Mariana Santos Vidal Tomás

Copy Number Variation Analysis in Retinal Angiomatous Proliferation

Master in Cellular and Molecular Biology

July 2016

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Faculty of Sciences and Technology of University of Coimbra

Master in Cellular and Molecular Biology

Copy Number Variation Analysis in Retinal Angiomatous Proliferation

Mariana Santos Vidal Tomás

Dissertation presented to the University of Coimbra in order to obtain the MSc degree in Cellular and Molecular Biology, performed under the scientific supervision of Professor Isabel Marques Carreira, Associated Professor with Aggregation of Faculty of Medicine of the University of Coimbra and co-orientation of Professor Maria Carmen Alpoim, Associated Professor of Faculty de Sciences and Technology of University of Coimbra.

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“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.”

Marie Curie

O júri:

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ABSTRACT

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly in developed countries. Clinically, AMD is a progressive disease of the macula, the central region of the retina, which usually starts by early asymptomatic stages, but can progress to the late blinding forms of the disease – geographic atrophy and choroidal neovascularization (NV). Among the last, stands out retinal angiomatous proliferation (RAP), a NV subtype characterized by the growth of abnormal vessels beginning in the retina, which presents the most reserved prognosis. AMD's etiology is multifactorial, combining genetic factors, which account for up to 70% of the reported cases, and non-genetic risk factors, such as smoking and ethnicity. Most genetic alterations associated with AMD incidence and progression are single nucleotide polymorphisms (SNPs) in *CFH* (chromosome 1q31.3) and *ARMS2/HTRA1* (chromosome 10q26) genes. Moreover, copy number variations (CNVs) involving *CFHR3* and *CFHR1* genes have also been extensively reported.

This is a cross-sectional study, aiming to evaluate copy number variation within the genes most commonly associated with AMD in patients with RAP. Taking advantage of the characteristics of MLPA (multiplex ligation probe amplification) technique, SALSA probemix P236 (MRC-Holland) was adapted to this project which pertained 99 samples, 32 RAP patients and 67 controls.

Of all evaluated genes and considering the studied cohort, the only one with significant differences between RAP and controls was *ARMS2* SNP A69S (rs10490924). The presence of this genetic variation is therefore a potential biomarker of RAP development prognosis. Whereas, the copy number variation in *CFHR3* and *CFHR1* as well as SNPs also evaluated by P236 did not show much implication with RAP.

These preliminary results contribute to a better understanding of RAP's genetic background being one further step towards a new diagnostic approach of this AMD subtype and ultimately, to prediction of this AMD's subtype development.

Key Words: Age-related macular degeneration, RAP, MLPA, CNV, *ARMS2*

RESUMO

Degenerescência macular relacionada com a idade (AMD) é a principal causa de cegueira em idosos nos países desenvolvidos. Clinicamente, AMD traduz-se por uma doença progressiva da mácula, a região central da retina. Esta está associada a estádios iniciais assintomáticos que podem progredir para estádios mais avançados da doença, com perda de visão: atrofia geográfica e neovascularização coroidal. Destas, destaca-se a proliferação angiomatosa da retina (RAP), um subtipo de neovascularização coroidal caracterizado pelo crescimento anormal de vasos sanguíneos a partir da retina e pelo prognóstico mais reservado. A AMD possui uma etiologia multifactorial, combinando fatores genéticos, que correspondem a cerca de 70% dos casos reportados, com fatores não genéticos, como por exemplo tabagismo e etnicidade. A maioria das alterações genéticas associadas com a incidência e progressão da AMD são polimorfismos de um só nucleótido (SNPs) nos genes *CFH* (cromossoma 1q31.3) e *ARMS2/HTRA1* (cromossoma 10q26). Para além destes, também têm sido reportadas variações no número de cópias dos genes *CFHR3* e *CFHR1*.

Este projeto é um estudo transversal cujo objetivo é avaliar a variação do número de cópias nos genes mais comummente associados com AMD, em doentes com RAP. Devido às vantagens da técnica de MLPA, o painel de sondas P236 (MRC-Holland) foi usada neste projeto para avaliação de 99 amostras: 32 doentes com RAP e 67 controlos.

De todos os genes analisados e para a amostragem testada, apenas foram detetadas diferenças significativas no SNP A69S (rs10490924) do gene *ARMS2*. A presença desta variação pode assim ser considerada um potencial biomarcador do desenvolvimento da RAP. Por outro lado, a variação do número de cópias nos genes *CFHR3* e *CFHR1*, bem como os restantes SNPs avaliados pela *probemix* P236 não demostraram possuir grande correlação no desenvolvimento de RAP.

Estes resultados preliminares contribuíram para um conhecimento mais aprofundado da base genética associadas à RAP, podendo ser um passo no desenvolvimento de uma nova técnica de diagnóstico e eventualmente, de prever o desenvolvimento deste tipo de AMD.

Palavras-chaves: Degenerescência macular com a idade, RAP, MLPA, CNV, *ARMS2*

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ABBREVIATION INDEX

aCGH	Array Comparative Genomic Hybridization
aHUS	Atypical Hemolytic Uremic Syndrome
AMD	Age-Related Macular Degeneration
APOE	Apolipoprotein E
ARMS2	Age-Related Maculopathy Susceptibility 2
BMI	Body Mass Index
BrM	Bruch's Membrane
C2-5	Complement Component 2-5
CFB	Complement Factor B
CFH	Complement Factor H
CFHR 1-5	Complement Factor H Related 1-5
CFI	Complement Factor I
CI	Confidence Interval
CNV	Copy Number Variation
CNP	Copy Number Polymorphism
CRP	C-Reactive Protein
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid
FDA	Food and Drug Administration
FFA	Fundus Fluorescein Angiography
GA	Geographic Atrophy
GWAS	Genome-Wide Association Studies
HTRA1	High Temperature Requirement Factor A serine peptidase 1
ICG	Indocyanine Green Angiography
IPA	Isopropyl Alcohol
LCR	Low-Copy Repeats
LIPC	Hepatic Lipase C
MAC	Membrane Attack Complex
MESA	Multi-Ethnic Study of Atherosclerosis
MHC	Major Histocompatibility Complex
MLPA	Multiplex Ligation-dependent Probe Amplification

NAHR	Non-Allelic Homologous Recombination
NHNES	National Health and Nutritional Examination Survey
NV	Neovascular AMD
OMIM	Online Mendelian Inheritance in Man
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PCV	Polypoidal Choroidal Vasculopathy
PED	Pigment Epithelial Detachment
RAP	Retinal Angiomatous Proliferation
RCA	Regulators of Complement Activation
ROS	Reactive Oxygen Species
RPE	Retinal Pigmented Epithelium
SNP	Single Nucleotide Polymorphism
TIMP3	Tissue Inhibitor of Metalloproteinases 3
VEGFA	Vascular Endothelial Growth Factor A
WHO	World Health Organization

1 INTRODUCTION

1.1 THE HUMAN EYE

The human eye is a complex and intricate structure that allows us to perceive the surrounding world. After entering the eye, the light is focused by the lens on the retina, where photoreceptors will transduce the stimuli into chemical signals - phototransduction. These will be transmitted along the retinal neural network, to the optical nerve and finally to the brain, where it will be decoded and interpreted.[1–3]

The retina is a highly organized neural network that covers the interior of the eye and is composed by different types of neurons: photoreceptors, bipolar cells and ganglion cells. Retinal photoreceptors (rods and cones) are polarized neurons that consist of an inner and an outer segment. The inner segment contacts with the bipolar cells. On the other hand, the outer segment interacts with the retinal pigmented epithelium (RPE), comprising light-sensitive photopigments (such as rhodopsin). After performing phototransduction, these need to be regenerated, having thus, high metabolic demands. RPE, Bruch's membrane (BrM) and choroidal vasculature work as a support system for photoreceptors.[1–6]

RPE is a monolayer of epithelial cells with microvilli that ease RPE interactions with the photoreceptors. RPE's main role is to phagocyte the shed outer segments of the photoreceptors in order to recycle the photopigments. BrM is an extracellular matrix (ECM) composed of collagen and elastin that separates RPE from the choroidal vessels. BrM and RPE work as a blood-retina barrier. This barrier is of major importance to the proper retina's homeostasis since it isolates the photoreceptors and transports nutrients and oxygen from choriocapillaris to photoreceptors as well as metabolites back to the blood vessels. Behind the BrM there is a complex net of capillaries (choriocapillaris) named choroid whose main function is, as previously explained, to support the physiological needs of the photoreceptors (Figure 1).[1–4]

The central part of the retina is called the macula. The fovea, a specialized region of the retina, localizes within the macula and is responsible for central high-

acuity vision, since it is constituted by a dense population of cone photoreceptors that receive direct light (Figure 2).[1–3]

Retina progressively changes with age: there is loss of rod photoreceptors, decrease of phagocytosis by the RPE, accumulation of retinoid metabolites and BrM's thickening.[1]

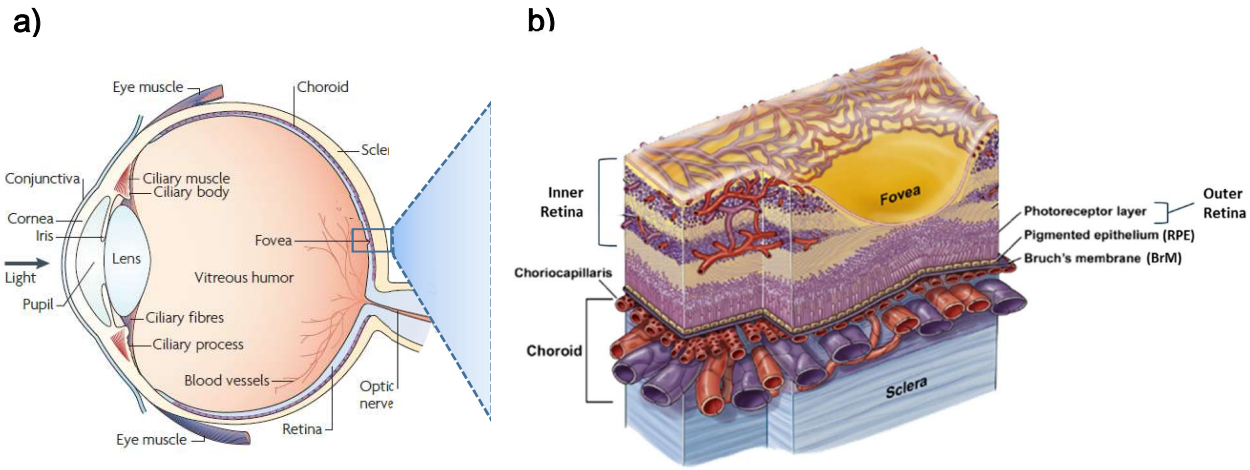


Figure 1 – Structure of the human retina. **a)** Schematic representation of the cross-section of the human eye. **b)** Schematic diagram of retina in the fovea and its support system: RPE, BrM and choroid. Adapted from Wright *et al.* (2010) and Anand-Apte *et al.* (2010).[7,8]

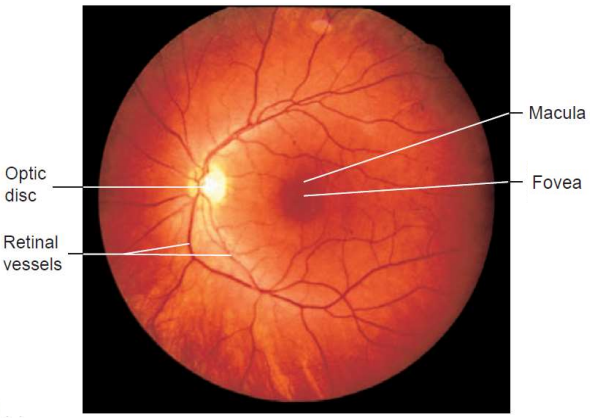


Figure 2 – Retina's fundus photograph of a healthy individual. Adapted from Seeley, Anatomy and Physiology, Sixth Edition (2004).[9]

1.2 AGE-RELATED MACULAR DEGENERATION

Age-related Macular Degeneration (AMD, OMIM #603075) is a late-onset multifactorial disease characterized by a progressive destruction of the retina's central region (Figure 3). AMD usually starts by early asymptomatic stages presenting two major hallmarks: the development of subretinal extracellular lipidic (lipofuscin) and proteic deposits, cellular debris (called *drusen*), and pigmentary abnormalities in RPE.[1,2,10–12]

Figure 3 – Schematic representation of AMD's main clinical feature, loss of central vision. Adapted from Carr *et al.* (2013).[13]

However, it can progress to the late blinding forms of the disease, which depending on the type of lesion can be classified into "dry" or "wet" (also called exudative) forms.

Dry AMD is described as geography atrophy (GA), as it is associated with a well delineated area of photoreceptor's degeneration within the retina, BrM thinning, atrophy of the RPE and exposure of the choroidal vessels beneath it.[3,4,14,15] Accumulation of lipofuscin, an auto fluorescent pigment resultant of an incomplete degradation of phagocytosed photoreceptor outer segments in RPE cells is also a feature of GA.[15] Its pathological mechanism is yet to be reported due to lack of molecular targets. So there are still no therapies approved for this subtype of AMD.[2,4]

On the other hand, exudative AMD is characterized by neovascularization, an abnormal growth of new blood vessels. This leads to fluid leakage into the retina, haemorrhage, pigment epithelial detachment (PED) and loss of the integrity of the blood-retina barrier.[4,10,12] Neovascular (NV) AMD represents the most aggressive form of AMD causing not only vision impairment, but also blindness. Furthermore, it is the only form with an FDA's approved treatment: intravitreal injections of antibody fragments that bind to vascular endothelial growth factor (VEGF) receptors, inhibiting angiogenesis.[16]

Light damage, lipid oxidation, mitochondrial impairment, complement activation and inflammation are some of the factors proposed to participate in AMD's pathogenesis.[2,7,17]

Retinal photoreceptors have high metabolic demands so they depend on the RPE phagocytosis function in order to recycle its by-products such as cholesterol esters, oxidized lipids and lipofuscin. When RPE integrity is compromised the by-products will accumulate in the retina and create *drusen*. This along with the formation of reactive oxygen species (ROS - due to mitochondria impairment) will increase RPE injury and might disrupt the blood-retinal barrier, allowing the recruitment of inflammatory cells. Consequently, there will be a stimulation in the production of angiogenic and chemotactic factors by the presence of macrophages since together with RPE, they are a major source of pro-angiogenic factors such as VEGF.[2,7,17]

1.3 Retinal Angiomatous Proliferation

At the present, AMD's subgroups are addressed based on retinal modifications, either atrophy or the presence of hemorrhages. However, due to AMD's late onset characteristics, initial asymptomatic stages and poor access to the retina, the study of the etiology of pathological changes is complex.

In the past decades, there has been an extensive debate concerning exudative AMD subtypes. In 2001, Yannuzzi and colleagues classified exudative AMD subtype's based upon the anatomical position of the origin of neovascularization in the macula (Figure 4). Thus, when the choroid is the source of pathological formation of new vessels, AMD can be divided into polypoidal

choroidal vasculopathy (PCV) and classical choroidal neovascularization, depending on the presence or absence of polypoidal structures at the terminus of these new vessels, respectively.[18,19]

Some researchers consider yet another classification: occult and classical choroidal neovascularization, depending on RPE's integrity, i.e. whether the vascularization has reached the RPE or if it is localized subretinally.[19]

On the other hand, if the vascularization begins within the retina, AMD is classified as retinal angiomatous proliferation (RAP). With the progression of the disease, new vessels can proliferate towards the inner retina (stage I – intraretinal neovascularization) or on the opposite way, into the subretinal space, causing PED due to fluid accumulation (stage II – subretinal neovascularization). In a final stage, proliferation can proceed into the choroid, causing a retinal-choroidal anastomosis and consequently massive haemorrhages (stage III) (Figure 4).[18–20]

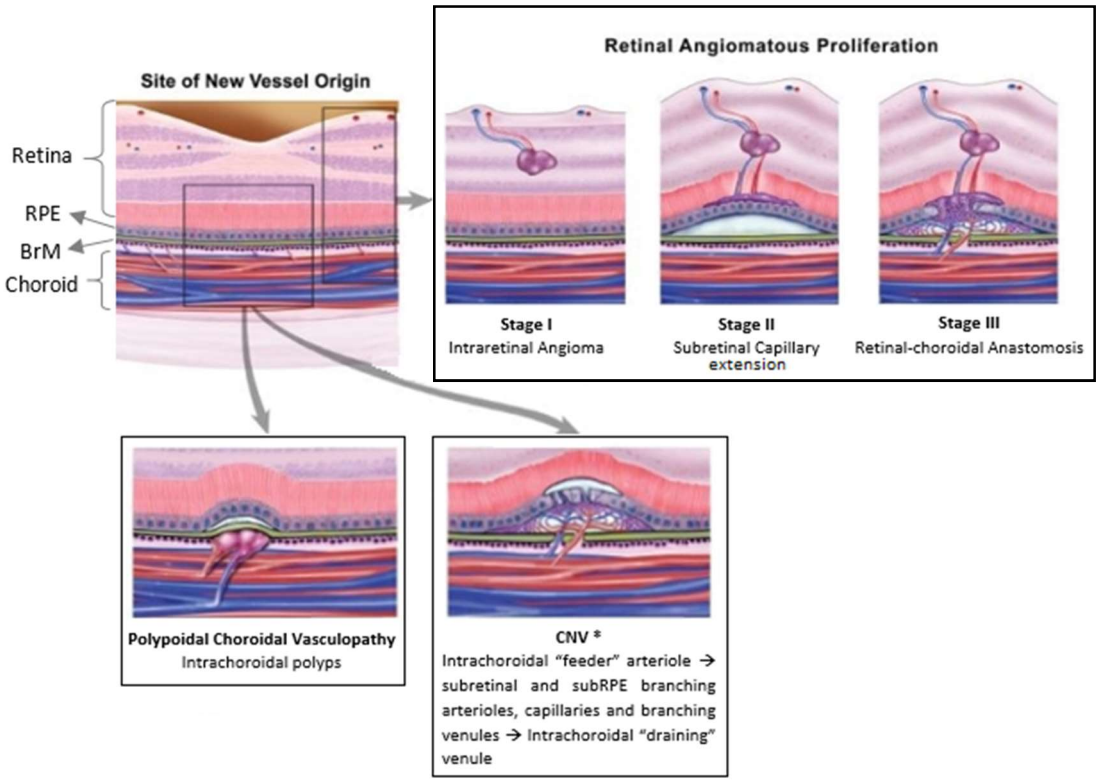


Figure 4 – Schematic representation of the pathophysiology of each exudative AMD's subtypes when compared to a healthy retina. Adapted from Koreen *et al.* (2010).[19]

*** Note:** Koreen et al. used the term CNV to address to choroidal neovascularization, which should not be confused with copy number variation.

1.4 EPIDEMIOLOGY AND RISK FACTORS

According to the World Health Organization (WHO), AMD is the third cause of vision loss worldwide (Figure 5) and the first among elderly people in developed countries.[21] Its prevalence has changed over the past 20 years, overcoming cataracts as the major cause of blindness in high-income countries.[22] Moreover, since population's average life expectancy is increasing, AMD incidence is expected to escalate in the next few years.[23]

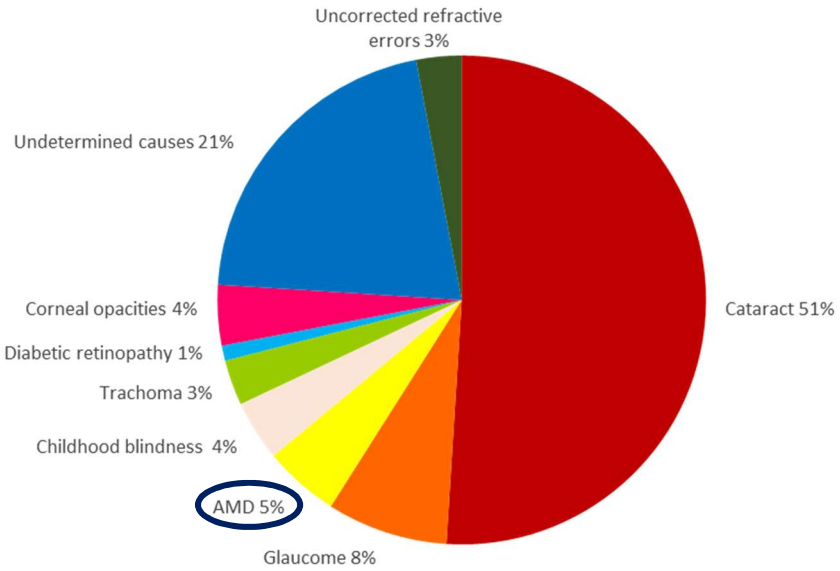


Figure 5 – Global causes of blindness worldwide, 2010. Adapted from WHO.[21]

AMD is a multifactorial disease with a complex etiology in which advanced age represents the strongest risk factor. In fact, according to the Three Continent AMD Consortium, population below 64 years do not represent a significant percentage of AMD (0.21%). Whereas after 85 years, AMD prevalence increases exponentially to 13.05%.[24]

Both environmental and genetic factors influence the risk of developing the pathology. It has been reported that genetic factors cause up to 71% of AMD cases whereas the remaining percentage is due to environmental factors.[25]

Concerning environmental factors, smoking has been extensively associated with a 2-fold increased risk of AMD development.[24,26–28] Oxidative stress is hypothesized to be the mechanism underlying this effect. Furthermore, increased body mass index (BMI), high sunlight exposure, among others have also been reported to modulate AMD's development and evolution.[3,10,29]

Multiple epidemiologic studies have addressed AMD's prevalence in different racial and ethnic groups, revealing significant variations between groups. Generally, the Caucasian population has a higher incidence of this pathology.[30,31] These conclusions might be explained due to AMD's large variability regarding risk factors.

Prevalence of late AMD have been studied in different countries throughout the years, ranging from 0.6 to 2.6% (Table 1).

Few articles have reported prevalence of dry and exudative AMD in different populations. The Three Continent AMD Consortium, reunited 14752 subjects with similar ethnicity from 3 different populations across the globe, and concluded that 4.22% of individuals older than 85 years with AMD showed "pure GA", whereas 5.76% had "pure neovascular AMD".[24] AMD's incidence in the Portuguese population has been recently analysed by Cachulo and colleagues (n=2975). They reported a 16.2% incidence of general AMD cases, 0.44% of exudative AMD and 0.27% of GA.[32]

Concerning RAP lesions, Cohen and colleagues (n=207) reported an incidence of 15.1% of neovascular AMD cases.[19] The Retina Study Group summarized RAP prevalence in different studies, in different populations (Table 2).[15] Different diagnostic techniques, cohort sizes and subjects age could explain major differences among studies.

Overall, further studies should be performed to assess AMD subtype's prevalence since there is a major discrepancy among statistic results.

Table 1 – AMD's prevalence in different populations assessed by various studies around the globe.[15]

Study	Country	Number of Subjects (n)	Age	Prevalence of late AMD (%)
Baltimore Eye Study	USA	2518	≥ 40 years	1.23
Beaver Dam Eye Study	USA	4752	≥ 40 years	1.64
Multi-Ethnic Study of Atherosclerosis	USA	2315	45 – 84 years	0.6
Blue Mountain Eye Study	Australia	3632	≥ 50 years	2.06
Rotterdam Study	Netherlands	6774	≥ 55 years	1.65
Coimbra Eye Study	Portugal	2975	≥ 55 years	0.67

Table 2 – RAP's prevalence assessed by different studies, in different populations.[15]

Country	Year	RAP prevalence (%)
France	1995	26.8
Israel	2002	28
USA	2002	5
Portugal	2004	9.4
Italy	2008	25

1.5 DIAGNOSIS

AMD's main target, the retina, is a tissue to which there is poor access to, making it difficult to study.

Currently, AMD clinical diagnosis is performed through retinal imaging, since there is still no test aiming to evaluate genetic markers. There are multiple

diagnostic techniques, but the routinely method is fundus fluorescein angiography (FFA). FFA is an invasive technique that evaluates choroidal and retinal blood flow. Fluorescein, a yellow dye, is injected intravenously and then retinal photographs are taken. In neovascular AMD, due to the abnormal growth of vessels, fluorescein will leak, causing a hyperfluorescence region, making it very useful to distinguish GA from exudative AMD. (Figure 6).[15,16]

Indocyanine green angiography (ICG) is similar to FFA, except it uses a dye with different characteristics, which improves the visualization of the choroidal circulation and morphology.[19,33] Therefore, ICG is more useful in the diagnosis of RAP.

The drawbacks of these methods are that the interpretation of the results depends on the clinic's sensibility (having to be analysed by a minimum of two experts) and it only offers a diagnosis of the disease, not allowing an anticipation of its development.

AMD is one of the major causes of blindness worldwide, with great impact on life's quality and high social and economic burden.[34] Since only expensive and invasive treatments are available, it is of utmost importance to identify genetic markers for AMD such as single nucleotide polymorphisms (SNPs) or copy number variations (CNVs) that allow to evaluate the risk of AMD progress at a pre-symptomatic stage.

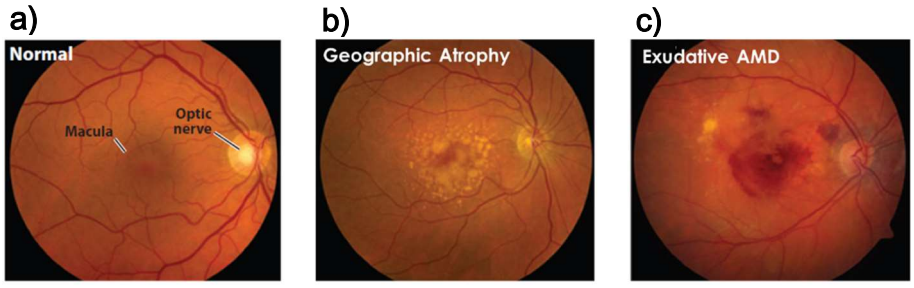


Figure 6 – Fundus photographs of **a)** a healthy individual, **b)** an individual with geographic atrophy (characterized by accumulation of drusen – yellow patches) and **c)** a person with exudative AMD (characterized by a hemorrhage within the macula). Adapted from Fritsche *et al.* (2014).[1]

1.6 GENETIC BACKGROUND

As previously mentioned, AMD's etiology is multifactorial, combining non-genetic risk factors, such as smoking and ethnicity, and genetic factors which account for up to 70% of the reported cases.[25]

The major role of genetics in AMD's development was established through twin studies, familial aggregation, and linkage analysis.[11,12,29,35] Twin studies demonstrated a high concordance for AMD in monozygotic twins, thus revealing AMD's hereditability.[12,29] On the other hand, linkage studies analyze multi-generational families in order to examine regions of the genome that present the linked markers observed in affected individuals.[12,29,35,36]

After verifying genetics' contribution to AMD, the next step was to describe, specifically, which genes were involved in it. Initially researchers began to investigate genes that were previously described in pathologies similar to AMD, such as Stargardt's disease.[12] However, since the study of candidate genes did not achieve great results, association studies began to be performed.

Association studies compare specific alleles in two populations, one comprising diseased individuals and other with healthy ones.[29,37] More recently, due to technological advances, genome-wide association studies (GWAS) allowed the possibility to evaluate the whole genome, instead of just specific regions.[3] Thus, GWAS is particularly useful in complex diseases such as AMD. These studies aim to find a connection between a higher frequency of a genetic variation and the pathology in question.[29,37]

All genetic studies discussed previously allowed the identification of some AMD susceptibility genes. Identification of AMD susceptibility genes, together with genetic variants behind AMD pathology might increase our ability to predict the risk of developing AMD. These genetic biomarkers could also help to develop new putative targets to new therapeutic approaches.

A major hallmark of AMD is drusen formation having been described to harbour major complement components encoded by AMD susceptibility genes.[38,39] Hence, deregulations of the complement pathway are thought to be associated with the development of AMD. Variations in complement factor H *(CFH),* complement factor H related *(CFHR)* genes and complement component 2 *(C2)* genes have been implied in complement abnormal behaviour. Variations

in age-related maculopathy susceptibility 2 *(ARMS2)* have also been extensively associated with this disease.

1.6.1 Regulators of Complement Activation (RCA) locus

The complement system is an innate mechanism of immune response that acts against pathogens, eliminates immune complexes and apoptotic cells and promotes inflammation. Upon activation, formation of a complement factor 3 (C3) convertase which cleaves C3 into C3a and C3b in order to form complement factor 5 (C5) convertase, induces ultimately the formation of the terminal membrane attack complex (MAC). There are three pathways that lead to complement activation: alternative, classical and lectin. All three lead to formation of C3 convertase.[40]

In the alternative pathway, C3 is spontaneously hydrolysed into C3(H_2O) which will interact with complement factor B (CFB) leading to the formation of a C3 convertase that will cleave C3 into C3a and C3b. This last molecule is very similar to C3(H_2O) so it will also bind to CFB generating more C3 convertase and creating a positive feedback that will continuously activate the alternative cascade (Figure 7).[40,41] Another characteristic of the alternative pathway is its lack of discrimination towards pathogens and host cells, therefore it as to be tightly regulated.[42]

Complement Factor H (CFH). *CFH* (OMIM #134370) encodes a major suppressor of the alternative complement cascade. CFH not only increases the dissociation of C3 convertase complex but also acts as a cofactor for complement factor I (CFI), inactivating C3b and therefore inhibiting the complement activation (Figure 7).[42,43] Plus, CFH binds to host cellular surfaces, C-reactive protein (CRP) and heparin, which facilitates its interaction with C3b.[44–46]

AMD's most commonly described genetic variation is a SNP in *CFH* gene that substitutes a thymine by a cytosine, leading to a change in the amino acid in position 402 from a tyrosine to a histidine (Y402H, rs1061170).[47,48] This alteration

diminishes CFH's binding properties and consequently, its ability to regulate the alternative pathway leading to an abnormal activation of the complement cascade and contribute to the accumulation of drusen.[2,40,42,47,49,50] It is associated with a 2,5 to 5,5-fold greater risk of AMD.[51]

CFH is located in chromosome 1q31.3 region, which is referred to as the regulators of complement activation (RCA) block that also comprises the complement factor H related *(CFHR)* genes (Figure 8).[52]

Complement Factor H Related 1–5 (CFHR1-5). These are five genes with high homology that individually encode different plasma proteins. Some authors suggest they are ancestrally related and may have arisen from exon shuffling and duplication of *CFH* during evolution.[15,40,52,53] Furthermore, they are flanked by short DNA sequences that share more than 96% of similarity between them - low-copy repeats (LCRs) - potentiating genomic rearrangements (Figure 8).[52,54]

CFHR1 (OMIM #134371) and CFHR3 (OMIM #605336) can both work as inhibitors and/or as activators of the complement cascade. CFHR1 solely inhibits MAC assembly and both CFHR1 and CFHR3 inhibit C5 convertase activity decreasing the levels of C5a, which inhibits the complement pathway.[53,55] On the other hand, it has also been reported that CFHR1 and CFH have a very similar C3 binding region and that CFHR3 acts as cofactor for CFI, acting both as CFH inhibitors, thus leading to complement activation.[52,53,55,56] There is still controversy concerning CFHR1 and CFHR3 function and little is known about CFHR2, CFHR4 and CFHR5 functions.

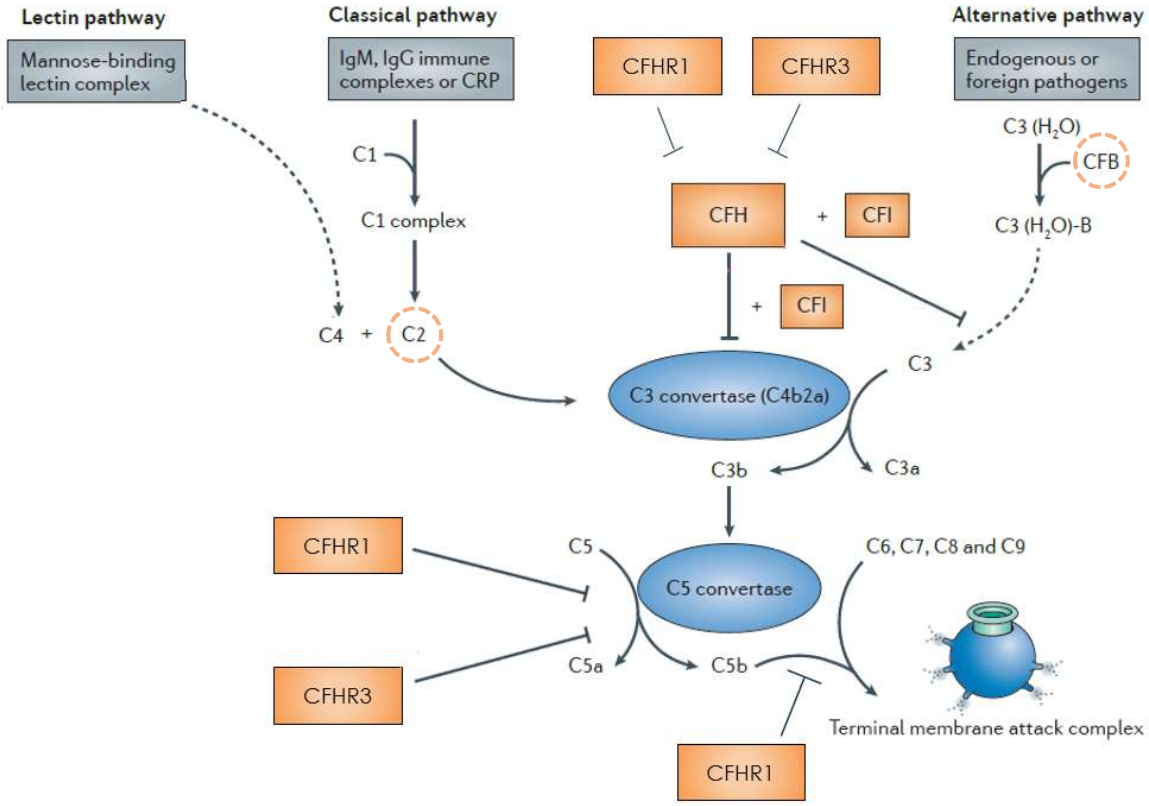


Figure 7 – Representative scheme of the three complement pathways' components and its regulators. Orange squares represent regulatory proteins of the complement cascade but that do not participate directly on the cascade. CFH and CFI are inhibitors of the complement system, whereas CFB and C3 stimulate it, leading to an immune response. C2 is also an activator of complement system, specifically the classical pathway. CFHR1 and CFHR3 are both activators and inhibitors of the complement cascade. Adapted from Zhang *et al.* (2012).[57]

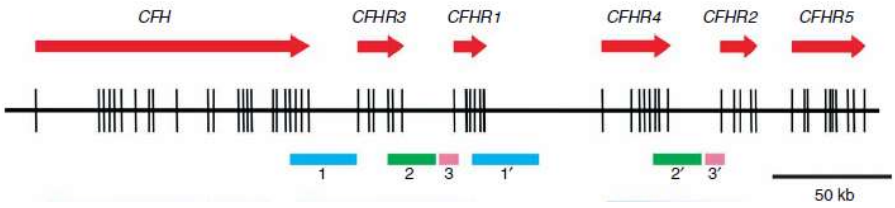


Figure 8 – RCA locus in chromosome 1q31.3. This region includes *CFH* and *CFHRs* genes (red arrows) which are flanked by DNA segments with high homology between them (only the three major LCRs pairs are described in blue, green and pink). The bars represent exons of each gene. Adapted from Hughes *et al.* (2006).[58]

1.6.2 Complement component 2 (C2)

Complement component 2 (*C2*) (OMIM #613927) integrates the lectin and the classical activation pathways and is encoded by the homologous gene located in chromosome 6p21.3, specifically the major histocompatibility complex class (MHC) III region.[29,50,59] It activates the complement system by interacting with C4 to form a C3 convertase (Figure 7).

C2 and *CFB* are neighbouring genes, separated by only 500 base pairs, and as such it has been difficult to determine which one contributes to AMD susceptibility. Their deregulation has been reported to reduce the risk of AMD.[59–62] C2 and CFB were found to be expressed in the neural retina, RPE and choroid. This emphasize the notion that an altered function in these genes may cause a decreased activation of the complement pathway in the eye.[59]

1.6.3 Age-Related Maculopathy Susceptibility 2 (ARMS2)

Age-Related Maculopathy Susceptibility 2 *(ARMS2)* (OMIM #611313) is located in chromosome 10q26.1 region and is a neighbouring gene of high temperature requirement factor A1 *(HTRA1)* (Figure 9).

ARMS2 functional role is still unknown but its presence has been reported within the mitochondria of photoreceptors.[63] The presence of the SNP A69S alters the protein's conformation deregulating its function. Thus, it is hypothesized that the *ARMS2* SNP A69S might lead to mitochondrial impairment and therefore cause oxidative stress.[50,63,64]

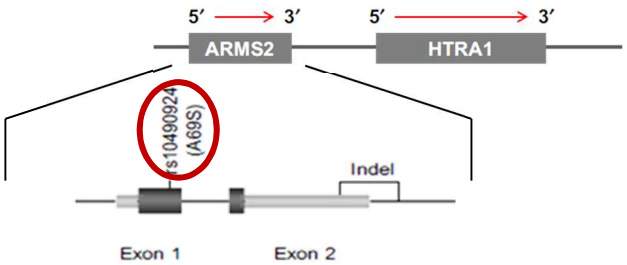


Figure 9 – Schematic representation of *ARMS2* gene in chromosome 10q26.1 and its most associated SNP A69S. Adapted from Wang (2014).[65]

The described genes are the most associated with AMD pathology even though *CFI*, *C3*, vascular endothelial growth factor A *(VEGFA),* hepatic lipase *(LIPC),* apolipoprotein E *(APOE),* metalloproteinase inhibitor 3 *(TIMP3),* together

with many other, were also reported as AMD susceptibility genes.[50] However, they still are a topic of extensive debate and additional studies are required to effectively validate their role in AMD.

Table 3 – Summary of the main genes and their contribution to AMD disease.

Genes	Location	Function	Alteration	AMD contribute	References
ARMS2	10q26.1	Possible antioxidant function	rs10490924	Increase risk	[63,64,66]
CFH	1q31.3	Inhibition of the complement pathway	rs1061170	Increase risk	[66–68]
CFHR3-CFHR1	1q31.3	Regulators of the complement pathway	CNP147	Protective	[53,56,58,67]
C2	6p21.3	Regulator of the complement pathway	rs9332739	Protective	[59–61]

1.7 COPY NUMBER VARIATION

Most reports on genetic influence in AMD susceptibility are focused on SNPs in *CFH* and *ARMS2/HTRA1* genes. However, these only account for about 60% of AMD hereditability.[69,70] Thus, there must be another genetic contribution underlying AMD pathology such as CNVs. These represent a common genetic structural variation which alters the number of copies of a DNA segment (longer than 1 kb).[11,50,71] This alteration might arise from non-allelic homologous recombination (NAHR), a mechanism in which there is a misalignment of homologous chromosomes due to the existence of LCRs (Figure 10).[54]

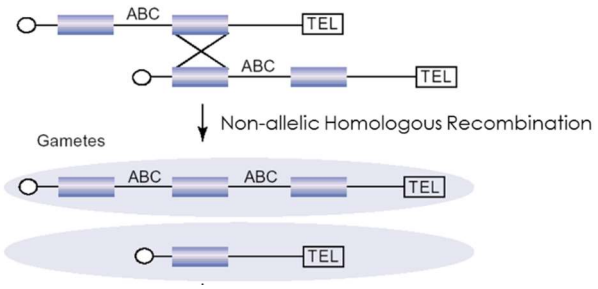


Figure 10 – Non-Allelic Homologous Recombination. Due to LCRs' (blue blocks) high similarity, chromosomes might misalign during meiosis, leading to erroneous crossing-over and consequently to duplications and deletions. Adapted from https://eichlerlab.gs.washington.edu/research.html (accessed on 29/06/2015).

CNVs are associated with several pathologies since they can alter gene dosage by interrupting or deleting a gene sequence or creating a fusion gene, among other mechanisms.[11,70] When present in more than 1% of the population, CNVs can be addressed as copy number polymorphisms (CNPs), representing a benign polymorphic variation.

RCA locus is rich in LCRs and the genes located within it show high homology between them, making it a favourable region to rearrangements. Among these, the deletion of *CFHR1* and *CFHR3* is the most reported alteration in AMD, presenting a protective effect of 1.75- to 4-fold.[49,53,58,72–74] However, this deletion is also associated with increased risk of developing atypical hemolytic uremic syndrome (aHUS).[75] These contrary effects might be explained due to CFHR1 and CFHR3's ability to both inhibit and stimulate the complement pathway and therefore, inflammation.

CNVs are widely associated with multiple pathologies. Array Comparative Genomic Hybridization (aCGH) and Multiplex Ligation-dependent Probe Amplification (MLPA) are two of the most common techniques applied in detection of CNVs.[76] aCGH allows a genome-wide screen evaluation of CNVs and due to that characteristic it is a very expensive technique. Hence, when specific genes are known to be associated with a certain disease, MLPA seems a good alternative in research. In fact, there is a SALSA probemix for MLPA, which comprises probes for the aforementioned genetic variations involved in AMD's development.

1.8 Multiplex Ligation-dependent Probe Amplification

Multiplex Ligation-dependent Probe Amplification (MLPA) is a molecular technique which allows CNV's evaluation of multiple DNA sequences in the same reaction. Even though it is based on multiplex PCR assay, both methods have significant differences concerning the amplifying product (in the multiplex PCR assay what is amplified is the DNA sample, whereas in MLPA what is amplified are the probes that hybridize with each DNA sequence) and the number of primers used (multiplex PCR assay needs one pair for each DNA target sequence while MLPA uses the same pair of primers to all probes).[76]

Each MLPA probe is composed by two oligonucleotides half-probes (5' and 3') that comprise, each, two sequences: a hybridization sequence (which is complementary to a specific DNA sequence) and a primer sequence (that allows the simultaneous amplification of multiple targets). Furthermore, one of the half-probes contains a stuffer sequence allowing separation of different size probes during capillary electrophoresis.[76]

Initially, DNA double helix denatures, allowing the access and hybridization of half-probes with the specific target sequence. Then, a DNA ligase will bind the two halves, forming the probe that will be amplified. The PCR products will then be separated by the size given by the stuffer sequence through capillary electrophoresis. Finally, each probe's fluorescence peak will be measured, normalized and compared with reference samples (Figure 11).[77]

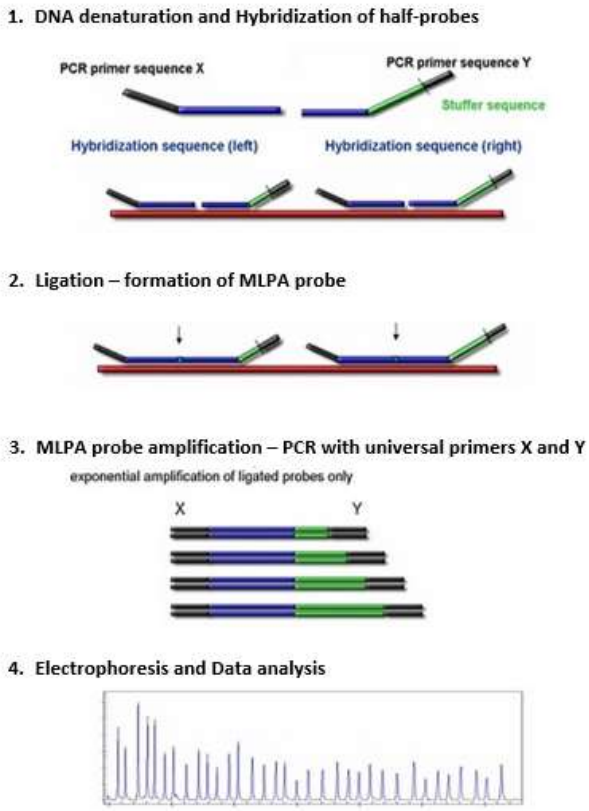


Figure 11 – Major steps in a MLPA reaction. **1)** DNA denaturation and hybridization of half-probes with the respective DNA sequences; **2)** Ligation reaction and formation of a MLPA probe; **3)** MLPA probes' amplification; **4)** Separation by capillary electrophoresis. Adapted from MRC-Holland - MLPA ® General Protocol (2014).[77]

One of the multiple advantages of this technique is the analysis of multiple target sequences using only one pair of primers, since what is amplified is the ligated probes instead of the DNA sequence. Another advantage is its sensitivity as the half-probes only bind and amplify in the presence of a perfect match between them and the DNA target sequence. This characteristic allows the detection of the presence or absence of SNPs (when we have specific probes to detect it, i.e. a probe that only amplifies when the rare allele is present). When it is not the case, deletions should be confirmed by another method such as DNA sequencing because, due to the technique sensitivity, an apparent deletion could be in fact a SNP or a point mutation.[76,77]

However, this assay has some disadvantages like the failure to detect balanced rearrangements, since there is no variation in the DNA quantity but in the order of DNA sequence. Also, since it is a targeted technique it only informs variations in the specific genes that hybridize with the MLPA probes, excluding important genes that have been associated with the disease but that are not included in the probemix. Furthermore, MLPA as comparative technique demands the use of controls with a defined and well characterized number of copies.[76,77]

2 AIMS

AMD is one of the major causes of blindness worldwide having a great impact on life's quality. Usually, patients with early AMD are asymptomatic, so when the disease is clinically detected it has already caused some damage in vision acuity. Considering this, and since only expensive and invasive treatments are available, prevention of this progressive disease is of utmost importance. However, due to AMD's late onset, high variability and multifactorial characteristics there still exist many unanswered questions that should be addressed, namely, AMD's genetic background.

Nowadays, the only diagnosis of AMD subtypes is performed through retinal imaging, an invasive technique that relies on the evaluation by a minimum of 2 clinicians. So, it is important to develop new diagnose procedures. Since MLPA is a quick, robust and relatively economic technique that only requires a DNA extracted from blood sample, it might be a promising approach to a putative test to eventually diagnose RAP.

Considering this, the main goal of this project is to detect specific genetic variations through MLPA and associate them with the development of RAP within a section of the Portuguese population.

3 MATERIAL AND METHODS

3.1 PATIENTS

This study was conducted with the approval of the ethics committee of the Faculty of Medicine of the University of Coimbra and with the informed consent of all patients undergoing this study. The Association for Innovation and Biomedical Research on Light and Image recruited a total of 99 patients (59-95 years), which were submitted to retinal imaging in order to evaluate the presence or absence of disease (RAP or control). It was verified that 32 of the 99 patients presented the disease and so the other 67 were used as the control group for this study. Afterwards, two EDTA blood collection tubes with approximately 9 mL of peripheral blood were collected from each patient and sent to the Cytogenetics and Genomics Laboratory, Faculty of Medicine of University of Coimbra, were all the subsequent experiments were performed.

3.2 SAMPLES

Upon arrival, all blood tubes were identified by the laboratory codes and stored at 4ºC. In order to separate the blood components (blood fractionation) each tube was centrifuged 10 minutes at 1600 x g. Thus 3 phases were obtained: erythrocytes (red blood cells – that are denser and so accumulate at the bottom of the tube); buffy coat (a thin layer of white blood cells and platelets) in the middle and plasma (a clear yellowish solution) above it (Figure 12). DNA is extracted from nucleated cells located within the buffy coat, i.e. from white blood cells.

Two buffy coats from each patient (800 – 1600 µL) were collected. One was stored at -20ºC and the other was used to extract DNA.

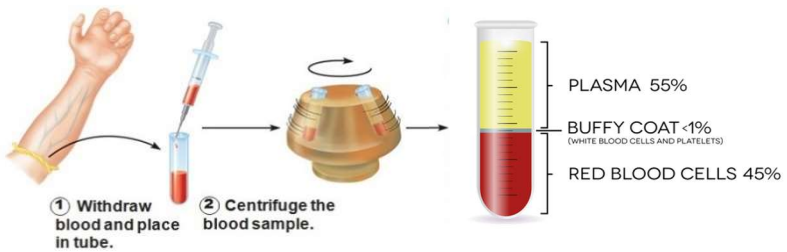


Figure 12 – Sample's collection and blood fractionation. Adapted from Marieb and Hoehn, Human Anatomy & Physiology - 9th Edition (2013).[78]

3.3 DNA EXTRACTION

Genomic DNA was extracted from 800 – 1200 µL of buffy coat using commercial extraction kits: *Jetquick blood and cell culture DNA Midi Spin Kit* (Genomed, Löhne, Germany) and *GeneCatcher™ gDNA 3-10 mL Blood Kit (Invitrogen, Carlsbad, USA),* according to the manufacture's recommendations.[79,80]

3.3.1 Jetquick blood and cell culture DNA Midi Spin Kit (Genomed, Löhne, Germany)

The standard protocol for this kit requires a volume of 3 mL of whole blood sample, however in order to maximize extraction's yield and purification, 800 – 1200 µL of buffy coat were used. The remaining volume was completed with PBS 1x.

The first steps of this procedure aim to lyse cells and remove the major contaminants such as proteins and RNA. Ethanol is added to the lysate in order to precipitate DNA. The sample is then applied to the spin columns that contain silica membranes, to which the DNA will bind. The next steps will wash the membrane and remove any remaining impurities. Finally, DNA is eluted with 800 µL of 10 mM Tris-HCl, pH 8.5 (Figure 13).

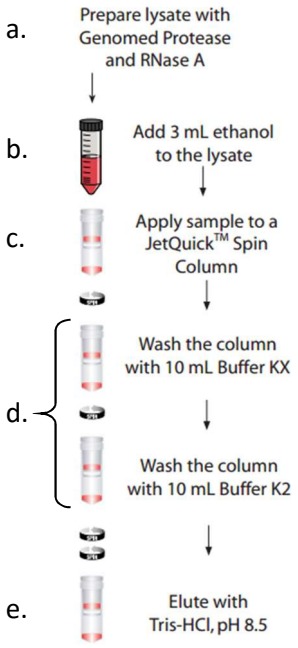


Figure 13 - Major steps of DNA extraction using Jetquick blood and cell culture DNA Midi Spin Kit (Genomed, Löhne, Germany). Adapted from JetQuick™ Genomic DNA Purification Kits (2012).[79]

3.3.2 GeneCatcher™ gDNA 3-10 mL Blood Kit (Invitrogen, Carlsbad, USA)

Due to discontinuation of the previous kit, a magnetic bead-based protocol was adopted.

This kit allows us to extract DNA from 3 to 10 mL of blood by adapting the reagent volumes. Thus, as described for the other extraction kit, 800 – 1200 µL of buffy coat were used and PBS 1x was added to a total volume of 5 mL.

The protocol has 4 main steps: DNA capture and purification, beads washing and finally, DNA elution (Figure 14). Summarily, cells are lysed in order to allow the DNA to bind to the magnetic beads. After digesting the remaining proteins, DNA will be precipitated with 100% isopropyl alcohol (IPA) and consequently an aggregate of beads will be formed and washed. Finally, DNA will be eluted in 1 mL of Elution Buffer.

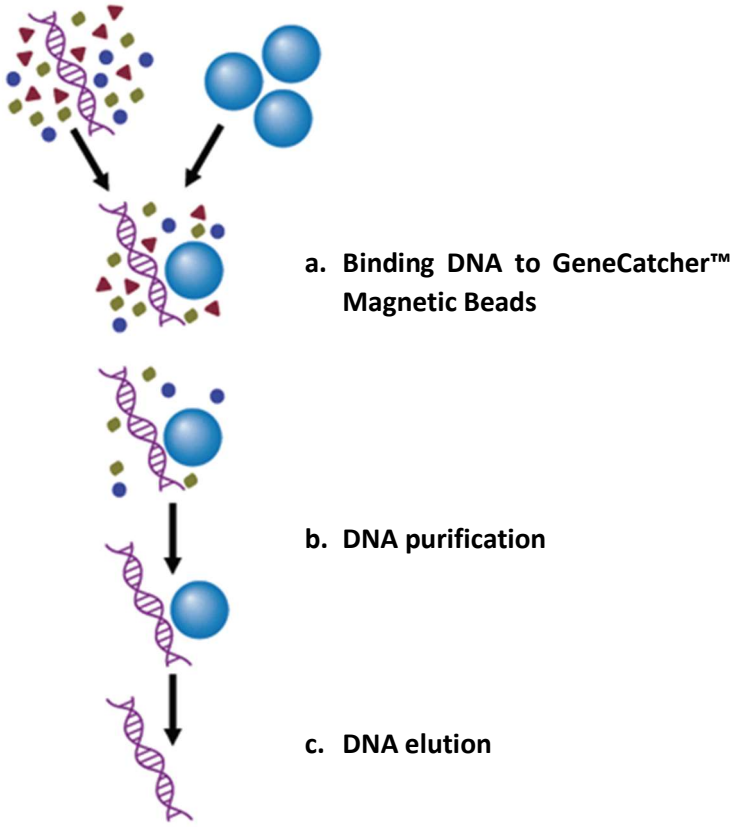


Figure 14 – Major steps of DNA extraction using GeneCatcher™ gDNA 3-10 mL Blood Kit (Invitrogen, Carlsbad, USA). Adapted from GeneCatcher™ gDNA Blood Kits - For purification of gDNA from human blood (2012).[80]

3.4 DNA QUANTIFICATION AND ASSESSMENT OF DNA PURITY

After DNA extraction, 2 µL of sample were used to assess DNA's concentration (ng/µL) and purity by spectrophotometer NanoDrop-1000 (Thermo Fisher Scientific, Wilmington, USA).

The ratios between absorbance at 260 nm and 280 nm (A260/A280) and between absorbance at 260 nm and 230 nm (A260/A230) were used to evaluate DNA purity. The ratio A260/A280 should be about 1.80-2.0 to ensure that our sample is not contaminated with proteins. Whereas, A260/A230 should be higher than A260/A280 and up to approximately 2.30 to ensure there is no other contaminators such as phenol and salts.

3.5 MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION

The presence of SNPs and CNVs was evaluated through MLPA assay according to the manufacturer's recommendations (MRC-Holland, 2014). The probe panel used was SALSA MLPA probemix P236 ARMD mix-1 (MRC-Holland, Amsterdam, The Netherlands) which contains 35 probes covering both RCA locus and flanking genes *KCNT2*, *CRB1* and *TNNT2*. P236 also presents 3 probes for the rare allele of rs1061170 *(CFH* Y402H), rs10490924 *(ARMS2* A69S) and rs9332739 *(C2* E318D), 1 for the common allele of rs1410996 *(CFH*- intron 15) and 8 reference probes located in different autosomal chromosomal locations.

Briefly, a total volume of 5 µL, with an input of approximately 90 ng of DNA was denaturated at 98°C for 10 minutes. Then, the probemix was added to each tube and were heated during 1 minute at 95°C and maintained at 60°C during 15 to 16 hours. After hybridization, in order to form the MLPA probes, the temperature was diminished till 54°C and a ligase mastermix was added and incubated for 15 minutes. In order to inactivate the ligase, samples were kept for 5 minutes at 98°C. Finally, a multiplex PCR amplification (35 cycles of 30s at 95ºC, 30s at 60ºC and 1min at 72ºC) was performed, followed by a final extension

step. All this steps were executed using the ABI 2720 (Applied Biosystems, Foster City, USA) thermocycler.

After the PCR reaction, 1 µL of PCR product was added to a 9,4 µL mixture of *Rox™* and HiDi formamide (Applied Biosystems, Foster City, USA) in order to perform fragment separation by capillary electrophoresis using ABI PRISM™ 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Data analysis was preformed recurring to GeneMapper v4.1 (Applied Biosystems, Foster City, USA) and to Coffalyser.Net (MRC-Holland, Amsterdam, The Netherlands) softwares in order to evaluate the electropherograms obtained and the ratio of copy numbers between samples and controls, respectively.

SALSA MLPA kits comprise nine control fragments that evaluate the quality of the MLPA experience: 4 Q-fragments (64, 70, 76 and 82 nt) ligase independent which controls if the DNA quantity was enough; 3 D-fragments (88, 92 and 96 nt) that allow to evaluate if the denaturation was successful; a X fragment (100 nt) and a Y fragment (105 nt) which allow us to determine the gender of the sample (Figure 15; Table 4).

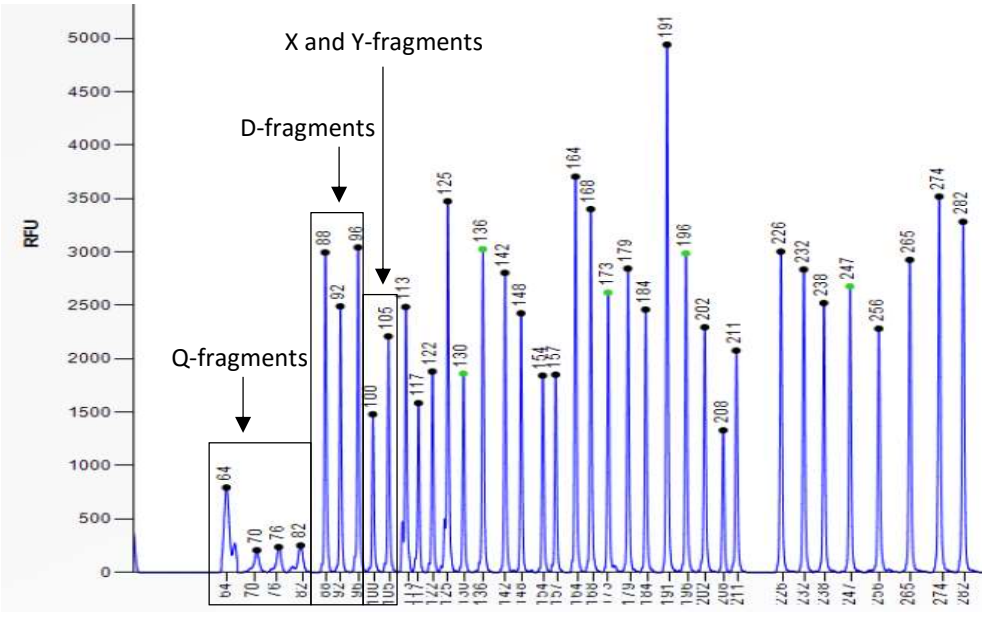


Figure 15 – Control quality fragments within a capillary electrophoresis pattern of a male DNA sample. Adapted from MRC-Holland, SALSA MLPA probemix P236-A3 ARMD mix-1 (2016).[81]

Table 4 – MLPA quality control fragments adapted from MRC-Holland, 2014.[77]

SALSA MLPA Probe	Length (nucleotides, nt)	Interpretation
92 nt benchmark probe	92	Normal probe. Reference to compare the other quality control fragments.
Q-fragments	64, 70, 76, 82	High when DNA amount is insufficient or the ligation reaction failed. When all Q-fragment signals are higher than ⅓ (33%) of the 92 nt control fragment means that DNA quantity is too low.
D-fragments	88, 96	Low when occurred a poor DNA denaturation. When the signal is inferior to 40% of the 92 nt control fragment means that there were problems in the DNA denaturation process.
X and Y fragments	100, 105	Control for sample swapping. Probes specific for the X and Y chromosome.

Three reference controls and one negative (without DNA) control were used for each MLPA experiment.

The results are presented as ratio between DNA sample and DNA control (Table 5). Besides analysing copy number variation, P236 probemix also evaluates the presence of the rare alleles of rs10490924 *(ARMS2*) and rs9332739 *(C2*). It has 2 probes for each SNP, one for the rare allele and other for the common. When the rare allele is present in homozygosity the probe for the rare allele will present a ratio of 1, whereas the probe for the common allele will present a ratio of 0. On the other hand, if only one rare allele is present the probe for the rare and common allele will each have a ratio of 0.5. Furthermore, there are two probes that detect SNPs in *CFH:* one for the rare allele of rs1061170 in exon 9 that will only generate signal if the rare allele is present; and other for the common allele of rs1410996 in intron 15 that will have its signal diminished if a rare allele is present.

Table 5 – Interpretation of MLPA results given by Coffalyser.Net and colour codification used to display the results.

Ratio	Copy Number Status	SNP Status	Representation
0	Homozygous deletion	Absence	0
0.25 ≤ 0.75	Heterozygous deletion	Heterozygous Presence	0.5
0.75 – 1.3	Normal	Presence	1
> 1.3	Numerical gain / Amplification	More than 2 copies	2

3.6 Statistical Analysis

Data analysis was performed on the SPSS (Statistical Package for the Social Sciences) program from IBM. Logistic regression was used to examine the effect of each genetic variation in RAP development. Values of $p \leq 0.05$ were considered statistically significant.

4 RESULTS AND DISCUSSION

A total of 99 samples (32 RAP and 67 controls) were studied through SALSA MLPA probemix P236 ARMD mix-1.

GeneMapper v4.1 software allowed to obtain electropherograms with a peak pattern specific for each sample (Figure 16), whereas Coffalyser.Net was used to obtain the ratio of copy numbers between samples and the defined controls.

This probemix evaluates SNPs and CNVs in genomic regions with great variability and therefore it was not possible to define perfect controls i.e. that presented only 2 copies for single probe tested. Thus, data analysis was performed in blocks, recurring to two sets of controls: one to analyse *CFH* SNPs (rs1061170 and rs1410996) and another to evaluate the remaining probes. In sample analysis of all 49 probes, only 19 presented variations in the ratio of their probe signal: the 6 probes concerning the 4 SNPs and all 13 probes for *CFHR3* and *CFHR1* genes (Tables 6 and 7).

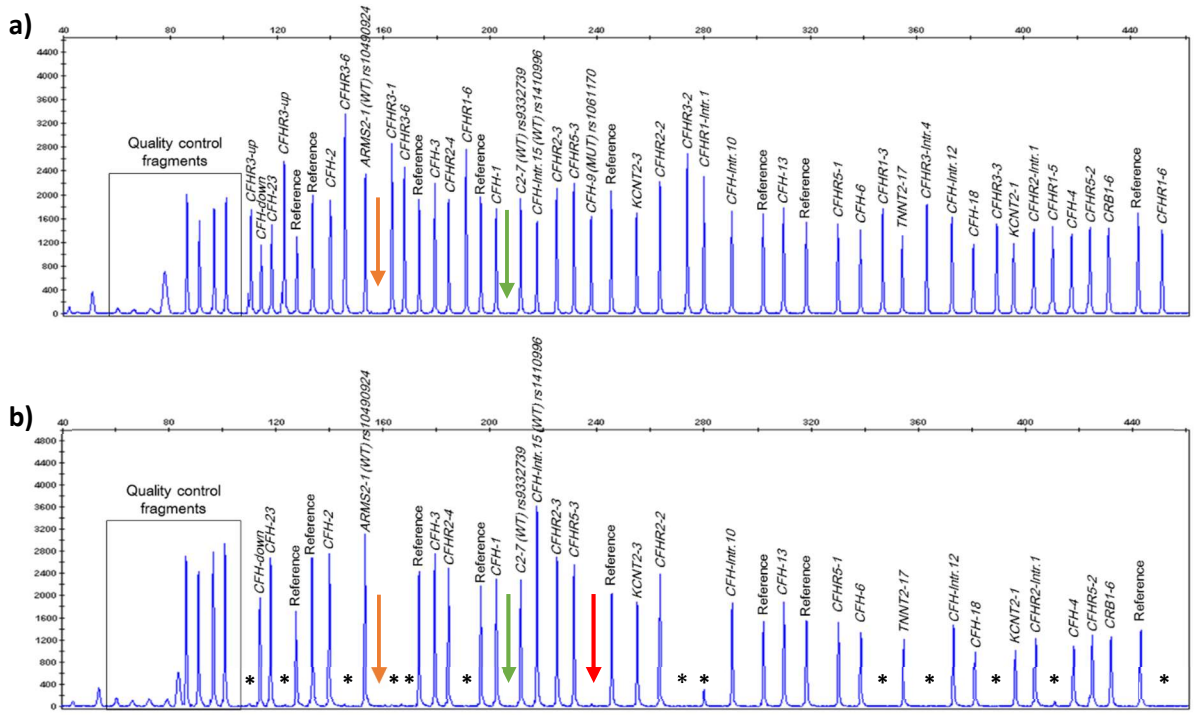


Figure 16 – Examples of electropherogram of female samples analyzed by MLPA using SALSA MLPA probemix P236. **a)** Common electropherogram, missing probe signals of rare alleles of SNPs in C2 (↓) and ARMS2 genes (↓). **b)** Electropherogram of an individual with a homozygous deletion of *CFHR3* and *CFHR1* (*) and common alleles for *C2, ARMS2* and *CFH-9* SNP Y402H (↓).

Table 6 – Summary of all alterations detected in RAP samples. The first two columns present the ratio of the probe signal of SNPs in *CFH*, Y402H (risk allele) and the intronic SNP (common allele). Since alterations in *CFHR3* and *CFHR1* occurred in block, i.e. in all probes for those genes, no ratio is displayed. The last 4 columns represent the alleles of *C2* and *ARMS2* SNPs. **Red** represents a decrease in MLPA probe signal, whereas **blue** represents increase. **Yellow** refers to the absence of the probe target. Probes with a normal ratio are represented in white.

	CFH Y402H (risk allele)	*CFH*-Intr. 15 (common allele)	*CFHR3*	*CFHR1*	*C2*		*ARMS2*	
					Common allele	Rare allele	Common allele	Rare allele
RAP 1	**0**	1.07	Deletion	Deletion	0.97	**0**	0.49	0.55
RAP 2	1.96	**0**			0.95	**0**	1.01	**0**
RAP 3	1.03	**0**			0.98	**0**	0.49	0.57
RAP 4	**0**	**0**			0.94	**0**	**0**	1.10
RAP 5	1.9	**0**			0.94	**0**	0.48	0.52
RAP 6	**0**	1.04	Deletion	Deletion	0.94	**0**	0.49	0.51
RAP 7	1.03	**0**			1.01	**0**	**0**	1.10
RAP 8	**0**	**0**			1.03	**0**	**0**	1.01
RAP 9	0.98	1.06			1	**0**	**0**	1.08
RAP 10	1.02	1.03	Deletion	Deletion	0.93	**0**	**0**	1.19
RAP 11	2.1	**0**			1.01	**0**	0.53	0.58
RAP 12	1.07	1.13			1.06	**0**	1.02	**0**
RAP 13	1.01	1.1	Deletion	Deletion	1.03	**0**	0.51	0.55
RAP 14	0.95	0.98	Deletion	Deletion	1.02	**0**	1.06	**0**
RAP 15	**0**	1.94	Deletion	Deletion	0.96	**0**	**0**	1.15
RAP 16	**0**	1.98			0.97	**0**	**0**	1.08
RAP 17	1.03	1.04			0.98	**0**	**0**	1.08
RAP 18	**0**	1.06	Deletion		1.06	**0**	0.58	0.73
RAP 19	0.95	**0**			1.07	**0**	1.08	**0**
RAP 20	1.05	**0**			1.08	**0**	0.57	0.72
RAP 21	**0**	2.27	Deletion	Deletion	0.97	**0**	0.52	0.53
RAP 22	1.06	**0**			1	**0**	**0**	1.01
RAP 23	**0**	2.06			1.03	**0**	0.49	0.50

Table 6 (continuation) – Summary of all alterations detected in RAP samples. The first two columns present the ratio of the probe signal of SNPs in *CFH*, Y402H (risk allele) and the intronic SNP (common allele). Since alterations in *CFHR3* and *CFHR1* occurred in block, i.e. in all probes for those genes, no ratio is displayed. The last 4 columns represent the alleles of *C2* and *ARMS2* SNPs. **Red** represents a decrease in MLPA probe signal, whereas **blue** represents increase. **Yellow** refers to the absence of the probe target. Probes with a normal ratio are represented in white.

	CFH Y402H (risk allele)	*CFH*-Intr. 15 (common allele)	*CFHR3*	*CFHR1*	*C2*		*ARMS2*	
					Common allele	Rare allele	Common allele	Rare allele
RAP 24	**0**	1.1			0.97	**0**	1.06	0
RAP 25	1	**0**			1	**0**	0	1.02
RAP 26	1.09	1.09			0.94	**0**	0.53	0.54
RAP 27	2.12	**0**			0.93	**0**	0.48	0.51
RAP 28	**0**	1.04	Deletion	Deletion	0.99	**0**	1.06	0
RAP 29	**0**	1.92	Deletion	Deletion	0.99	**0**	1.07	0
RAP 30	2.06	**0**			0.99	**0**	1.03	0
RAP 31	1.04	**0**			0.73	0.48	1	0
RAP 32	1.08	1.04	**0**	**0**	0.99	**0**	0	1.15

Table 7 – Summary of all alterations detected in control samples. The first two columns present the ratio of the probe signal of SNPs in *CFH*, Y402H (risk allele) and the intronic SNP (common allele). Since alterations in *CFHR3* and *CFHR1* occurred in block, i.e. in all probes for those genes, no ratio is displayed. The last 4 columns represent the alleles of *C2* and *ARMS2* SNPs. **Red** represents a decrease in MLPA probe signal, whereas **blue** represents increase. **Yellow** refers to the absence of the probe target. Probes with a normal ratio are represented in white.

	CFH Y402H (risk allele)	*CFH*-Intr. 15 (common allele)	*CFHR3*	*CFHR1*	*C2*		*ARMS2*	
					Common allele	Rare allele	Common allele	Rare allele
Ctrl 1	1.01	1	Deletion	Deletion	0.93	**0**	0.99	**0**
Ctrl 2	0.97	0.94	Deletion	Deletion	0.98	**0**	1.01	**0**
Ctrl 3	0.98	**0**			1.01	**0**	0.98	**0**
Ctrl 4	**0**	1.99	Deletion	Deletion	1	**0**	0.96	**0**
Ctrl 5	0.96	1.01	Deletion	Deletion	1.01	**0**	0.51	0.56
Ctrl 6	1.03	1	Deletion	Deletion	0.97	**0**	0.5	0.58
Ctrl 7	0.97	1.02	Deletion	Deletion	0.96	**0**	0.51	0.53
Ctrl 8	1.02	1.02	Deletion	Deletion	0.98	**0**	0.49	0.51
Ctrl 9	1	1.03			0.73	0.53	1.01	**0**
Ctrl 10	0.97	0.79	Deletion	Deletion	0.94	**0**	0.98	**0**
Ctrl 11	**0**	1.02	Deletion	Deletion	0.99	**0**	**0**	1.22
Ctrl 12	**0**	2.11	Deletion	Deletion	1	**0**	0.97	**0**
Ctrl 13	1.88	**0**			1.01	**0**	1.03	**0**
Ctrl 14	**0**	2.02	Deletion	Deletion	1.05	**0**	0.97	**0**
Ctrl 15	1.02	1.04	Deletion	Deletion	1.03	**0**	1	**0**
Ctrl 16	**0**	1.91	**0**	**0**	1.01	**0**	0.51	0.59
Ctrl 17	**0**	1.96	Deletion	Deletion	0.98	**0**	0.98	**0**
Ctrl 18	1.02	1.01			0.99	**0**	0.97	**0**
Ctrl 19	0.83	0.92	Deletion	Deletion	0.67	0.44	1.09	**0**
Ctrl 20	**0**	1.91	Deletion	Deletion	0.94	**0**	0.47	0.5
Ctrl 21	1.01	1.02	Deletion	Deletion	0.97	**0**	0.51	0.5
Ctrl 22	1.07	1.08			1.04	**0**	1.05	**0**
Ctrl 23	2.02	**0**			1.04	**0**	1.04	**0**

Table 7 (continuation) – Summary of all alterations detected in control samples. The first two columns present the ratio of the probe signal of SNPs in *CFH*, Y402H (risk allele) and the intronic SNP (common allele). Since alterations in *CFHR3* and *CFHR1* occurred in block, i.e. in all probes for those genes, no ratio is displayed. The last 4 columns represent the alleles of *C2* and *ARMS2* SNPs. **Red** represents a decrease in MLPA probe signal, whereas **blue** represents increase. **Yellow** refers to the absence of the probe target. Probes with a normal ratio are represented in white.

	CFH Y402H (risk allele)	*CFH*-Intr. 15 (common allele)	*CFHR3*	*CFHR1*	*C2*		*ARMS2*	
					Common allele	Rare allele	Common allele	Rare allele
Ctrl 24	1.02	**0**			1.09	**0**	0.51	0.64
Ctrl 25	**0**	2.12			1.02	**0**	1.03	**0**
Ctrl 26	**0**	**0**			1.16	**0**	1.12	**0**
Ctrl 27	1.11	**0**			1.11	**0**	1.08	**0**
Ctrl 28	**0**	**0**			1.02	**0**	1.03	**0**
Ctrl 29	1.05	1			1.07	**0**	1.07	**0**
Ctrl 30	**0**	1.03			1.04	**0**	1.13	**0**
Ctrl 31	1	0.98	Deletion	Deletion	0.99	**0**	0.97	**0**
Ctrl 32	**0**	2.01	Deletion	Deletion	1.01	**0**	0.54	0.68
Ctrl 33	**0**	0.99			1.06	**0**	1.07	**0**
Ctrl 34	1.02	**0**		Deletion	1.01	**0**	0.53	0.58
Ctrl 35	1.14	**0**			1.01	**0**	1.18	**0**
Ctrl 36	2.03	**0**			1	**0**	0.57	0.66
Ctrl 37	**0**	**0**			1.08	**0**	1.17	**0**
Ctrl 38	2.08	**0**			1.09	**0**	1.12	**0**
Ctrl 39	**0**	1.02			1.04	**0**	1.06	**0**
Ctrl 40	**0**	1.04			1.02	**0**	1.09	**0**
Ctrl 41	1.01	1.02	Deletion	Deletion	1.04	**0**	1.1	**0**
Ctrl 42	0.98	**0**			1.09	**0**	1.14	**0**
Ctrl 43	2.09	**0**			1.11	**0**	0.57	0.70
Ctrl 44	1.03	1.01			1.07	**0**	0.58	0.70
Ctrl 45	1.02	1.05			1.06	**0**	1.11	**0**
Ctrl 46	1.07	1.08			1.03	**0**	1.09	**0**

Table 7 (continuation) – Summary of all alterations detected in control samples. The first two columns present the ratio of the probe signal of SNPs in *CFH*, Y402H (risk allele) and the intronic SNP (common allele). Since alterations in *CFHR3* and *CFHR1* occurred in block, i.e. in all probes for those genes, no ratio is displayed. The last 4 columns represent the alleles of *C2* and *ARMS2* SNPs. **Red** represents a decrease in MLPA probe signal, whereas **blue** represents increase. **Yellow** refers to the absence of the probe target. Probes with a normal ratio are represented in white.

	CFH Y402H (risk allele)	*CFH*-Intr. 15 (common allele)	*CFHR3*	*CFHR1*	*C2*		*ARMS2*	
					Common allele	Rare allele	Common allele	Rare allele
Ctrl 47	**0**	1.11	Deletion	Deletion	1.14	**0**	0.55	0.73
Ctrl 48	**0**	1.13			1.1	**0**	0.59	0.73
Ctrl 49	1.07	**0**			1.03	**0**	1.13	**0**
Ctrl 50	**0**	2.11	**0**	**0**	1.06	**0**	1.12	**0**
Ctrl 51	**0**	2.09	**0**	**0**	1.05	**0**	1.08	**0**
Ctrl 52	2.06	**0**			1.04	**0**	1.08	**0**
Ctrl 53	**0**	1.03			1.08	**0**	1.14	**0**
Ctrl 54	**0**	1.06			1.04	**0**	1.1	**0**
Ctrl 55	**0**	1.24			0.97	**0**	0.98	**0**
Ctrl 56	1.21	**0**			1.04	**0**	0.94	**0**
Ctrl 57	1.07	1.14	Deletion	Deletion	0.96	**0**	0.95	**0**
Ctrl 58	**0**	2.29	Deletion	Deletion	0.73	0.44	0.51	0.50
Ctrl 59	0.99	1			0.93	**0**	0.95	**0**
Ctrl 60	1.15	1.16			1.01	**0**	0.97	**0**
Ctrl 61	1.11	**0**			1	**0**	0.48	0.52
Ctrl 62	**0**	2.02	**0**	**0**	0.96	**0**	0.99	**0**
Ctrl 63	1.07	**0**			0.94	**0**	1.01	**0**
Ctrl 64	2.05	**0**			0.93	**0**	0.95	**0**
Ctrl 65	**0**	2.09	Deletion	Deletion	0.9	**0**	0.95	**0**
Ctrl 66	**0**	1.1	Deletion	Deletion	0.97	**0**	0.96	**0**
Ctrl 67	2.01	**0**			1.01	**0**	**0**	1.03

Taking into account the particularity of the results obtained from this probemix, specifically the evaluation of CNVs and SNPs, the analysis was performed on each genetic variation individually, followed by a global interpretation of the results.

4.1 COMPLEMENT SYSTEM AND INFLAMMATION

One major hallmark of AMD is the development of drusen, an agglomerate of extracellular debris containing, among others, immunoglobulins and complement components. Thus, these deposits have been reported to induce inflammatory response.[2,39,40,82–84]

The complement system is an innate mechanism of immune response in which inflammation is one of the outcomes. It is a sequential process that relies in the cleavage and activation of its components, amplification of the signal and finally formation of the MAC.[40]

Almost every complement components and regulators such as CFH, CFHR1-5, C2/CFB, C3 and C5 have been found in drusen, which increases the theory that the complement pathway is over activated in AMD. Moreover, these components have already been reported has having genetic variations associated with AMD's development.

4.1.1 CFH

CFH is a major inhibitor of the alternative complement cascade as it increases the dissociation of C3 convertase complex and inactivates C3b by acting as a CFI cofactor. Furthermore, CFH binds to pathogens and host cellular surfaces such as BrM which facilitates its interaction with C3b and consequent inhibition of the complement system.

CFH gene is rich in SNPs that have been associated with AMD. Even though SALSA probemix P236 has 13 probes targeting this gene, it only accesses two of the susceptibility SNPs: rs1061170 (exon 9) and rs1410996 (intron 15). Further, these were the only probes that presented variations in their ratios.

4.1.1.1 *CFH-9* Y402H – rs1061170

The presence of rs1061170 was classified considering the presence/absence of the allele detected by the probe, the rare allele: 0 (no rare allele was detected), 1 (rare allele is present in heterozygosity), 2 (rare allele is present in homozygosity) and >2 (abnormal number of rare alleles copies, i.e. more than 2).

Considering both populations studied (n=99) a total of 60 samples (RAP and controls) presented 2 or more rare alleles (60.6%), a condition more frequently observed than the presence of the common allele. Yet, having 2 rare alleles (n=47) is more frequent than having more (n=13), 47.5% vs 13.1%, respectively. However, the percentages for each classification in RAP and controls did not differ much. Heterozygosity for this SNP was not observed (Table 8).

Table 8 – Results obtained for *CFH*-9 Y402H SNP (rs1061170) variations.

Number of Rare Alleles	RAP (n=32)	CONTROLS (n=67)	p-value	OR [a]	CI [b]
0	12 (37.5%)	27 (40.3%)	0.874		
1	0 (0%)	0 (0%)	-	-	-
2	15 (46.9%)	32 (47.8%)	0.909	1.055	0.422 – 2.635
> 2	5 (15.6%)	8 (11.9%)	0.609	1.406	0.380 – 5.201

Data are presented as *n* (%) for all RAP and controls frequencies.

[a] OR: Odds ratio; [b] 95% CI (confidence interval)

The rs1061170 SNP was the first to be associated with increased risk of AMD. This SNP leads to a change in the encoded amino acid, which decreases in CFH's binding properties. Consequently, its ability to regulate the alternative pathway will be affected, leading to an abnormal activation of the complement cascade.

Even though it is one of the most described genetic variations associated to AMD in general, when considering specific subtypes, like RAP, it seems to exist little association between the SNP and the development of RAP.[47,66,85,86]

Taking this into account, the results obtained are in agreement with previous reports, since the percentage of each genotype was very similar between controls and RAP.

4.1.1.2 *CFH*-Intr.15 – rs1410996

Besides Y402H, a noncoding SNP, rs1410996, located in intron 15 has also been reported to increase the risk of AMD.[68,87,88] Due to its location, rs1410996 function is not fully stablished but it is thought to regulate the expression of *CFH*.[88]

As opposed to what was analysed for the previous SNP, the presence of rs1410996 was classified considering the presence/absence of the common allele: 0 (no common allele was detected), 1 (common allele is present in heterozygosity), 2 (common allele is present in homozygosity) and >2 (abnormal number of rare alleles copies, i.e. more than 2) (Table 9).

The common allele is present in about 64.6% of all samples. Homozygous present of the common allele is more frequent in controls (47.8%) whereas not having none is more frequent in RAP (43.8%). Since only one subject displayed the alleles in heterozygosity, this sample was not accounted for statistical analysis.

Table 9 – Results obtained for *CFH* intronic SNP (rs1410996) variations.

Number of Common Alleles	RAP (n=32)	Controls (n=67)	P-VALUE	OR [a]	CI [b]
0	14 (43.8%)	21 (31.3%)	0.299	1.641	0.645 – 4.177
1	0 (0.0%)	1 (1.5%)	-	-	-
2	13 (40.6%)	32 (47.8%)	0.514		
> 2	5 (15.6%)	13 (19.4%)	0.930	0.947	0.281 – 3.195

Data are presented as *n* (%) for all RAP and controls frequencies.

[a] OR: Odds ratio; [b] 95% CI (confidence interval)

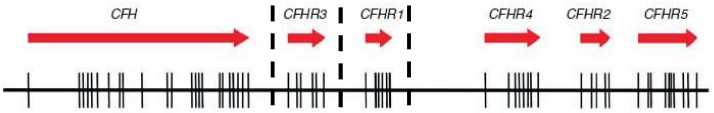
Similarly, as observed for Y402H, there were no significant variations between RAP and controls, which suggests that rs1410996 may not be a relevant genetic marker for this particular disease.

4.1.2 *CFHR3-CFHR1* CNV

Around 60% of all reports that studied the genetic influence in AMD are about SNPs in *CFH* and *ARMS2*. However, reports of CNV comprising CFH-related genes such as *CFHR1*, *CFHR3* and *CFHR4* are becoming more and more common.

CFHR1 and CFHR3 can both work as positive and negative regulators of the complement pathway, although it seems that the inhibition of the complement has a greater effect.[53,55]

In this study, this region was analysed as a block since copy number variation detected was common to all probes targeting *CFHR1* and *CFHR3.* Thus, samples were classified as having 0, 1 or 2 copies of both genes. However, partial deletion of only one of the genes was also detected in the study case, thus all the combinations presented in Figure 17 were observed.



Homozygous deletion (n=5)	0 0
Heterozygous deletion (n=33)	
Heterozygous deletion of *CFHR1* (n=1)	
Heterozygous deletion of *CFHR3* (n=1)	
No copy number variation (n=59)	

Figure 17 – Combinations of *CFHR3* and *CFHR1* copy number observed. Considering *CFHR3-CFHR1* as a block, it can present 2 copies of each gene (no copy number variation - white) or 1 copy of each gene (heterozygous deletion - red). A complete absence of *CFHR3-CFHR1* was also verified (homozygous deletion – yellow). However, two of all samples displayed a partial deletion of the block, presenting only one copy of *CFHR1* (heterozygous deletion of *CFHR1*) or of *CFHR3* (heterozygous deletion of *CFHR3*).

The results obtained for these genes are accounted in Table 10. Just one RAP and one control samples presented the partial deletion of only *CFHR3* or *CFHR1* gene, respectively and therefore these 2 samples were regarded into Table 10 has having only 1 copy.

Considering the entire population (n=99), as expected, the presence of 2 copies of these genes was more common (n=59; 59.6%) than copy number variations (n=60, 40.4%), considering that only deletions were observed. The

presence of only one copy was detected in 31.3% and 37.3% of RAP and controls, respectively. On the other hand, homozygous deletions were only observed in 4 controls and in one RAP sample.

Table 10 – Results obtained for *CFHR3* and *CFHR1* CNVs.

Number of *CFHR3-CFHR1* copies	RAP (n=32)	Controls (n=67)	p-value	OR [a]	CI [b]
0	1 (3.1%)	4 (6.0%)	0.655		
1	10 (31.3%)	25 (37.3%)	0.491	0.452	0.047 – 4.314
2	21 (65.6%)	38 (56.7%)	0.485	0.724	0.292 – 1.792

Data are presented as *n* (%) for all RAP and controls frequencies.

[a] OR: Odds ratio; [b] 95% CI (confidence interval)

Deletion of *CFHR3* and *CFHR1* has been reported as a protective factor, being associated with a 43% reduction in the odds of developing AMD.[49,56,67]

No significant variations between RAP and controls were observed. Even though the results were not statistically significant, the tendency was of a higher frequency of deletions in controls compared to RAP. This is clearer as only one in 32 RAP samples presented a homozygous deletion.

Few articles report *CFHR3* or *CFHR1*-only deletions, and when detected they are present in a very small minority of the subjects evaluated.[53,73] Our results corroborate those findings since, of all 99 samples analyzed, only 1 RAP and 1 control presented a *CFHR3* and *CFHR1*-only deletion, respectively.

A larger cohort and additional functional studies are needed to enlighten the consequence of these genotypes in the risk of AMD development.

4.1.3 *C2*-7 E318D – rs9332739

C2's influence in AMD has been extensively debated. First, it is located very close to *CFB*. Second, the frequency of SNPs within this gene differs between ethnic groups. Moreover, an altered function in these genes may cause a decreased activation of the complement pathway in the eye.[59]

Several articles have described rs9332739 (E318D) as having a protective effect in AMD in Caucasians, nevertheless in Korean population the frequency of the protector allele is almost null.[59,60,62,89]

Taking into account that this is a cross-sectional study of the Portuguese population, which is majorly Caucasian, it was unexpected to find the rare allele of this genetic variation in only 4 subjects: 3 control samples and in 1 RAP, always in heterozygosity.

These results suggest that this SNP may not be useful in RAP diagnosis.

4.2 *ARMS2* A69S – RS10490924

As *C2*, *ARMS2* is also located very close to its neighboring gene *HTRA1*, and for that reason it was difficult to evaluate which one affected AMD susceptibility.

A SNP located in exon 1 of *ARMS2*, A69S (rs10490924), is the most reported genetic variation associated with increased risk of developing AMD and when in homozygosity the risk increases from 2.8 to 8.1-fold.[15] Moreover, of all AMD's subtypes, it has the strongest association with RAP.[63,65,66,85]

In fact, rs10490924 was the genetic alteration that displayed more variability between the two populations.

The presence of 1 or 2 rare alleles was higher in RAP (71.9%) than in controls (28.4%), with only 2 controls (3.0%) presenting 2 rare alleles. Heterozygosity was also more common in RAP patients (37.5%) than in control samples (25.4%). On the other hand, homozygosity of the common allele was clearly higher in controls (71.6%) than in RAP (28.1%) (Table 11).

Table 11 – Results for *ARMS2* SNP A69S (rs10490924) variations.

Number of Rare Alleles	RAP (n=32)	Controls (n=67)	p-value	OR [a]	CI [b]
0	9 (28.1%)	48 (71.6%)	<0.001		
1	12 (37.5%)	17 (25.4%)	0.011	3.765	1.349 – 10.504
2	11 (34.4%)	2 (3.0%)	<0.001	29.333	5.542 – 155.260

Data are presented as *n* (%) for all RAP and controls frequencies.

[a] OR: Odds ratio; [b] 95% CI (confidence interval)

A69S was the only genetic variation evaluated that presented significant differences between controls and RAP samples ($p<0.001$). The rare allele of this SNP was considerably more present in RAP than in controls, suggesting that it might be a strong genetic marker for this AMD subtype.

ARMS2 function is not fully stablished, however Fritsche *et al.* reported its presence in mitochondria of the photoreceptors, suggesting that it might be involved in mitochondrial homeostasis, oxidative stress and ROS formation.[50,63,64] Therefore, its potential role as an antioxidant may be related to the increased risk that the presence of the SNP has in the development of AMD. On the other hand, it has also been described as a constituent of the extracellular matrix, what might suggest its involvement in the development of hemorrhages within the macula, a hallmark of RAP.[90] More functional studies should be performed in order to clarify the role of this genetic alteration.

4.3 *ARMS2* A69S AND *CFHR3-CFHR1* CNV

The association between *CFHR3-CFHR1* CNV and *ARMS2* SNP was also evaluated. Each population was subdivided by the number of copies of *CFHR3* and *CFHR1* and the presence or absence of the rare allele.

In RAP it was more recurrent to have 2 copies of *CFHR3* and *CFHR1* with at least one rare allele (46.9% vs 10.4% in controls), whereas in controls the presence of normal copy numbers with no rare allele was more frequent (46.3% vs 18.8% in RAP). On the other hand, having one or none copies of *CFHR3* and *CFHR1* and 2 common alleles is more usual in controls (25.4%) than in RAP (9.4%) (Table 12).

The presence of the *ARMS2* rare allele combined with a normal copy number of *CFHR3-CFHR1* genes has significant more presence in RAP than in controls. However, no conclusion can be formulated when *CFHR3-CFHR1* deletion is associated with the *ARMS2* common allele.

Table 12 – Association of *CFHR3-CFHR1* number of copies with the presence of *ARMS2* different alleles in RAPs and controls.

	***CFHR3-CFHR1* copies**			
	RAP (%)		Controls (%)	
ALLELE OF *ARMS2* A69S SNP	No copy number variations	Copy number variations (deletion)	No copy number variations	Copy number variations (deletion)
Common allele (GG)	18.8	9.4	46.3	25.4
Rare allele (TG; TT)	46.9	25.0	10.4	17.9
TOTAL	65.6	34.4	56.7	43.3

4.4 OVERALL PERSPECTIVE OF THE RESULTS

The multifactorial nature of the development of AMD demands the analysis of the prevalence of both risk and protection factors and its correlation with the presence of RAP. Consequently, a single genetic variation will not be sufficient to cause AMD. Thus, when studying AMD samples, an analysis englobing multiple risk factors should be performed.

With this in mind, each population was clustered by the number of risk and protective factors reported in each sample and compared with the presence or absence of the disease. To not underestimate the effect of homozygosity, each allele in homozygosity were accounted as 2 risk factors and homozygous deletion of *CFHR3-CFHR1* were considered as 2 protective factors (Figure 18).

Furthermore, one risk factor should not be sufficient to induce the disease's development. Having this in consideration, the vast majority of RAP samples present 2 or more risk factors (78.1%) whereas they are present in only 50.7% of controls (p=0.012).

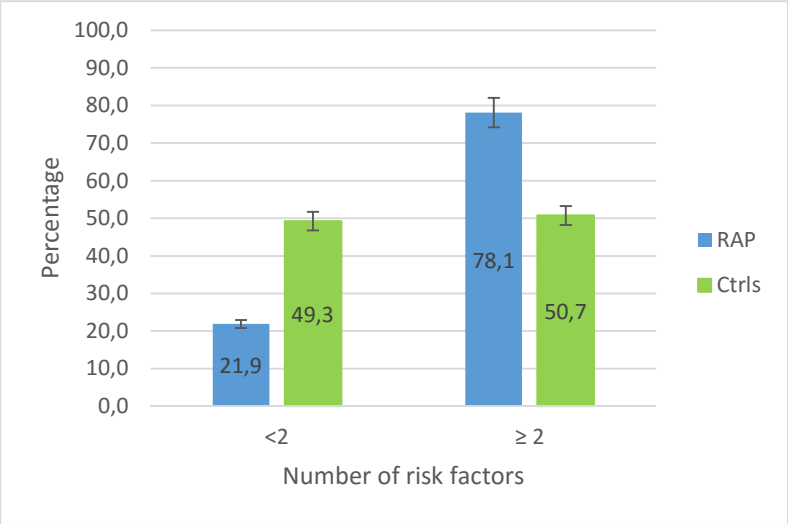


Figure 18 – Percentage of patients presenting risk factors in RAP and controls. The samples are divided according to the number of risk factors, "less than 2" ("<2") and "2 or more" ("≥ 2"), in agreement with AMD's multifactorial characteristics.

The frequency of protective factors does not differ between RAP and control patients, whereas having the presence of 2 or more risk factors is more common in RAP samples. In other words, the results obtained seem to suggest that the influence of risk factors in RAP's development are more significant than the effect of the protective factors in the progression of the disease. However, some results presented on Table 6 and Table 7 were not as expected.

One of the controls (Ctrl 67) presented the higher number of risk factors of all the samples. Besides displaying 2 risk alleles in both *CFH* Y402H and *ARMS2* SNPs, it did not present a decrease in *CFHR3-CFHR1* copy number, a reported protective factor.[49,52,53,67,73] In spite of the above mentioned results, this patient is only 59 years old and since AMD risk increases considerably with age he might still develop the disease.[24] Thus, this patient should be clinically followed and its classification as a control should be further evaluated.

RAP 28 and RAP 29, two of the oldest subjects evaluated (95 and 91 years-old, respectively), did not present any of the studied risk factors. AMD's multifactorial etiology might explain the development of RAP in these women. Smoking, nutrition, oxidative stress and inflammatory levels are potential factors (not evaluated in this study) that may lead to AMD.[91–94] Other major risk factor is gender, since it has been demonstrated that women have more predisposition to develop the disease.[95]

RAP 32, an 82-year-old female subject, presented 2 risk alleles for the *ARMS2* SNP and 1 risk allele for the *CFH*-9 SNP. Coincidently, it also manifested homozygous deletion of the *CFHR3-CFHR1* block, an occurrence highly reported as protective against AMD development. This, strengthens the hypothesis that risk factors, particularly *ARMS2* SNP, have more influence in AMD progression than protective factors.

As of above, a 62-year-old female control (Ctrl 11) also displayed homozygosity of the *ARMS2* risk allele but in opposition with RAP 32, it presented *CFHR3* and *CFHR1* in heterozygosity. Due to her young age, regarding the incidence age of this disease, and her gender she should be followed clinically since she could still develop RAP in the future.

Ctrl 50, 51 and 62 did not present any SNP that might induce AMD and furthermore, copies of *CFHR3* or *CFHR1*. These subjects should also be monitored to ensure that their condition will not deteriorate.

The development of RAP is a complex system, where the effect of one factor is not enough to trigger the disease. In Figure 19 are portrayed the most important factors involved in AMD's pathophysiology. Both the alteration of AMD susceptibility genes and the influence of aging and environmental factors will affect key pathways present in the ocular structure, such as photoreceptors, RPE, Bruch's membrane and choroidal capillaries. The abnormal function of these support systems will induce a cascade of cellular alterations that will cause pathology phenotypes, represented in red. The amount of cumulative pathologies will then dictate the appearance of AMD, whether CNV, where RAP is incorporated, or GA.

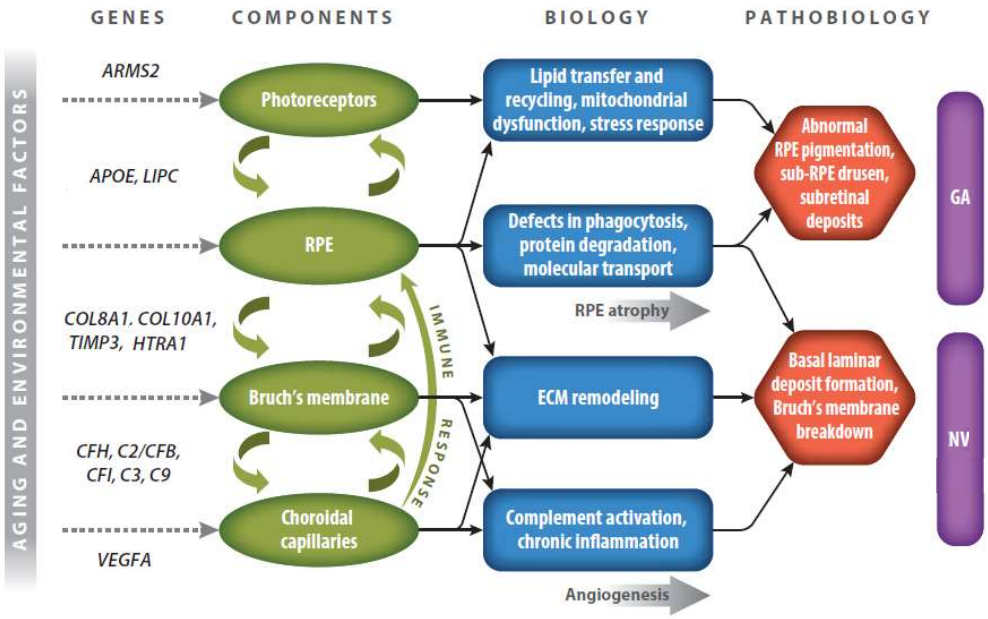


Figure 19 – Multiple-hit "threshold" integrative model of AMD's pathophysiology. Age-related changes associated with environmental insults act in a synergistic way that lowers the threshold for the disease. At that point, genetic alteration in AMD susceptibility genes will lead to alterations in photoreceptors and their support systems (green) and induce cellular alterations (blue). These changes may induce the development and progression of AMD. RPE: Retinal Pigmented Epithelium; GA: Geographic Atrophy; NV: Neovascular AMD. Adapted from Fritsche *et al.* (2014).[1]

5 CONCLUSION

As mentioned throughout this work, the study of AMD is affected by multiple factors. First, it is a late-onset disease with asymptomatic initial stages, which difficult its early diagnosis. Second, is a multifactorial disorder with genetic, environmental and behaviour factors contributing to its development. Third, genetic background of this disease can diverge on account of ethnicity of the population in study. At last, AMD's development is a result of the combination of multiple genetic variations, which means that only one genetic alteration might not be sufficient to trigger the disease.

Identifying susceptibility variations within each population might be a method to predict AMD's development and even to establish putative targets to new therapeutic approaches.

This study main objective was to evaluate RAPs genetic background within the Portuguese population taking in advantage of MLPA's characteristics, using P236 probemix. So far this study has allowed us to conclude that:

→ Even though *CFH* Y402H, rs1061170, is a major susceptibility factor for AMD disease in general, no association was found in the RAP subtype;

→ The results obtained for the *CFH* intronic SNP suggest that it might not be a good biomarker for RAP;

→ Of all evaluated genes, the only one with significant differences between RAP and controls was *ARMS2* SNP A69S (rs10490924). The presence of this genetic variation is therefore a potential biomarker for the prognosis of RAP development.

→ The absence of statistical significance might be a reflection of the small cohort. Additionally, the controls had an average age of 68.9 (± 5.6) years old. Considering AMD's late onset characteristic, the controls are young and might still develop the disease, which can also influence the results.

→ P236 probemix was designed to evaluate specific alterations associated with AMD, however it is still in need of improvement.

This is a pilot, cross-sectional study and even though there were some drawbacks, these are promising findings, which contribute to a better

understanding of RAP's genetic background. Ultimately, it is a step further towards considering a new diagnostic approach of this AMD subtype.

6 FUTURE PERSPECTIVES

AMD as one of the major causes of blindness worldwide has a great impact on life's quality. Furthermore, it represents a high social and economic burden, having reached in 2010, a worldwide cost around 316 billion euros.[34]

Usually, patients with early AMD are asymptomatic, so when the disease is clinically detected it has already caused some damage in vision acuity. Considering this, and since only expensive and invasive treatments are available, prevention of this progressive disease is of utmost importance.

Even though MLPA P236 probemix evaluates some of the most associated alterations with AMD, there are other that were not comprised. Since it is a major pro-angiogenic regulator, *VEFGA* has been related with AMD, specifically with neovascular subtypes. *ApoE*, *CFI* or *CFHR4* are also genes that should be included in future analysis. On the other hand, SNP *C2* might be removed from the probemix since it appears to have no variance between populations.

An increase of the cohort numbers may be beneficial to consolidate the results.

Since AMD is a late-onset disease, another aspect to be improved is the age of the controls, in order to assure that they will not develop the disease. Also a long-term follow up should be acquired to guarantee that, in the younger controls, there is no modification in the classification as controls.

Finally, as a multifactorial disease, it is beneficial to analyse the diverse environmental and behaviour factors that can affect AMD's development such as smoking habits and nutritional intake.

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