision of: Prof. Hassan ME Azzazy

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DEDICATION

To my mother and father

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ABSTRACT

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Association of Single Nucleotide Polymorphisms in the *CFH, ARMS2* and *HTRA1* genes with risk of developing Age related macular degeneration in Egyptian patients

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Age related macular degeneration (AMD) is one of the leading causes of blindness in the elderly worldwide. Due to earlier clinical observations that AMD concentrates in families, a genetic component to the disease has been suggested. Several genetic studies have identified areas of association with AMD on chromosomes 1q31 and 10q26. On chromosome 1q31 the most famous single nucleotide polymorphism (SNP) to be linked to AMD was rs1061170 on the complement factor H gene (*CFH*). This SNP was studied in several populations and was found to be highly associated with AMD in most Caucasian populations with odds Ratio (OR) reaching 9.79 for CC homozygous and 4.36 for CT heterozygous in some cases. However in Japan, China, Korea and South Africa no or weak association was found. Other SNPs on chromosome 10q26 were also associated with AMD. SNP rs10490924 (Ala69Ser) on the age related maculopathy susceptibility 2 gene (*ARMS2*) was associated with AMD in the USA, Germany, China, Turkey and India with OR values reaching 8.61 for homozygotes. On the *HTRA1* (HtrA *serine peptidase* 1) gene rs11200638 was associated with AMD in China, India and the USA with OR reaching up to 7.9 for individuals homozygous for the risk allele. Due to varying results of association in different populations we aimed to examine the association between AMD and SNPs on *CFH, ARMS2* and *HTRA1* in Egyptian patients, a previously unstudied population. We recruited 26 individuals diagnosed with AMD and twenty unrelated age matched controls. Genotyping was carried out through polymerase chain reaction (PCR) followed by allele-specific restriction digestion and direct sequencing for some cases. We found all three SNPs to be significantly associated with AMD. For rs1061170 the OR for heterozygous TC genotype was 5.5 (95% CI: 1.145-26.412). While for the combined TC+CC genotypes the OR was 8 (95% CI: 1.726-37.090). Similarly, for rs10490924 the OR for heterozygous TG genotype was 4.667 (95% CI: 1.187-18.352) and for the combined TG+TT genotypes it was 7 (95% CI: 1.852-26.461). In *HTRA1* rs11200638 OR for GA genotype was 5 (95% CI: 1.195-20.922) and for combined GA+AA genotypes it was 6 (95% CI: 1.456-24.733). We conclude that our study results indicate a trend of association between the three polymorphisms studied and AMD in agreement with findings in Caucasian populations.

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LIST OF ABBREVIATIONS

AMD: Age related macular degeneration

AP: Alternative Pathway

AREDS: Age Related Macular Degeneration Study

ARMS2: Age related Maculopathy Susceptibility 2

CFH: Complement Factor H

CI: Confidence Interval

CNV: Choroidal Neovascularization

CRP: C-reactive Protein

DMSO: Dimethyl Sulfoxide

FA: Fluorescein Angiography

GA: Geographic Atrophy

LOD: Logarithm of Odds

MAC: Membrane Attack Complex

NF-kB: Nuclear Factor kB

NVAMD: Neovascular Age related Macular Degeneration

OCT: Optical Coherence Tomography

OR: Odds Ratio

PCR: Polymerase Chain Reaction

PDT: Photodynamic Therapy

PEDF: Pigment Epithelium Derived Factor

POS: Photoreceptor Outer Segments

RCA: Regulation of Complement Activation

RFLP: Restriction Fragment Length Polymorphism

RISC: RNA-induced Silencing Complex

ROS: Reactive Oxygen species

RPE: Retinal Pigment Epithelium

SD: Standard Deviation

SNP: Single Nucleotide Polymorphism

TGCE: Temperature gradient capillary electrophoresis

VA: Visual Acuity

VEGF: Vascular endothelial growth factor

WARMDGS: Wisconsin Age Related Macular Degeneration Grading System

CHAPTER 1. INTRODUCTION

Anatomy of the eye and retina

The eye is a sensory organ concerned with detecting and converting light energy into neuronal signals and transmitting them to the brain. It is composed of seven main regions; the cornea, sclera, aqueous humor, vitreous humor, lens, iris and the retina (see figure 1). The retina is the photosensitive region of the eye mainly concerned with the detection and conversion of light signals. The retina also includes the "Choroid" a vascular system controlling up to 95% of the ocular blood flow. The cornea is covered with a tear film which acts as a lubricant protecting the eye during blinking and is composed of three layers; aqueous, mucoid and lipid [2].

The retina is made up of a thin layer of neuronal cells covering the back of the eye and acting as a part of the central nervous system. Three main types of cells make up the retina; photoreceptors (rods and cones), bipolar and ganglion cells. The photoreceptors are the part that converts light energy into neuronal signals which are passed into bipolar cells which in turn pass them to ganglion cells and out of the eye. Other cells present in the retina include amacrine cells, horizontal cells and interplexiform neurons which are responsible for modifying signals before they leave the eye [2]. Blood is supplied to the retina through two routes; the choroid and retinal blood vessels. Retinal vessels supply blood to the inner two thirds of the retina while the outer one third is avascular and receives nutrients and oxygen from the choroid [3].

Two main regions span the retina; the peripheral region contains mostly rods, occupies most of the retina and is responsible for detecting gross motion [2]. The central part of the retina is called the "macula" and is responsible for high resolution Visual Acuity (VA) and detection of fine details. The macula is made up of cones mainly, has a higher ratio of ganglion cells than any other area and covers a small portion of the retina [2, 4]. At the centre of the macula is the "fovea" which contains no blood vessels, any small injury to the fovea leads to loss of vision, while damage at any part of the macula leads to loss of central vision (see figure 2) [2, 5].

In cross section, ten main layers make up the retina, they are (arranged from the inside to outside); the inner limiting membrane, nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear

layer, external limiting membrane, photoreceptor layer and the retinal pigment epithelium (RPE) which is closely attached to the Choroid (see figure 3) [2]. Separating the RPE from the choroid is a semi-permeable membrane called "Bruch's membrane". Cells of the RPE, photoreceptors and choriocapillaries are highly interdependent physiologically and thus damage to one highly affects the other. Photoreceptors are responsible for photo transduction through reisomerization of vitamin A derivative (11-cis retinal) upon detection of light photons. The RPE performs several functions including; phagocytosis of the outer limits of photoreceptors in balance with their synthesis, nutrient transport, cytokine secretion and regeneration of (11-cis) retinal. Finally, choriocapillaries are responsible for supplying the RPE and photoreceptors with blood [4, 5].

Age related Macular degeneration (AMD)

Clinical features and classification

With ageing the retina undergoes several biochemical and anatomical changes in Bruch's membrane. These changes include; collagenous thickening, calcification and lipid infiltration. However, during AMD a major histopathological feature is displayed, that is the appearance of lipid rich deposits under the RPE known as "Drusen". These appear as yellowish lesions in the macula during fundoscopic examination and may be classified as small, medium and large or as soft and hard depending on the appearance of their margin. Hard drusen have well defined margins, while soft drusen have blurred edges as shown in figures 4 and 5 [4, 6]. According to the stage of the disease AMD is classified as early or late. Early AMD involves the development of few medium sized drusen and hyperplasia of the RPE, which results in little visual impairment and is often asymptomatic. However if symptoms occur they include; blurred vision, scotomas and abnormal dark adaptation [4]. Late AMD is classified into two types; dry AMD/ geographic atrophy (GA) and wet/ neovascular AMD (NVAMD). Since AMD involves damage to the macula even the most advanced cases of AMD rarely exhibit loss of peripheral vision (see figure 6) [7].

<u>Dry AMD or geographic atrophy (GA)</u>

GA develops gradually and usually involves the centre of the fovea leading to loss of central vision over the course of months or even years. As expected with AMD, GA

involves the development of drusen. However, the main feature leading to loss of vision in GA is cell death in the RPE leading to atrophy of the overlying retina and underlying RPE (see figure 7) [8, 9].

Exudative, wet or neovascular AMD (NVAMD)

This form of AMD involves the development of new pathological choroidal blood vessels called Choroidal Neovascularization (CNV). These blood vessels invade the sub-RPE or sub-retinal spaces and may leak serous or lipid exudates. This in turn hinders supply of nutrients to and removal of wastes from the retina. Clinically they appear as greenish gray lesions surrounded by a ring of hyperpigmentation [6, 9]. If these blood vessels hemorrhage, the release of toxic iron from hemoglobin may lead to further damage to the retina[7]. In CNV loss of vision is sudden and may take place over days or weeks[4]. At its end, CNV may develop into fibrovascular or atrophic macular scar and permanent changes to central vision [9]. Several types of CNV occur including; classic, occult or mixed (which could be either predominantly classic or predominantly occult). In fluorescein angiography (FA) early stages of classic CNV show hyperfluorescnce with well defined boundaries. Leakage of the dye occurs at later stages and the boundaries become blurred. Early phases of occult CNV show irregular elevation of the RPE showing ill-defined area of hyperfluorescence followed by spotted hyperfluorescence as a result of fibrovascular pigment epithelium detachment. Later phases show leakage of fluorescein which may be of an unknown source [6, 9].

Pathophysiology

AMD shows interplay between various processes including; oxidative damage, abnormal lipid metabolism, immune system irregularities, apoptosis and CNV [5]. The visual cycle involves re-isomerization of trans-retinol into (11-cis) retinal by the RPE as shown in figure 9. As mentioned earlier RPE is also responsible for phagocytosis of apical photoreceptor outer segments (POS). As well as maintaining the blood-retina barrier. With ageing, RPE dysfunction may result in accumulation of visual cycle intermediates that may be toxic to photoreceptors. Accumulation of lipo-proteinaceous granules called "Lipofuscin" may be also involved in the oxidative stress component of AMD[3, 5].

Role of oxidative Stress

Respiration in the mitochondria involves the release of a number of reactive oxygen species (ROS) including superoxide anions O_2^- and singlet oxygen. With ageing the number of ROS released through the mitochondrial walls increases. These ROS then attack proteins altering their structures and resulting in loss of function. They also attack lipids with unsaturated fatty acids peroxidizing them as shown in figure 10. Peroxidized lipids themselves later on act as ROS, attacking proteins and other lipids in a "propagation reaction". These peroxidized lipids can be found in lipofuscin granules and increase with increased oxygen exposure of the RPE. The eye is especially prone to ROS attacks due to several reasons. Photoreceptors contain the most unsaturated fatty acids of the body and are prone to photo-oxidative reactions due to their constant exposure to light. They also contain high concentrations of retinoids in the RPE which are used to capture light and are rich in double bonds. Finally, Bruch's membrane is rich in lipids and is exposed to high oxygen flow. Areas of hyperpigmentation showing autofluorescence have been noted in CNV, suggesting that lipofuscin plays a role in its development.

Protective mechanisms in the eye against ROS attacks are present at three levels; molecular, cellular and large scale tissue protection. Cellular antioxidant enzymes include; superoxide dismutase, catalase, glutathione transferase, glutathione reductase, and glutathione peroxidase. Vitamins such as C and E and antioxidant carotenoids act on the molecular level. Cellular protection involves increased activation of transcription factors such as nuclear factor kB (NF-kB) which affects gene expression of antioxidant enzymes. At a large scale level, presence of ROS and peroxidized lipids results in increased production of vascular endothelial growth factor (VEGF) which is involved in the production of vascular endothelial cells [5, 8, 10]

Drusen and drusogenesis

Drusen are the pathogenic component most highly associated with AMD and appear as amorphous deposits that develop extracellularly in the area between the RPE and Bruch's membrane. Normally, 95% of the aged population exhibit few small (< 63 μm) hard drusen. However when drusen are more numerous or when soft drusen (≥ 125–250 μm) develop in the macula, especially if they appear together with pigment

irregularities or depigmentation, they are considered a major risk factor for advanced AMD especially NVAMD.

Drusogenesis involves the interplay between various factors and is a complex process that takes place over many years as shown in Figure 11. The pathogenic effect of drusen is exerted by both their physical action through displacement of the RPE and receptors and also through their indirect effect which involves immune activation and inflammation. Many immune associated proteins such as dendritic cell processes, immunoglobulins, class II antigens, and most importantly components of the complement cascade have been found in drusen. The most important of these are complement proteins that include; activators and inhibitors, activation specific complement fragments, and terminal pathway components such as the membrane Attack Complex (MAC). MAC is known to attack both pathogens and host cells and tissues including the RPE and photoreceptors. This indicates that local inflammation and activation of the complement cascade may play a role in drusogenesis, RPE/photoreceptor degeneration, and Bruch's membrane disruption [3].

Choroidal neovascularization (CNV)

Blood vessels that develop during CNV are usually weak, leaky and curled, they usually bleed forming dense macular scars that lead to vision loss. Endothelial cells of choroidal blood vessels are normally under strict balance between pro-angiogenic factors such as VEGF and anti-angiogenic factors such as pigment epithelium derived factor (PEDF) and as a result are called "silent". However, during CNV loss of this balance occurs either through increased production of pro-angiogenic factors or decreased production of anti-angiogenic factors. The causes for this loss of balance are numerous and may include retinal hypoxia or ischemia or immune and inflammatory response [3].

Epidemiology

AMD is one of the most common eye diseases that affect the elderly and is estimated to affect 50 million people worldwide contributing to about 8.7% of cases of blindness worldwide (see figure 12) [11, 12]. In the US it was estimated that nearly seven million persons suffered from AMD in the year 2007 [5]. In the early nineties the population of the USA included 30 million persons aged 65 or older. Out of this

population 150-200'000 persons developed CNV in one or both eyes, with a smaller number experiencing loss of vision due to GA [13].

The epidemiology of AMD was extensively studied in the 90s by three major studies; the Beaver Dam study in the USA, the Blue Mountains eye study in Australia and the Rotterdam study in the Netherlands. The Beaver Dam Wisconsin study included 4926 people aged 43-86 years, who were examined to assess the relationship between drusen, RPE abnormalities and macular degeneration with age and sex. The study found that 95.5% of the population showed one or more drusen in the macula of at least one eye. People above the age of 75 had higher frequencies of the disease compared to those 43 to 54 years old [14]. In Australia, the Blue Mountains study examined 71 patients over 49 years of age to assess the prevalence of early and late AMD. Patients were subjected to detailed eye examination and grading of the disease according to the Wisconsin age related macular degeneration grading system (WARMDGS) to assess the presence and severity of lesions. End stage AMD was found in 1.9% of the population with 0% being contributed by people below 55 years of age and 18.5% contributed by those 85 or older. Prevalence rates for all lesions were lower than those found in the Beaver Dam study [15]. Finally in Rotterdam, Netherlands a study was conducted to determine the prevalence of AMD in the elderly population. The study population included 6251 patients aged 55 to 98 who were examined for the presence of drusen, pigmentary abnormalities and atrophic NVAMD. Drusen were found in 40.8% of persons aged 55 to 64 and increased to 52.6% for people above the age of 85. Drusen, RPE hypopigmentation and increased retinal pigment also increased with age. Atrophic or NVAMD were found in 1.7% of the population and also increased with age. NVAMD was twice as common as atrophic AMD. Overall the disease was found to be less common than in the United States [16].

Results from other ethnic groups show that the prevalence and incidence of AMD varies remarkably between different ethnicities. For example; NVAMD is more common than GA in Caucasians as reported in; the beaver Dam, Blue Mountains and Rotterdam studies. However, GA was found to be more common in Caucasians in Iceland and Norway. GA is also more common than NVAMD in other racial groups such as the Inuits of Greenland. Recent population based studies have also shown that GA was more common in Caucasians than blacks. Data from the National Health and

Nutrition Examination Survey III as well as the Los Angeles Latino Eye study and the Proyecto VER also showed higher prevalence of NVAMD in whites compared to Mexican Americans [17].

Risk factors

The risk factor most highly associated with AMD is age. Another highly implicated risk factor is smoking. Other risk factors include family history and hypertension and the minor risk factors; increased C-reactive protein (CRP), increased white blood cell count, increased intake of fat and being a female. Most studies found no link between elevated lipids or clinical manifestations of atherosclerosis, such as myocardial infarction or stroke, with advanced AMD. The Beaver Dam study also found no association with hypertension. No relationship with diabetes mellitus was also found. A link to cataract surgery has been found and thus it has been recommended that ophthalmologists notify their patients of this risk before surgery and follow up with them post-operatively. Several factors were found to be protective of AMD including; black race, higher intake of fish, nuts and dark green leafy vegetables [4, 8, 17].

Smoking

Smoking affects several metabolic pathways that have been implicated in the pathology of AMD. Smoking lowers levels of the antioxidant carotenoids in the eye as well as antioxidants in general in the serum. The Beaver dam, Rotterdam and Blue mountain studies have shown that smoking increases odds of CNV with Odds Ratio (OR) ranging from 2.5 to 5.6. Follow up studies have also found that incidence of AMD increase with the number of packs smoked with risk ratios of 2.0 or larger for 40-45 packs/year or more [13, 17].

Alcohol

An association between alcohol consumption and AMD has been established especially for heavy drinkers who consume more than three drinks a day. However, study results were not conclusive as most alcohol consumers also smoked and as a result separating the effects of alcohol from those of smoking was difficult [18].

Diagnosis of AMD

Clinically AMD may present as distortion or blurring of vision and scotoma in central vision which may be sudden in CNV and more gradual in GA. Patients with bilateral GA and unilateral CNV may be asymptomatic. Patients with drusen and mild pigmentary changes may have normal visual acuity while late AMD usually results in reduced visual acuity in the affected eye. An Amsler grid, which is a grid of vertical and horizontal lines, may be used to detect central distortion. Scotomas may be also detected using the Amsler grid, these are areas of loss or degradation of VA within a normal field. Stereoscopic fundal examination (usually using slit lamp microscopy) usually shows drusen, pigmentary, exudative, hemorrhagic or atrophic changes to the macula. Fluorescein angiography (FA) is usually used to confirm the presence of AMD and may be used to assess the nature of NVAMD [9]. FA involves injecting a fluorescent dye intravenously which is then imaged using different filters [6]. An additional diagnostic tool is use of Optical Coherence Tomography (OCT) which involves cross sectional imaging across the retina, RPE and choroid. OCT helps in assessing presence of pigment epithelial detachments and for patients allergic to sodium fluorescein [9].

Management and Treatment

Prophylaxis

Due to the role played by oxidative damage in development of AMD, endogenous enzymes and compounds as glutathione help in protecting the retina against oxidative damage. The antioxidant effects of vitamins C and E, β-carotenes, flavonoids and polyphenol may also help in protection against AMD. Antioxidant enzymes present in the RPE require micronutrients as Selenium, Zinc, Manganese and Copper to perform their action [19]. The age related macular degeneration study (AREDS) investigated the use of four nutritional treatments for the prevention of AMD, study groups included placebo, zinc, anitoxidants (vit. C, vit. E and beta carotene) and zinc plus antioxidants. It was found that zinc plus antioxidants reduced the risk of patient's progressing to advanced AMD (OR 0.72) or moderate vision loss (OR 0.73) over a six year period. The benefit is greatest for patients with early AMD in one eye and late in the other [9].

Non-exudative AMD

No therapy for non-exudative AMD is available to stabilize or reverse disease progression. Laser coagulation has been used to remove drusen but its value in lowering the progression of AMD is doubtful. Measures that reduce risk of developing AMD include lowering or avoiding cigarette consumption. Other factors include diet changes since the AREDS study found that vitamin E, beta carotene, vitamin C, zinc and copper can reduce the risk of visual loss in patients with moderate to severe dry AMD. Also found to reduce the risk are the carotenoids; lutein and zeaxanthin which are the only carotenoids concentrated in the macula [7].

Exudative AMD

1) *Conventional laser treatment (laser photocoagulation therapy*)

Conventional laser treatment was the go-to method for treating CNV before the year 2000. It involves removal of areas of CNV which may result in improved VA. However, this method has been shown to have nearly 54% recurrence rate 5 years after treatment. The method also involves exposing all areas of CNV to intense laser which usually includes the fovea and leads to loss of fine vision [7, 9, 20].

2) *Photodynamic therapy (PDT) with Verteporfin*

This method approved by the FDA in the year 2000 involves IV injection of Verteporfin (Visudyne®, Novartis) a photosensitizing drug, followed by exposing the affected area to diode laser (689nm). The photosensitive molecules of Verteporfin absorb light energy and release free radicals to oxidize the lipid membranes and proteins resulting in disruption of cellular structures, thrombosis and occlusion of vasculature in active CNV. This treatment is not effective on minimally classic lesions or occult CNV. The treatment course usually involves therapy every 3 months for two years (nearly five treatments over two years). Phase three studies have showed that PDT reduced risk of severe vision loss by 66% compared to placebo. However, only 13% of patients gained vision compared to 7% for placebo, and most treated patients still suffered from vision loss even if it was less than expected for the natural course of the disease [4, 7, 9].

3) Anti-angiogenic drugs

a. Anti-vascular endothelial growth factor (anti-VEGF)

Due to the role played by VEGF in the development of new blood vessels anti-angiogenic drugs have been employed in the treatment of CNV. Two anti-VEGF drugs have been FDA approved for treatment of CNV. Pegaptinib sodium (Macugen-Eyetech Inc.) is an anti-VEGF oligonucleotide (RNA aptamer) conjugated to polyethylene glycol; it exerts its action by binding to the major human soluble VEGF isoform ($VEGF_{165}$). Ranibizumab (Lucentis-Genentech Inc.) is a recombinant anti-VEGF monoclonal antibody fragment that binds all isoforms of VEGF-A. Another anti-VEGF agent used off label for treatment of CNV is Bevacizumab (Avastin-Genentech Inc.) used originally as anticancer agent for colorectal, lung and breast cancer). Bevacizumab is a full length humanized monoclonal antibody against all isoforms of VEGF-A. Several cases of blindness resulting from microbial contamination of Avastin vials have been reported in the year 2011[21]. Pegaptinib costs $995/dose every 6 weeks while Bevacizumab costs $10/dose, and Ranibizumab costs $1950/dose [4, 7, 9, 20, 22].

b. VEGF trap

A decoy receptor for VEGF, it has higher affinity than naturally occurring receptors. It is a recombinant protein with the key ligand-binding sites present in natural receptors together with the Fc portion of human IgG. It acts on all members of the VEGF family. It is currently in Phase II/III of clinical trials [23].

c. Tyrosine kinase inhibitors

VEGF binding to its receptors results in a downstream reaction cascade involving tyrosine kinase as shown in figure 13. Activation of this pathway leads to the pro-angiogenic activity of VEGF with increased cell proliferation and permeability. Vatalanib is a multi-VEGF receptor inhibitor that binds the intracellular kinase domain of all three VEGF receptors (VEGFR 1-3). It is an oral product and thus avoids the commonly employed intravitreal pathway which involves the drug being injected directly into the eye. It is currently in phase II/III of clinical trials. [23].

d. Pigment epithelium derived factor (PEDF)

PEDF is a potent anti-angiogenic factor. Adenovirus containing PEDF encoding DNA is injected into the vitreal or retinal space. Trials on animals showed regression of CNV following therapy [23].

e. Nicotinica acetylcholine receptor antagonists

Involvement of a cholinergic pathway in angiogenesis has been recently discovered. Thus, pharmacologic inhibition of nicotinic acetylcholine receptor (nAChR) has been under investigation with phase I trials showing that mecamylamine (an nAChR antagonist) shows reduced CNV lesion size upon intravitreal administration [23].

f. Small interfering RNA (siRNA)

Upon entry of double stranded RNA into the cell they are detected as means of viral infection and are processed by enzyme dicers into single stranded nucleotides called small interfering RNA (siRNA). These strands bind RNA-induced silencing complex (RISC) which as a result degrade the complementary messenger RNA (mRNA) resulting in reduced VEGF production in the cell. Usually siRNA are directly injected into the cell to avoid induction of antiviral defenses through injection of dsRNA. Examples of proteins targeted by siRNA therapy in CNV are VEGF and VEGF receptors. One example of this therapy is Bevasiranib (Cand5) a siRNA that targets VEGF-A mRNA. Since siRNA therapy aims at reducing production of VEGF, it may not be effective in reducing already produced VEGF and thus concomitant administration of an agent that binds VEGF may show higher efficacy (such as combining Bivasiranib and Lucentis) [23].

4) Steroids

Include Triamcinolone and Anecortave acetate and act through preventing vascular growth by inhibiting proteases necessary for endothelial cell migration [9].

5) Surgical treatment

a. Excision of Choroidal neovascular membranes

Excision of Choroidal neovascular membranes involves vitrectomy followed by removing CNV by retinotomy incision which could help preserve photoreceptors. This is more suitable for occult CNV as the boundaries are not delineated [9].

b. Retinal pigment epithelial cells transplantation

Could be exogenous or autologous both methods are showing non-encouraging results with high complications [9].

c. Evacuation of submacular hemorrhage

Evacuation of submacular hemorrhage involves removing submacular hemorrhage secondary to CNV to prevent subretinal fibrosis and permanent vision loss [9].

d. Macular translocation

Macular translocation involves relocating the fovea to a neighboring area with healthy RPE in patients of subfoveal CNV, it may lead to retinal detachment, increased lens opacity and tilted image [9, 20].

Combination therapy

Currently under investigation and involves combining anti-VEGF therapy with other methods of treatment such as PDT or IV Triamcinolone [9].

CHAPTER 2. LITERATURE REVIEW

Genetics of Age related macular degeneration

AMD is a complex disease that involves interplay between genetics and the environment. The genetic component of AMD has been proven to include not only a single gene but to be of a polygenic nature with multiple genes playing a role in the development of AMD [24].

Familial aggregation and Twin studies

For a long time clinical observations have noted that AMD clusters in families and thus a genetic component has been suggested to play a role in developing the disease[24]. In the 1990s Silvestri et al conducted a study to examine the level of genetic predisposition to AMD and to define a mode of inheritance for the disease. They examined 36 AMD patients (both CNV and GA) as well as sex matched controls and their immediate families. They found that 20 of the 81 siblings of the patients had AMD compared to one of the 78 control siblings. These were statistically significant results that supported the involvement of hereditary factors in the etiology of AMD [25]. The next logical step was conducting familial aggregation studies to figure out if an individual had a higher risk of developing AMD if he was related to a patient [24]. The findings by Seddon et al concluded that first degree relatives of AMD patients especially those with CNV had higher prevalence of AMD compared to relatives of healthy individuals, thus indicating a genetic component together with the environmental component of AMD [26]. Klaver et al used data from the Rotterdam study using first degree relatives of 87 patients of late AMD and first degree relatives of 135 control subjects without AMD. They also found that first degree relatives of patients with AMD have increased rate of developing the disease at a relatively young age. Their results showed that nearly one fourth of all late AMD is genetically determined thus validating the importance of the genetic component of the disease [27]. Twin studies followed where the balance between genetic and environmental factors is assessed through examining a trait in both identical and non-identical twins [24, 28]. Meyers et al compared the concordance of AMD in monozygotic and dizygotic twins who are 40 years or older with AMD between 1986 and 1991. They found that AMD was concordant in 100% (25 of 25) monozygotic twins and in five out of 12 (42%) of

dizygotic twins. The 100% concordance in monozygotic twins may indicate a strong a non-genetic component if repeated in dizygotic twins, however since this is not the case a major genetic component is suggested [29].

Linkage studies and Genome scans

At this point linkage studies to identify chromosomal regions carrying DNA markers that occur at a higher frequency of a certain disease allele in patients compared to healthy individuals were conducted. Linkage is measured through measuring the LOD score (logarithm of odds) between the marker and the disease. In 1998 a linkage study conducted by Klein et al found linkage between AMD and chromosome 1q25-q31 in a large family with dry AMD [30]. Kenealy et al found linkage between AMD and locus 10q26 in 70 multiplex families (consisting of 133 affected sibpairs) [31]. Genome studies followed to identify chromosomal regions associated with AMD. In the year 2000, Weeks et al genotyped 225 AMD families with 212 affected sibpairs for 386 markers and found linkage at chromosome 10 [32]. In 2001 they expanded their study to increase the linkage detecting power and included 860 affected individuals from 391 families including families containing at least two siblings affected with AMD. The study confirmed linkage to the locus on 1q31 which was previously reported by Klein et al [33]. Schick et al in 2003 found a weak signal on chromosome 1 and confirmed previously reported linkage to markers on chromosome 12, they also found linkage to a locus on chromosome 6 [34]. Seddon et al also confirmed the results of Klein et al for Chromosome 1, Weeks et al for chromosome 10 and Schick et al for Chromosome 6 [35]. Genome-wide scan and genetic linkage analysis by Majewski et al linked the following loci to AMD; 1q31, 3p13, 4q32, 9q33, and 10q26 [36]. High resolution genome scan by Abecasis et al also confirmed linkage to 1q31 but surprisingly found no linkage to the previously reported 10q26 locus [37]. While the genome scan by Iyengar et al in 2004 showed a major linkage locus on chromosome 15q and a total of 13 regions on 11 chromosomes (1q31, 2p21, 4p16, 5q34, 9p24, 9q31, 10q26, 12q13, 12q23, 15q21, 16p12, 18p11, and 20q13). These results Confirmed association with previously reported loci including 1q31 and 10q26 [38].

Association studies

Complement system

Association studies were employed to examine if there is an increased presence of a certain allele in persons affected by the disease in comparison with the general population. In the case of AMD they were used to fine-map regions of significant linkage on chromosomal region 1q25-32 [24]. In the year 2005, association studies undertaken by three separate groups were able to establish a relationship between the Single Nucleotide Polymorphism (SNP) rs1061170, sequence T1277C, protein Y402H on the Complement Factor H gene (*CFH*) and risk of developing AMD (for gene view see figure 14).

Haines et al genotyped 44 SNPs on Chromosome 1q31 for association in two independent data sets (the first with 182 families and the other with 495 AMD cases and 185 controls). In both data sets the *CFH* gene showed the most significant association results. They then screened 24 patients and controls for risk associated variants in the coding region of *CFH*, and found a greater presence for rs1061170 in cases as compared to controls. The odds ratio for AMD was 2.45 (95% CI: 1.41 to 4.25) for carriers of one C allele and 3.33 (95% CI: 1.79 to 6.20) for carriers of two C alleles. When the analysis was restricted to only neovascular AMD, these odds ratios increased to 3.45 (95% CI: 1.72 to 6.92) and 5.57 (95% CI: 2.52 to 12.27), respectively [39]. Klein et al carried out an association study using 96 cases and 50 controls from the AREDS study. They tested SNPs for allelic association with the disease status and found rs1061170 to be strongly associated with AMD [40]. Finally Edwards et al carried out an allele association study on case-control studies with a discovery sample of 224 cases and 134 controls and a repeat sample of 176 cases and 68 controls. The most significant association was found for rs1061170 out of 86 SNPs genotyped in the Regulation of Complement Activation (RCA) complex and its flanking regions. The relative risk for developing AMD for a person carrying at least one C allele was 2.7 (95% CI: 1.9 to 3.9) [41]. Hageman et al conducted an association study for sequence variants in AMD on two independent cohorts of nearly 900 AMD patients and 400 controls. The study found association for 8 common *CFH* SNPs with the mis-sense variant at Y402H having a highly significant association. The study was also able to identify an at risk haplotype (that includes the Y402H variant) that was found in 50% of AMD cases and 29% of controls with OR= 2.46 (95% CI 1.95-

3.11)[42]. Further discussion of the complement system and its genetic role in AMD can be found below.

Several other studies testing the association between AMD and the Y402H polymorphism on different populations are summarized in table 1. Strong associations were detected in the populations of the US, Australia, Brazil, Finland, France, Israel, Italy, the Netherlands, Turkey and Russia with OR reaching 9.79 for CC homozygous and 4.36 for CT heterozygous [43-56]. On the other hand studies in Japan, Korea, South Africa and China have found no or weak association between the disease and *CFH* Y402H polymorphism. In Japan three studies concluded that there was no association between Y402H and the disease [57-59]. In Korea Kim et al found that Y402H was marginally associated with NVAMD [60]. While in South Africa Ziskind et al found no association between Y402H allele and the disease in the Black South African Xhosa population [61]. In China results were conflicted with Lau et al and Gao et al finding an association between Y402H and the disease with OR reaching 4.4 [62, 63]. On the other hand several other studies found no association between this specific SNP and risk of developing AMD in Chinese patients [11, 64-66]. Ng et al found several other SNPs in the *CFH* to be associated with exudative AMD including; rs3753396, rs3753394, rs551397, rs800292, rs2274700, and rs1329428 [64]. Chen et al also identified strong association at the SNPs; rs3753394, rs800292 and rs1329428 but not at rs1061147, rs1061170 or rs380390 [11]. These results show that different ethnic groups and backgrounds showed different associations between Y402H and the risk of developing AMD.

Chromosome 10q26

Several association studies were conducted to identify the relationship between AMD and two loci on chromosome 10q26 which was previously identified through linkage analysis. First of these loci is LOC387715 (later named the age related maculopathy susceptibility 2 gene/*ARMS2* whose function is yet to be identified (for gene view see figure 15). Expression of the protein related to this gene has been shown to be limited to the placenta and under normal conditions transcripts from LOC387715 don't appear in the retina and it is likely that its expression is weak. The gene has two exons and is transcribed into an 818bp mRNA encoding a 107 amino acid protein that has no matches in the public protein or protein motif database, rs10490924 occurs in the

coding sequence for this gene [67-69]. Significant association between rs10490924 polymorphism (Ala69Ser) and AMD was found in the USA, Germany, China, Turkey and India with OR values reaching 8.61 for homozygous and 3.35 for persons heterozygous for the risk allele (see table 2) [45, 54, 56, 65, 68-70]. While in Russia the association was weak and only detected in cases of CNV [55]. [68]

Another locus is the *HTRA1* (HtrA *serine peptidase* 1) which encodes a family of heat shock serine proteases which are expressed in mouse retina and human RPE gene (for gene view see figure 16). These proteases are responsible for the regulation of extracellular matrix proteoglycans which in turn facilitate access of other degradative matrix enzymes as collagenases to their substrates. *HTRA1* also binds and inhibits transforming growth factor β (TGF-β) an important regulator of angiogenesis. Altering the levels of *HTRA1* could allow degradation of the extracellular matrix thus allowing the infiltration of choroidal blood vessels and loss of RPE as characteristic of CNV. The rs11200638 polymorphism occurs in the promoter for *HTRA1* and is nearly 6.1Kb downstream of LOC387715. Some studies have shown that this polymorphism is associated with increased levels of *HTRA1* mRNA and protein [65, 68, 71]. Studies conducted in China, India and the USA have found significant associations between the rs11200638 and risk of developing AMD with OR reaching up to 7.9 for individuals homozygous for the risk allele (see table 3) [54, 65, 66, 68, 70, 72]

The complement system

The complement system plays an important role in the interplay between adaptive and innate immunity, as well as contributing to physiological homeostasis in general to remove damaged, necrotic, and apoptotic cells. The complement system functions to eliminate pathogens from the bloodstream by disrupting their membranes through pore formation. This system functions through both an antibody-directed fashion (the classical pathway) and independent of specific antigenic recognition (the alternative pathway) [10, 28, 71, 73]. The complement system is composed of several soluble and membrane bound factors and is active in intravascular spaces, body fluids and tissues. The system is activated by one of four pathways the classical, lectin or alternative Pathways (AP) which all converge at the C3 activation step, as well as the intrinsic pathway [71]. The intrinsic pathway involves cleavage of C3 or C5 by serine proteases

associated with coagulation/ fibrinolytic cascades which results in the direct activation of the complement pathway. The classical pathway is initiated through interaction between antibodies or molecules released by inflammatory processes with pattern recognition receptors on CIq. While the lectin pathway is initiated by interaction between carbohydrates on microbes and lectin proteins such as mannose binding lectin. CIq and mannose binding lectin form complexes with serine proteases (Clr/Cls and MASP, respectively), to cleave C4 into its functionally active components C4a and C4b. C4b then binds C2 helping its cleavage into active C2a and then binding it into the complex C2aC4b which is a C3 convertase. The alternative pathway is a spontaneous pathway and thus it provides as quick response to pathogens and cellular damage. The alternative pathway forms a different C3 convertase through spontaneous hydrolysis of an internal C3 thioester. This forms C3(H_2O) a fluid-phase version of C3b which binds C3 changing its conformation and facilitating further cleavage by factor D into Ba and Bb fragments. C3(H_2O) binds with Bb, forming the initial alternative pathway convertase C3(H_2O) Bb [71]. All pathways meet at the C3 activation step where C3 convertase cleaves C3 into C3a an anaphylatoxin and C3b. C3b then binds factor B to form C3bB which facilitates its cleavage by factor D into C3bBb itself a C3 convertase in a step that amplifies C3 activation. C3bBb either binds C4b2a of the lectin pathway or C3b of the alternative and classical pathways to form C4b2aC3b and C3bC3bBb respectively which both act as C5 convertases. C5 convertase cleaves C5 into the anaphylatoxin C5a and C5b which undergoes several downstream changes to form the membrane attack complex (MAC). MAC is responsible for target cell lysis and death through formation of pores in their cell membranes [71, 73]. Regulation of the alternative pathway occurs through a number of structurally similar complement proteins such as the membrane bound CPH, decay accelerating factor, membrane cofactor protein, C4-binding protein, and complement receptor 1 and the fluid phase complement factor H (*CFH*). *CFH* blocks activation of C3 into C3b and also aids the Factor1 mediated degradation of C3b (see figure 17) [10, 28, 71, 73]. Abnormal structure or functioning of complement pathway regulators can lead to imbalance in the normal homeostasis of the complement system which as a result can lead to damage of healthy cells. Since *CFH* is the only fluid phase regulator its aberrant functioning may lead to serious changes in the pathway [71].

Complement and AMD

As mentioned earlier drusen were found through immunohistochemical and biochemical studies on eyes of AMD patients to contain a large concentration of complement proteins. This is why it was deduced that drusen are a by-product of complement pathway related inflammatory response [71].

CFH Y402H

It has been suggested that the change of positively charged Histidine into an uncharged hydrophobic Tyrosine caused by the Y402H SNP results in aberrant binding of *CFH* to the anionic surfaces of retinal capillaries as well as problems with binding to C3b leading to problems with the formation of MAC [63, 73]. Y402H is located at a site on *CFH* that influences its binding to C-reactive protein (CRP) a biomarker for inflammatory response. Patients of AMD show accumulation of CRP in the RPE which suggests problems in its binding to *CFH* [43, 49, 73].

Other complement SNPs

SNPs other than the *CFH* Y402H SNP have been found to be involved in the development of AMD. Li et al found stronger association between AMD and 20 other SNPs in the *CFH* with 3 SNPs showing the strongest association namely; rs2274700 in exon 10 and the two intronic SNPs, rs1410996 and rs7535263 [74]. SNPs outside the *CFH* gene include SNPs in *CFE*, *C2*, *C3* and deletions on *CFHRI* and *CFHRJ* genes. Since *C3* acts as a meeting point for all complement pathways, SNPs in C3 have been identified as risk factors for AMD. These include the rs2230199 polymorphism where individuals heterozygous for this SNP had OR= 1.7, and homozygotes had OR= 2.6 [10]. SNPs on the *C2* and *CFB* genes were shown to be protective of AMD and can to an extent counteract the risk alleles on some other genes [44, 71, 75, 76].

Complement based therapies

Several potential therapies targeting the complement system are currently under investigation with none released into the market yet. These include inhibition of C3 activation or C3 convertase assembly, promoting C3 convertase decay, promotion of factor l-mediated C3B proteolysis, inhibition of C3 and/or C5 convertase activities,

inhibition of membrane attack complex assembly by CD5-9, and promoting the protective action of *CFH* [71].

CHAPTER 3. MATERIALS AND METHODS

Patients and control individuals

Two independent groups including AMD patients and non-AMD controls were recruited from the retina clinics at the National Eye Research Center in Road el Farag, Mansoura University Medical School and The Research Institute Of Ophthalmology in Giza over the period from June 2010 till July 2011. IRB approval of the study protocol was obtained from the American University in Cairo. A total of 46 individuals including 26 patients of AMD (25 with CNV and one patient with GA) and 20 non-AMD controls participated in the study. The patient group included 9 females and the control group containing 11 females. All members of both groups were above 50 years of age. Patients were included in the study if they exhibited sufficient media clarity for fundus photography and signs of AMD including macular lesions in one or both eyes not related to other eye diseases. Patients with diabetic retinopathy, ocular trauma, retinal detachment, high myopia and or choroidal or retinal dystrophy were excluded from the study.

Ophthalmic examination

Members of both groups received standard ophthalmic examination by a retinal specialist including visual acuity measurement and slit lamp biomicroscopy. AMD patients underwent FA and for some cases OCT was performed to confirm diagnosis. Controls were confirmed not to show evidence of AMD in both eyes.

DNA extraction and genotyping

Eight milliliters of peripheral venous blood samples were collected into K_3EDTA blood collection tubes. DNA was extracted from the buffy coat using a commercially available blood extraction and purification kit (QIAamp blood mini extraction kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -80ºC. Genotyping was carried out through PCR followed by allele-specific restriction digestion and direct sequencing. Primer sequences for PCR and sequencing reactions are shown in table 4. PCR reactions were slightly adjusted from the method by Xu et al and performed using Applied Biosystem's Verity 96 well Thermal cycler [68]. PCR reaction volume was 25µL including 10µL buffer (including 25mM/L $MgCl_2$, 25µM

of each dNTP, 1pmol of each primer and 0.5 units *Taq* polymerase) as well as 50ng genomic DNA. Amplification of *HTRA1* involved the addition of 5% dimethyl sulfoxide (DMSO) to the reaction mixture. Samples were exposed to denaturing at 95ºC for 5 minutes followed by 35 cycles of denaturing at 95ºC for 15 seconds, annealing at 57ºC for 15 seconds and extension at 72ºC for 45 seconds with the final extension extended to 3 minutes. PCR products were resolved on 2% agarose gel electrophoresis (Seakem LE Agarose, Lonza, Basel, Switzerland) and visualized using Ethidium Bromide and ultraviolet light.

Restriction digestion and sequencing

Amplification products were then subjected to restriction enzyme digestion according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Fifteen microlitre reaction mixtures were prepared including; 10µL PCR product, 0.25µL enzyme and 4.75µL 1X enzyme buffer. The enzymes and conditions are shown in the table 5 and all digestions were conducted for 3 hours. Products were resolved on 2% agarose gel electrophoresis (Seakem LE Agarose, Lonza) and visualized using Ethidium Bromide and ultraviolet light. Genotypes were determined using restriction patterns and were further confirmed by direct sequencing. Sequencing was conducted using forward and reverse primers and combined Sanger and Roche 454 technologies on ABI 3730xl DNA sequencer. Sequencing results were viewed using Bioedit sequence Alignment editor (Ibis Biosciences, Carlsbad, CA, USA). Alignment and identification of SNPs was done using Serial Cloner 2.1 software (Serial basics, www.serialbasic.com), sequences for genes and SNPs were obtained from the NCBI database [77].

Statistical analysis of data

Numerical data was examined using the student t test. Hardy Weinberg Equilibrium (HWE) χ^2 values were calculated to identify genotyping errors using the formula $(p^2) + (2pq) + (q^2) = 1$. Linkage Disequilibrium (D') values were calculated using Haploview software version 4.2. Genotype and allele frequencies were compared using χ^2 test, Odds Ratio (OR) and 95% Confidence intervals (95% CI) were calculated using calculators available on the website of the Institute of Human Genetics using logistic regression analysis [78]. P value below 0.05 was considered statistically significant.

CHAPTER 4. RESULTS

A total of 26 individuals diagnosed with AMD were recruited for this study. 25 cases suffered from CNV and only one case suffered from GA. Twenty unrelated, ethnically matched controls were recruited. Characteristics of the study population are shown in table 6. Mean age of cases was 65± 8 versus 60 ± 8 for controls (p= 0.079 using student t-test). Percentage of males in cases was 65.3% and for controls 45% (p=0.279 $\chi 2$ test). Genotypes were determined successfully using RFLP and sequencing for confirmation for 20 patients and 15 controls in rs1061170 (*CFH*), 24 patients and 20 controls in rs10490924 (*ARMS2*) and 24 patients and 15 controls for rs11200638 (*HTRA1*). RFLP Band size and distribution are shown in the table 7, a sample of RFLP, alignment and sequencing results are shown in figures 18-32. Genotype frequencies were in Hardy Weinberg Equilibrium as shown in tables 8-10.

CFH rs1061170 was significantly associated with AMD with the frequency of the risk C allele being 0.53 in cases and 0.17 in controls (p< 0.005, $\chi 2$ test). Genotype distributions were significantly different between the AMD and control groups (p<0.05, $\chi 2$ test). Compared to the wild type TT genotype the OR for heterozygous TC genotype was 5.5 (95% CI: 1.145-26.412). The OR for the CC homozygous genotype was not calculated as this genotype was not detected in the control group. For the combined TC+CC genotypes the OR was 8 (95% CI: 1.726-37.090) as shown in table 8.

Similarly, *ARMS2* rs10490924 was significantly associated. The risk allele T was found at a frequency of 0.5 in the AMD and 0.15 in control populations (p< 0.005, $\chi 2$ test). Genotype distributions were significantly different between the AMD and control groups (p<0.05, $\chi 2$ test). Compared to the wild type GG genotype the OR for heterozygous TG genotype was 4.667 (95% CI: 1.187-18.352). The OR for the TT homozygous genotype was not calculated as well since it was not detected in the control group. However the OR for the combined TG+TT genotypes was 7 (95% CI: 1.852-26.461) as shown in table 9.

In *HTRA1* the rs11200638 was found to be significantly associated with AMD, with the risk allele A found at a frequency of 0.44 in patients and 0.17 in controls (p< 0.05, $\chi 2$ test). Genotype distributions were significantly different between the AMD

and control groups ($p<0.05$, $\chi 2$ test). Compared to the wild type GG genotype the OR for heterozygous AG genotype was 5 (95% CI: 1.195-20.922). The OR for the AA homozygous genotype was not calculated due to its absence in the control group. While the OR for the combined AG+AA genotypes was 6 (95% CI: 1.456-24.733) as shown in table 10.

Combinations of genotypes between rs10490924 and rs11200638 are shown in table 11. Carriers of the GT+AG genotype combination had an OR of 4.583 (95% C.I= 0.995-21.119) when compared to carriers of the wild type genotype combination (GG+GG) as shown in table 12. The two SNPs were found to be in high linkage disequilibrium with D'= 0.899, LOD= 5.22 and r^2= 0.737 in cases and D'=1, LOD=2.83 and r^2= 0.769 in controls

CHAPTER 5. DISCUSSION

AMD is a widespread eye disease that has been implicated as a major cause of worldwide blindness especially in the elderly. Clinically, it had been noted for a long period of time that AMD clusters in families, indicating a genetic component to AMD. This was followed by extensive genetic studies that were able to associate several polymorphisms with risk of developing AMD. Of the highest importance are complement system polymorphisms including the most famous rs1061170 in the *CFH* (Y402H), as well as other SNPs in the *CFH* such as rs2274700, rs1410996 and rs7535263. Other SNPs in the complement outside the *CFH* gene include SNPs in *CFE, C2, C3* and deletions on *CFHRI* and *CFHRJ* genes. Outside the complement system and on chromosome 10q26 two other SNPs were identified. One is rs10490924 on the coding sequence of the *ARMS2* gene whose function is yet to be identified. The other is rs11200638 which occurs on the promoter for *HTRA1* gene which encodes a family of heat shock serine proteases. However, during the conduction of association studies it became clear that a certain SNP associated with risk of AMD in one population is not necessarily associated in another. This case is strongly exhibited for the most famous of SNPs *CFH* Y402H which showed strong associations with populations in the US, Australia, Brazil, Finland, France, Israel, Italy, the Netherlands and Russia. While no association was found in Japan and South Africa and weak or conflicting results were reported in Korea and China.

We attempted to study the association between AMD and three major SNPs that have been previously associated with AMD namely; rs1061170 in the *CFH* gene, rs10490924 on *ARMS2* and rs11200638 on *HTRA1*. The study was conducted on an Egyptian population a previously unstudied and ethnically distinct group. We found that the association between rs1061170 and AMD was significant with the frequency of the risk C allele being 0.53 in cases compared to 0.17 in controls ($p< 0.005$, $\chi 2$ test). The OR for the heterozygous TC genotype was 5.5 (95% CI: 1.145-26.412) compared to the wild type TT genotype. Also the OR for the combined TC+CC genotypes the OR was 8 (95% CI: 1.726-37.090). Similarly, rs10490924 was significantly associated with the risk T allele found at a frequency of 0.5 in patients compared to 0.15 in controls ($p< 0.005$, $\chi 2$ test). OR for the heterozygous TG genotype was 4.667 (95% CI: 1.187-18.352) and OR for the combined TG+TT

genotypes 7 (95% CI: 1.852-26.461). Finally rs11200638 was found to be significantly associated with AMD, with the risk allele A found at a frequency of 0.44 in patients and 0.17 in controls ($p< 0.05$, $\chi 2$ test). Heterozygous AG genotype had an OR of 5 (95% CI: 1.195-20.922) while the AG+AA genotypes had an OR of 6 (95% CI: 1.456-24.733). Even though our study sample was small it was able to indicate significant association between the polymorphisms studied and AMD. These results agree with findings in Caucasian populations which exhibited ORs reaching 4.3 for CT in rs1061170, 3.3 for TG in rs10490924 and 1.86 for AG on rs11200638 (see tables 1-3) [45, 50, 65]. The study found that the HTRA1 and ARMS2 genes are in high linkage disequilibrium in our patient group which is in agreement with findings in previous populations [46, 68].

Obviously the main drive behind AMD genetic research is to be able to develop a genetic diagnostic test or platform that allows identification of patients that are at risk of developing the disease. Recent research indicates that identifying genetic risk may be of benefit to patients as it may affect disease progression, treatment outcome and lifestyle decisions. The interplay between environment and genetics in developing AMD has been under scrutiny lately. One of the environmental factors that have been strongly linked to AMD is smoking. This is why several studies have been conducted to find if the combination of smoking and carrying genetic risk factors leads to a higher OR than each factor alone. Seddon et al found that for individuals with wet or dry AMD the CC genotype in rs1061170 in *CFH* conferred OR of 10.2 when combined with smoking [79]. Baird et al found OR of 2.39 for individuals with the CC genotype who were smokers [80]. Chu et al found that risk for another non coding SNP in *CFH* rs1410996 was also modified by smoking [81]. These findings are of great importance since they indicate that being a smoker and carrying genetic risk factors increases risk of developing AMD significantly. As a result if a person is identified as a carrier of risk genes a change in lifestyle and stopping smoking may result in reduced possibility of developing AMD.

Another benefit of genetic testing for AMD that has been under study with mixed results is the effect of SNPs associated with AMD and disease severity. Seddon et al found that both the *CFH* Y402H and ARMS2 polymorphisms were independently associated with disease progression into late AMD [82]. They also found

that the presence of both polymorphisms, BMI> 25 and smoking increased risk of progression 19 folds. Francis et al found similar association between *CFH* and development of CNV or GA [72]. Other studies were not able to confirm this relationship and found no link between SNPs and disease progression [52, 83, 84]. Other studies found an association between genetic variants and the type of neovascular lesions in patients. Brantley et al found significant association between the C allele in rs1061170 with predominantly classic lesions [85]. Wegsheider et al also found that the CC genotype was more commonly present in predominantly classic lesions without occult CNV rather than other types of CNV [86].

Studies were also conducted to indicate whether genetic changes affect response to therapy whether through PDT, Bevacizumab, Ranibizumab or antioxidants and zinc. Studies on PDT showed mixed results with Brantley et al finding that rs1061170 TT genotypes showed lower improvement in Visual Acuity (VA) post PDT treatment when compared to CT or CC genotypes [87]. While on the other hand, Goverdhan et al found an association between CC genotype and both the likelihood of resorting to PDT as well as VA outcome following PDT [88]. Others such as Feng et al and Seitsonen et al showed that genotypes don't affect PDT outcome [89, 90]. Brantley et al also examined the effect of polymorphisms on response to Bevacizumab injections and found that CC genotypes did worse with therapy than TT and TC genotypes [91]. Klein et al found that CC genotypes were more likely to progress into advanced AMD after antioxidant and zinc supplementation than other genotypes [92]. While examining the effects of genotypes on Ranibizumab treatment, Lee et al found that genotypes didn't affect significantly VA post treatment. However they affected the number of injections over a 9 months period with CC genotypes showing higher risk of requiring extra injections [93].

For genetic testing to be carried out, it has become apparent that use of a single SNP is not specific since differences in genotypes between patients and controls may be marginal. Also since studies show that the association varies greatly between different ethnic groups [94]. This is why use of a panel of SNPs has been suggested. Maller et al investigated the association between five *CFH* variants, LOC387715, and C2IFB genes and risk of developing AMD in a large case-control study. They found that persons homozygous for all risk variants had a 285-fold

greater risk than the lowest risk group [44]. However Depspriet et al argued that these results are not accurate and proposed that studies need to be repeated in populations representative for the settings in which the genetic testing will be applied. They also proposed that the discriminative accuracy of testing these SNPs for the prediction of end-stage AMD is 80%. This value is considerably high when taking into account that the discriminative accuracy of high serum cholesterol for the prediction of cardiovascular disease is 77% [95].

Recently labs that offer genetic testing for factors associated with risk of AMD development are starting to emerge in various countries. Using the Gene tests website, an NIH related website that allows for searching for Independent laboratories conducting genetic testing for a certain disease [96], several independent laboratories (mostly university related) were found to carry out genetic testing for AMD as shown in table 13 [97]. For example, the Bay area genetic laboratory offers targeted mutational analysis for *CFH* haplotype groups, C3, *ARMS2*, mitochondrial A4917G and adjusts results for smoking using the Macula risk test® [98]. The Molecular Genetics lab in Bayern Germany offers targeted mutational analysis for *CFH* Y402H and *HTRA1* using RFLP [99]. Similarly, the Molecular Diagnostics Laboratory at Duke University uses RFLP to conduct targeted mutational analysis for *CFH* Y402H and *ARMS2* genes [100]. And finally, the Laboratory of Human Genetics in Warsaw Poland works only on the Y402H *CFH* SNP [101]. Commercially available or soon to be available genetic tests include the Macula risk® tests for SNPs rs1061170 in *CFH*, rs2230199 in C3, rs10490924 in *ARMS2* and A4917G in the ND2 gene as well adjusting for smoking. The predictive value of this test was found to be 83% in a large, independent, prospective clinical trial [102]. Also, the AMD risk assessment test by Asperbio analyses 3 SNPs associated with high risk in *CFH* and *ARMS2*, as well as 3 SNPs associated with lower risk in CFB and C2 [103]. Recently, Optherion based in Connecticut, USA has completed an exclusive worldwide licensing agreement with Sequenom, Inc. Under which Sequenom's labs will develop and commercialize diagnostic tests for AMD [104, 105]. Sequenom developed the RetnaGene™ AMD test for nine SNPs in; the regulators of complement activation (RCA) locus including, CFH, complement factor H-related 4 (CFHR4), complement factor H-related 5 (CFHR5) and coagulation factor XIII B subunit (F13B) genes; as well as SNPs in complement component 2 (C2), complement factor B (CFB), complement component 3 (C3) and

ARMS2 genes. The test focuses only on genetic markers only since they remain static throughout life and thus no variable factors are introduced which may affect accuracy. Clinical validation of the test showed that the test yielded 82% sensitivity and 63% specificity [106, 107].

Genetic studies on AMD are not focused solely on developing diagnostic tests. Therapeutic agents derived from knowledge about the genetics of AMD are currently under investigation. Using findings implicating errors in the alternative complement pathway in development of AMD, Rohrer et al examined the therapeutic use of novel recombinant Factor H to inhibit the AP. They found that specific inhibition reduced angiogenesis in mouse CNV [108]. Tortajada et al examined the protective role conferred by the Factor H- Ile61 variant through ELISA based methods and found that this variant binds more efficiently to C3b and competes better in proconvertase formation. They also showed through functional analysis that this variant had greater cofactor activity for the factor I-mediated cleavage of fluid phase and surface bound C3b [109]. To identify the role played by the *ARMS2* gene in AMD Chen et al expressed a recombinant protein encoded by the gene in *E.coli*. This approach would allow identification or isolation of an antibody against this protein, which allows identification of any natural protein encoded by this gene in human cells [110].

CHAPTER 6. CONCLUSION AND RECOMMENDATIONS

We conclude that our data demonstrates significant association between AMD and rs1061170 on *CFH*, rs10490924 on *ARMS2* and rs11200638 on *HTRA1* in Egyptian patients. Frequencies of the risk alleles were 0.53 in cases compared to 0.17 in controls, 0.5 in patients compared to 0.15 in controls and of 0.44 in patients and 0.17 in controls for rs1061170, rs10490924 and rs11200638 respectively. The ORs for carriers of one risk allele compared to homozygous wild type were 5.5 (95% CI: 1.145-26.412), 4.667 (95% CI: 1.187-18.352) and 5 (95% CI: 1.195-20.922) for rs1061170, rs10490924 and rs11200638 respectively. These findings are the first for the Egyptian population an ethnically distinct group. Findings are in agreement with findings in Caucasian populations which exhibited ORs reaching 4.3 for CT in rs1061170, 3.3 for TG in rs10490924 and 1.86 for AG on rs11200638. The utility of genetic testing for AMD has been under an intense debate. Some physicians believe that due to the uncertain nature of the role played by genetics in AMD, genetic testing may serve no purpose but to increase patients' worries. There are also concerns that if information about the patients' risk of developing the disease becomes publicly available it could lead to problems in obtaining medical insurance. However, recent research indicates that identifying genetic risk may be of benefit to patients as it may affect disease progression, treatment outcome and lifestyle decisions. To examine this, studies on the effect of genetic risk factors on disease progression, treatment outcome and the interplay between genetic factors and smoking should be conducted on Egyptian patients. Expanding the study to include a more diverse sample including patients and controls from various parts of Egypt would also add to our understanding of the genetics of AMD. Finally, association with other complement SNPs should be examined. These include; rs1410996 and rs7535263, SNPs on *CFE*, *C2*, *C3* and deletions on *CFHRI* and *CFHRJ* genes, as well as protective SNPs on the *C2* and *CFB* genes.

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CHAPTER 8. LIST OF FIGURES

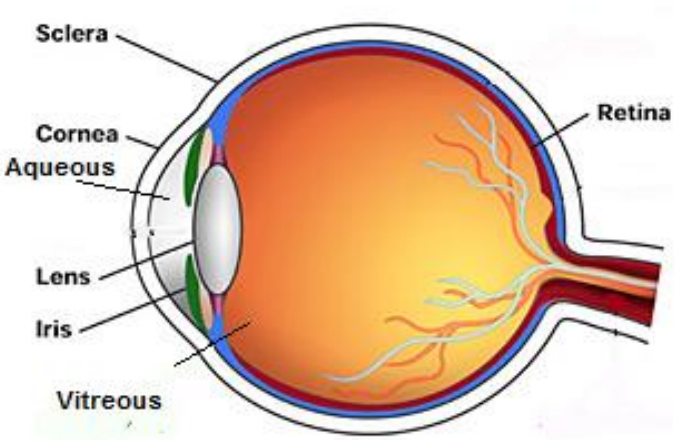

Figure 1. Structure of the eye. The figure shows the seven main regions of the eye; the cornea, sclera, aqueous humor, vitreous humor, lens, iris and the retina[111].

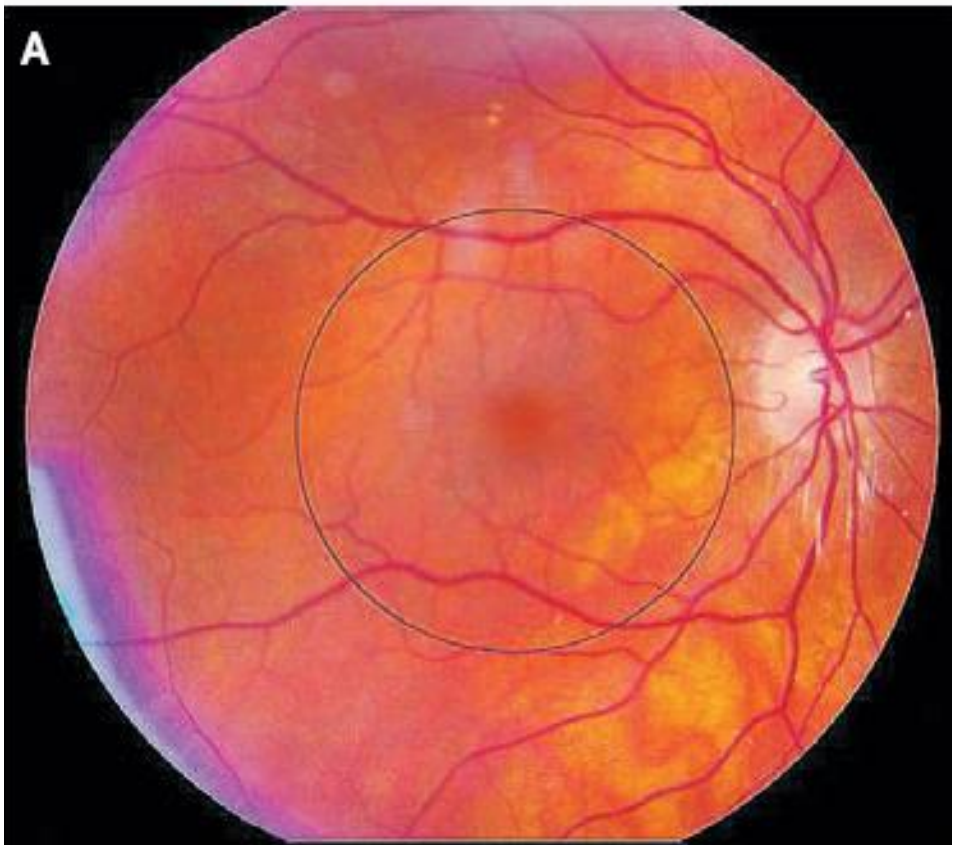

Figure 2. Normal retina with the macula encircled. The macula is the central region of the retina and is responsible for high resolution visual activity and detection of fine details[4].

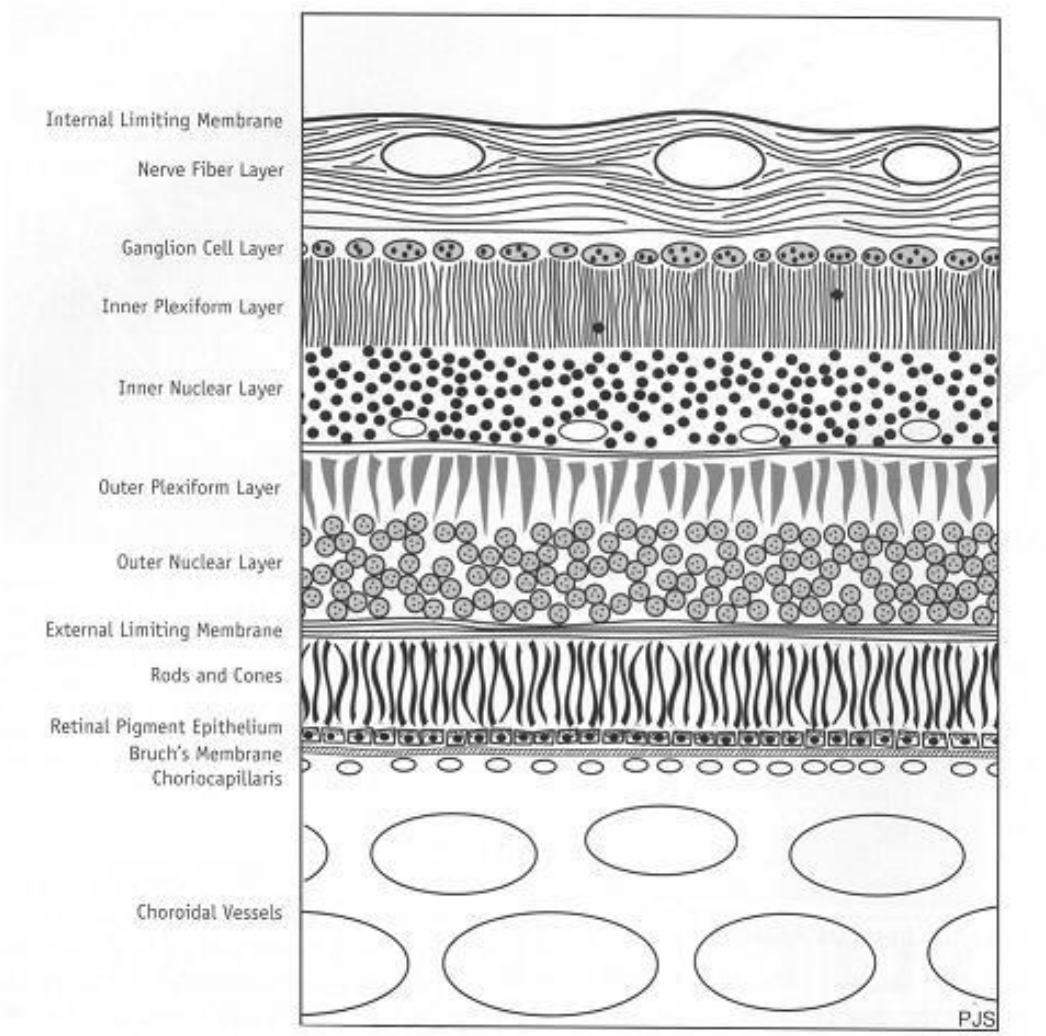

Figure 3. The ten layers of the retina. These are; the inner limiting membrane, nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, external limiting membrane, photoreceptor layer and the retinal pigment epithelium (RPE)[112].

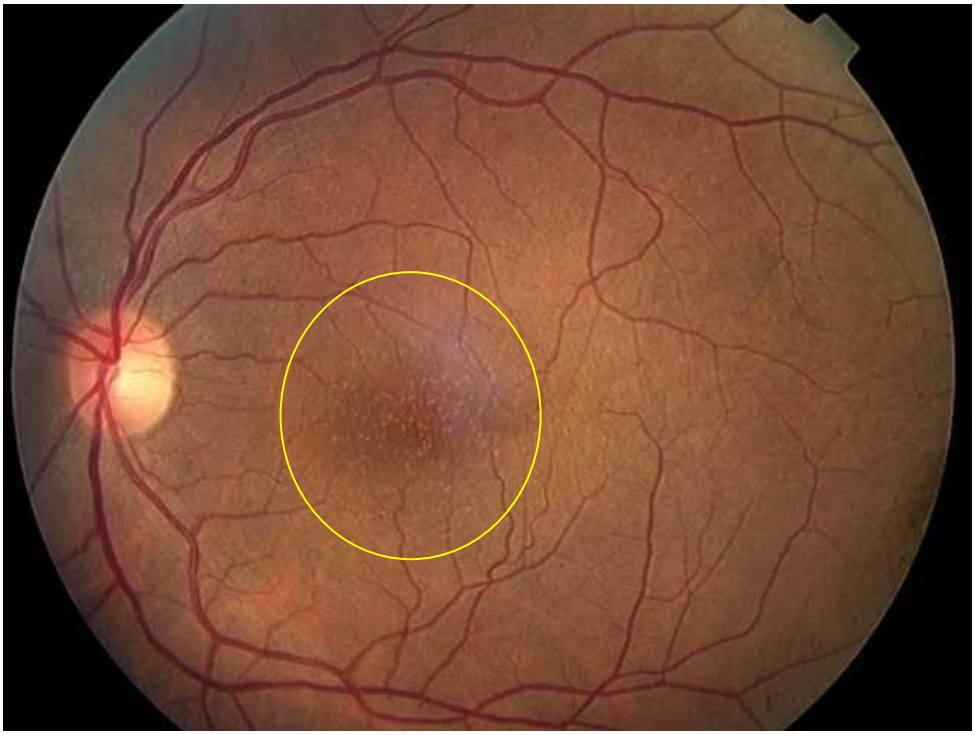

Figure 4. Retina showing soft drusen[113].

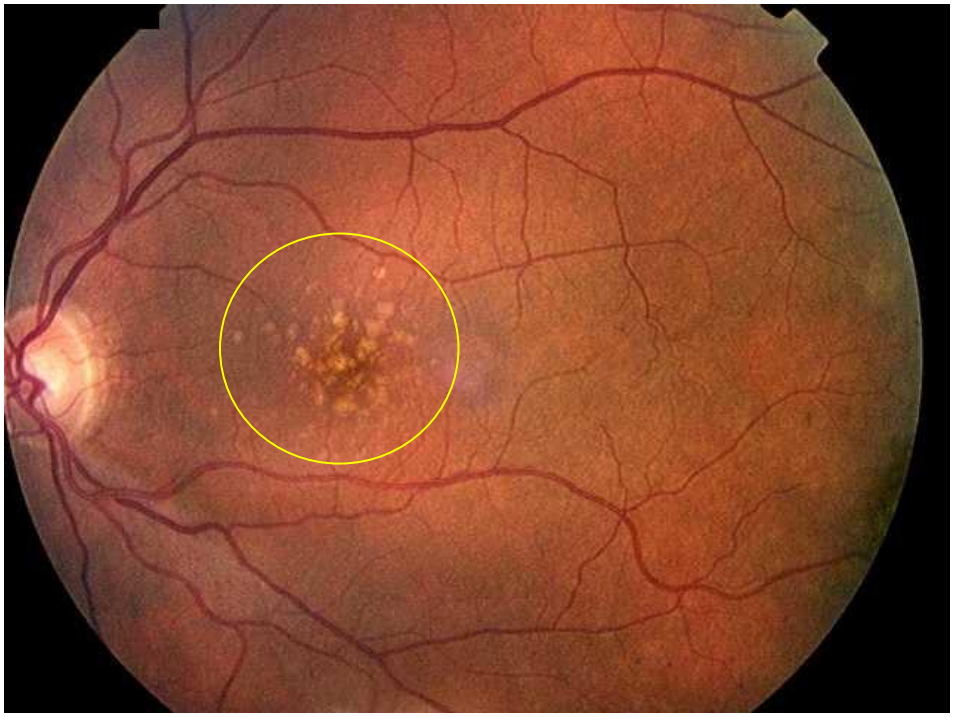

Figure 5. Retina showing hard drusen[113].

Figure 6. Loss of vision in AMD. Damage to the macula occurs and thus even the most extreme cases of AMD rarely exhibit loss of peripheral vision[114].

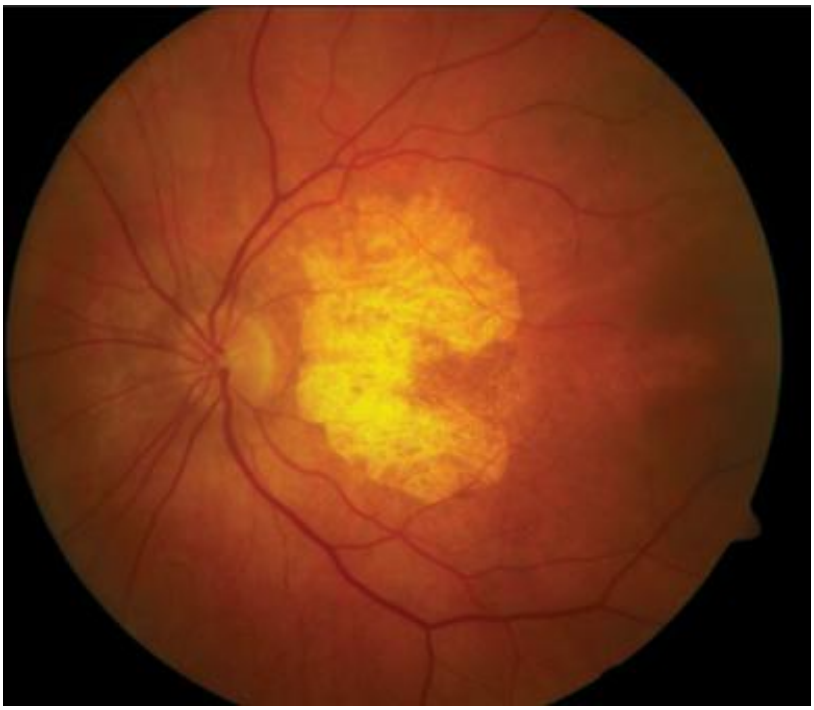

Figure 7. Geographic atrophy. This is an advanced form of AMD that involves RPE cell death resulting in atrophy of the overlying retina and underlying RPE[115].

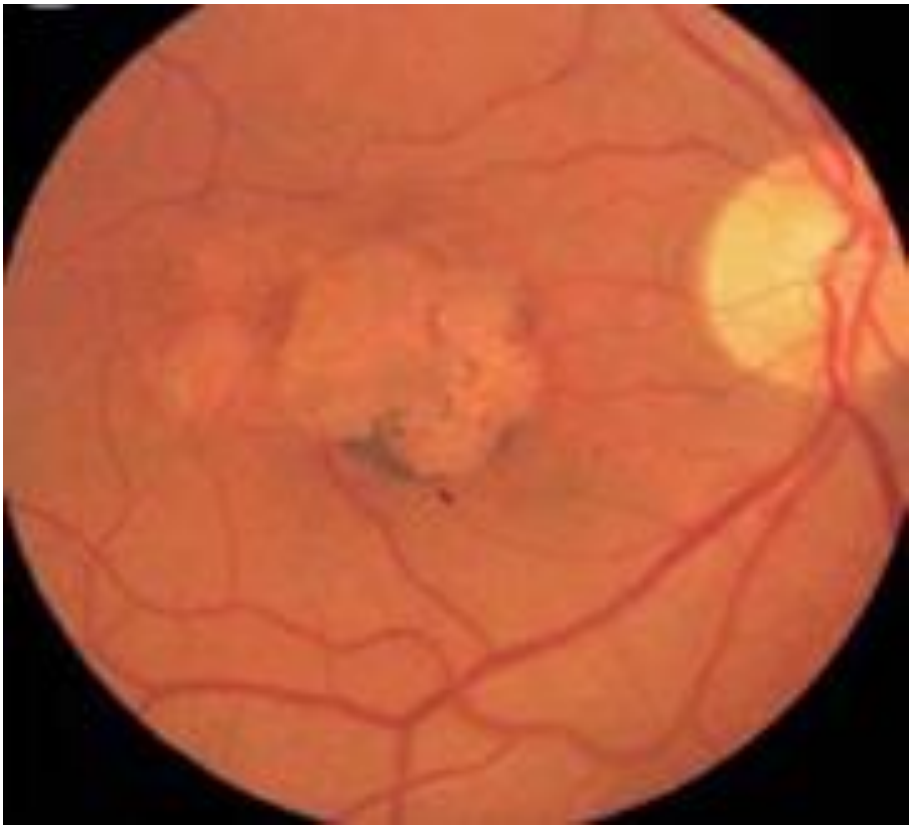

Figure 8. Choroidal neovascularization. This is an advanced stage of AMD which develops in NVAMD and involves growth of pathological blood vessels[116].

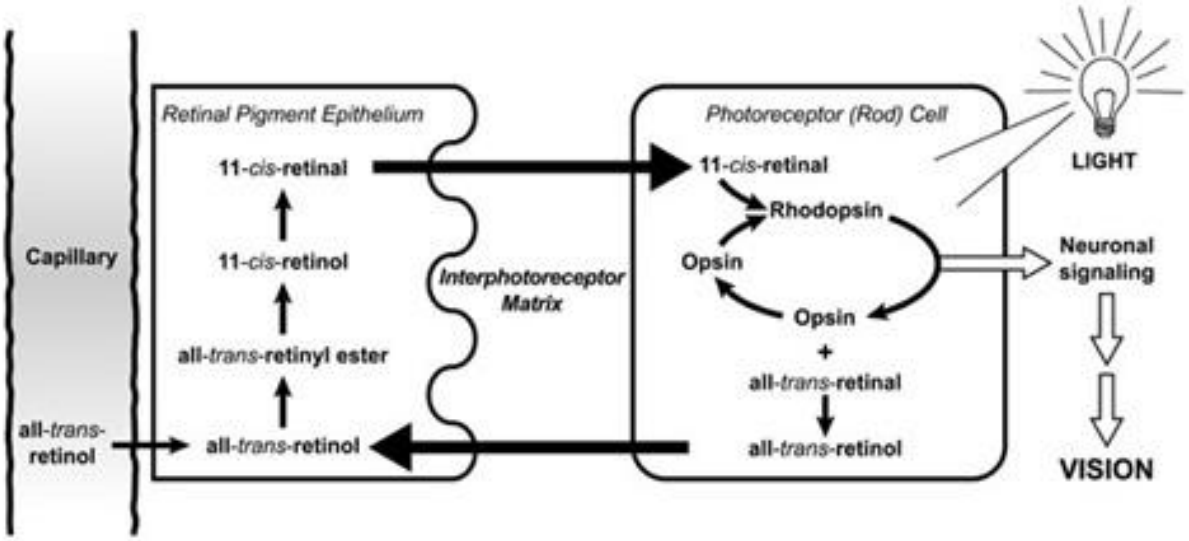

Figure 9: The visual cycle. Showing reisomeration of trans-retinol into 11-cis-retinal by the RPE[117]

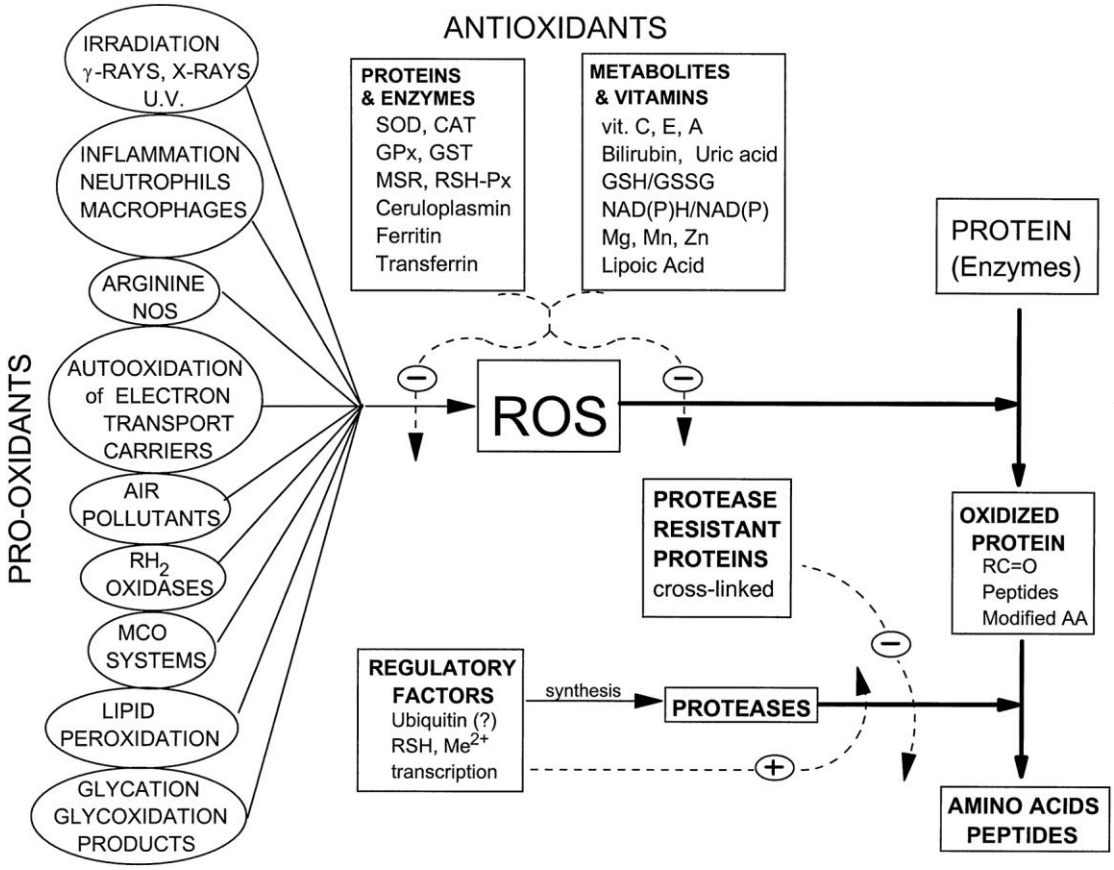

Figure 10: Oxidative stress and the role played by antioxidant enzymes and vitamins[118].

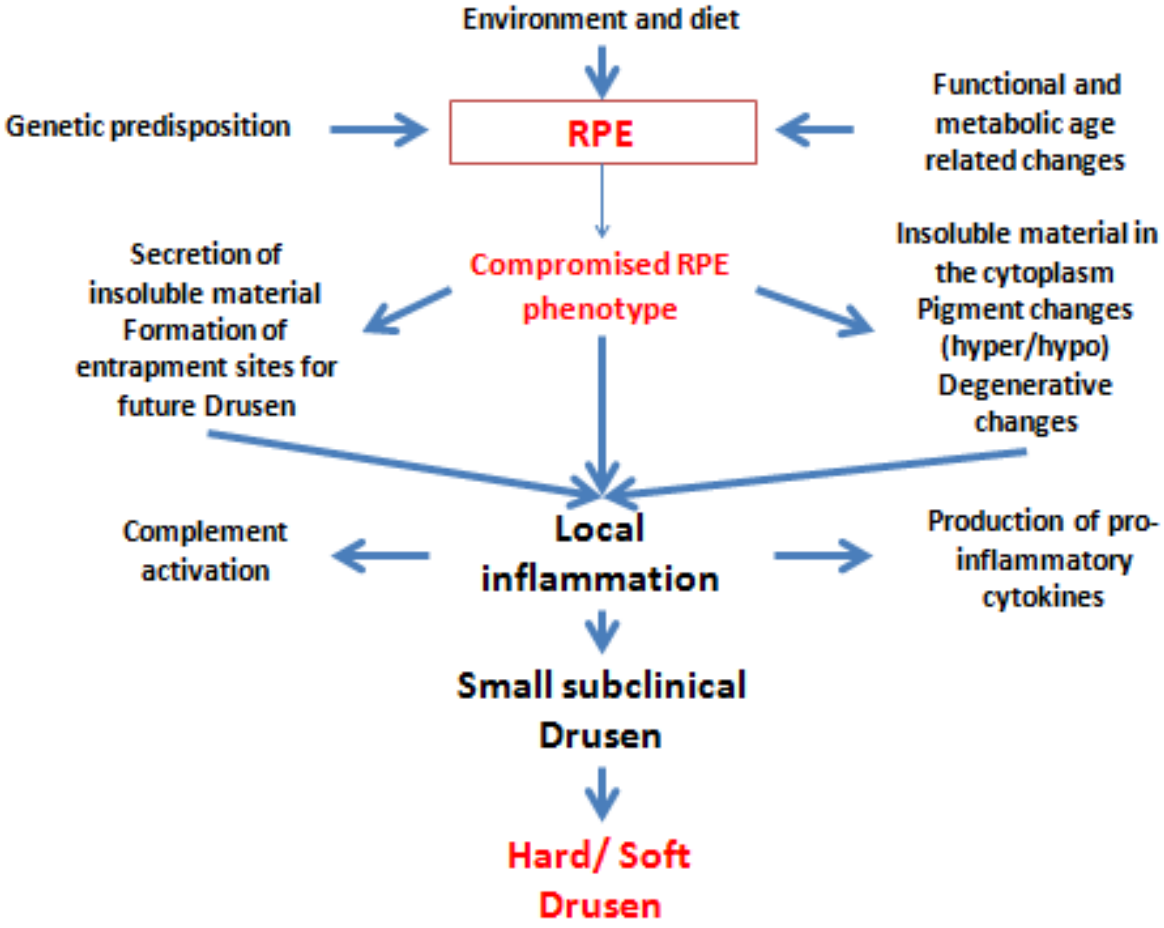

Figure 11. Schematic diagram of drusogenesis. This is a complex process that occurs over the years and is affected by several factors including; genetic predisposition, environment and diet and functional and metabolic age related changes (modified from[3]).

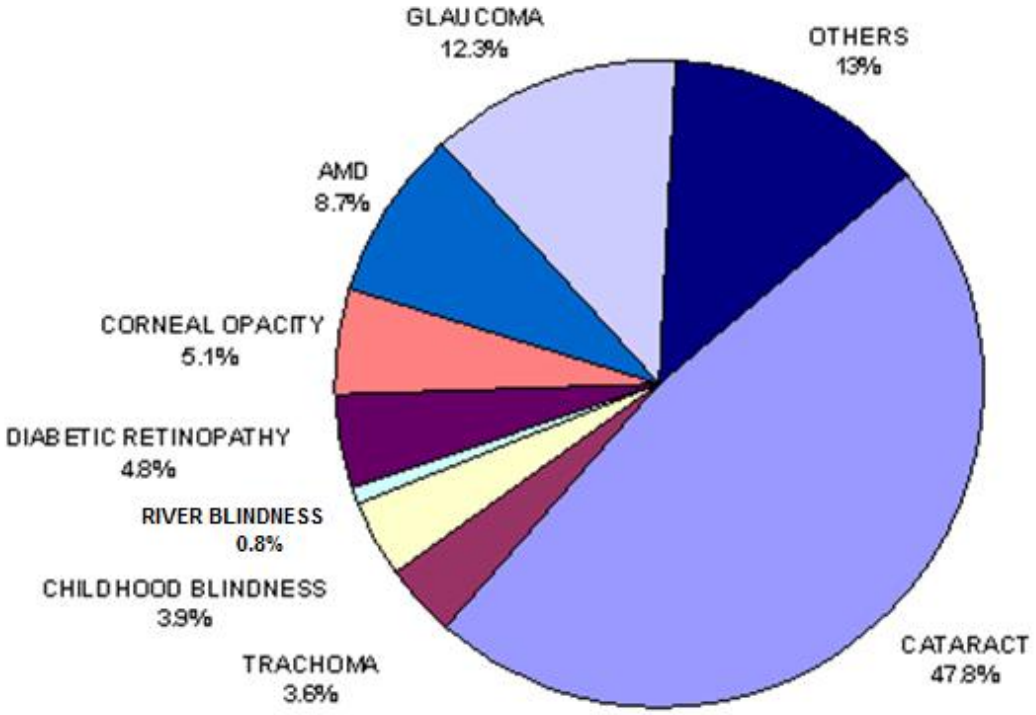

Figure 12. Worldwide causes of blindness according to the World Health Organization. AMD contributes to 8.7% of worldwide blindness[119].

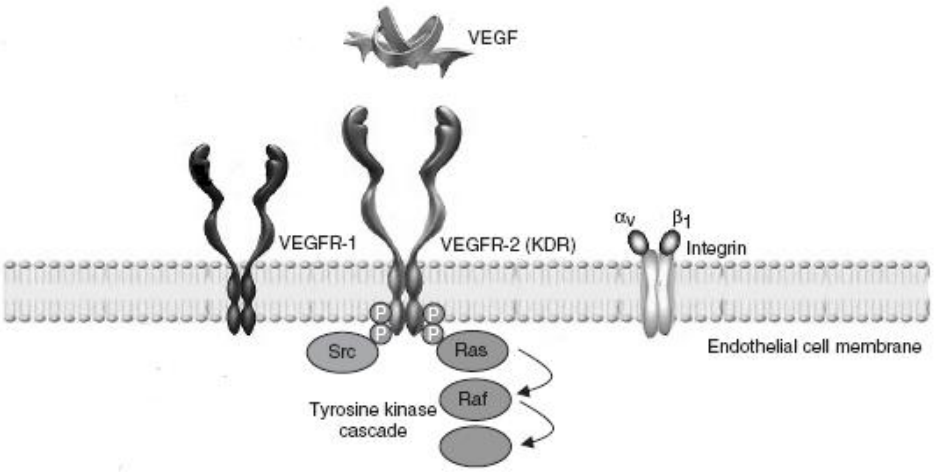

Figure 13: Interaction of VEGF with VEGF receptors. This interaction triggers an intracellular tyrosine kinase reaction (modified from [120]).

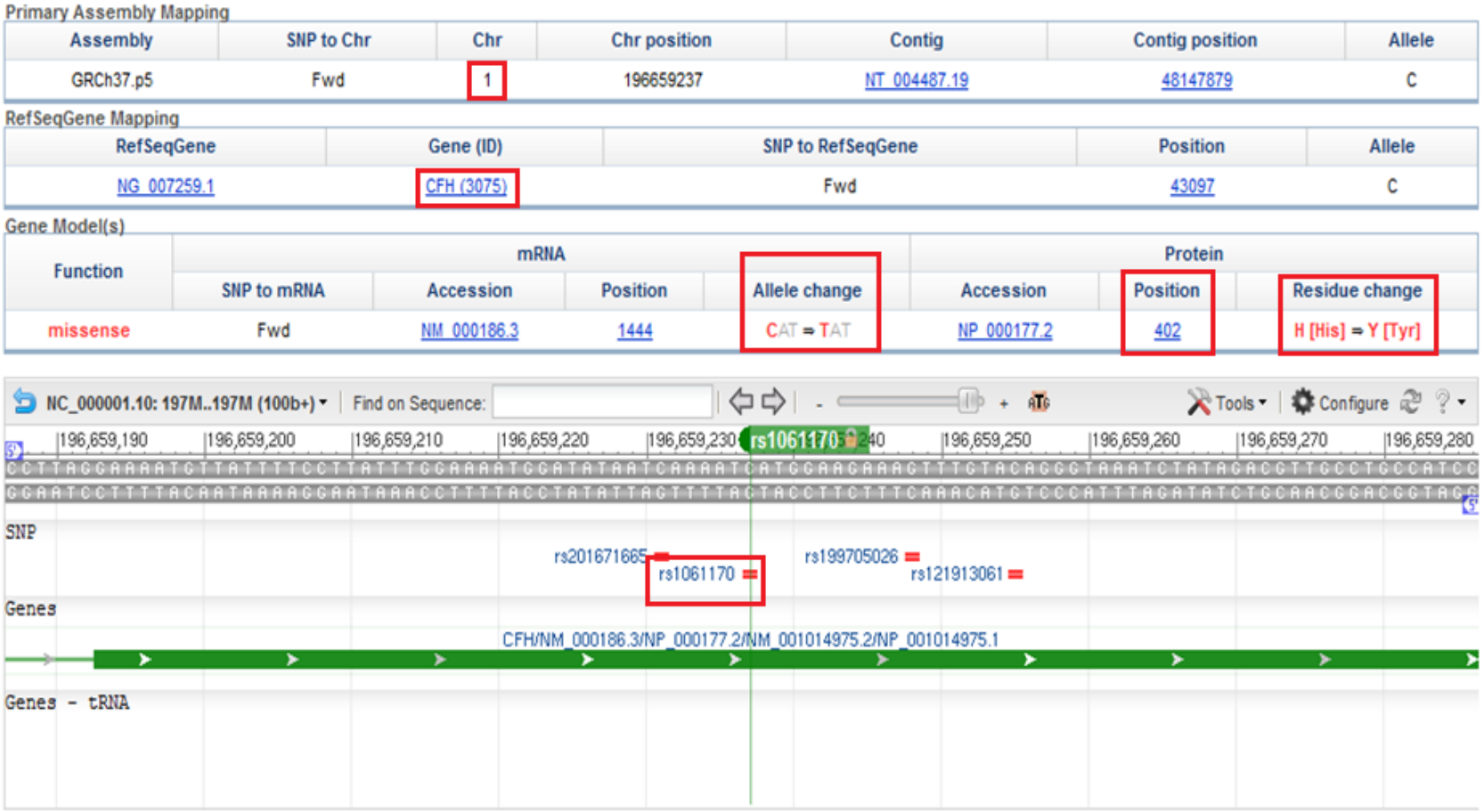

Figure 14. Gene view of rs1061170. This SNP is on Chromosome 1q31 leading to allele change C→T with amino acid change H→Y at position 402 of the *CFH* protein[77].

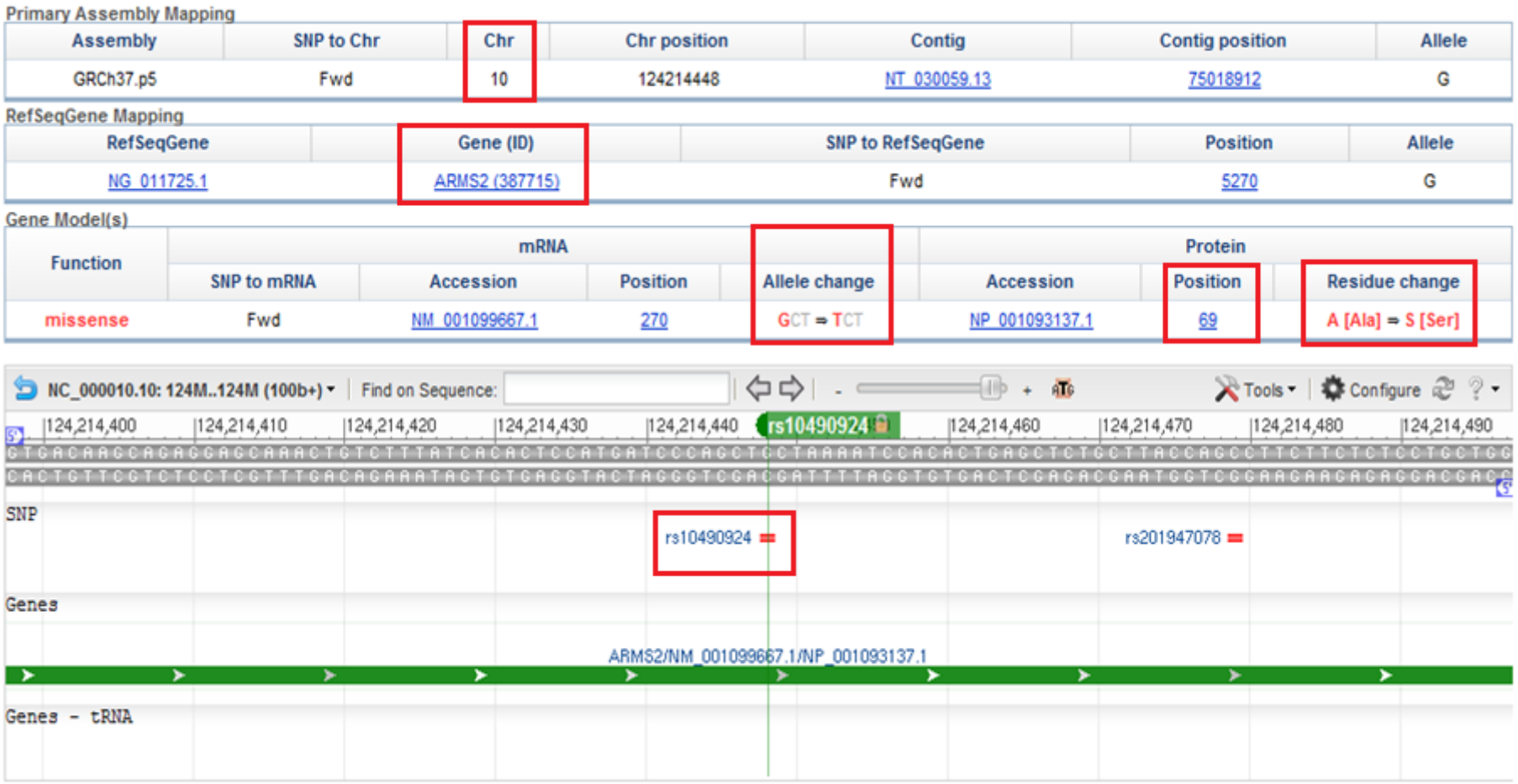

Figure 15. Gene view of rs10490924. This SNP is on Chromosome 10q26 leading to allele change G→T with amino acid change A→S at position 69 of the *ARMS2* protein[77].

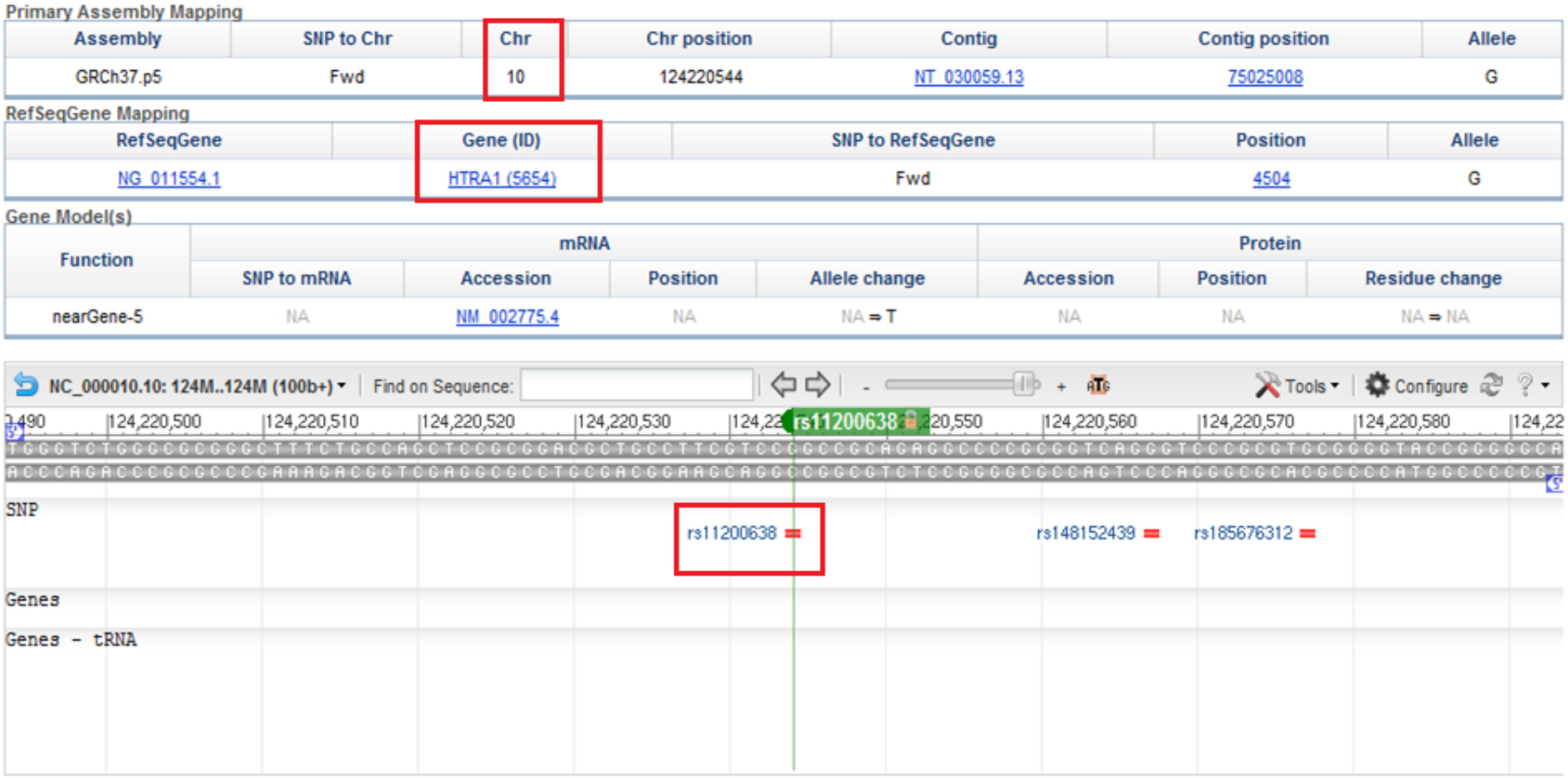

Figure 16. Gene view of rs11200638. This SNP is Chromosome 10q26 leading to allele change A→T on the *HTRA1* gene[77].

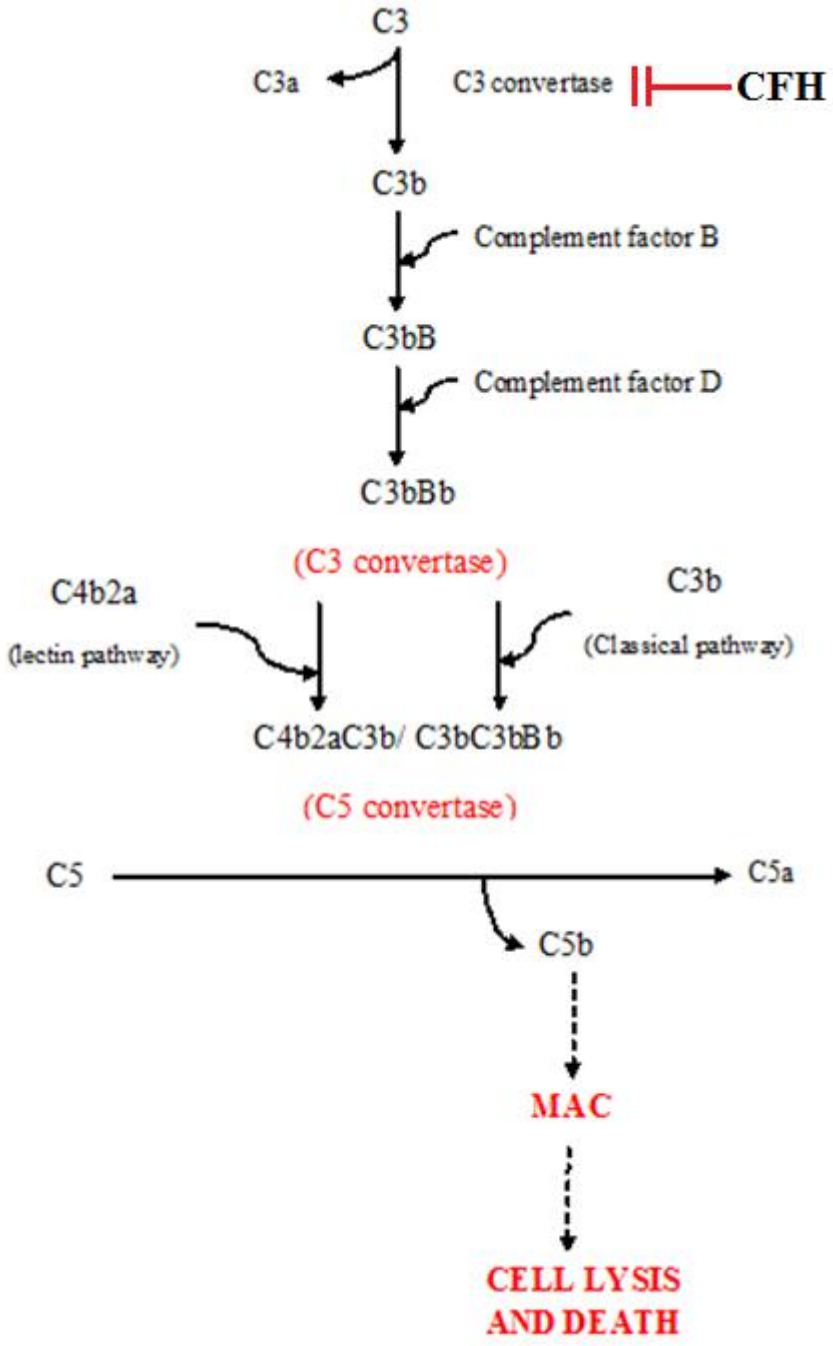

Figure 17. The complement pathway. *CFH* regulates complement through blocking activation of C3 into C3b and also aiding Factor1 mediated degradation of C3b (figure modified from[71]).

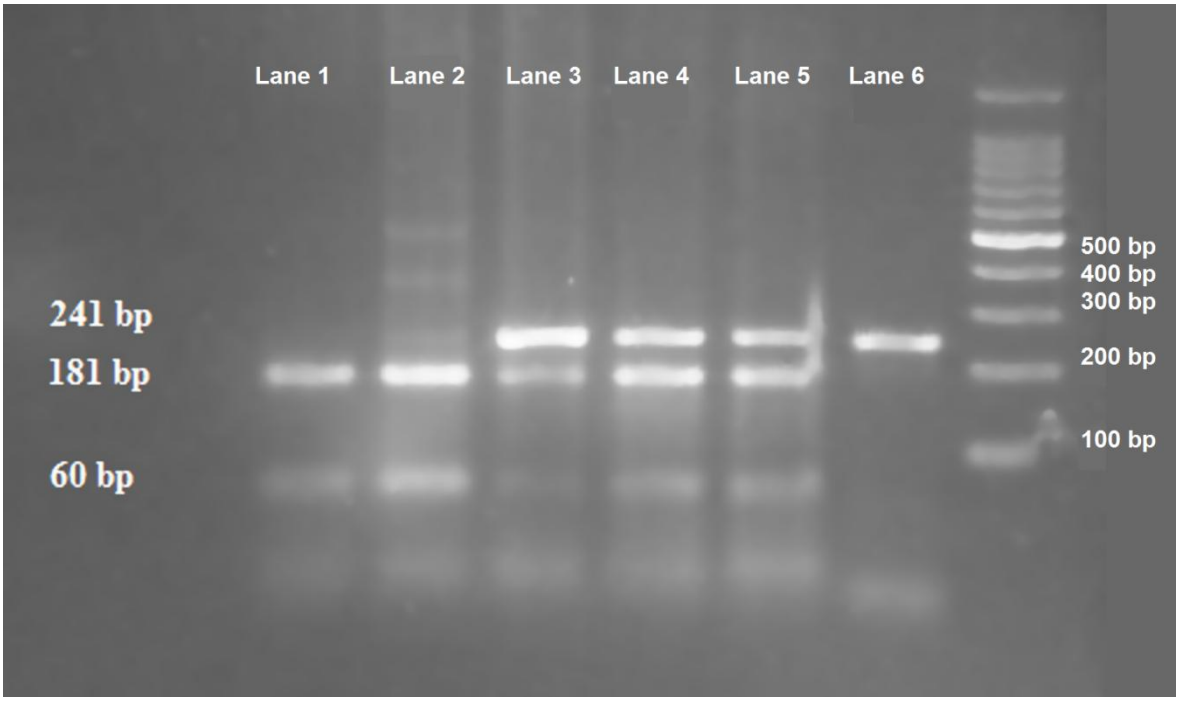

Figure 18. RFLP for *CFH* rs1061170. Digestion using *Tsp509*I shows 241bp band for homozygous CC, 181bp band + 60bp band for homozygous TT and 241bp +181bp + 60bp bands for heterozygous TC. Lanes 1 and 2 are TT, lanes 3,4 and 5 are TC and lane 6 is CC.

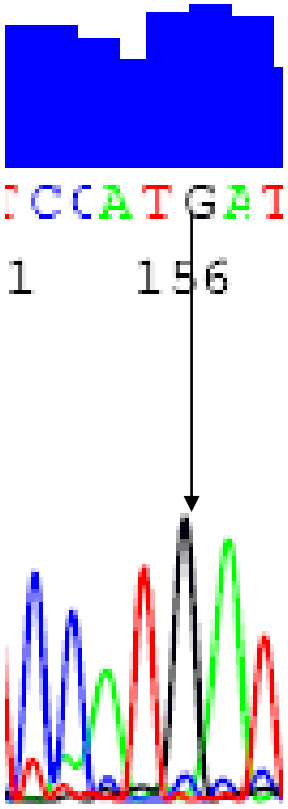

Figure 19. Sequencing of *CFH* rs1061170 CC genotype using backward primer. The CC genotype shows one black peak. C, A, T and G show Blue, green, red and black peaks respectively.

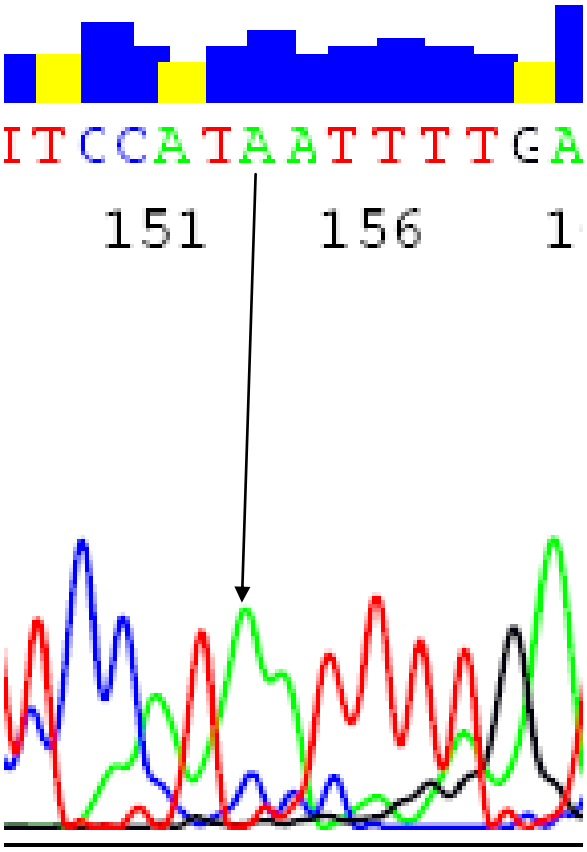

Figure 20. Sequencing of *CFH* rs1061170 TT genotype using backward primer. The TT genotype shows one green peak. C, A, T and G show Blue, green, red and black peaks respectively.

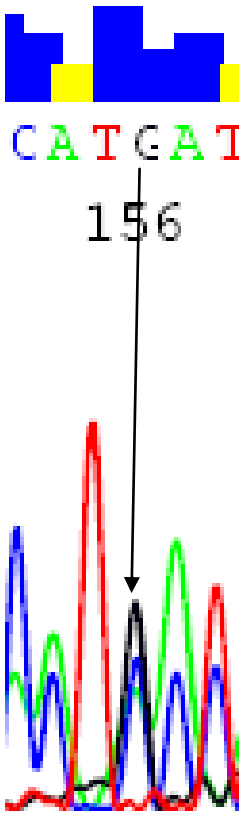

Figure 21. Sequencing of *CFH* rs1061170 TC genotype using backward primer. The TC genotype shows both black and green peaks. C, A, T and G show Blue, green, red and black peaks respectively.

a)

b)

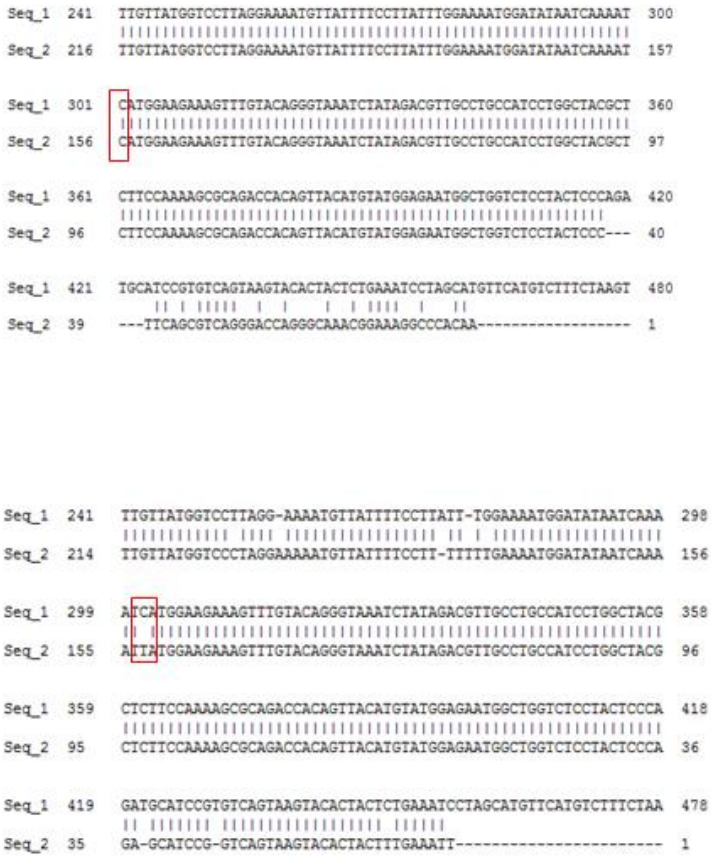

Figure 22. Alignment results for *CFH* gene. a) shows results for TC genotype, b) for CC Genotype and c) for TT Genotype, to differentiate between homozygous and heterozygous genotypes we refer to the sequencing figures.

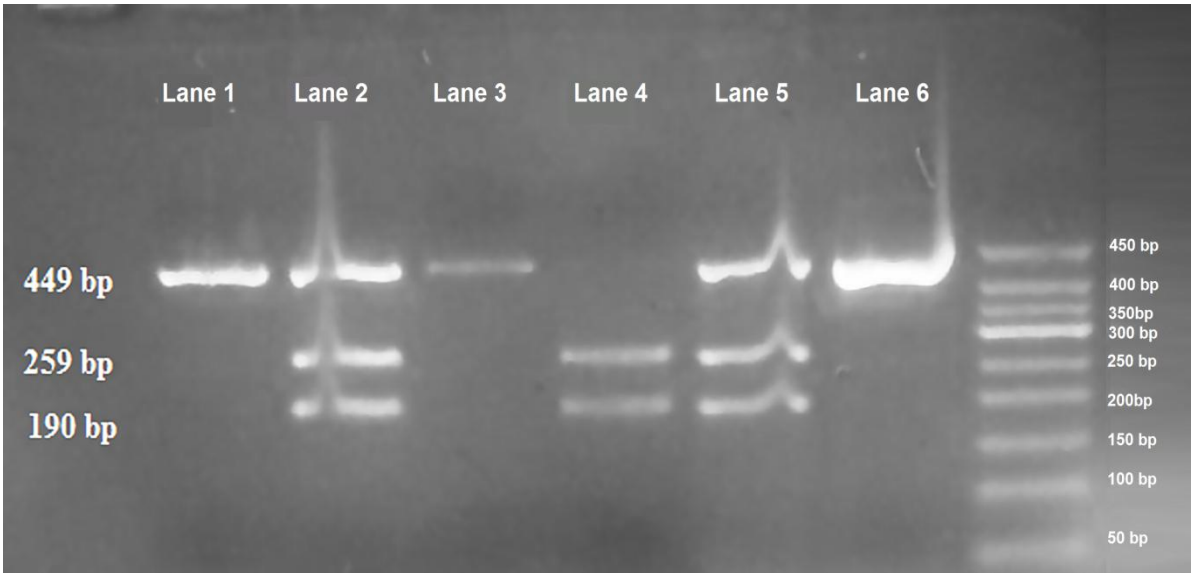

Figure 23. RFLP for *ARMS2* rs10490924. Digestion using *Pvu*II shows 449bp band for Homozygous TT, 259bp band + 190bp band for Homozygous GG and 449bp +259bp + 190bp bands for heterozygous TG. Lanes 1, 3 and 6 show TT, lanes 2 and 5 show TG and lane 4 shows GG.

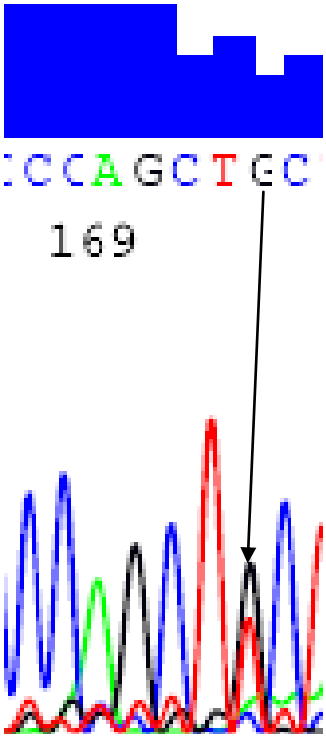

Figure 24. Sequencing of *ARMS2* rs10490924 TG genotype using forward primer. TG genotype shows both black and red peaks. C, A, T and G show Blue, green, red and black peaks respectively.

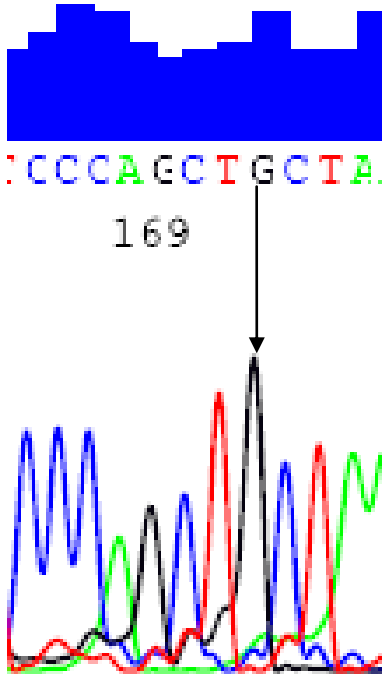

Figure 25. Sequencing of *ARMS2* rs10490924 GG genotype using forward primer. The GG genotype shows one black peak. C, A, T and G show Blue, green, red and black peaks respectively.

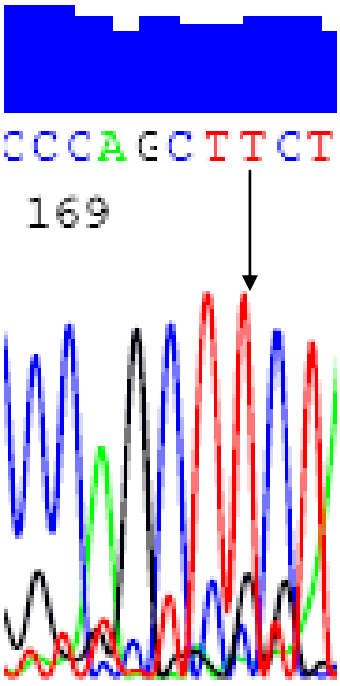

Figure 26. Sequencing of *ARMS*2 rs10490924 TT genotype using forward primer. The TT genotype shows one red peak. C, A, T and G show Blue, green, red and black peaks respectively.

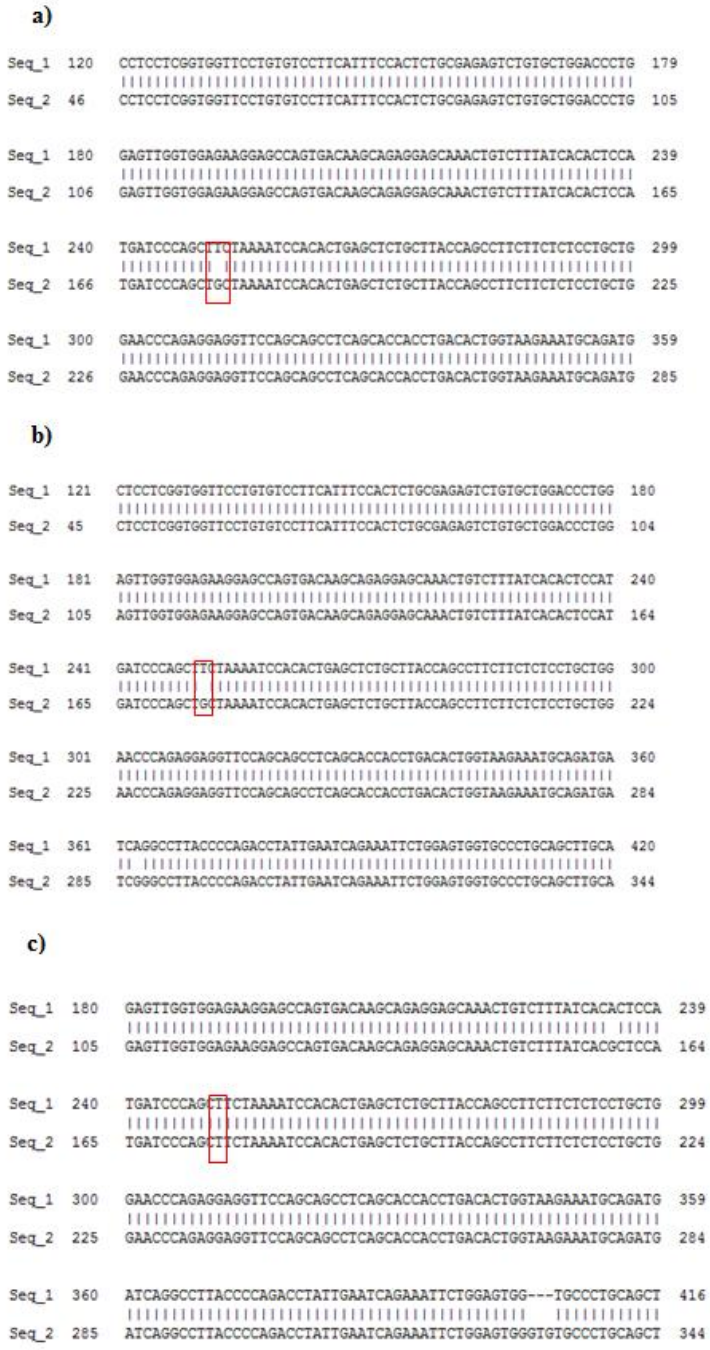

Figure 27. Alignment results for *ARMS2* gene. a) shows results for TG genotype, b) for GG Genotype and c) for TT Genotype , to differentiate between homozygous and heterozygous genotypes we refer to the sequencing figures.

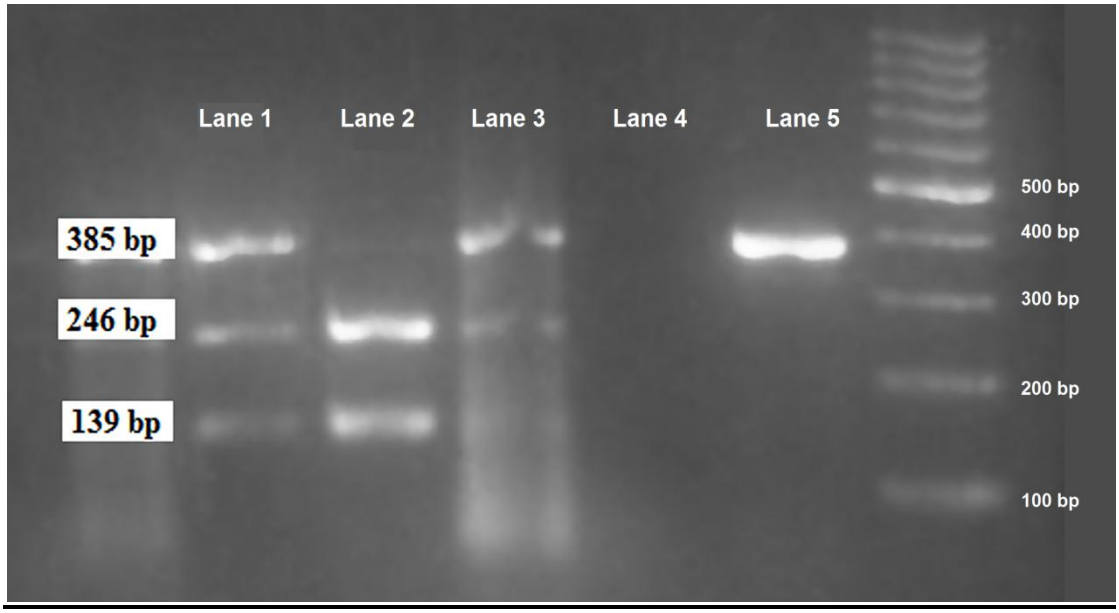

Figure 28. RFLP for rs11200638 in *HTRA1*. Digestion using *Eag* I shows 385bp band for Homozygous AA, 246bp band + 139bp band for Homozygous GG and 385bp +246bp + 139bp bands for heterozygous AG. Lane 1 is AG, Lane 2 is GG, lane 3 is a smear, lane 4 there was no amplification and lane 5 is AA.

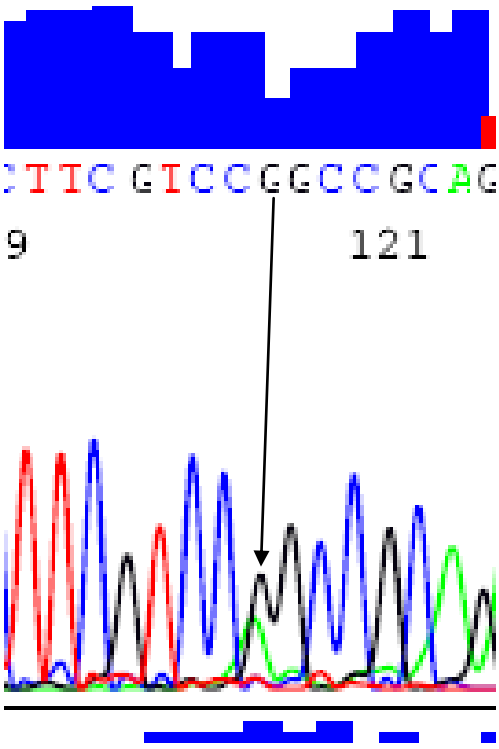

Figure 29. Sequencing of *HTRA1* rs11200638 AG genotype using forward primer. The AG genotype shows both black and green peaks. C, A, T and G show Blue, green, red and black peaks respectively.

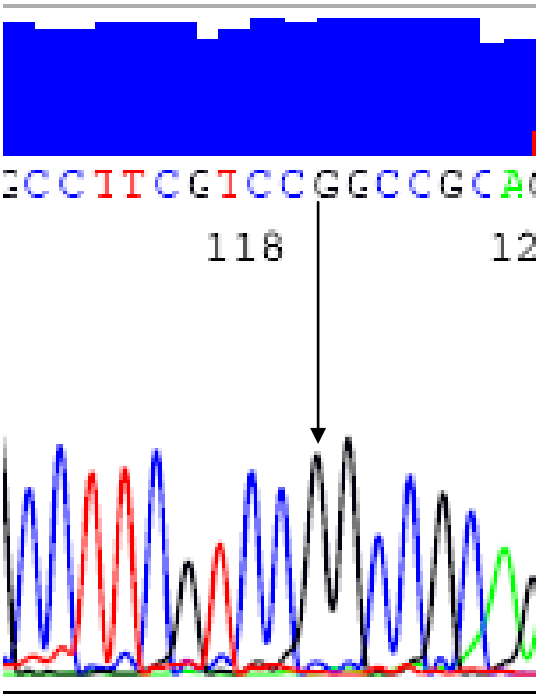

Figure 30. Sequencing of *HTRA1* rs11200638 GG genotype using forward primer. The GG genotype shows one black peak. C, A, T and G show Blue, green, red and black peaks respectively.

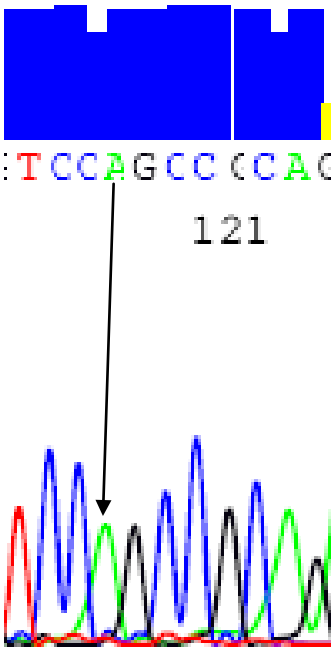

Figure 31. Sequencing of *HTRA1* rs11200638 AA genotype using forward primer. The AA genotype shows one green peak. C, A, T and G show Blue, green, red and black peaks respectively.

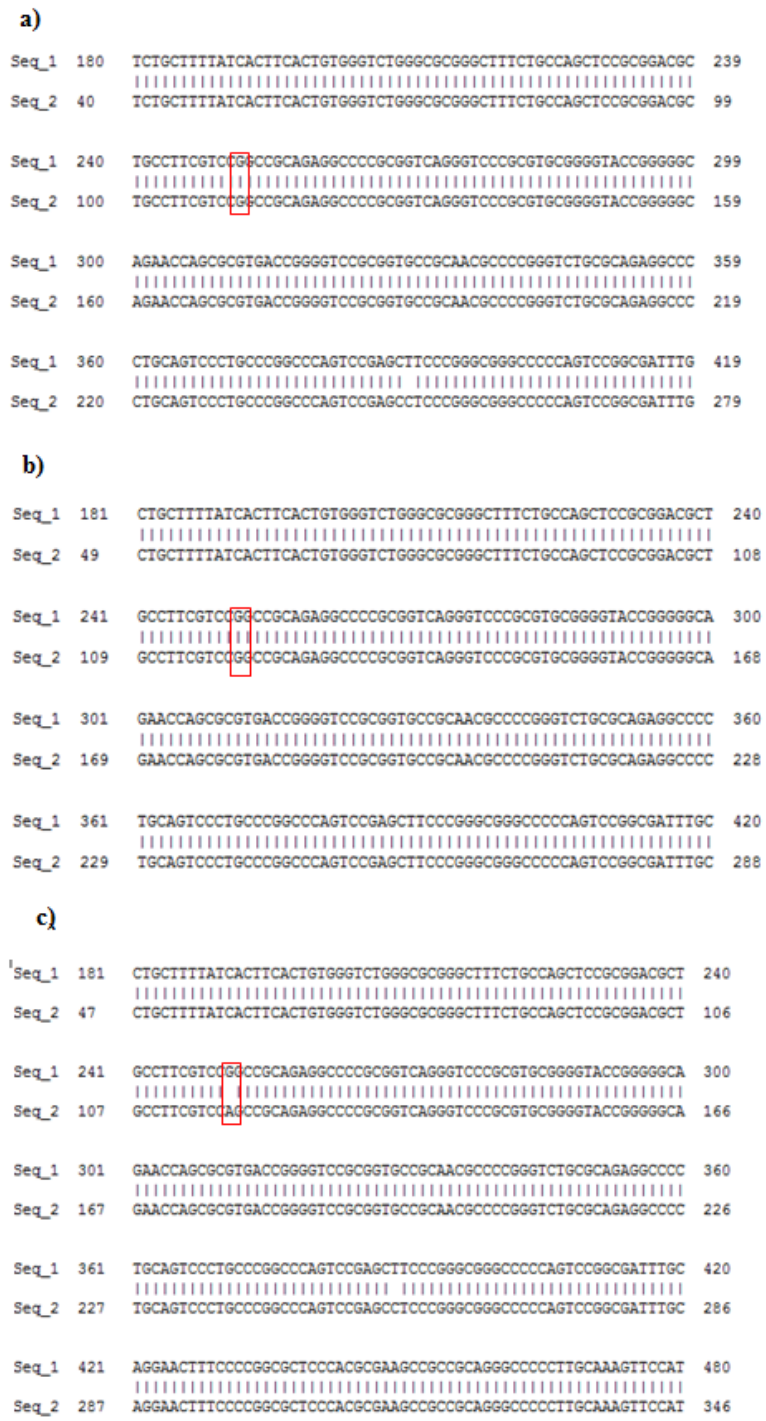

Figure 32. Alignment results for *HTRA1* gene. a) shows results for AG genotype, b) for GG Genotype and c) for AA Genotype, to differentiate between homozygous and heterozygous genotypes we refer to the sequencing figures.

CHAPTER 9. LIST OF TABLES

Table 1: Association studies between AMD and *CFH* rs1061170 polymorphism

***CFH* (rs1061170)**				
Study/ year	**Country/ Patients/ Controls**	**Genotyping method**	**Results**	**Conclusions**
Zareparsi et al 2005 [50]	USA/616/275	Not specified	OR TC= 4.36 (95% CI 3.13–6.08) CC= 5.52 (95% CI 3.54–8.59).	Y402H polymorphism significantly associated with AMD
Schaumberg et al 2006 [43]	USA/111/401	Not specified	OR TC=1.46 (95% CI: 1.05–2.04) CC=2.13 (95% CI: 1.10–4.16)	Y402H polymorphism significantly associated with AMD
Maller et al 2006 [44]	USA/ 1238 NVAMD/ 934	Illumina BeadArray	TC= 2.7-fold increased risk for AMD CC= 7.6-fold increased risk	Y402H polymorphism significantly associated with AMD
Francis et al 2007 [72]	USA/ 333 advanced AMD (108 GA, 205	PCR followed by TGCE or direct	OR= 2.399 (95% CI; 1.768, 3.256)	Y402H polymorphism significantly associated with AMD

	CNV)/ 171	sequencing		
Pulido et al 2007 [45]	USA/ 89 NVAMD/ 230	PCR-RFLP	OR TC= 1.16 (95% CI 0.64 to 2.09). CC= 4.97 (95% CI 2.52 to 9.79	Y402H polymorphism significantly associated with AMD
Baird et al 2006 [53]	Australia/ 236/ 144	MALDI-TOF validated through sequencing	OR TC= 1.86 (95%CI= 1.1-3.16) CC= 9.26, (95% CI= 4.52-18.98)	Individuals with at least one copy of the C allele showed increased risk of AMD
Teixeira et al 2010 [49]	Brazil/ 119/ 152	PCR sequencing	TC= increased risk by 1.36-folds CC= increased the risk by 4.63-folds	The Y402H polymorphism is a risk factor for developing AMD in the Brazilian population
Lau et al 2006 [63]	China/ 163 NVAMD/ 232	PCR-RFLP and verified through sequencing	C allele OR= 4.4 (95% CI= 2.3– 8.5; P < 0.00001).	Y402H polymorphism significantly associated with AMD
Gao et al 2010 [62]	China/ 208/ 140	PCR-RFLP	OR= 2.29 (95% confidence interval, 1.06-4.95).	Association of Y402H with AMD though to a lower extent than in the white population

Chen et al 2006 [11]	China/ 163 NVAMD/ 244	Taqman assay	Significant association at rs3753394, rs800292 and rs1329428 but not at rs1061147, rs1061170 or rs380390	Three SNPs of the *CFH* gene, rs1329428 and rs800292 (I62V) and for the first time rs3753394 increased risk for exudative AMD Y402H not associated with AMD
Ng et al 2008 [64]	Hong kong (Chinese)/ 163 NVAMD/ 155	Sequencing	Significant association at rs3753396 (novel), rs3753394, rs551397, rs800292, rs2274700, and rs1329428 (previously reported).	*CFH* related to susceptibility to AMD
Xu et al 2008 [68]	China/ 121 NVAMD/ 132	PCR-RFLP and sequencing	Y402H was not associated with exudative AMD. Frequencies for the risk C allele were 10.3% in AMD cases and 8.0% in controls (p=0.353). No significant difference between the *CFH* genotypes in AMD group and control group (p=0.496).	Y402H not associated with AMD
Yang et al 2010 [66]	China (Han Chinese)/109 NVAMD/ 150	PCR-RFLP	*CFH* rs800292 significantly associated with a reduced risk for exudative AMD. *CFH* Y402H had similar allele and genotype frequencies in the case and control groups	Y402H not associated with AMD. rs800292 (*CFH* V62I) highly associated with reduced risk of AMD

			(p>0.05).	
Ziskind et al 2008 [61]	Black South African Xhosa population/16 (early AMD)/98	PCR-RFLP	C allele frequency in AMD = 53.1%, in controls= 41.8%. CC frequency in AMD= 31%, in controls= 13.3%	No association between Y402H allele and the disease
Okamoto 2006 [59]	Japan/ 96 NVAMD/ 89	Sequencing	Using the four SNPs reported by Hageman et al. five haplotype blocks were identified. Haplotype 5, which contains Y402H was found at a frequency of 4% for both controls and AMD cases	No association between Y402H allele and the disease
Gotoh et al 2006 [58]	Japan/ 146 NVAMD/ 105	Sequencing	The frequency of the C allele of rs1061170 is as low as 0.04 in Japanese.	No association between Y402H allele and the disease
Fuse et al 2006 [57]	Japan/ 80 dry AMD/ 196	Sequencing	The frequencies of Y402H were not significantly higher in the AMD group than in the control group	No association between Y402H and the disease

Seitsonen et al 2006 [46]	Finland/ 181 familial cases, 154 sporadic cases/ 105 non-AMD, 350 blood donors	Sequencing	combined analysis of familial and sporadic AMD cases CC showed relative risk= 5.31 (95% CI:3.35-8.40) compared to blood donor controls = 9.79 (95% CI: 4.76-20.1) compared to non-AMD controls TC risk =2.34 (95% CI:1.54-3.57) compared to blood donor controls = 2.51 (95% CI: 1.46-4.31) compared to non-AMD controls	Y402H significantly associated with AMD
Souied et al 2005 [47]	France/ 141 NVAMD (81 familial and 60 sporadic cases)/ 91	Sequencing	OR TC =3.0 (CI 95% 1.6-5.6). CC= 6.8 (CI 95% 3.0-15.2) After adjustment for age and sex ($p<0.0001$): OR TC=3.0 (CI 95% 1.6-5.6) CC=6.9 (CI 95% 3.1-15.6).	Y402H associated with NVAMD
Kim et al 2008 [60]	Korea/ 114 NVAMD/ 187	Sequencing	The difference between the frequencies of Y402H in cases and controls was not statistically	Y402H marginally associated with NVAMD

			significant (P =0.071). Other SNPs (-275C>T, I62V, and IVS15) were found to be associated with an elevated risk of exudative AMD.	
Chowers et al 2008 [52]	Israel/ 240 NVAMD/ 118	MALDI-TOF	OR TC=2.1 (95% CI=1.3 −3.4) CC= 3.4 (95% CI= 1.7–6.8). Combined OR= 2.4 (95% CI 1.5–3.8, p=0.0005)	Y402H associated with NVAMD
Simonelli et al 2006 [48]	Italy/ 104/ 131	Taqman Assay	OR CC= 3.9 (95% CI: 1.9 to 8.2	Y402H associated with AMD
Despriet et al 2006 [51]	Netherlands	Taqman Assay	OR CC increased in an allele-dose manner; = 2.00 (95% CI 1.56-2.55) for stage 2 AMD, = 4.58 (95% CI, 2.82-7.44) for stage 3 AMD, =11.02 (95% CI, 6.82-11.81) for stage 4 (late, vision threatening)	Y402H associated with AMD
Fisher et al 2007 [55]	Russia/ 155/ 151	Sequencing	OR TC=2.01 (95% CI 1.14 to 3.56) CC=2.71 (95% CI 1.25 to 5.90)	Y402H associated with AMD (at a lower level than western population)
Soysal et al	Turkey/ 147/ 105	PCR- RFLP	OR	Y402H associated with AMD

2012 [56]			TC= 2.22 (95% CI: 1.23-4.00) CC= 4.12 (95% CI: 1.96-8.65)	

Table 2: Association studies between AMD and *ARMS2* rs10490924 polymorphism

***ARMS2* (rs10490924)**				
Study/ year	**Country/ Patient/ Controls**	**Genotyping method**	**Results**	**Conclusions**
Yang et al 2006 [65]	USA/ 442/ 309	Not specified	OR TG= 1.35 (95% CI; 0.99, *1.86)* TT=6.09 (95% CI; 3.27, 11.34)	ARMS2 significantly associated with AMD
Pulido et al 2007 [45]	USA/ 87 NVAMD/ 232	PCR-RFLP	OR TG= 3.35 (95% CI= 1.91-5.90). TT= 7.75 (95% CI; 3.46-17.35).	ARMS2 significantly associated with AMD
Francis et al 2007 [72]	USA/ 333 (108 GA, 205 CNV)/ 171	PCR followed by TGCE or direct sequencing	OR= 4.671 (3.245, 6.722)	ARMS2 significantly associated with AMD

Fisher et al 2007 [55]	Russia/ 155/ 151	Sequencing	The frequency of the risk allele was not significantly different between AMD cases (31.6%) and controls (28.5%) In late AMD, risk for the disease increased with TG OR=2.55 (95% CI 1.24-5.24) TT OR=3.47 (95% CI 1.01- 11.88)	Contribution of ARMS2 to risk is weak and found only in late stage AMD
Rivera et al 2005 [69]	Germany/ 1166/ 922	MALDI-TOF	TT= 7.6-fold increased risk OR= 57.6 (95% CI: 37.2, 89.0) for individuals homozygous for risk alleles at both *CFH* Y402H and LOC387715.	ARMS2 significantly associated with AMD
Xu et al 2008 [68]	China/ 121 NVAMD/ 132	PCR-RFLP and sequencing	OR TG= 1.56 (95% CI; 0.80–3.03) TT= 5.45 (95% CI; 2.59–11.49)	ARMS2 significantly associated with AMD
Kaur et al 2008 [70]	India/ 250/ 250	Sequencing	Significant associations were found with the risk alleles of rs10490924 (T allele; P = 5.34 x 10^{-12})	ARMS2 significantly associated with AMD
Soysal et al 2012 [56]	Turkey/ 147/ 105	PCR-RFLP	OR TG= 1.88 (95% CI: 1.07 to 3.30) TT= 8.61 (95% CI: 3.55 to 20.87)	ARMS2 significantly associated with AMD

Table 3: Association studies between AMD and *HTRA1* rs11200638 polymorphism

***HTRA1* (rs11200638)**				
Study/ year	**Country/ Patient/ Controls**	**Genotyping method**	**Results**	**Conclusions**
Yang et al 2006 [65]	USA/ 442/ 309	Not specified	OR GA=1.86 (95% CI; 1.35, 2.56) AA= 6.56 (95% CI; 3.23,13.31) p = 1x l0 $^{-9}$	rs1l200638 significantly associated with AMD
Xu et al 2008 [68]	China/ 121 NVAMD/ 132	PCR-RFLP and sequencing	OR GA= 2.75 (95% CI; 1.34– 5.64), AA=7.90 (95% CI; 3.61–17.26)	rs1l200638 significantly associated with AMD
Francis et al 2007 [72]	USA/ 333 (108 GA, 205 CNV)/ 171	PCR followed by TGCE or direct sequencing	OR 3.973 (95% CI; 2.928, 5.390)	rs1l200638 significantly associated with AMD
Yang et al 2010 [66]	China (Han Chinese)/ 109 NVAMD/ 150	Taqman assay	OR for GA and AA combined was 2.02 (95% CI; 1.20-3.39)	rs1l200638 significantly associated with AMD

Kaur et al 2008 [70]	India/ 250/ 250	Sequencing	Significant associations were found with the risk alleles of rs11200638 (A allele; $p = 4.32 \times 10^{-12}$)	rs1l200638 significantly associated with AMD

Table 4: PCR primers for genotyping the three SNPs

	Forward (5'-3')	**Backward (5'-3')**
***CFH* rs1061170** [63]	TCA TTG TTA TGG TCC TTA GGA AA	TTA GAA AGA CAT GAA CAT GCT AGG
***ARMS2* rs10490924** [68]	TAC CCA GGA CCG ATG GTA AC	GAG GAA GGC TGA ATT GCC TA
***HTRA1* rs11200638** [68]	ATG CCA CCC ACA ACA ACT TT	CGC GTC CTT CAA ACT AAT GG

Table 5: Restriction digestion reaction conditions [68]

Locus	Enzyme	Conditions
***CFH* rs1061170**	*Tsp* 509I	65˚C, buffer 1, for 3 hours
***ARMS2* rs10490924**	*Pvu* III	37˚C, buffer 2, for 3 hours
***HTRA1* rs11200638**	*Eag* I	37˚C, buffer 3, for 3 hours

Table 6: Characteristics of the study population

	AMD patients (n=26)	Controls (n=20)	All subjects (n=46)	p-value
Age (mean ± SD)	65 ± 8 years	60 ± 8 years	63 ± 8 years	0.079
Sex Male Female	 17 (65.4%) 9 (34.6%)	 9 (45%) 11 (55%)	 26 20	0.279

Table 7: Results of restriction digestion for the three SNPs showing band distribution and size

	Wild type homozygous	**Risk homozygous**	**Heterozygous**
CFH **rs1061170**	TT	CC	TC
	Two bands	One band	Three bands
	181+60bp	241bp	241+181+60bp
ARMS2 **rs10490924**	GG	TT	TG
	Two bands	One band	Three bands
	259+190bp	449bp	449+259+190bp
HTRA1 **rs11200638**	GG	AA	GA
	Two bands	One band	Three bands
	246+139bp	385bp	385+246+139bp

Table 8: Genotype and allele frequencies of patients and controls in *CFH* rs1061170

Genotypes	AMD cases (n=20)	Controls (n=15)	χ^2	p-value	OR (95%CI)
CFH rs1061170 Genotype					
TT	4 (20%)	10(66.7%)			1.0 (ref)
TC	11 (55%)	5 (33.3%)	4.82	0.02811	5.5 (1.145-26.412)
CC	5 (25%)	0			
CC + TC	16 (80%) HW p=1.0	5 (33.3%) HW p=1.0	7.78	0.00529	8 (1.726-37.090)
Allele frequency					
C	0.53 +/- 0.075	0.17 +/-0.061	9.43	0.00214	5.526 (1.762-17.336)

HW: Hardy Weinberg Equilibrium, OR: Odds ratio, CI: Confidence interval

Table 9: Genotype and allele frequencies of patients and controls in *ARMS2* rs10490924

Genotypes	AMD cases (n=24)	Controls (n=20)	χ^2	p-value	OR (95%CI)
ARMS2 rs10490924 Genotype					
GG	6 (25%)	14 (70%)			1.0 (ref)
TG	12 (50%)	6 (30%)	5.11	0.02380	4.667 (1.187-18.352)
TT	6 (25%)	0			
TT+TG	18 (75%)	6 (30%)	8.91	0.00284	7 (1.852-26.461)
	HW p=1.0	HW p=1.0			
Allele frequency					
T	0.50 +/- 0.072	0.15 +/-0.051	11.90	0.00056	5.667 (2.011-15.969)

HW: Hardy Weinberg Equilibrium, OR: Odds ratio, CI: Confidence interval

Table 10: Genotype and allele frequencies of patients and controls in *HTRA1* rs11200638

Genotypes	AMD cases (n=24)	Controls (n=15)	χ^2	p-value	OR (95%CI)
HTRA1 rs11200638 Genotype					
GG	6 (25%)	10 (66.7%)			1.0 (ref)
GA	15 (62.5%)	5 (33.3%)	5.14	0.02334	5 (1.195-20.922)
AA	3 (12.5%)	0			
AA+GA	18 (75%)	5 (33.3%)	6.62	0.01006	6 (1.456-24.733)
	HW p=1.0	HW p=0.403			
Allele frequency					
A	0.44 +/-0.061	0.17 +/-0.061	6.09	0.01357	3.889 (1.273-11.880)

HW: Hardy Weinberg Equilibrium, OR: Odds ratio, CI: Confidence interval

Table 11: combination of genotypes of ARMS1 and *HTRA1* in patients and controls

			HTRA1		
			GG	GA	AA
AMD cases	ARMS1	GG	6 (27.3%)	0	0
		TG	0	11 (50%)	0
		TT	0	2 (9%)	3 (13.6%)
Controls	ARMS1	GG	10 (66.7%)	1 (6.7%)	0
		TG	0	4 (26.7%)	0
		TT	0	0	0

Table 12: Genotype and allele frequencies of patients and controls in carriers of the (GT+AG) *ARMS2, HTRA1* combination

Genotypes	AMD cases (n=20)	Controls (n=14)	χ^2	p-value	OR (95%CI)
HTRA1 rs11200638 Genotype					
GG+GG	6 (30%)	10 (71.4%)			1.0 (ref)
GA+TG	11 (55%)	4 (28.6%)	4.01	0.04513	4.583 C.I.=[0.995-21.119]
AA +TT	3 (15%)	0			
	HW p=1.0	HW p=1.0			
Allele frequency					4.435
A+T	0.42 +/- 0.073	0.14 +/-0.060	6.14	0.01320	(1.296-15.176)

HW: Hardy Weinberg Equilibrium, OR: Odds ratio, CI: Confidence interval

Table 13: Independent laboratories conducting genetic testing for AMD

Laboratories offering clinical testing	Sequence analysis of the entire coding region	Targeted mutational analysis	Deletion/ duplication analysis	Website
Molecular Genetics Laboratory Salt Lake City, UT		✓		http://www.aruplab.com/guides/ug/tests/0051674.jsp
Bay Area Genetic Laboratory Hamilton, Ontario, Canada		✓		http://www.bagl.ca/
CeGaT GmbH Tuebingen, Germany	✓			http://www.cegat.de/
Molecular Genetics Martinsried, Bayern, Germany		✓		http://www.medizinische-genetik.de/
UCD DNA Diagnostic Laboratory Aurora, CO	✓			http://www.ucdenver.edu/academics/colleges/medicalschool/programs/genetics/Pages/DenverGenetics.aspx

Medical Genetics Unit Paterna, Comunidad Valenciana, Spain	✓			https://www.sistemasgenomicos.com/web_sg/webing/areas-biomedicina-ugm.php
Laboratory of Human Genetics Warsaw, Mazowieckie, Poland		✓		http://nzoz.genomed.pl/index.php?str=dim&lng=en
Advanced Diagnostic Laboratories (ADx) Denver, CO		✓		http://www.nationaljewish.org/professionals/clinical-services/diagnostics/adx/
Casey Eye Institute Molecular Diagnostic Laboratory Portland, OR	✓			http://www.ohsu.edu/xd/health/services/casey-eye/diagnostic-services/cei-diagnostics/index.cfm
Molecular Diagnostics and BioBanking Marshfield, WI	✓		✓	http://www.preventiongenetics.com/
Molecular Genetics Laboratory San Juan Capistrano, CA		✓		http://www.questdiagnostics.com/home.html

Sequenom Center for Molecular Medicine - Grand Rapids Grand Rapids, MI		✓		http://www.sequenomcmm.com/