Universidade de Aveiro
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Departamento de Ciências Médicas

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Testing a dementia risk group for polymorphisms in inflammation-related genes

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Ana Gabriela Henriques, Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro.

Este trabalho foi desenvolvido no grupo de Neurociências e Sinalização, e contou com o apoio do Instituto de Biomedicina Molecular (iBiMED), UID/BIM/04501/2013, da Universidade de Aveiro e da FCT (projeto PTDC/DTP-PIC/5587/2014 e JPND-BIOMARKAPD).

o júri

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palavras-chave

Doença de Alzheimer; neuroinflamação; Polimorfismos genéticos; Clusterina; Receptor do complemento 1.

resumo

A doença de Alzheimer (AD) é uma perturbação degenerativa multifatorial associada com a idade que ocorre no sistema nervoso central. Após a sua descrição inicial em 1907, numerosas teorias foram propostas para elucidar quais as principais causas associadas. A hipótese da inflamação tem sido recentemente reconhecida pela comunidade científica, uma vez que muitos estudos em modelos e doentes de Alzheimer propuseram fortes evidências da ativação do sistema imunológico e de processos inflamatórios durante o curso da doença. De facto, a acumulação de β-amilóide (Aβ) e proteína tau provocam uma resposta inflamatória cerebral como resultado do desenvolvimento patológico da AD. Atualmente, os estudos de associação genómica genética (GWAS) proporcionaram a identificação de diversas variantes genéticas que influenciam por exemplo processos inflamatórios e as vias do sistema imunitário na AD, estando as regiões polimórficas *CLU* rs11136000 e *CR1* rs3818361 entre elas. Além disso, ambos os polimorfismos de um único nucleótido (SNPs) parecem ter um papel colaborativo relativamente à eliminação de Aβ e à ativação do sistema imunitário através da estimulação do complemento.

No trabalho aqui descrito, foram realizadas análises bioinformáticas de genes de risco para a AD, principalmente o *CLU* e o *CR1*. As informações obtidas foram usadas para criar uma rede de interação proteína-proteína, bem como para realizar análises de enriquecimento de Ontologia Genética. A nossa análise bioinformática indica que ambos os genes *CLU* e *CR1* estão envolvidos numa variedade de vias de sinalização que compreendem a regulação do processo inflamatório e ativação do sistema imunológico.

A expressão genética de cada alelo de risco das SNPs *CLU* rs11136000 e *CR1* rs3818361 foi ainda avaliada em amostras de doentes "Putativos AD" e Controlos por testes de PCR e análises de sequenciação de Sanger. Adicionalmente, as frequências genotípicas e alélicas também foram determinadas com o intuito de criar um perfil genético dos grupos estudados.

Os nossos resultados demostraram que no grupo de doentes "Putativos AD" analisado para a variante *CLU* rs11136000, o alelo de risco C apresentou maior frequência (64%) quando comparado com o grupo Controlos (40%). O grupo de Controlos apresentou uma frequência de 60% para o alelo de não-risco. Para a variante *CR1* rs3818361, o alelo de risco A apresentou frequências semelhantes entre grupos, apesar do aumento da percentagem de homozigóticos de risco (6%) no grupo de doentes "Putativos AD".

Este trabalho auxilia na compreensão da relação entre estes polimorfismos genéticos e demência. Estudos adicionais devem avaliar o uso destas SNPs como ferramentas potencialmente úteis no diagnóstico da AD.

keywords

Alzheimer's disease; neuroinflamamtion; Genetic polymorphisms; Clusterin; Complement-receptor 1.

abstract

Alzheimer's disease (AD) is a multifactorial age associated degenerative disorder that occurs in the central nervous system. After its initial report in 1907, numerous theories have been proposed to elucidate on what are the related main causes. The inflammation hypothesis has been recently acknowledged by the scientific community since several studies in AD models and patients strongly supported the activation of the immune system and of inflammatory processes during disease development. In fact, the accumulation of amyloid-β (Aβ) and tau-neurofibrillary tangles provokes a brain inflammatory response as a consequence of the pathological development of AD. Currently, genome-wide association studies (GWAS) have provided several genetic variants that impact inflammation and immune system pathways in AD, being the polymorphic regions *CLU* rs11136000 and *CR1* rs3818361 among them. Furthermore, both single-nucleotide polymorphisms (SNPs) appear to have a collaborative role regarding Aβ clearance and immune system activation via complement stimulation.

In the work here described, bioinformatics analyses of AD risk-related genes, focusing on *CLU* and *CR1* were performed and the retrieved information used to rise a protein-protein interaction network, as well as to perform Gene Ontology enrichment analyses. Our bioinformatics analysis indicates that *CLU* and *CR1* are involved in a variety of signaling pathways that comprise activation and regulation of immune system process.

CLU rs11136000 and *CR1* rs3818361 genetic expression of each SNP risk allele was further evaluated in whole blood samples from "Putative AD" and Controls groups by PCR assays and Sanger sequencing analyses. Additionally, the genotyping and allelic frequencies were also determined in order to create a genetic profile of the studied groups.

Our results showed that on the "Putative AD" group analyzed for *CLU* rs11136000 variant, the C-risk allele presented a higher frequency (64%) when compared to Controls (40%). The Controls group displayed a 60% frequency for the non-risk allele. For the *CR1* rs3818361 variant, the A-risk allele showed similar frequencies among groups, although an increase in the percentage of homozygous risk carriers (6%) was observed in the "Putative AD" group.

This work aids into the understanding of the relation between these genetic polymorphisms and dementia. Additional studies should address the use of these SNPs as potential tools in AD diagnostics.

LIST OF CONTENTS

Abbreviations

Aβ	Amyloid beta-peptide
AD	Alzheimer´s disease
AICD	APP intracellular domain
APP	Amyloid precursor protein
APOE	Apolipoprotein E
APOEε4	APOE allele 4
ApoJ	Apolipoprotein J
CD	Cluster of differentiation
CD2AP	CD2-associated protein
CLU	Clusterin
CNS	Central nervous system
CR1	Complement receptor-1
CCR	C-C chemokine receptor type
CSF	Cerebrospinal fluid
CTF	C-terminal fragment
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EOAD	Early-onset familial Alzheimer's disease
GFAP	Glial fibrillary acidic protein
GWAS	Genome-wide association study
IC	Immune complexes
IDE	Insulin-degrading enzyme
IG	Immunoglobulins
IL	Interleukin
iNOS	Nitric oxide synthase
LOAD	Late-onset Alzheimer's disease
LB	Loading buffer
LPS	Lipopolysaccharide
MAC	Membrane attack complex

MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
NaOH	Sodium Hydroxide
nCLU	Nuclear clusterin
NFTs	Neurofibrillary Tangles (NFTs)
NF-kB	Factor-nuclear kappa-B
NO	Nitric oxide
PCR	Polymerase chain reaction
PET	Positron emission tomography
PSEN1	Presenilin-1
PSEN2	Presenilin-2
PPI	Protein-protein interaction
RAGE	Advanced-glycosylation end-products
RCA	Complement activation regulators
ROS	Reactive oxygen species
SCARA	Scavenger receptor A
sCLU	Secretory clusterin
SNPs	Single nucleotide polymorphisms
TAE	Tris-acetate-EDTA
TGF	Transforming growth factor
Th	T-helper
TNF	Tumor necrosis factor

1. Introduction

1.1 Alzheimer's disease and its neuropathological hallmarks

Alzheimer's disease (AD) is the primary form of age-related neurodegenerative dementia, which embodies an emerging global health crisis. In 2015, an estimated 46.8 million people worldwide lived with dementia, and the majority of these were diagnosed with AD (Figure 1) [1,2].

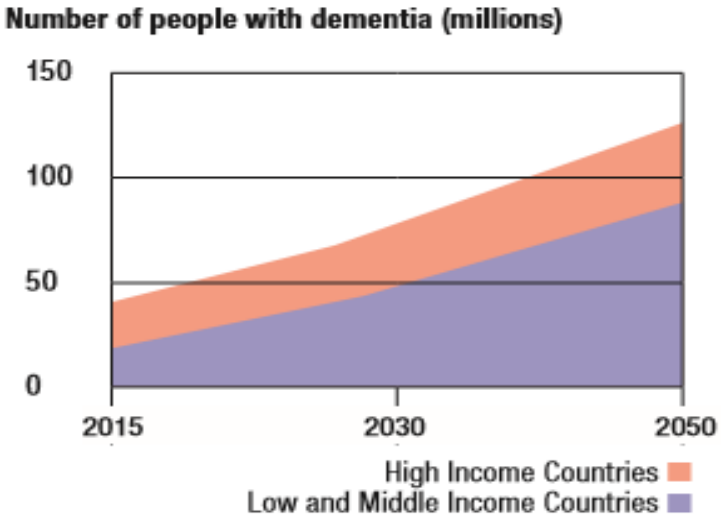

Figure 1: Expected number of people with dementia until 2050, according to the World Alzheimer Report of 2015. This analysis shows that high income countries have more affected individuals with dementia compared to low and middle income countries. Nevertheless, both will have a strong increase in the next 34 years. From[1].

This amount will nearly double every 20 years, reaching 74.7 million in 2030 and 131.5 million in 2050. The fastest expansion in the elderly population is occurring in China, India, and their south Asian and western Pacific neighbors. Recent data showed that over 153000 people in Portugal have dementia, 90000 of AD type. In other words, at least 1% of the Portuguese population has been diagnosed with this illness, representing a striking impact on the national public health system[1,3].

AD was first described in 1907 by a Bavarian psychiatrist with expertise in neuropathology named Alois Alzheimer, regarding his observations in a 51-year-old patient,

Auguste D. Her symptoms included short-term memory loss, unusual behavior and neuropathological characteristics, that later became recognized as the hallmarks of the disease[4].

The initial symptoms of this disease are frequently mistaken as part of standard aging processes, or indicators of stress. With AD development, symptoms may comprise aggression, irritability, confusion, and language problems, as well as loss of long-term memory and ultimately death[5].

Neuropathological alterations are represented by loss of neurons and synapses in the cerebral cortex and certain subcortical regions, resulting in gross atrophy of the affected areas and degeneration of specific brain areas, including temporal and parietal lobes and parts of the frontal cortex and cingulate gyrus[5]. Magnetic resonance imaging (MRI) and positron emission tomography (PET) techniques reveal specific atrophied brain regions, predominantly in the entorhinal cortex, the hippocampus and the temporal cortex, as patients followed the typical disease development stages: from asymptomatic, to mild cognitive impairment (MCI), and finally AD[6,7].

Certainly, the clinical development of AD is diverse and there are numerous features adding to the typical neuropathological extracellular senile plaques and neurofibrillary tangles (NFTs). Senile plaques, which are also known as amyloid plaques, consist of extracellular deposits of amyloid beta (Aβ) peptide, mainly represented by Aβ40, and Aβ42.

Aβ peptides derive from the proteolytic processing of amyloid precursor protein (APP), which represents an integral membrane protein with extensive expression through the body[8]. Moreover, is mostly concentrated in neuronal synapses and it is associated with neurite extension and synaptic plasticity. By contrast, NFTs are intracellular lesions entailing structures of paired helical filaments composed of hyperphosphorylated tau protein[8]. The latter is expressed predominantly in neurons, and it is responsible for microtubules stabilization and axonal transport (Figure 2)[8].

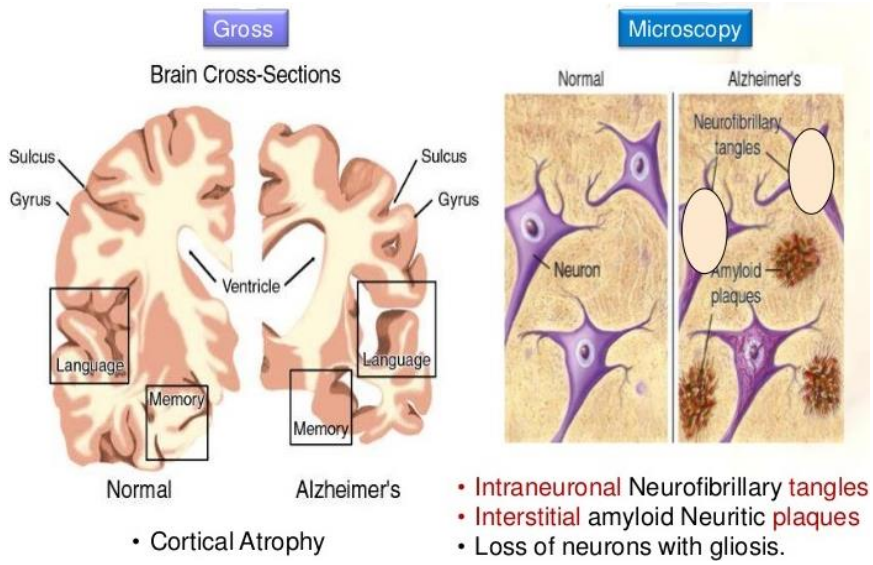

Figure 2: **Alzheimer disease and the associated morphological alterations in the brain.** This image shows gross and microscopic differences between normal and Alzheimer brain. It is possible to observe that the specific areas regarding the language and the memory are affected in the brain cross-sections in Alzheimer, compared to normal brain, which ultimately leads to cortical atrophy. Additionally, there are microscopic accumulations of intraneuronal neurofibrillary tangles, interstitial amyloid neuritic plaques and loss of neurons with gliosis in Alzheimer compared to normal brain. From[9].

Amyloid markers, such as cerebrospinal fluid (CSF) Aβ42 and PET amyloid tracer uptake are the primary modifications of AD progression, nonetheless they reach a plateau level by the MCI stage. On the other hand, functional and metabolic markers are identifiable by task-dependent stimulation on functional MRI and ^{18}F-fluorodeoxyglucose PET, being unusual indicators for the MCI stage, and continuing to alter going into the dementia stage. The final structural alterations are followed by a temporal pattern that is associated with increased tau pathology accumulation (Figure 3)[7].

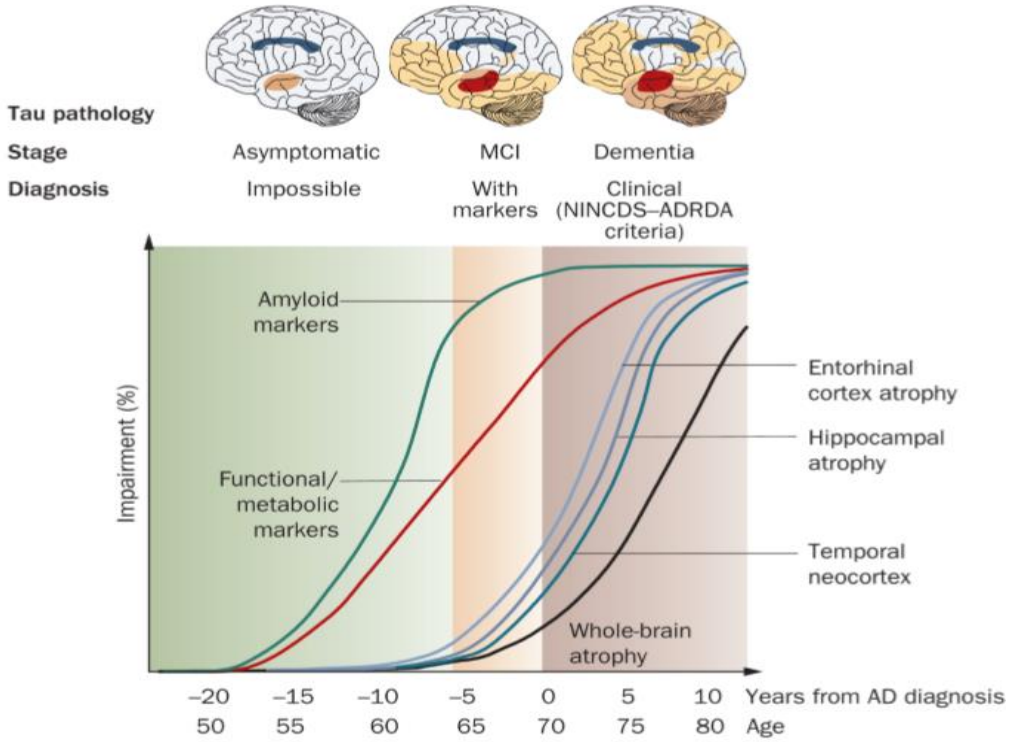

Figure 3: **The expected evolutionary model of cognitive and biological markers of AD**. Abbreviations: AD, Alzheimer disease; MCI, mild cognitive impairment; NINCDS–ADRDA, National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association. From [7].

1.2 Genetics of Alzheimer's disease and Genome-wide association studies (GWAS)

Epidemiological research discovered several risk factors for AD, mainly divided into vascular (such as, smoking, obesity, dyslipidemia, diabetes, hypertension and asymptomatic cerebral infarction), psychosocial (such as lower education, poor social engagement and physical activities) and genetic factors[8]. The latter has, in fact, a notorious role in AD and positive family history is a solid risk factor. Studies on identical twins established a high heritability for AD, being about 58% to 80%[8].

Therefore, AD is characterized by two forms centered on genetic features, namely, early-onset familial Alzheimer's disease (EOAD) and late-onset Alzheimer's disease (LOAD)[10,11,12]. The familial EOAD form is inherited by Mendelian genetic distribution in an autosomal dominant pattern and the usual age of onset is typically before 60[13]. The loci of causative genetic mutations include the *APP* gene (located in chromosome 21) and the *Presenilin-1* (*PSEN1*) and *Presenilin-2* (*PSEN2*)

genes (located on chromosomes 14 and 1, respectively), all of which have complete penetrance[10]. The EOAD genes mutations and their molecular phenotypes on AD patients, such as increase Aβ formation and aggregation are summarized in Table 1[14].

Table 1. EOAD genes and their pathogenic effects on Alzheimer disease.

Gene	Protein	Chromosome	Molecular Phenotype
APP	Amyloid-β protein percursor	21q21	Increased $A\beta_{42}/A\beta_{40}$ ratio Increased Aβ production Increased Aβ aggregation
PSEN1	Presenilin -1	14q24	Increased $A\beta_{42}/A\beta_{40}$ ratio
PSEN2	Presenilin -2	1q31	Increased $A\beta_{42}/A\beta_{40}$ ratio

On the other hand, LOAD covers more than 90% of AD patients, and still lacks a well-defined way of transmission[15]. This form shows a genetically complex pattern of inheritance of joint mechanism between genetic risk factors, aging, environmental factors and life exposure events, that together regulate lifetime risk for AD[16]. Furthermore, *APOE* (apolipoprotein E) gene, which encodes the APOE protein, has been established as a major risk factor for LOAD[17,18] and has an important role in mediating cell signaling, synaptic plasticity, neuroinflammation (inflammation in the central nervous system (CNS)), as well as in cholesterol and lipid transport[20]. APOEε4 (APOE allele 4) amplifies the risk of AD in Caucasians and Asians populations and individuals with two APOEε4 alleles have 15 to 17 times higher risk to AD than those lacking this allele[21,22]. Nevertheless, the APOE genotype describes merely 20% of AD risk[20].

Although EOAD mutations led to AD, APOEε4 allele represents a genetic risk factor for LOAD by increasing the age of onset in a dose-dependent manner and it is not a direct cause for AD development. Subsequently to the initial studies of *APOE* as a genetic risk factor for LOAD, hundreds of genes have been published with an association to AD, making it difficult to track and analyze the available data.

Currently, the best approach to search for new AD genes has been the genome-wide association study (GWAS). Several GWAS reports have identified many genes (Table S1, Appendix 1) based on numerous genetic markers as single nucleotide polymorphisms (SNPs) to find the genetic association with disease risk and/or endophenotypes such as age-of-onset, biomarkers, imaging results and neuropathological endpoints[16].

These genes are associated with numerous biological processes, including cell migration, lipid transport and endocytosis, amyloidogenesis, tauopathy, synaptic and cytoskeletal function, and are also related to immunological mechanisms and neuroinflammation[23] (Figure 4).

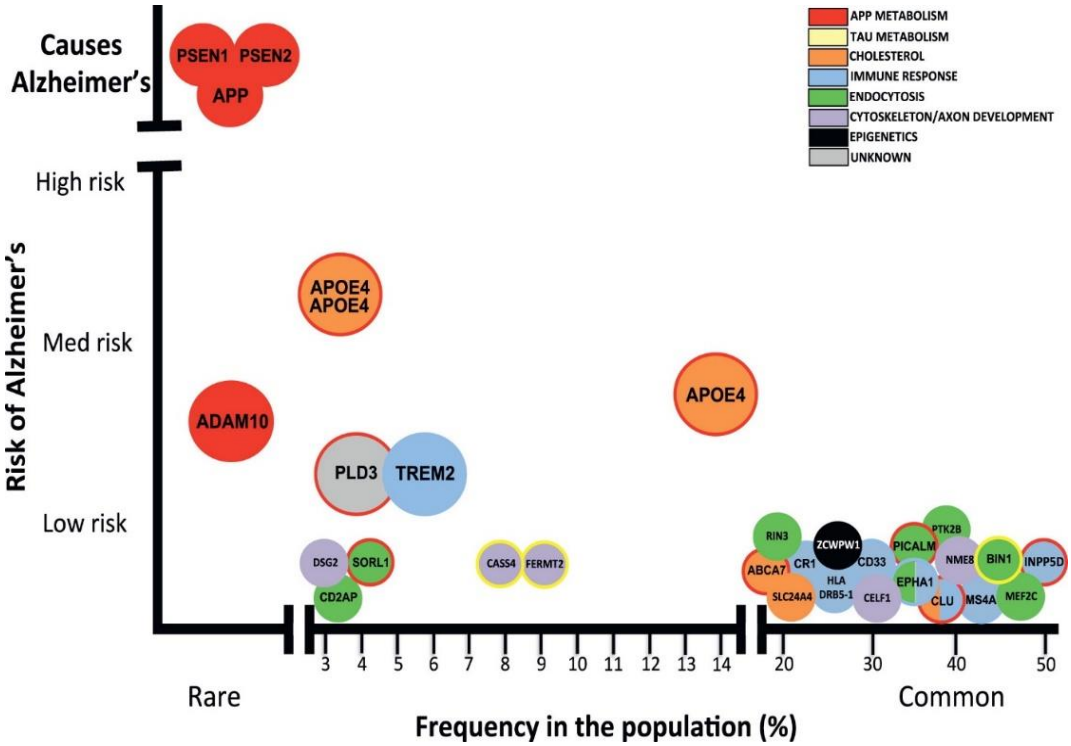

Figure 4: Rare and common variants contribute to Alzheimer's disease risk from GWAS. This illustration shows the association between the frequency of these rare and common variants in the population obtained from GWAS with the risk of developing Alzheimer's disease. The variants can be evaluated in a progressive scale going from low risk to medium and high risk. Additionally, the variants are illustrated in different colors according to their respective biologic function. Abbreviations: *ADAM10*, disintegrin and metalloproteinase domain-containing protein 10; *APP*, amyloid precursor protein; *PS1*, presenilin-1; *PS2*, presenilin-2; *APOE4*, apolipoprotein E4; BIN1, myc box-dependent-interacting protein 1; *CD2AP*, CD2-associated protein; *EPHA1*, ephrin type-A receptor 1; *MS4A6A*, membrane-spanning 4-domains subfamily A member 6A; *CLU*, clusterin; *CR1*, complement receptor-1; *PICALM*, phosphatidylinositol-binding clathrin assembly protein; *ABCA7*, ATP-binding cassette sub-family A member 7; *CD33*, myeloid cell surface antigen CD33; *HLA-DRB1*, HLA class II histocompatibility antigen, *DRB1*-1 beta chain; *HLA-DRB5*, HLA class II histocompatibility antigen, DR beta 5 chain; PTK2B, protein-tyrosine kinase 2-beta; *SORL1*, sortilin-related receptor; SLC24A4, sodium/potassium/calcium exchanger 4; RIN3, ras and rab interactor 3; *DSG2*, desmoglein-2; *INPP5D*, phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1; *MEF2C*, myocyte-specific enhancer factor 2C; NME8, thioredoxin domain-containing protein 3; *ZCWPW1*, zinc finger CW-type PWWP domain protein 1; *NYAP1*, neuronal tyrosine-phosphorylated phosphoinositide-3-kinase adapter 1; *CELF1*, CUGBP Elav-like family member 1; *FERMT2*, fermitin family homolog 2; CASS4, cas scaffolding protein family member 4; PLD3, phospholipase D3; *TREM2*, triggering receptor expressed on myeloid cells 2. From[24].

GWAS use genotyping shared ancestral polymorphisms that typically arise in 0.5% of the overall population[16]. Even though a large amount of the genetic variance of LOAD continues inexplicable, several loci containing genetic variants influencing both immunological response and inflammatory pathways in AD patients have been identified by GWAS. Among these new genes are *Clusterin* (*CLU*) located on chromosome 8, which is a powerful regulator of complement activation and *Complement receptor-1* (*CR1*) placed on chromosome 1, which is a vital player on immune system. These genes both participate in Aβ clearance, and both are major players within inflammatory response that is activated as part of the brain's innate immune system in AD (Figure 5)[16,25]. CLU rs11136000 and CR1 rs3818361 SNPs were reported to contribute for AD pathogenesis, with both variants having a strong risk for LOAD[26,27]. *CLU* and *CR1* involvement in AD will be further discussed.

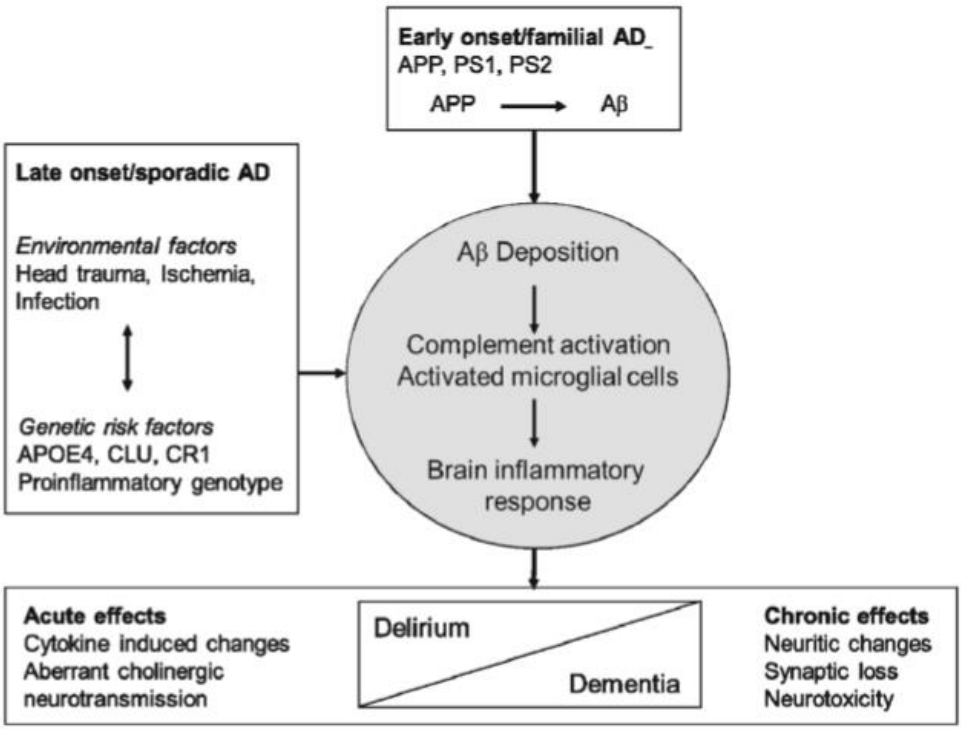

Figure 5: **Genetic risk factors and inflammation in AD.** This representation shows the association among innate immunity-related genetic risk factors and inflammation-inducing events (brain trauma, ischemia and infection) that influence the multifactorial etiology of LOAD. Both delirium and dementia encode a neuroinflammatory response that may describe the vulnerability of AD patients to additional cognitive deterioration. Subsequently, this response provokes a delirium occurrence related to a systemic inflammatory reaction. Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer's disease; *APOE4*, apolipoprotein E4; *APP*, amyloid precursor protein; *CLU*, clusterin; *CR1*, complement receptor-1; *PS1*, presenilin-1; *PS2*, presenilin-2. From[25].

The genetic findings on the etiology and pathogenesis of AD will continue to improve our knowledge on the pathological mechanisms underlying AD. Additionally, the search for further rare sequence variants in genes that prompt AD development will also aid in this understanding. Ultimately, these discoveries will support the development of new therapeutic approaches for preventing, stopping and even reversing AD course[27].

1.3 Inflammation and immune response in Alzheimer's disease

Many studies confirmed the presence of immune-related antigens and cells around amyloid plaques in the brains of individuals with AD, in addition to Aβ and tau protein aggregates[28]. Several evidences support the involvement of inflammation and immune responses in the pathogenesis of psychiatric disorders such as dementia, depression, and schizophrenia[29]. The pathogenesis of AD is also affected by immunological and inflammation mediated mechanisms. In the 1990s, studies addressing the activated complement factors, cytokines and a wide range of receptors in the brain of AD patients confirmed the association between immunological and inflammatory courses with this disease and that immune system-mediated activities promote AD pathogenesis[30,31].

1.3.1 Aβ and Neuroinflammation

As previously described, the two main pathological hallmarks for AD, namely Aβ plaques and NFTs, are key players in AD course. According to the amyloid cascade hypothesis, Aβ accumulation in the brain — resulting from aberrant processing of APP or dysfunctional clearance of Aβ peptide — is the initiating event in AD. Additionally, every EOAD related mutation influences the formation or aggregation predisposition of the Aβ peptide. In fact, the majority of cases regarding EOAD are triggered by dominant genetic mutations, mainly inked to the PSEN1 and PSEN2 proteins, that constitute part of the γ-secretase complex involved in the proteolytic process of APP[8].

Furthermore, the cleavage of APP occurs by two main ways: the non-amyloidogenic or the amyloidogenic pathway, although just the later leads to Aβ formation.

In the non-amyloidogenic via, that precludes Aβ formation, APP is initially cleaved by α-secretase, which generates a soluble N-terminal ectodomain named secreted amyloid precursor protein-α (sAPPα) - that is release from the cell surface -, and a truncated APP C-terminal fragment (CTF) (αCTF or C83). This fragment is subsequently cleaved by γ-secretase, leading to the secretion of a small non-pathogenic peptide p3 and a free APP intracellular domain (AICD), which is released into the cytosol[32]. On the other hand, in the amyloidogenic pathway, the β-secretase initiates Aβ

generation by shedding a large part of the ectodomain of APP (sAPPβ) and generating an APP CTF (βCTF or C99). This fragment is then cleaved by γ-secretase, generating Aβ and AICD. This final cleavage regulates the formation of the predominant Aβ40 or the more aggregation-prone and neurotoxic Aβ42 species (Figure 6)[32,33].

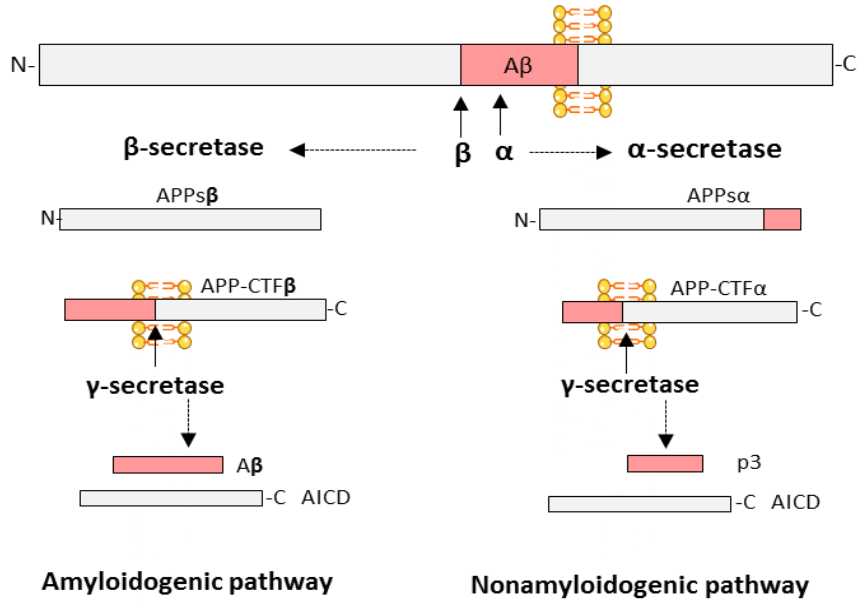

Figure 6: **APP proteolytic processing.** APP processing involves proteolytic cleavage by several secretases. The amyloidogenic pathway releases Aβ peptides through cleavage by β- and γ-secretases. The non-amyloidogenic pathway is initiated by α-secretase cleavage, which cuts the middle of the Aβ domain, resulting in the release of several soluble APP fragments. Abbreviations: Aβ, β-amyloid; APP-CTF, APP C-terminal fragment; AICD, APP intracellular domain. Reproduced from[34].

The amyloidogenic pathway products are the main pathological findings in AD[35]. Numerous (soluble and insoluble) species and aggregation states of Aβ peptide can occur, comprising monomers, oligomers, protofibrils, fibrils and Aβ plaques. Further, the latest understandings about their biology indicate that these forms most likely have variable levels of pathogenic effects. Genetic findings sustain the hypothesis that atypical formation or increase of Aβ is a pathogenic feature in both EOAD and LOAD[8] and reports confirmed the development of Aβ pathology on transgenic mice with human APP mutations, as well as in cell lines[36,37].

Additionally, the presence of Aβ can instigate microglial cells, which are the local macrophages of the CNS. These are the main cellular foundations of the innate immune system, not only by promoting the homeostasis regulation of other cells such as astrocytes and neurons in the CNS[38,39], but also by remodeling the brain being involved in the removal of apoptotic neurons and the reduction of tissue impairment[40,41].

Microglia ensure the adequate examination of their local microenvironment and protection by attacking pathogens via phagocytosis, generation of cytokines, oxyradicals, and initiation of the complement cascade[42]. Additionally, these CNS cells may also induce neural development, influence vascular growth and dissipate toxic senile plaques by phagocytosis[43]. Microglia can destroy soluble Aβ peptides via proteolytic degradation in endolysosomal compartments[44] and sphingolipid-modulated exosomes can activate microglia-mediated clearance of Aβ[45]. Conversely, senescence of microglia and failure to correctly transform the microglial phenotype could impact the success of microglial amyloid clearance[46,47,48].

In AD, Aβ endures chronic activation of stimulated microglia (due to the peptide's accumulation), which produces a continuous level of inflammatory cytokines, chemokines, reactive oxygen and nitrogen species. In addition, these substances are responsible for preserving the activation of the stimulated cells, further discussed. Consequently, this mechanism leads to a vicious loop that eventually damages microglia. This damage also results from affected adjacent resident cells of the CNS, such as astrocytes, oligodendrocytes and neurons, and perhaps aggravated tau pathology as well, which lastly causes neurodegeneration and neuron loss. Moreover, if these courses extend over a continued period, it compels the microglia cells into a senescent, 'burn-out'-like (dystrophic) phenotype, which is assumed to be permanent (Figure 7)[49].

The amyloid cascade hypothesis supports a crucial pathogenic role for Aβ, a fundamental neurotoxic peptide in AD[50]. Immunological machinery and neuroinflammation triggered by Aβ have likewise a critical role in AD development[51,52].

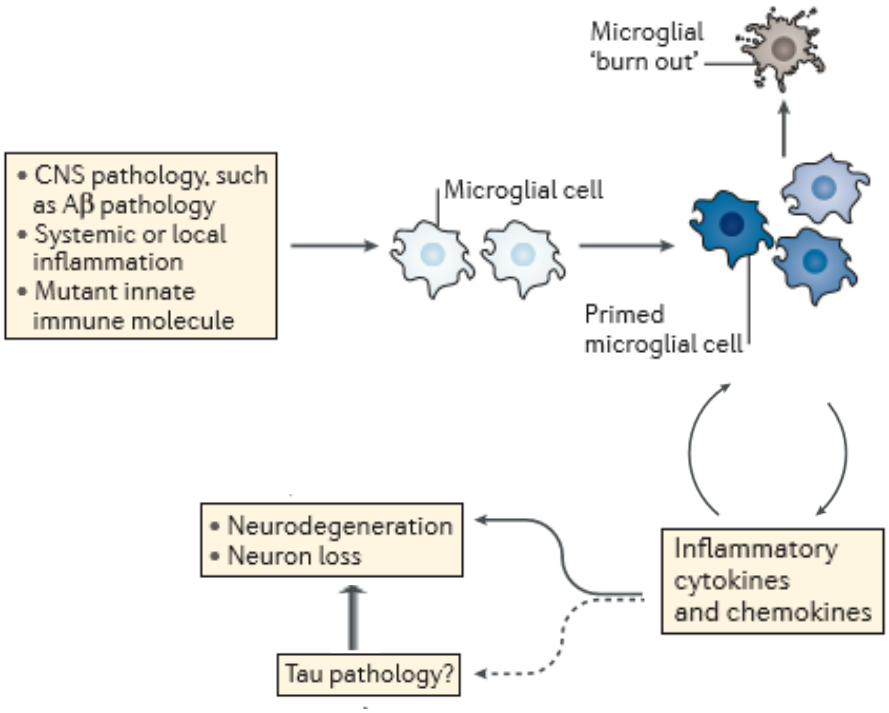

Figure 7: **Microglial phenotypes prompted by Aβ.** Generation of different microglia phenotypes stimulated by Aβ and other pathological protein deposits (alterations in the CNS, systemic or local inflammation, and mutations in genes encoding innate immune molecules). This process leads towards the formation of inflammatory cytokines and chemokines, that ultimately causes neurodegeneration, neuron loss and probably intensified tau pathology. From[49].

Clustered microglia and fibrous astrocytes surrounding extracellular deposits of Aβ plaques were mentioned in various publications. The accumulation of Aβ peptides, particularly aggregation-prone Aβ42 species, foment the formation of amyloid fibrils and Aβ oligomers in AD. Misfolded Aβ peptides can generate chemokines and similarly have a direct role as microglial attractants. For instance, chemokine receptors such as C-C chemokine receptor type-2 (CCR2), C-C chemokine receptor type-3 (CCR3), C-C chemokine receptor type-5 (CCR5), and CX3C chemokine receptor-1 (CX3CR1) exist in the microglia and brain of AD patients, and CXC chemokine receptor-2 (CXCR2) and CXC chemokine receptor-3 (CXCR33) are within the proximity of neuritic plaques[53].

Microglia are not only observed within amyloid plaques, but they also connect to Aβ oligomers and Aβ fibrils by cell surface receptors, such as cluster of differentiation (CD) CD36[54], integrin-associated protein (IAP)/CD-47, α6β1 integrin[55], scavenger receptor A-1 (SCARA1)[56], Toll-like receptors (TLRs)[57] and associated receptors (e.g., CD14)[58]. In fact, the association between receptor and Aβ fibrils promotes the *in vitro* phagocytosis by microglia cells of Aβ fibrils and the destruction of soluble Aβ species via extracellular proteases, neprilysin, and insulin-degrading

enzyme (IDE)[59]. Successively, the connection of CD36 and TLRs 4 and 6 with Aβ peptides contribute to the generation of proinflammatory cytokines and chemokines by microglia[60].

Additionally, the complement system, a crucial feature of the innate immune system, comprises anaphylatoxins and opsonins, essential for Aβ clearance. In fact, genetic removal of complement factor C3 in mice has shown to increase Aβ deposition and neurodegeneration[61]. The assembly of proteins from the complement system is also one role of microglia cells and astrocytes and reduced clearance of Aβ peptides by microglia adds to the pathogenesis of AD.

Chronic microglial activation is present in AD pathogenesis. For example, a positive feedback loop among inflammation and increased Aβ production leads to chronic, non-resolving inflammation[62,63]. Chronic inflammation leads to massive and continued release of proinflammatory cytokines, chemokines, and other inflammation mediators. This process contributes to neuroinflammation and consequently to neuronal impairment. High levels of proinflammatory cytokines such as TNFα versus low levels of anti-inflammatory cytokine TGF-β induced the progression of dementia from MCI to AD[64]. Furthermore, microglial stimulation and inflammatory reactions lead to activation of mitogen-activated protein kinase (MAPK) and intensifies hyperphosphorylation of tau protein in mouse models[63,65]. Cytokine-release impacts on tau pathology, affects neuronal transport and injures neurons[66].

On the other hand, microglia activation elevates nitric oxide (NO) and reactive oxygen species (ROS) production. In turn, ROS and reactive nitrogen species (RNS) can disrupt proteins, lipids, carbohydrates, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA), which causes several forms of injury including cell death[67]. Oxidative stress can also intensify Aβ production and aggregation process[68]. Additionally, inducible nitric oxide synthase (iNOS) is expressed in the brain of patients with AD, and its genetic removal in mice is protective regarding AD course[69].

Like microglia, astrocytes have a rapid reaction towards pathology, altering their morphology, antigenicity, and activity[70]. Astrocytes have an indispensable neuro-supportive role in the brain and their functions include the release and reutilization of transmitters, ion homeostasis, energy metabolism control, synaptic remodeling, and modulation of oxidative stress. In fact, the disturbance of the neuro-supportive astrocyte features has highly damaging magnitudes for the CNS[71].

Responsive astrocytes reside in peri-plaque areas of the brain in AD patients and in AD transgenic mouse models, with a characteristic form that resonant glial scarring - a mechanism by which the cells deliver a barrier among healthy tissue and areas of injury or infection[72]. The MCP1 (Monocyte chemoattractant protein-1 (MCP-1/CCL2)) chemokine is prominently concentrated in

Aβ plaques and acts as chemotactic for adult astrocytes. These astrocytes express receptors that associates with Aβ, including low density lipoprotein receptor-like protein, membrane-associated proteoglycans, and scavenger receptor-like receptors. Such system is responsible for the high accumulation of reactive astrocytes situated within Aβ deposits[72].

Additionally, numerous studies concluded that plaque-localized, responsive astrocytes can degrade Aβ peptides[73]. Moreover, in Tg2576 transgenic mice this feature is related to insulin degrading enzyme (IDE), crucial for Aβ degradation. IDE levels are higher in immunoreactive astrocytes around Aβ deposits[74].

Astrocyte secretion of matrix metalloproteinases may also play a key role in extracellular clearance of Aβ[75]**.** Aβ disturbs astrocyte calcium homeostasis[76] and elevates GFAP, a process associated with degeneration of neurons in astrocyte-neuron co-cultures[77]. Responsive astrocytes induce neuropathology via expression or overexpression of several of inflammation-related factors. For instances, S100 calcium-binding protein-b (S100b), a neurotropin that promotes neurite growth, is overexpressed in AD brain, due to countless dystrophic neurites within Aβ deposits[78]. Further, Aβ can also lead to IL-1β, TNF-α, iNOS, and NO formation in astrocytes[79].

Most of these results may be suggestive of modifications regarding astrocyte transcription factors. The proinflammatory cytokines IL-1β, IL-6, and TNF-α are regulated by nuclear kappa-B (NF-kB) and CCAAT-enhancer-binding proteins (C/EBP) transcription factors. Astrocyte NF-kB is also induced upon Aβ exposure, which augments IL-1β and IL-6 levels. Hence, the release of chemokines and adhesion molecules in astrocytes can be regulated by NF-Kb mechanisms, which possibly allows the incursion of peripheral leukocytes and adds to an inflammatory reaction in the brain[80].

1.3.2 Immunity effects

The microglial activation process is very diverse and certainly influences the evolution of neurodegenerative diseases[81].

Infiltrated blood macrophages from periphery and perivascular macrophages have an important role in the pathogenesis of AD[82]. For instance, activated perivascular macrophages decrease the cerebral amyloid angiopathy and Aβ deposition in cerebral blood vessels and leptomeninges in a mouse model of AD[83], an event involving the CCR2 chemokine, that is crucial for Aβ clearance by perivascular macrophages[84]. Indeed, elevated infiltration levels from peripheral macrophages were reported in the CNS of AD model mice[85], which may partly contribute to Aβ clearance.

Several in vitro studies of oligodendrocyte toxicity has been described for Aβ25-35[86], Aβ40[86,87], and Aβ42[88]. Oligodendrocytes express mRNAs and show immunoreactivity towards complement components C1q, C1s, C2, C3, C4, C5, C6, C7, C8, and C9. This fact proposes that these may possibly be a major cause for complement augmentation in pathologically-vulnerable areas of the AD brain[89]. Complement stimulation takes place on oligodendrocytes via C1q association to myelin oligodendrocyte protein and results from reduced levels of C1 inhibitor and membrane cofactor protein expressed by these cells.

Aβ can also impact at the white blood cells. Aβ fibrils function as an altered self-antigen, improving Aβ specific T lymphocytes. Blood vessels contiguous to Aβ fibrils display elevated levels of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which induces extravasation of stimulated Aβ specific T lymphocytes[91].

Additionally, the population of lymphocytes was shown to be altered in peripheral immune system with low levels of peripheral T and B lymphocytes in AD patients[92]. In particular advanced stages, it is possible to detect reduced levels of naive CD4+ T lymphocyte subpopulations, CD19+ B lymphocytes and naive B cells (immunoglobulin (Ig)-D+CD27−) and also increased double negative (IgD−CD27−) memory B cells detection. Further, a reduction of CD8+CD28− suppressor T lymphocytes and IL-10 production in AD was also reported, which suggests lower immunosuppressive skills[93].

In AD, chronic inflammation promotes the reduction of naïve lymphocytes and the expression of altered chemokine receptors in lymphocytes. Furthermore, the elevated expression of pro-inflammatory chemokine receptors, C-C chemokine receptor type 6 (CCR6) and C-C chemokine receptor type 7 (CCR7) on B lymphocytes can contribute to chronic inflammatory condition[94].

1.4 Clusterin

Clusterin (CLU) is an approximately 80kDa chaperone glycoprotein that can be dissociated in two 40kDa chains under reducing conditions. It is present in most of biological fluids[95].

The human *CLU* gene is located on chromosome 8 and contains 9 exons presented with numerous transcriptional isoforms[95,96]. The mature protein matches a secretory form of clusterin (sCLU), being a heterodimer with α and β subunits connected with five disulphide links. Individually, these subunit expresses three N-glycosylation locations with a carbohydrate heterogeneity, which improves the variety of glycoforms. In addition to sCLU that was initially identified, a nuclear form (nCLU) was described in human and murine cell lines exposed to many stress promoters such as radiations, heat shock, drugs, as well as in carcinoma cells. Furthermore, nCLU assembly was also observed in other conditions such as ethanol-induced cell death and hypoxia in the neonatal rodent brain[97].

Contrarily to sCLU cell survival effects, nCLU is related to cell death by apoptosis. The sCLU is extensively expressed in the body, being mostly secreted in CSF[98]. Elevated plasma clusterin in healthy centenarians (older than 105 years) suggests a potential function for this protein through longevity[99]. These mechanisms are still not completely understood, although additional reports acknowledged the involvement of other molecules that also function as binding ligands of sCLU such as complement factors, immunoglobulins, leptin, Aβ, alpha-synuclein (α-synuclein), prion protein, transforming growth factor beta (TGFβ)-receptors and stressed unfolded proteins[100,101].

1.4.1 Clusterin in Alzheimer's disease

Clusterin is associated with lipid transport and metabolism, membrane protection, cell-cell interaction, apoptosis, and complement regulation. Regarding the complement system, clusterin controls the membrane attack complex and limits complement stimulation related to inflammatory reaction[102].

Under normal conditions, some neuronal subpopulations, particularly at the pontobulbar and spinal cord motor nuclei have high expression levels of CLU. Moreover, latest investigations in postmortem human brain reveled plenty populations of neurons expressing low levels of clusterin mRNA in the neocortex. CLU is also present within a few subpopulations of astrocytes, specifically over the hippocampal and in some neocortical subdivisions[103].

Nowadays, it is known that CLU levels in the brain fluctuate throughout healthy aging. In line with this evidence significant age-related difference in clusterin expression expression were observed in glial cells [105].

Reduced levels of CLU in high-density lipoproteins is linked to insulin resistance and obesity, which are also known risk factors for AD. In AD, several studies reported the co-localization of CLU in NFTs and extracellular Aβ deposits. Further, GWAS and meta-analyses indicated *CLU* as one of the LOAD susceptibility genes. These studies also described that increased clusterin plasma levels may be associated with brain atrophy, disease severity, and disease progression[106].

In AD, the *CLU* variants may obstruct the usual function of clusterin as a protective factor against oxidative stress, by inhibiting the normal role of clusterin as a sensor and chaperone of ROS. Among different reported SNPs the variant rs11136000 has a particular connection with plasma CLU levels and contributes to AD with similar genetic risk effects in both the Asian and Caucasian populations. The T-allele is assumed to be associated with reduced risk in AD development and better cognitive performance, whereas the C-allele represents a risk factor for AD development. It was reported that homozygous expression of the C-allele facilitates neural hyperactivity in frontal and posterior cingulate cortices as well as hippocampus, throughout a visual working memory task, a hyperactivation that at long term can be detrimental[107,108,109].

Furthermore, additional findings revealed reduced or even absent hippocampal–prefrontal connectivity during memory retrieval in AD patients and also in subjects suffering from MCI[110]. For the risk variant, a C-allele dosage-dependent variation of the efficient combination in hippocampus and prefrontal cortex during episodic memory tasks was observed applying genetic and imaging methods on healthy subjects (Figure 8)[103].

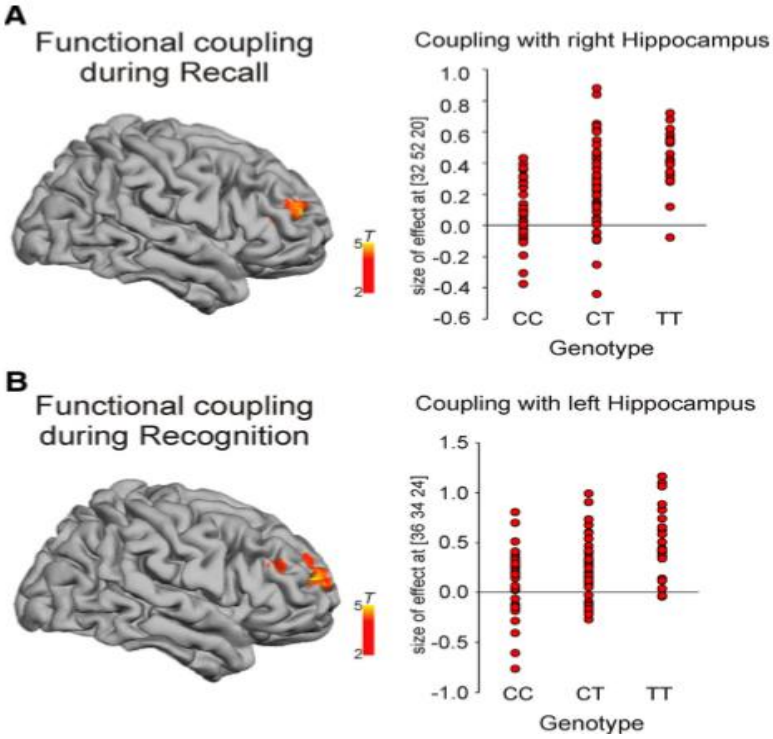

Figure 8: **Altered functional coupling in carriers of rs11136000 risk variant for *CLU***. **A**: carriers of the C-risk allele reveal considerably reduced allele dosage-dependent coupling of the right dorsolateral prefrontal cortex (DLPFC) with the right hippocampus seed region through recall. Each red dot indicates size of effect in one subject and reproduces the connectivity among right DLPFC and right hippocampal seed area. **B**: carriers of the C-risk allele reveal considerably reduced allele dosage-dependent coupling of the right dorsolateral DLPFC with the left hippocampus seed region through recognition. Each red dot indicates size of effect in one subject and reproduces connectivity among the right DLPFC and the right hippocampal seed area Adapted from[103].

Further reports showed altered white matter integrity in healthy carriers of the rs11136000 risk variant, which translates to neurodevelopmental susceptibility[111]. Additionally, young healthy carriers of the C-allele presented a diminished fractional anisotropy, caused by myelin integrity loss rather than axonal injuries, using diffusion tensor MRI imaging. Microstructural alterations in long interhemispheric and intrahemispheric associations such as the corpus callosum, the fornix and the cingulum, are related to a prodromal vulnerability in AD[112,113].

As mentioned, individuals expressing the genotype related to greater risk (CC homozygous genotype) have higher neural activation in a task related network. A possible explanation would be that carriers of the protective T-allele would achieve an equivalent level of task performance, although with less brain stimulation and that greater cognitive effort is necessary in at-risk

individuals to complete equivalent levels of performance[102]. Moreover, additional reports described that carriers of this recently discovered rs11136000 AD risk variant of *CLU*, had longitudinal increases in neural activity resting state in brain regions critical to memory processes, in order to maintain their physiological function in cognitively normal at-risk individuals. Supporting these supposed compensatory boosts in neural response in some individuals may be the threshold beyond which primary cognitive damage initiates, setting their conversion from MCI to AD[114]. Increased hippocampal stimulation throughout memory processes in MCI individuals has also been observed in rs11136000 risk carriers, which may ultimately express earlier rates of decline in memory performance comparative to non-carriers at the pre-symptomatic stages of AD[115,116].

Besides, increased levels of CLU were identified on cerebrospinal fluid of AD patients, which may represent a relevant peripheral marker associated with disease development.

In vitro and *in vivo* experimental models indicated a potential linkage between CLU and Aβ aggregation and clearance. Clusterin levels improve the degree of Aβ clearance and efflux via the blood brain barrier[117]. Furthermore, extracellular sCLU mutually limits Aβ40 monomers aggregation and also the toxic and inflammatory effects of oligomers by sequestration previous to peptides degradation. In addition, a recent meta-analysis demonstrates that sporadic coding variants affecting sCLU β-chain are mostly observed in AD[118].

1.5 Complement receptor-1

Complement receptor-1 (*CR1*), also known as C3b/C4b receptor or CD35, is situated on chromosome 1q32 and encodes CR1 protein. It is associated with the complement response and is part of the regulators of the complement activation (RCA) family of proteins[119].

The largest production of complement proteins occurs in the liver, but is also highly present in the serum. Nevertheless, glial cells and neurons in the CNS can synthesize these proteins as well during injury and neurodegeneration[120,121-125.]

The complement system identifies molecular arrangements on pathogens or molecular patterns associated with damaged tissues and dying cells. Recognition may occur via C1q or mannose binding proteins, which have collagen-like receptor binding domains, or over interfaces with the multifunctional protein C3. After being triggered, a cascade of several proteins from the complement pathway can attract and stimulate immune cells, amplify antigen-specific immune responses, induce phagocytosis, support complement-mediated cytolysis by the membrane attack complex (MAC), and control cell proliferation and differentiation[120].

Furthermore, both classical and alternative pathways activation can form catalytic multiproteins, namely the C3 convertases, producing two proteolytic C3 fragments: C3a, which is associated with inflammation and phagocyte recruitment, and C3b that could trigger the lytic pathway related to C5, C6, C7, C8, and C9, which ultimately generates MAC, C5b-9, and may induce cytotoxicity. This pathway also leads to C5a activation, an additional small proinflammatory peptide. Then, C3b binding to specific molecules, by a mechanism entitled opsonization, facilitating phagocytosis by a number of cells through their surface expression of complement receptors (Figure 9)[111].

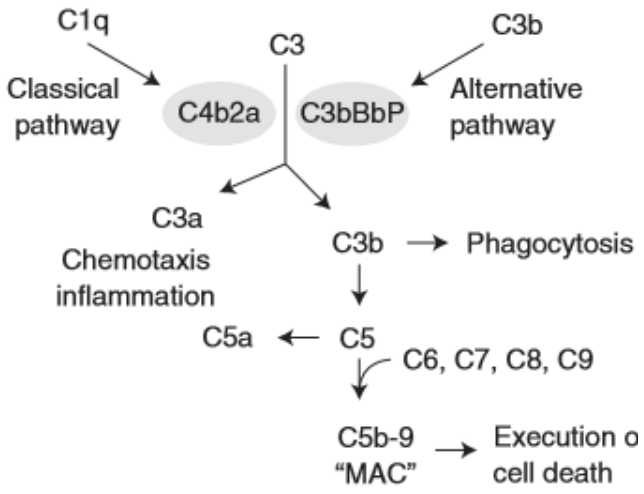

Figure 9: Schematic interaction of the complement system components. Abbreviations: complement proteins, C1q, C3, C3b, C3a, C5, C5a, C5b, C6, C7, C8, C9; membrane attack complex, MAC. From[111].

1.5.1 Complement receptor 1 in Alzheimer's disease

Many polymorphisms for *CR1* have been studied, demonstrating that SNPs may affect the expression of CR1 molecules on the cell surface[119]. In AD, these SNPs on *CR1* are reported to be associated with greater risk for disease development[120]. In fact, Aβ can activate the complement system[121] and large GWAS confirmed that rs3818361 within the *CR1* locus is associated with LOAD susceptibility in Caucasians[122]. The relationship between *CR1* markers and LOAD in European and American population is additionally confirmed by associations between disease and the rs3818361 variant[26,27]. This variant is also associated with neuroimaging measures and neuritic plaques burden in AD brains.

Studies have explored inter-group variances in brain amyloid burden among risk (AG heterozygous genotype/AA homozygous genotype) and non-risk (GG homozygous genotype) carriers of the AD rs3818361 SNP in *CR1* gene. Recent GWAS results showed a larger risk in carriers of the A-risk allele of this SNP in AD individuals. Significantly greater variance in brain amyloid deposition was observed in the non-risk group (GG homozygous genotype), which appeared to be associated in part with *APOE* genotype[120]. Consequently, GG homozygous genotype and *APOE* ε4 carriers had more amyloid deposition in numerous brain areas, comparative to *APOE*ε4 non-carriers.

Although the *CR1* A-risk allele was linked to reduced fibrillar amyloid in non-demented individuals, these authors have also observed elevated brain amyloid levels in carriers of the APOEε4 allele relative to non-carriers in cognitively normal older individuals[120]. Additional studies confirmed that individuals expressing GG homozygous genotype and APOEε4 positive results exhibited reduced episodic memory, which is a common endophenotype of LOAD. This indicates that there is a greater susceptibility for AD development in individuals caring both the risk variant rs3818361 and the APOEε4 allele[26].

Additionally, *CR1* is pointed as a vital AD susceptibility gene, since the expression of complement factors are upregulated in affected regions of AD brains[126].

CR1 protein regulates the complement activity and high complement cascade activity can exacerbate AD pathology. Essentially, the presence of CR1 on phagocytic cells induces them to phagocyte specific particles, by stimulating the complement reaction. Of note, high CR1 expression levels are related to elevated cognitive deterioration in AD. Further, Aβ clearance in the brain is associated with CR1 expression[127]. Clearance of plasma Aβ42 is dependent on binding of CR1, which therefore leads to glial activation, ultimately promoting neural degradation and chronic inflammatory cascade activation[127,128]. CR1 protein expression may modulate microglia, leading to an amplified clearance rate of immune complexes[129]. Since genetic variance in CR1 modifies the volume of Aβ clearance by the immune response, inadequate clearance of Aβ and amplified chronic inflammation by *CR1* polymorphisms can contribute to AD pathogenesis[130].

2. Aims of the Thesis

Both *CLU* and *CR1* are two of the most common genes from Alzgene database. These genes comprise risk alleles polymorphisms involved in AD development and pathogenesis, but limited evidences are known about the molecular mechanisms involved.

CLU encodes for the protein clusterin, which is highly expressed in the brains of individuals with AD. It can be related to Aβ aggregation, and complement response activation. In fact, in individuals with MCI and AD, elevated plasma clusterin is correlated with a more rapid cognitive decline. On the other hand, *CR1* is a complement receptor present in the cerebral cortex. Emerging evidence supports the notion that the complement cascade is involved in neurodegeneration process prompted by astrocyte-mediated opsonisation by *CR1*. Thus, *CLU* and *CR1* have emerged as very interesting targets for AD.

The aim of this thesis was to address both inflammation-related gene variants in a dementia subpopulation group from a primary care-based cohort previously established by the Neuroscience and Signalling group. Hence the specific aims of this thesis were to:

- Perform a literature survey and a bioinformatic analysis on AD inflammation related-genes;
- Amplify the polymorphic regions rs11136000 and rs3818361 of *CLU* and *CR1* genes respectively, from blood samples of the study group;
- Determine the genotype and the allelic frequency of the polymorphic regions rs11136000 and rs3818361 of *CLU* and *CR1* in the study group;
- Correlate genotypic and allelic frequencies with dementia.

3. Materials and Methods

3.1 Bioinformatics analyses

3.1.1 Literature survey on AD risk-related genes and construction of a protein-protein interaction network

The genes identified for this study were collected from a literature survey of review articles published between 1/1/2015 to 31/12/2015 at PubMed database[131] (using the key words Alzheimer's disease and risk genes, article types), that complemented the data available on AD risk related genes. Only the interactions that were curated in *Homo sapiens* have been included.

The genes were identified by their own ID: gene symbol (www.genecards.org/)[132] and UniProtKB identifier (www.uniprot.org/)[133]. The gene symbol identifier of the collected proteins was loaded into STRING database (http://string-db.org)[134] to generate a protein-protein interaction network. The network was generated using data from "Gene Ontology (GO) Biological Processes", "GO Molecular Functions" and "GO Cellular Components" as a network enrichment method. Markov Cluster algorithm (MCL) was also applied in order to calculate the powers of the associated adjacency of this network.

3.1.2 Gene ontology analysis

The analysis (adjusted P-value < 0.05) was carried out using STRING database according to GO annotation. Data regarding *CLU* and *CR1* genes were collected for 'biological processes', 'molecular functions' and 'cellular component', particularly focusing on GO in which these genes were involved.

3.2 Characterization of the study group

Our pilot study group included a population of 63 individuals from a cross-sectional population-based study on a Portuguese population from the Aveiro region: 32 of which were individuals with dementia, based on cognitive evaluation tests, herein referred as "Putative AD" group and 31 individuals that forms the Controls group. This is part of a project approved by the ethics committee for health of the central regional administration in Coimbra (Comissão de Ética para a Saúde da ARS Centro, protocol 0128804-04.04.2012), and by the National Committee for Data Protection.

These 63 individuals represent a subgroup of a total of 590 individuals (568 fulfilled the inclusion criteria), nominated by primary care-based cohort (pcb-Cohort). Individuals undertook a

survey and were submitted to several cognitive evaluations and dementia screening tests during an interview, regardless of the clinical diagnosis. Screening tests comprised Clinical Dementia Rate (CDR), Mini Mental State Examination (MMSE), Geriatric Depression Scale (GDS), Katz of Activities of Daily Living (ADL) and Instrumental Activities of Daily Living (IADL). A subset of individuals that were both CDR positive (CDR≥1, that represents a dementia cognitive rate from moderate to severe) and MMSE positive, were designated as "Putative AD" group, which in fact included individuals clinically diagnosed for AD. The "Putative AD" group came from a dementia risk group of 68 individuals based on CDR classification (Martins *et al.* 2016, Dementia geriatric cognitive disorder, in press), that were also MMSE positive. Controls and "Putative AD" individuals were age and sex matched. All participants provided written informed consent for the study. The age and gender variables are shown on Table 2.

Table 2. Representation of the gender and age variables between the "Putative AD" group and the Controls group.

	GENDER		**AGE**			**MEAN AGE**
	N	**%**	**INTERVAL**	**N**	**%**	
CONTROLS	**MALE**					76
	10	32%	**50-64**	3	30	
			65-74	1	10	
			≥75	6	60	
	FEMALE					
	21	68%	**50-64**	2	10	
			65-74	4	19	
			≥75	15	71	
"PUTATIVE AD"	**MALE**					77
	10	31%	**50-64**	2	20	
			65-74	2	20	
			≥75	6	60	
	FEMALE					
	22	69%	**50-64**	2	9	
			65-74	4	18	
			≥75	16	73	

According to the gender variable, the entire study group was mainly represented by female individuals (69% for "Putative AD" group and 68% for the Controls group, against 31% and 32% for males from the "Putative AD" group and Controls group, respectively). A large portion of the samples were from female individuals over 75 years old for both "Putative AD" and for Controls groups, comparative to male individuals. There were no significant differences regarding the mean ages for both analysed groups.

3.3 PCR analyses

3.3.1 Blood samples collection

Blood samples were collected in EDTA-tubes according to standard procedures and were promptly aliquoted and frozen at -80°C, upon arrival in the laboratory.

3.3.2 Genotyping test

PCR technique is a scientific tool used to replicate DNA from a template, generating numerous copies of a specific sequence. This technique was used to amplify a specific portion of *CLU* and *CR1* polymorphic regions, respectively rs11136000 (with 685bp) and rs3818361 (with 664bp) from each patient. For the PCR reaction, Phusion Blood Direct PCR Master Mix (ThermoFisher Scientific, USA) was employed according to the manufacturer's instructions. 1µl of whole blood was used in the amplification of each gene and no previous gDNA extraction was required.

Primers were designed as previously described[169] and tested for uniqueness using the NCBI BLAST W search engine (Table 3).

Table 3: **Sequence of the primers and the concentration used for the genotyping of the *CLU* and *CR1* polymorphism**[135].

Gene	SNP ID	Primer name	Sequence (5'-3')	Concentration of primer in AS-PCR (µM)
CLU	rs11136000	CLU-Fw CLU-Rv	CCTGGCTTAAAGAATCCACTCATC CAGGGGATTCCTTTGAGATAGAGT	0.1µM 0.1µM
CR1	rs3818361	CR1-Fw CR1-Rv	TTCAACTACTGGTTATGGAGCA CACTCACCCTTCATCGCAAA	0.1µM 0.1µM

PCR technique was performed in a final volume of 20 μL, per gene reaction. The PCR round comprised 10 μL of 2XPhusion Blood II DNA Polymerase Master Mix (ThermoFisher Scientific, USA), 1μL of primer forward (Fw) (Eurogentec, Belgium) and 1μL of primer reverse (Rv) (Eurogentec, Belgium) of each corresponding gene, 1 μL of whole blood and 7 μL of ultrapure DNase/RNase-free distilled H_2O (ThermoFisher Scientific, USA).

The PCR program included an initial denaturation step at 98°C for 5min, followed by 35 cycles of amplification at 94°C for 1min, 63°C for 30sec and 72°C for 46sec. The final extension was performed at 72°C for 5min.

Following amplification, the PCR products were centrifuged at 1000×g during 1 to 3 min to sediment all blood residues and DNA samples were precipitated as described below.

3.3.3 Precipitation of DNA fragments

1/10 of sodium acetate (pH 3M, pH 5.2), followed by 2.5 volumes of 100% Ethanol were added to the solution, which was then mixed and placed at -20°C overnight. Afterwards, the tubes were centrifuged at 14000rpm for 20 min at 4°C. The supernatant was discarded and 100 μL of Ethanol 70% was added to the remaining *pellet*. The tubes were placed at -20°C for 20 min, and later on centrifuged at 14000rpm during 5 min. The supernatant was discarded and the DNA pellet was dried out completely at 37°C. Finally, 13 μL of ultrapure DNase/RNase-free distilled H_2O was added to each tube in order to dissolve the *pellet*. The purified DNA fragments were stored at -20°C for long term storage and/or 4°C for short term storage.

In order to verify the successful precipitation of the DNA fragments, 2 μL of each sample were analysed by agarose gel electrophoresis. DNA was precipitated from 10 μL of the previous amplified PCR product.

3.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a technique used for separation of DNA fragments. As DNA is a negatively charged molecule, it migrates from cathode to anode by the porous matrix of agarose, via electric field. The separation process occurs according to the fragment size, leading to a faster migration by the shorter molecules. The percentage of the agarose gel depends on the DNA band size that we intend to examine.

Subsequently to the precipitation of the DNA products, conventional electrophoresis was executed at 100V for 35 min in 1XTris-acetate-EDTA buffer on 1.5% agarose gel stained with 4 µL of GreenSafe Premium® (Nzytech, Portugal)/100ml of agarose (Nzytech, Portugal) solution. From the PCR products 2 µL was used to run on the gel. Loading buffer (LB) was added in a ratio of 1:6 to increase sample density. The 1Kb Plus DNA Ladder (ThermoFisher Scientific, USA) was used as a molecular weight marker. The purified DNA fragments were visualized under ultraviolet (UV) light and further analysed with ImageLab 5.2.1 (Bio-Rad Laboratories, Inc., USA) software.

After DNA precipitation, 10 ml of the product was analyzed by Sanger Sequencing, using the previous primers to allow *CLU* and *CR1* polymorphisms detection.

3.4 Sequencing analysis

Sanger sequencing technique uses a DNA polymerase that copy single-stranded DNA templates by assembling nucleotides to a growing chain (extension product). This process occurs in the 5' -> 3' direction. At the 3' end of a primer starts the chain elongation, being the place were an oligonucleotide anneals to the template. The product extension occurs by additional deoxynucleotides incorporation by complementary to the template. The extension product grows by the formation of a phosphodiester bridge among the 3'-hydroxyl group on the primer and the 5'-phosphate group of the incoming deoxynucleotide, which enables the development of the extension product.

Further, DNA polymerases can also add analogues of nucleotide bases, as the dideoxy method of DNA sequencing that uses 2',3'-dideoxynucleotides as substrates. When dideoxynucleotides are combined at the 3' end of the developing chain, chain elongation is completed selectively at A, C, G, or T. After the binding of the dideoxynucleotide, the chain is absent of a 3'-hydroxyl group therefore further elongation of the chain is prevented.

The sequencing results were analysed with BioEdit (Ibis Biosciences, Carlsbad USA) software.

4. Results

4.1 Bioinformatics analyses of *CLU* and *CR1* genes in AD pathology

In the field of AD, there are several GWAS emphasising novel potential susceptibility loci, beyond the well-established *APOEε4* association with disease. The markers on a GWAS array comprise selected gene polymorphic variants based on their aptitude to cover common variation in the human genome. An increasing amount of other GWAS risk genes for AD involved in inflammation have been discovered, among those are *CLU* and *CR1*.

To better understand the involvement of these genes in AD, a search for the available AD related-genes and their interacting proteins was performed in databases and in the literature. The retrieved information was further analysed by raising a protein-protein interaction (PPI) network, and by performing a Gene Ontology (GO) (adjusted P-value<0.05) term enrichment, a pathway analysis for gene characterization. Particular emphasis was additionally given to inflammation and immune response related-genes, as *CLU* and *CR1*.

4.1.1 Interaction network of AD risk-related genes

During the literature search, information regarding several genes involved in AD pathogenesis but also associated with inflammation and immune response were collected. To identify the common inflammation and immune response interactors of AD risk-related genes, human protein ID were collected from several reports from the public database PubMed. Furthermore, in order to evaluate the AD related genes interaction network, human protein ID's of those genes were introduced in STRING database, which raised a PPI network, where only curated interactions have been considered.

A total of 32 genes were identified as AD interacting genes, among which 14 genes were associated with immune response. As expected, both *CLU* and *CR1* were identified within the 14 gene list. The full list of AD related-genes and its association with inflammation and immune response pathways is presented in Table S1 (Appendix 1).

A network was generated using "Biological process based" as the network weighting method. A total of 32 proteins (UniProtKB identifier corresponding to the above mentioned genes) were used as input on STRING. The extracted network consists of 32 protein nodes and 68 predicted associations between these proteins (Figure 10).

The originated network showed multiple proteins and clusters organized by different sizes and colours. Such clustering indicates that the proteins are at least partially biologically connected, as a group. Coloured lines between the proteins indicated the various types of interaction evidence. Each interaction between nodes was created and represented by different colours, being separated by boundaries with no flow. This network was the result of clustering proteins by common features (high-flow regions). The obtained network showed 5 separated clusters highlighted by different colours (bue cluster, yellow cluster, red cluster, green cluster and brown cluster), that were obtained according to the MCL clustering (MCL=3) applied algorithm analysis. (Figure 10).

There are also several disconnected nodes in this network including CELF1, CASS4, PLD3, TREM2, MEF2C and NYAP1, which mean that these nodes have no proven direct association to date. Still, these proteins are represented in the network by a respective colour, which links these nodes to specific clusters within this network.

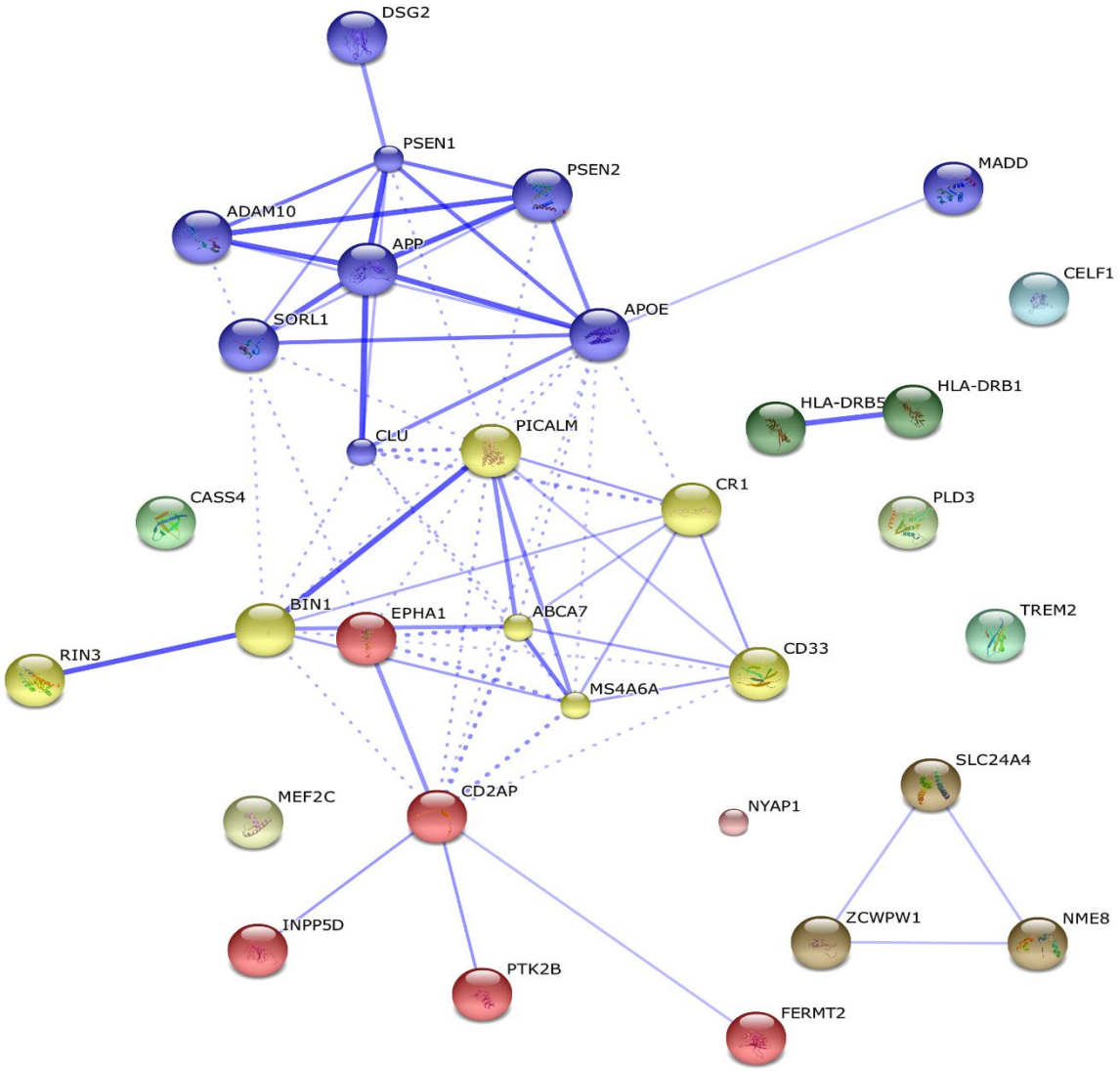

Figure 10: Interaction network of AD risk related-genes. This protein interaction network consists of 32 protein nodes and 68 protein interactions analysed by String database. The network nodes are proteins and the edges are represented by predicted confidence associations represented by blue line thickness or blue dots, as an indication of the available data support between the nodes. Five separated clusters were obtained using the clustering algorithm MCL (MCL=3). (Low edge confidence (dots): 0.150; Medium edge confidence: 0.400; High edge confidence: 0.700; Highest edge confidence (darker blue line: 0.900).

The highest edge confidence cluster in the network (blue cluster) was associated with the most numbers of nodes, including DSG2, PSEN1, PSEN2, ADAM10, MADD, SORL1, APOE, APP and CLU. The disconnected node CELF1 is likewise associated with this cluster. As expected, this cluster includes the most well-known genes contributing to AD pathogenesis, such as APP, PSEN1, PSEN2 and APOE. CLU, a protein associated with Aβ clearance, was strongly linked to the proteins APP, APOE and PSEN1.

CR1 was associated with the cluster that included the nodes RIN3, PICALM, BIN1, ABCA7, MS4A6A and CD33 (yellow cluster), which represent common proteins associated with LOAD. CR1, a protein related to immune response and also to Aβ clearance was directly linked to BIN1, ABCA7, MS4A6A, PICALM and CD33 by medium confidence edges. It was also connected to CLU by a low confidence edge.

HLA-DRB1 and HLA-DRB5 nodes, two members of the major histocompatibility complex, were the only proteins that represented the green cluster in this network. Despite being disconnected nodes, CASS4, PDL3 and TREM2 (represented by green coloured nodes) are also related to this cluster.

The nodes EPHA1, CD2AP, INPP5D, PTK2B and FERMT2 were included in the red cluster, as the disconnected NYAP1 node. This cluster is centrally represented by CD2AP, which is linked to EPHA1 by high edge confidence association, and to INPP5D, PTK2B and FERMT2 by medium edge confidence. Still, SLC24A4, ZCWPW1 and NME8 were the only members of the brown cluster, being all linked by medium edge confidence.

4.1.2 GO analysis of *CLU* and *CR1* within the AD risk-related genes network

CLU and *CR1* AD risk-genes identified in the present study were analyzed by STRING database according to GO annotation by grouping them into 3 categories: biological processes, molecular functions and cellular components. The statistical method using the Bonferroni correction was applied and only GO terms with a p-value < 0.05 were considered.

Particular emphasis was given to *CLU* and *CR1* and to the inflammation related processes in which these genes were involved. The results obtained from the network regarding the biological process revealed 15 GO terms, where *CLU* and *CR1* were found. All of these GO terms were also associated features within inflammation and immune response processes (Table 4).

Table 4: GO terms regarding biological processes associating *CLU* and *CR1* within the AD risk-related genes network. "Count in gene set" indicates the total number of counted proteins that were categorized from the network. p-value < 0,05.

BIOLOGICAL PROCESSES			
GO term	**Count in gene set**	**Gene ID**	**p-value**
Adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	5	*INPP5D, HLA-DRB1, MEF2C,* ***CR1****,* ***CLU***	3.159e-4
Positive regulation of immune system process	9	*ADAM10, PSEN1,* ***CLU****,* ***CR1****, HLA-DRB1, HLA-DRB5, MEF2C, TREM2, PTK2B*	4.459e-4
Humoral immune response	5	***CLU****,* ***CR1****, HLA-DRB1, MEF2C, TREM2*	4.499e-4
Activation of immune response	7	***CLU****,* ***CR1****, PSEN1, HLA-DRB1, HLA-DRB5, INPP5D, MEF2C*	5.890e-4
Immune response-activating cell surface receptor signalling pathway	6	***CR1****, PSEN1, HLA-DRB1, HLA-DRB5, INPP5D, MEF2C*	5.890e-4
Immunoglobulin mediated immune response	4	***CLU****,* ***CR1****, INPP5D, HLA-DRB1*	6.740e-4
Immune response	10	*APP,* ***CLU****, PSEN1,* ***CR1****, MEF2C, TREM2, HLA-DRB1, HLA-DRB5, INPP5D, PT2KB*	1.250e-3
Humoral immune response mediated by circulating immunoglobulin	3	***CLU****,* ***CR1****, HLA-DRB1*	2.799e-3
Immune effector process	6	***CLU****,* ***CR1****, PSEN1, HLA-DRB1, INPP5D, PT2KB*	3.650e-3
Innate immune response	8	*APP,* ***CLU****,* ***CR1****, PT2KB, HLA-DRB1, HLA-DRB5, MEF2C, TREM2*	4.040e-3
Immune system process	11	*APP,* ***CLU****,* ***CR1****, PSEN1, PT2KB, HLA-DRB1, HLA-DRB5, MEF2C, TREM2, PICALM, ADAM10*	4.579e-3
Leukocyte activation	5	*ADAM10, PSEN1,* ***CLU****, MEF2C, PT2KB*	1.679e-2
Myeloid leukocyte activation	3	*ADAM10,* ***CLU****, PSEN1*	2.380e-2
Regulation of immune response	6	*PSEN1,* ***CLU****,* ***CR1****, MEF2C, HLA-DRB1, HLA-DRB5*	4.050e-2
Complement activation, classical pathway	2	***CLU****,* ***CR1***	4.700e-2

Both *CLU* and *CR1* were associated with the following GO terms: Adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains (p=3.159e-4), Positive regulation of immune system process (p=4.459e-4), Humoral immune response (p=4.499e-4), Activation of immune response (p=5.890e-4), Immunoglobulin mediated immune response (p=6.740e-4), Immune response (p=1.250e-3), Humoral immune response mediated by circulating immunoglobulin (p=2.799e-3), Immune effector process (p=3.650e-3), Innate immune response (p=4.040e-3), Immune system process (p=4.579e-3), Regulation of immune response (p=4.050e-2) and Complement activation, classical pathway (p=4.700e-2). The GO term Immune response-activating cell surface receptor signaling pathway (P=5.890e-4) was found for *CR1*, whereas *CLU* was observed with Leukocyte activation (p=1.679e-2) and Myeloid leukocyte activation (p=2.380e-2). The GO term Complement activation, classical pathway (p=4.700e-2) was found only for the two proteins, *CLU* and *CR1* as expected.

The results obtained from the network regarding the molecular function revealed 1 GO term, where *CLU* and *CR1* were found (Table 5). This GO term was associated with protein binding processes (p=4.419e-3).

Table 5: GO terms regarding molecular function associating *CLU* and *CR1* within the AD risk-related genes network. "Count in gene set" indicates the total number of counted proteins that were categorized from the network. p-value < 0,05.

MOLECULAR FUNCTION			
GO term	**Count in gene set**	**Gene ID**	**p-value**
Protein binding	20	*APP, RIN3, BIN1, **CR1**, **CLU**, CD2AP, EPHA1, PICALM, APOE, SORL1, PSEN1, PSEN2, ADAM10, PTK2B, MADD, DSG2, HLA-DRB1, TREM2, MEF2C, NME8*	4.419e-3

The results obtained from the network regarding the cellular component revealed 5 GO terms, where *CLU* and *CR1* were presented (Table 6).

Table 6: GO terms regarding cellular component associating *CLU* and *CR1* within the AD risk-related genes network. "Count in gene set" indicates the total number of counted proteins that were categorized from the network. p-value < 0,05.

CELLULAR COMPONENT			
GO term	**Count in gene set**	**Gene ID**	**P-value**
Neurofibrillary tangle	2	***CLU**, PICALM*	2.490e-3
Plasma lipoprotein particle	3	***CLU**, APOE, SORL1*	2.969e-3
Cell surface	9	*APP, ADAM10, PSEN1, ABCA7, **CR1**, CD33, DGS2, FERMT2, HLA-DRB1*	2.200e-4
Intracellular	22	*RIN3, CELF1, BIN1, **CLU**, PLD3, HLA-DRB1, HLA-DRB5, ABCA7, PICALM, SORL1, APP, APOE, ADAM10, TREM2, MEF2C, INPP5D, PTK2B, NYAP1, MADD, CASS4, SLC24A4, NME8*	8.549e-1
Plasma membrane part	16	*ADAM10, APP, PSEN1, SORL1, BIN1, PICALM, EPHA1, ABCA7, **CR1**, HLA-DRB1, HLA-DRB5, CD33, SLC24A4, DSG2, FERMT2, PTK2B*	2.010e-5

The GO terms Neurofibrillary tangle (p=2.490e-3), Plasma lipoprotein particle (p=2.969e-3) and Intracellular (p=8.549e-1) were associated with *CLU*, while *CR1* was related to Cell surface (p=2.200e-4) and Plasma membrane part (p=2.010e-5).

4.2 *CLU* polymorphic region rs11136000 and *CR1* polymorphic region rs3818361 amplification by PCR

Another aim of this thesis was to evaluate the frequency of *CLU* and *CR1* polymorphic regions rs11136000 and rs3818361 respectively in Controls and dementia individuals (here referred as "Putative AD" individuals) from a population-based subgroup of individuals, the pcb-cohort. The final aim was to address the potential risk association between these susceptibility SNPs and the disease risk group.

In order to perform *CLU* and *CR1* genotyping, the SNPs were amplified by PCR. The results were subsequently visualized in 1.5% agarose gels. The expected fragment of 685bp for *CLU* SNP (Figure 11) and the expected fragment of 664bp for *CR1* SNP (Figure 12) were obtained for all cases.

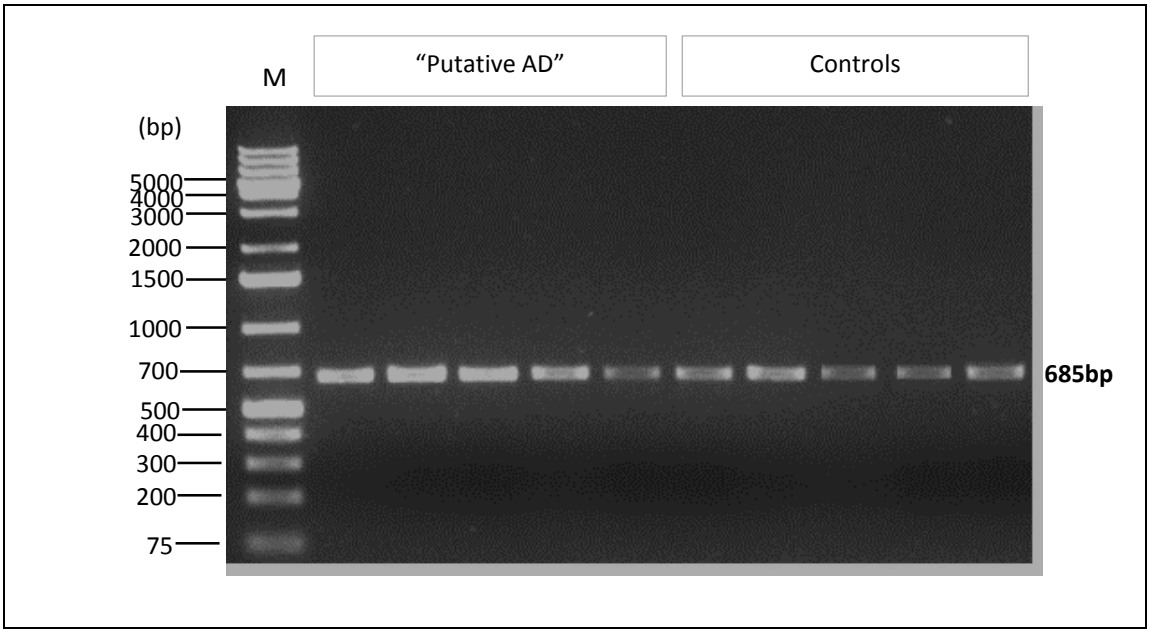

Figure 11: Representative gel of a set of "Putative AD" and Controls individuals. *CLU* SNP amplified by PCR technique. The expected product band has 685bp and was visualized in 1.5% agarose gel.

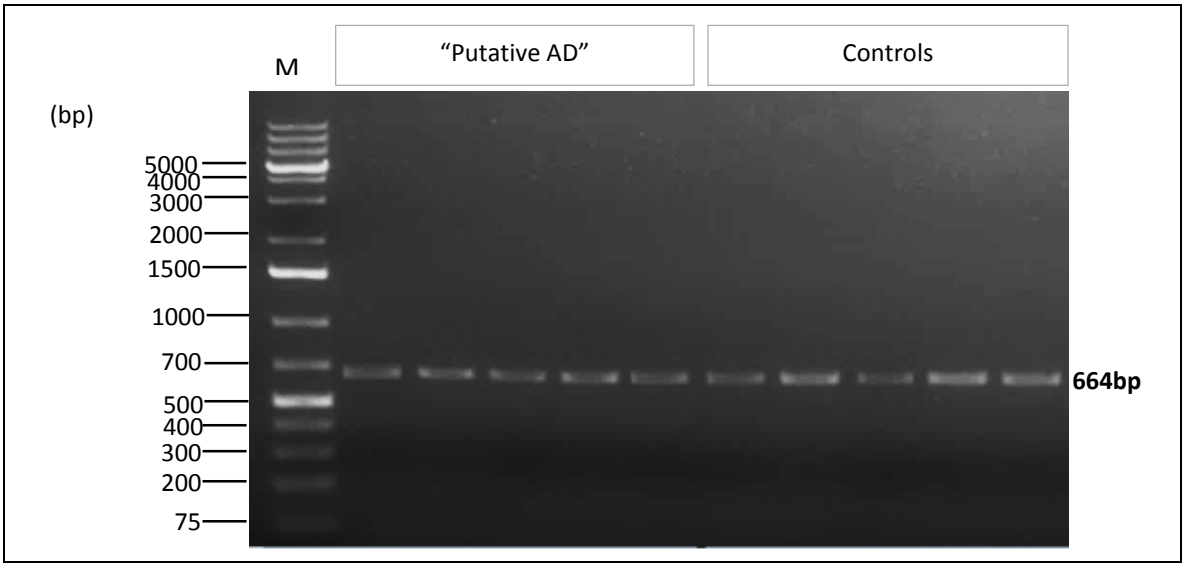

Figure 12: Representative gel of a set of "Putative AD" and Controls individuals. *CR1* SNP amplified by PCR technique**.** The expected product band has 664bp and was visualized in 1.5% agarose gel.

4.3 Associating the *CLU* and *CR1* SNP genotype with AD

Following PCR, all DNA samples were purified and successively evaluated by Sanger Sequencing. Representative sequencing patterns of each SNP for *CLU* and *CR1* genes are presented in Figure 13 and Figure 14, respectively.

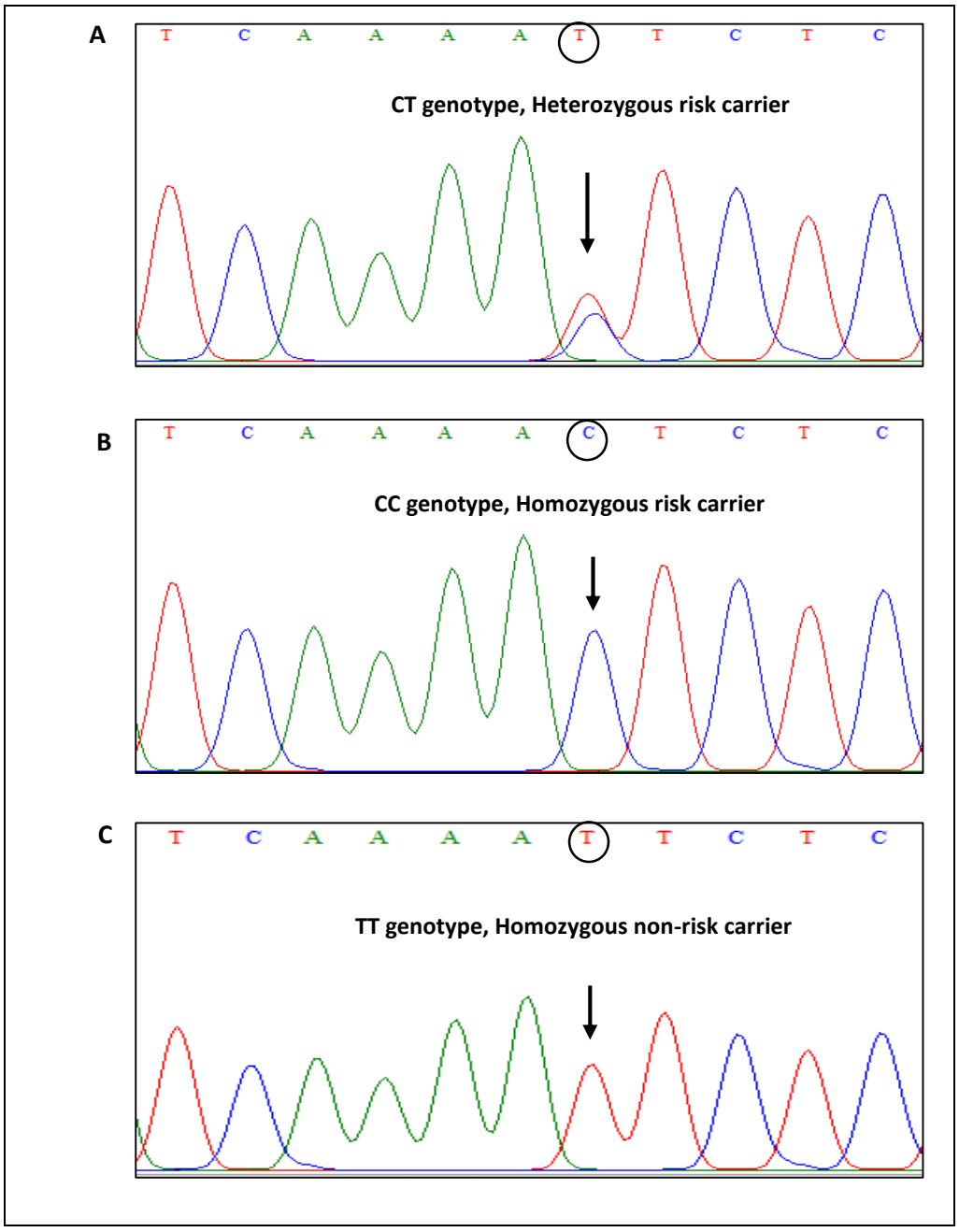

Figure 13: Examples of Sanger-sequencing results for *CLU* rs11136000 SNP from the studied individuals. A - CT genotype, Heterozygous risk carrier. **B –** CC genotype, Homozygous risk-carrier. **C -** TT genotype, Homozygous non-risk carrier.

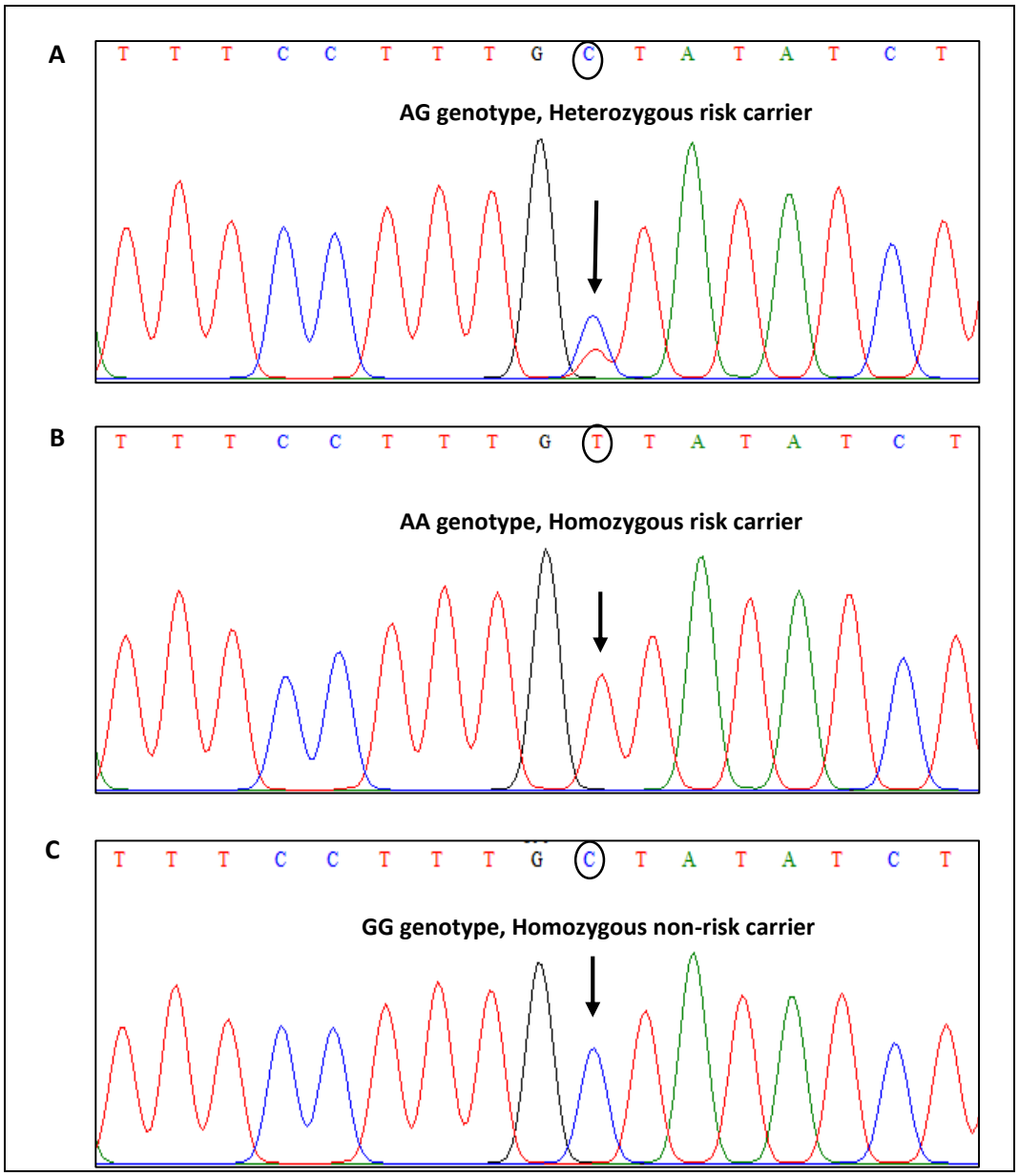

Figure 14: Examples of Sanger-sequencing results for *CR1* rs3818361 SNP from the studied individuals. The gene was sequenced with the primer reverse, which explains the difference between the complementary nucleotide on the sequence and the indicated genotype nucleotide. **A -** TC that corresponds to AG genotype, Heterozygous risk carrier. **B -** TT that corresponds to genotype AA, Homozygous risk-carrier. **C –** CC that corresponds to GG genotype, Homozygous non-risk carrier.

4.4 Determination of *CLU* and *CR1* SNPs genotypic frequencies

4.4.1 *CLU* rs11136000 SNP genotypic frequencies

After sequencing results analysis from *CLU* rs11136000 SNP, it was possible to identify in the "Putative AD" group: 14 homozygous risk-carriers with CC genotype, 13 heterozygous risk carriers with CT genotype and 5 homozygous non-risk carriers with TT genotype. From the Controls group sequencing analysis, it was identified 14 homozygous risk-carriers with CC genotype, 11 heterozygous risk carriers with CT genotype and 7 homozygous non-risk carriers with TT genotype. The Entire study group showed a frequency of 63 individuals. All data is represented as a percentage for each group in Figure 15.

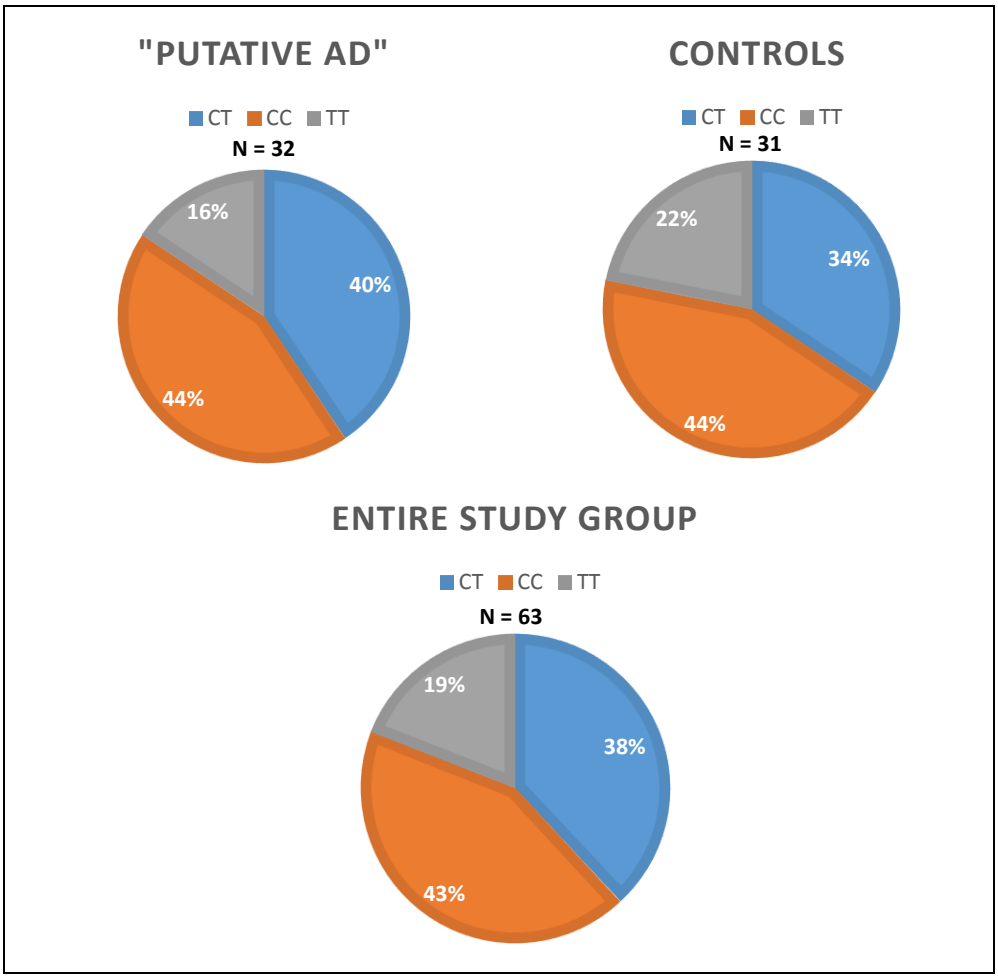

Figure 15: Analysis of the genotyping frequencies for *CLU* rs11136000 SNP in "Putative AD", Controls and Entire study group. CT - Heterozygous risk carriers with CT genotype; CC - Homozygous risk-carriers with CC genotype; TT - Homozygous non-risk carriers with TT genotype.

According to the data obtained, the most frequent group in "Putative AD" are homozygous-risk carriers being represented by CC genotype (44%), followed by the heterozygous risk carriers with CT genotype (40%). Additionally, the less frequent group of "Putative AD" group are homozygous non-risk carriers with TT genotype (16%). The most frequent group in Controls are likewise homozygous risk-carriers being represented by CC genotype (44%), followed by the heterozygous risk carriers with CT genotype (34%). Furthermore, the less frequent group of Controls are homozygous non-risk carriers with TT genotype (22%), although at a higher percentage than in "Putative AD" group. Although no percentage differences were obtained for the homozygous risk-carries among groups, a slight increase was obtained for the heterozygous risk-carriers in AD group.

Assuming all the sequencing information of the entire study group (Controls and "Putative AD"), the *CLU* genotyping frequencies did not change much: 27 homozygous risk carriers being represented by CC genotype (43%), 24 heterozygous risk carriers with CT genotype (38%) and 12 homozygous non-risk carriers with TT genotype (19%) (Figure 15). In fact, considering all 63 analysed individuals in total, the order of the genotypes frequencies was: CC > CT > TT, the same frequency observed for the individual groups.

4.4.2 *CR1* rs3818361 SNP genotypic frequencies

Regarding the sequencing results for the *CR1* rs3818361 SNP, in the "Putative AD" group it was identified: 2 homozygous risk carriers with AA genotype, 4 heterozygous risk carriers with AG genotype and 26 homozygous non-risk carriers with GG genotype. For the Controls group: no homozygous risk-carriers with AA genotype, 6 heterozygous risk carriers with AG genotype and 26 homozygous non-risk carriers with GG genotype. A 6% increase in homozygous risk-carriers were found in "Putative AD" group. In total, it was possible to identify from the sequencing analysis of the Entire study group ("Putative AD" and Controls) that 10 individuals were heterozygous risk carriers with AG genotype, 51 individuals were homozygous non-risk carriers with GG genotype and 2 individuals were homozygous risk carriers with AA genotype. Data is represented in percentages in Figure 16.

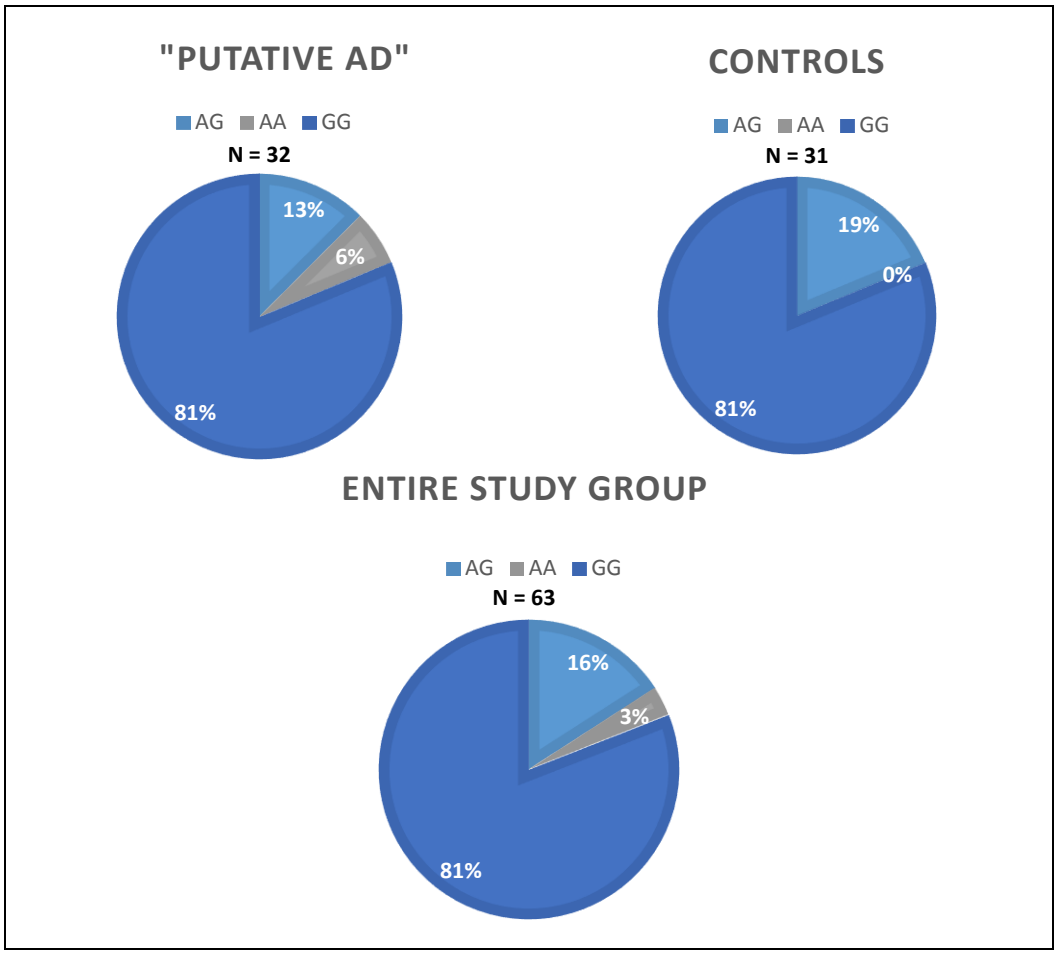

Figure 16: Analysis of the genotyping frequencies for CR1 rs3818361 SNP in "Putative AD", Controls and Entire study group. AG - Heterozygous risk carriers with AG genotype; AA - Homozygous risk carriers with AA genotype; GG - Homozygous non-risk carriers with GG genotype.

According to the data, the most frequent group in "Putative AD" group are homozygous non-risk carriers with GG genotype (81%), followed by the heterozygous risk carriers with AG genotype (13%). Additionally, the less frequent group of "Putative AD" individuals are homozygous risk carriers with AA genotype (6%). For the Controls group, the most frequent are homozygous non-risk carriers with GG genotype (81%), and the less frequent are heterozygous risk carriers with AG genotype (19%). Of note, no homozygous risk carriers with AA genotype were present in the Controls group.

Assuming the sequencing information from both “Putative AD” patients and Controls, this study identified in the Entire study group 2 homozygous risk carriers represented by AA genotype (3%), 10 heterozygous risk carriers with AG genotype (16%) and 51 homozygous non-risk carriers with GG genotype (81%) (Figure 16). Considering all individuals analysed, the frequency of the genotypes was: GG > AG > AA.

4.5 Evaluation of *CLU* rs11136000 SNP and *CR1* rs3818361 SNP allelic frequencies

4.5.1 *CLU* rs11136000 SNP allelic frequencies

The most common allelic frequencies of *CLU* rs11136000 SNP in “Putative AD” group was the risk allele (C-allele), with 41 alleles. The T-allele was the less frequent (23 from a total of 64 alleles). By contrast, for the Controls group, out of 62 alleles, the T-allele was the most frequent (37 alleles) and the C-allele was much less frequent than in “Putative AD” group (only in 25 alleles). By analysing *CLU* rs11136000 SNP sequences in both groups, it was possible to recognize that the C-allele was the most frequent with 66 alleles from a total of 126. The T-allele was the less frequent, since it was only present in 60 alleles from a total of 126 alleles. Data is represented in percentages in the following Figure 17.

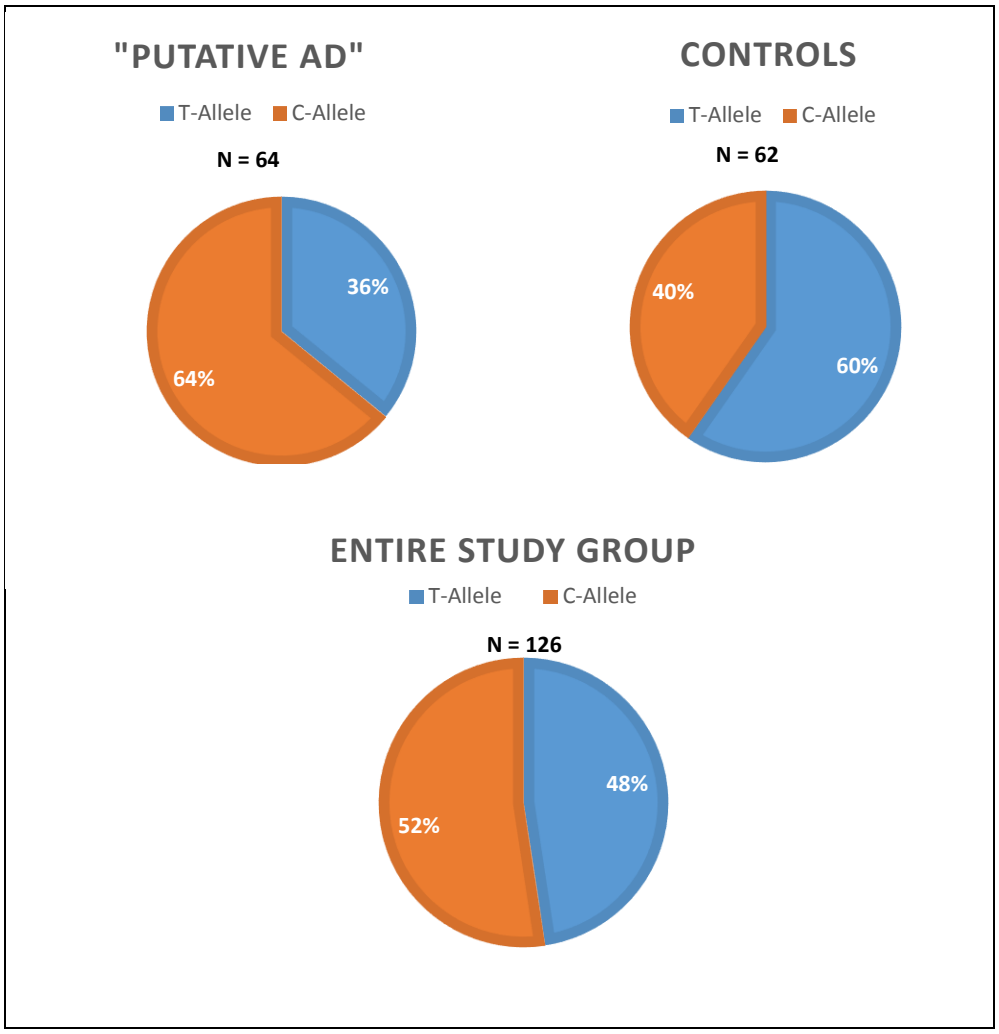

Figure 17: Sequencing results for *CLU* rs11136000 SNP allelic frequencies from all the studied groups. T-allele, non-risk allele; C-allele, risk allele.

In fact, by calculating the allelic frequencies for the "Putative AD" group, the T-allele represents a frequency of 36%, while the C-allele represents a higher frequency of 64%. Relatively to the Control group, the T-allele exhibited a frequency of 60% and the C-allele of only 40%. By analysing all individuals of both groups, the C-allele is slightly more frequent than the T-allele of 52% versus 48%, respectively.

4.5.2 *CR1* rs3818361 SNP allelic frequencies

According to the allelic frequencies for *CR1* rs3818361 SNP, the most frequent allele in the "Putative AD" group was the G-allele, with 56 alleles. Moreover, the A-allele was the less frequent (8 from a total of 64 alleles). Similarly, for the Controls individuals, the G-allele was the most frequent (56 alleles out of 64), and the A-allele was only 6 alleles. For the *CR1* rs3818361 SNP in the Entire study group, the G-allele was the most frequent with 112 alleles from a total of 126. Likewise, the A-allele was the less frequent, since it was 14 alleles from a total of 126 alleles. All data is represented as a percentage for each group in Figure 18.

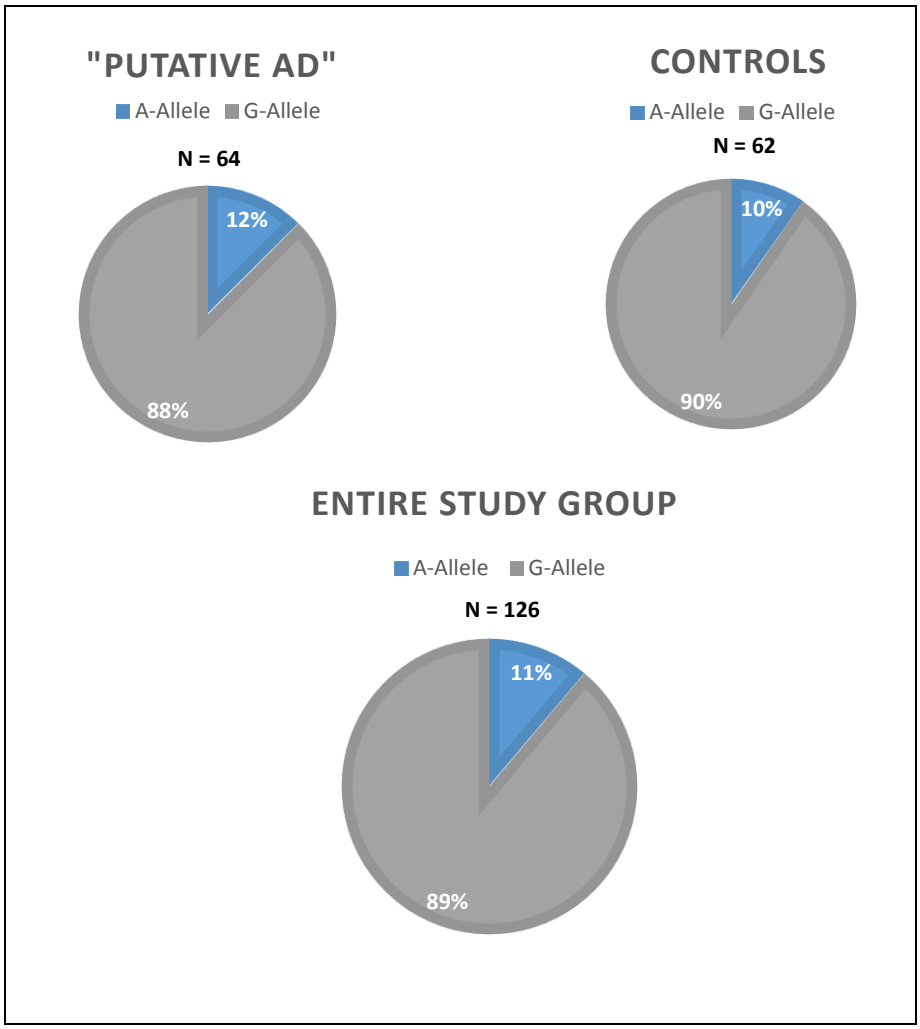

Figure 18: Sequencing results for *CR1* rs3818361 SNP allelic frequencies from all the studied groups. A-allele, risk allele; G-allele, non-risk allele.

The allelic frequencies of *CR1* rs3818361 SNP in "Putative AD" group were higher for the G-allele (88%) than for the A-allele (was only 12%). Similarly, results were obtained for the Controls group were the A-allele was associated with a lower frequency (10%) and the G-allele with higher frequency (90%). The allelic frequencies analysed of the entire study group did not change much when compared with the individual group frequencies already described. The G-allele showed a frequency of 89% and the A-allele lower frequency of 11%.

5. Discussion

The incidence and prevalence of AD increases with age and amplifies exponentially every five years after the sixth decade of life. Demographic aging is a reality worldwide, being expectable that the sum of AD cases will escalate in the near future[24].

Considering the restricted efficacy of potential interventions and diagnostics tools for the current dementia cases, studies have centred on the understanding of the molecular basics of the disease, to promote the development of premature diagnostic tools and disease-modifying effective therapeutic approaches. Understanding the interface among environmental and genetic aspects that control risk factors and outcomes of neurologic disease can deliver valuable information on dealing with this devastating disorder[16].

In this perspective, the work here presented aimed to study AD-related risk genes, in particular the *CLU* rs11136000 and *CR1* rs3818361 risk variants, which were analysed in a pilot study group of 63 individuals.

5.1 AD risk-related genes network

The first step of this work was to perform a bioinformatics analysis of the AD associated risk-genes.

A powerful approach for inquiring the biological relationships of a protein of interest is to analyse the protein-protein interactions networks. In our network, 5 separated clusters were obtained, exhibiting proteins that are already known to be associated with AD pathogenesis.

One cluster included the APP node and is associated with Presenilin genes (*PSEN1* and *PSEN2*), *APOE*, *SORL1*, *ADAM10* and *CLU*, linked by highest edge confidence (blue cluster). These genes centrally connected to APP node represent key players in APP processing and Aβ clearance in EOAD and LOAD, in agreement with literature data. *CLU* is also linked to *APOE* node by high edge confidence, which may be associated to the fact that both genes are inflammatory-related apolipoproteins involved in lipid metabolism. CELF1 is also related with this cluster. This gene may mediate tau toxicity and neuronal development[24].

Another cluster included *BIN1*, *PICALM* and *RIN3* (yellow cluster), all strongly associated by highest edge confidence. This may indicate that all these genes share common pathway features. In fact, all these genes associate with synapse function and neural

development[24]. *CR1* is also present in this cluster and it was associated by medium edge confidence to *CD33*, *ABCA7*, *PICALM* and *MS4A6A,* suggesting that these genes might at some point interplay in synapse function and immune response processes[24].

The *CD2AP* is a central node (red cluster), which may indicate that this gene has an important function associating *EPHA1*, *INPP5D*, *PTK2B* and *FERMT2* to synapse function and endocytosis processes[24].

HLA-DRB1 and *HLA-DRB5* were also linked in this network (green cluster). Both genes are HLA class II molecules and may act as an antigen-presenting molecule by regulating the specific immune response and innate immune response, potentially contributing to the alteration of these processes in AD[24].

NME8, *ZCWPW1* and *SLC24A4* were all associated by medium edge confidence (brown cluster). To date very little is known to date about these genes, but they have been associated to LOAD and may be important players in neural development[24].

5.2 GO analysis of *CLU* and *CR1* within the AD risk-related genes network

Next, we identified functional terms for the studied genes, *CLU* and *CR1*, within the AD risk-related genes network by using Gene Ontology (GO) analysis. In the obtained network, it was possible to identify 21 significantly enriched terms: 15 biological process, 1 predominant molecular function and 5 cellular components in total. Some of these are consistent with the current knowledge of the biological functions and compartments linked to these genes and implicated in AD processes, such as neuroinflammation and the immune system responses[24,122].

For instance, it was demonstrated that *CLU* and *CR1* are associated with several inflammation and immune response biological processes within AD risk-related genes network. By the network results, *CR1* was associated with immune response-activating cell surface receptor signaling pathway, while *CLU* was linked to leukocyte activation and myeloid leukocyte activation. Both genes were common players in complement activation, classical pathway and are also involved with Aβ clearance[24]. In AD, Aβ deposition induces complement activation and activates microglial cells, which ultimately stimulates the brain inflammatory response[25,98].

Regarding the molecular function, both genes were associated with protein binding features. For instance, CLU actions as an extracellular chaperone, were shown to prevent the aggregation of non-active proteins[117]. CR1 has been described as a mediator in cellular binding of particles and immune complexes that activated the complement[111].

About the cellular component data, CLU may be a part of the neurofibrillary tangle in association with PICALM and a plasma lipoprotein particle in association with SORL1. It could also be related to other intracellular components. On the other hand, CR1 may be associated with cell surface and plasma membrane part components. These results are in line with previous findings[111], which revealed that the recognition of immune-complexes by the complement protein might occur by collagen-like receptor binding domains of C1q or mannose binding proteins, which after triggered, will activate a cascade of several proteins from the complement pathway.

5.3 *CLU* rs11136000 profile

Neuroinflammation is recognized as a downstream event of the amyloid cascade hypothesis. In fact, Aβ within the CNS promotes microglia activation and instigates a pro-inflammatory cascade that ultimately contributes to the release of inflammatory and neurotoxic substances, such as cytokines, chemokines, reactive oxygen and nitrogen species, and various proteolytic enzymes, that lead to neuroinflammation and degenerative outcomes in neurons. This data supports the need to discover and analyse new risk variants of genes encoding immune system molecules, such as *CLU* and *CR1,* that have been shown to be elevated in tissues and body fluids of individuals with AD or prodromal forms of this disease. Furthermore, GWAS have shown that both genes may be linked to AD pathogenesis and increased risk[26,27].

CLU is a widely expressed multifunctional glycoprotein, present in amyloid plaques and significant elevated in AD patients. It is one of the primary chaperones for removal of Aβ from the brain, by modulating astrocyte and microglia activation[114]. According to several studies, CLU can likewise interact with Aβ peptides, playing a central function in Aβ aggregation, toxicity and clearance as a potential controller of inflammation in AD pathogenesis.

In our study, the genotype frequencies for *CLU* were similar for both groups: CC > CT > TT, with the respective percentages being: 43%, 38% and 19%, when we look at the entire analysed group. These results are in agreement with the genotype frequencies obtained in an Asian CLU rs11136000 cross-sectional population reports from ALZgene Database[136].

According to our data for the Entire study group, 43% of tested individuals expressed the risk genotype (CC Homozygous genotype) while 19% presented the protective genotype (TT Homozygous genotype). Previous studies showed that the former individuals may have increased task-related activation[26,102], which could reflect alteration in brain function, while nonrisk genotype individuals exhibited less brain activation potentially by using a more proficient and resourceful approach. Further, despite the fact that no differences were obtained for the homozygous risk carriers frequencies between "Putative AD" and Controls groups, a slight increase in heterozygous risk carriers was observed in the former group.

On the other hand, according to our allelic frequencies results, differences arise between the "Putative AD" group and the Controls group. In fact, the "Putative AD" individuals exhibited a greater frequency for the C-risk allele of 64%, comparatively to the Controls group (40%). Inversely, the T-non risk-allele had a higher frequency in the Controls group of 60%. Data is consistent with previous studies which demonstrated that rs11136000 polymorphic region of *CLU* is associated with increased risk of AD1[27]. Besides this study, others had consistently reported that the C-risk allele carriers demonstrated earlier rates of decline in memory performance relative to T-non-risk allele carriers. Furthermore, the T-allele of the *CLU* variant rs11136000 is presuming related with low risk to AD development and enhanced cognitive performance, while the homozygous expression of the C-allele is accepted as risk factor for AD[104]. Likewise, *CLU* also yields effects on early, pre-symptomatic stages of disease progression by hastening deterioration in memory performance in risk carriers who ultimately evolve to MCI/AD. These data implicate *CLU* in initial stages of AD pathogenesis and supports the notion that it may be essential in controlling disease development in carriers of this common risk variant.

Furthermore, by examining our data from the Entire study group, the C-risk allele ultimately expresses a frequency of 52% and the T-non-risk-allele showed a lower frequency of only 48%, which is in accordance with the Caucasian *CLU* rs11136000 cross-sectional population reports from ALZgene Database[136]. Despite the small size of our study group, it was possible to sustain the association between *CLU* rs11136000 C-risk allele carriers and the "Putative AD" individuals.

5.4 *CR1* rs3818361 profile

The *CR1* gene is located in a genetic cluster of complement activation genes. It is a multifunctional protein, which is broadly expressed by the brain defensive cells. Furthermore, *CR1* inhibits complement activation by diminishing the immune response and limiting adjacent tissue impairment. In the brain, it is hypothesized that *CR1* could act by reducing inflammation levels in AD through clearance of Aβ deposits and by protecting healthy neurons from inflammation-mediated damage[126]. Further, *CR1* may act as a brain receptor for Aβ and its levels are increased in AD patients[24]. These observations support the notion that *CR1* is involved in AD pathogenesis.

Additional, the rs3818361 polymorphic region of *CR1* gene has been associated with increased risk of AD in GWAS[26]. Nonetheless, for the rs3818361 polymorphism, risk carriers (A-allele) exhibited lower brain amyloid burden relative to non-risk carriers (G-allele), an effect that can be influenced by *APOE* genotype[120]. The later observation suggests that the clinical utility of this variant as disease risk factor may be limited. Hence in our study the relation of this *CR1* variant with dementia was also addressed.

Regarding the *CR1* rs3818361 polymorphism and considering the Entire study group, the genotype frequencies were: GG > AG > AA. These results agree with the genotypic frequencies obtained in an European *CR1* rs3818361 cross-sectional population study (Ensemble Database studies (http://www.ensembl.org/))[137].

According to our data, 81% of the tested individuals expressed the non-risk GG homozygous genotype while 19% expressed the risk AA homozygous genotype and the risk AG heterozygous genotype carriers (respectively, 16% and 3%). Although no differences were detected between the "Putative AD" group and the Controls group relatively to the homozygous non-risk genotype frequencies, the "Putative AD" group exhibited an increased frequency of homozygous risk-carriers, consistent with previous reported observations.

For this genetic variant, similar results were obtained for the allelic frequencies for all groups, with the non-risk allele being the most frequent. In fact, on the "Putative AD" group, the A-risk allele had the lowest frequency (12%), when compared to the G-non-risk allele. Additionally, for the Controls group, the A-allele also displayed a 10% frequency, similarly to that observed for the "Putative AD" group. This rs3818361 polymorphic region of *CR1* gene was previously associated with increased risk of AD. Furthermore, inadequate clearance of Aβ and amplified chronic inflammation by *CR1* genetic variation appears to be associated with AD pathogenesis of AD, with a current report proposing that forms of *CR1* with lower clearance are associated with higher risk of AD[26].

By examining all individuals from both groups (Entire study group), the G-non-risk-allele expresses a superior frequency (89%) than the A-risk-allele (11%), which is consistent with the European *CR1* rs3818361 cross-sectional population report from Ensemble Database studies (http://www.ensembl.org/)[137]. No significant association of *CR1* rs3818361 risk-allele with "Putative AD" individuals were identified, only 2% difference between groups. This may be related with the small number of samples analysed.

Further analysis with a higher number of individuals may help to confirm if these SNPs could actually have an impact on our population or relevance in dementia/"Putative AD" cases.

6. Conclusions

- Both *CLU* and *CR1* are common interactors in inflammation and immune response processes;
- For CLU polymorphism the percentage of homozygous risk-carriers individuals was the same in both groups. Nonetheless, the percentage of heterozygous risk-carriers was higher in the "Putative AD" group;
- Regarding the *CLU* allelic frequencies of the variant, a higher percentage of individuals in the "Putative AD" group exhibited the C-risk allele;
- For the *CR1*, an increase in the percentage of homozygous risk carriers in the "Putative AD" group was observed comparatively to the Controls group;
- Allelic frequencies of the *CR1* variant did not change much among both groups;
- For both genetic variants, the genotypic frequencies obtained for the Entire study group are in agreement with previous observations;
- Although some correlations could be identified between these polymorphisms and the "Putative AD" group, strong evidences should be supported by additional studies with increased number of individuals.

7. Future Perspectives

- Increase the number of samples in the entire group, including the number of "Putative AD" individuals;
- Analyze and estimate the genotypic frequencies and the allelic frequencies of *CLU* rs11136000 in MCI individuals;
- Establish correlations analysis with APOEε4 allele, since it is one of the major risk factors for LOAD;
- Perform a plasma analysis of CLU in demented and non-demented older individuals, correlate it with the genetic profiles, and ideally with amyloid β-deposition.

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Appendix 1

Table S1. AD risk associated genes (black) and AD inflammation associated genes (grey) provided from GWAS to date[24].

Gene (Protein)	Known function	Potential Effects on APP and Tau	Pathways	Gene Expression	UniProt ID
APP (Amyloid beta (A4) precursor protein)	Neurite outgrowth, adhesion and axonogenesis	Cleavage yields Aβ	APP processing	-	P05067
PSEN1 (Presenilin-1)	Component of catalytic subunit of gammasecretase complex. Proteolytic cleavage of integral membrane proteins	Cleaves APP	APP processing	-	P49768
PSEN2 (Presenilin-2)	Component of catalytic subunit of gammasecretase complex. Proteolytic cleavage of integral membrane proteins	Cleaves APP	APP processing	-	P49810
APOE (Apolipoprotein E)	Mediates binding, internalization and catabolism of lipoproteins	Aβ clearance	Lipid metabolism	-	P02649
ADAM 10 (Disintegrin and metalloproteinase domain-containing protein 10)	Proteolytic cleavage of integral membrane proteins	Cleaves APP	APP processing	-	O14672
CR1 (Complement receptor type 1)	Mediates cellular binding of immune complexes that activate complement	Aβ clearance	Immune response	Increased	P17927
BIN1 (Myc box-dependent-interacting protein 1)	Regulation of endocytosis of synaptic vesicles	Mediates tau toxicity	Synapse function	Increased	O00499
CD2AP (CD2-associated protein)	Scaffold molecule regulating actin cytoskeleton	Mediates tau toxicity	Synapse function & Endocytosis	No change	Q9Y5K6
EPHA1 (Ephrin type-A receptor 1)	Brain and neural development. Angiogenesis, cell proliferation, and apoptosis	N/A	Immune response & Neural development	No change	P21709
CLU (Clusterin)	Chaperone. Regulation of cell proliferation	Aβ clearance	Immune response &	Increased	P10909

Gene (Protein)	Known function	Potential Effects on APP and Tau	Pathways	Gene Expression	UniProt ID
			Lipid metabolism		
MS4A6A (Membrane-spanning 4-domains subfamily A member 6A)	Signal transduction	N/A	Immune response	No change	Q9H2W1
PICALM (Phosphatidylinositol-binding clathrin assembly protein)	AP2-dependent clathrin mediated endocytosis	APP trafficking & Aβ clearance	Synapse function & Endocytosis	No change	Q13492
ABCA7 (ATP-binding cassette sub-family A member 7)	Lipid homeostasis. Phagocytosis of apoptotic cells by macrophageS	Aβ clearance	Immune response & Lipid metabolism	Increased	Q8IZY2
CD33 (Myeloid cell surface antigen CD33)	Mediates sialic acid-dependent binding to cells	Aβ clearance	Immune response	Increased	P20138
HLA-DRB1 (HLA class II histocompatibility antigen, DRB1-1 beta chain)	Immunocompetence and histocompatibility	N/A	Immune response	N/A	P04229
HLA-DRB5 (HLA class II histocompatibility antigen, DR beta 5 chain)	Immunocompetence and histocompatibility	N/A	Immune response	N/A	Q30154
PTK2B (Protein-tyrosine kinase 2-beta)	Induction of long term potentiation in hippocampus	N/A	Synapse function & Neural development	N/A	Q14289
SORL1 (Sortilin-related receptor)	APOE receptor. Binds LDL and RAP and mediates endocytosis of the lipids to which it binds	APP trafficking	Lipid metabolism. Synapse function & Endocytosis	Decreased	Q92673
SLC24A4 (Sodium/potassium/Calciu	Brain and neural development	N/A	Neural development. Synapse	N/A	Q8NFF2

Gene (Protein)	Known function	Potential Effects on APP and Tau	Pathways	Gene Expression	UniProt ID
m exchanger 4)			function & Endocytosis		
RIN3 (Ras and Rab interactor 3)	Stimulates & stabilizes GTP-Rab5 in protein transport from plasma membrane to early endosome	N/A	Neural developme nt. Synapse function & Endocytosis	N/A	Q8TB24
DSG2 (Desmoglein-2)	Mediates cell-cell junctions between epithelial and other cell type	N/A	N/A	N/A	Q14126
INPP5D (Phosphatidyl inositol 3,4,5-trisphosphate 5-phosphatase 1)	Negative regulator of myeloid cell proliferation and survival	N/A	Immune response	N/A	Q92835
MEF2C (Myocyte-specific enhancer factor 2C)	Controls synapse formation during activity-dependent refinement of synaptic connectivity and facilitates hippocampal-dependent learning and memory	N/A	Neural developme nt. Synapse function & Immune response	N/A	Q06413
NME8 (Thioredoxin domain-containing protein 3)	Ciliary functions	N/A	N/A	N/A	Q8N427
ZCWPW1 (Zinc finger CW-type PWWP domain protein 1)	Epigenetic regulation	N/A	Neural developme nt	N/A	Q9H0M 4
NYAP1 (Neuronal tyrosine-phosphorylat ed phosphoinosi tide-3-kinase adapter 1)	Brain and neural development	N/A	Neural developme nt	N/A	Q6ZVC0
CELF1 (CUGBP Elav-like family member 1)	Regulates pre-mRNA splicing	Mediates tau toxicity	Neural developme nt	N/A	Q92879

Gene (Protein)	Known function	Potential Effects on APP and Tau	Pathways	Gene Expression	UniProt ID
MADD (MAP kinase-activating death domain protein)	Long-term neuronal viability	Mediates tau toxicity	Neural development	N/A	Q8WXG6
FERMT2 (Fermitin family homolog 2)	Actin assembly and cell shape and mediator of angiogenesis	Mediates tau toxicity	Cytoskeleton & Axonal transport	N/A	Q96AC1
CASS4 (Cas scaffolding protein family member 4)	Docking protein in tyrosine-kinase signaling involved in cell adhesion and spreading	N/A	Cytoskeleton & Axonal transport	N/A	Q9NQ75
PLD3 (Phospholipase D3)	Unknown	APP trafficking and cleavage	Unknown	Increased	Q8IV08
TREM2 (Triggering receptor expressed on myeloid cells 2)	Immune response, triggers production of inflammatory cytokines	Aβ clearance	Unknown	Increased	Q9NZC2

Appendix 2

Solutions

- 50x TAE (Tris-acetate-EDTA buffer)

To 600ml of deionised H_2O add:

- 242g Tris Base
- 57.1ml Glacial Acetic Acid
- 100ml 0.5M EDTA (pH 8.0)

Mix until the solutes have dissolved and adjust the volume to 1L with deionised H_2O. Store at room temperature.

- 0.5M Ethylenediaminetetraacetic acid (EDTA) (pH 8.0)

To 80ml of deionised H_2O add:

- 14.612 g EDTA

Mix until the solutes have dissolved and adjust the pH to 8.0 with Sodium hydroxide (NaOH). Adjust the volume to 100ml with deionised H_2O. Store at room temperature.

- 3M Sodium Acetate pH5.2

To 100ml of deionised H_2O/80ml of deionised H_2O add:

- 40.8g Sodium Acetate Trihydrate/24.6g Sodium Acetate Anhydrous

Adjust the pH to 5.2 with glacial acetic acid. Store at room temperature.

- Bromophenol Blue

  To 7ml of deionised H_2O add:

  - 0.025g Bromophenol Blue (0.25%)
  - 3ml glycerol (30%)

  Mix and store at 4°C.