Universidade de Aveiro Secção Autónoma de Ciências da Saúde
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Perfil genético do *ApoE* na Demência

Genetic profiling of *ApoE* in Dementia

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Dissertação 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 da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro.

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Dedico este trabalho, de uma forma muito especial, aos meus pais e irmãos, por serem as pessoas que sempre me acompanharam e apoiaram em todos os momentos da vida.

o júri
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palavras-chave

Apolipoproteina E, demência, alzheimer, patologia amilóide, diagnóstico neuroquímico, teste genético.

resumo

A demência é uma das principais causas de incapacidade entre os idosos, afetando mais de 36 milhões de pessoas em todo o mundo. É caracterizada pela deterioração progressiva das funções cognitivas, resultando em dificuldades no desempenho das atividades diárias do indivíduo. A idade de aparecimento dos sintomas, bem como a sua taxa de progressão, são variáveis entre a maior parte das demências, sendo estas geralmente caracterizadas por uma natureza progressiva, aumentando de gravidade ao longo do tempo. Entre os tipos mais frequentes de demência encontram-se a Doença de Alzheimer (DA), Demência Vascular, Demência de Corpos de Lewy e Demência Frontotemporal. O diagnóstico diferencial das demências é realizado tipicamente por testes neuro-psicológicos (para a exclusão de outras demências) e por exames imagiológicos. Contudo, muitos dos sintomas clínicos característicos podem sobrepor-se entre os diversos tipos de demência, o que pode constituir um problema devido a falta de especificidade e erros de diagnóstico. A compreensão dos fatores de risco ambientais e genéticos que podem modular o aparecimento e/ou progressão de doenças abre novas perspetivas relativamente à gestão destas neuropatologias.

O gene da apolipoproteína E (*ApoE*) é reconhecido como o maior fator de risco na demência, desempenhando um papel central em particular no desenvolvimento da DA, sendo que os portadores do alelo ε4 são mais suscetíveis para a doença. Além disso, possíveis associações foram também propostas entre este gene e outras doenças neurológicas, sendo no entanto estes dados ainda controversos. Assim, o objetivo principal deste trabalho consistiu em determinar as frequências alélicas e genotípicas do gene *ApoE* num grupo de estudo piloto de pacientes com demência na região de Aveiro. Este grupo foi subdividido com base no diagnóstico neuroquímico, no qual foram avaliados os níveis de $A\beta_{1-42}$, Tau-total e fosfo-Tau 181 no líquido cefalorraquidiano dos pacientes. Como resultado, observou-se que o alelo ε3 foi o mais frequente no grupo total, independentemente do tipo de patologia, e que o alelo ε2 foi o menos comum. O alelo ε4 foi de facto mais frequente em pacientes com DA do que em pacientes com outras neuropatologias, o que está de acordo com a relação proposta por outros autores. Adicionalmente, foi possível verificar que a frequência deste alelo nos pacientes com patologia amilóide é semelhante à observada no grupo DA, sugerindo um papel relevante para o *ApoE* no metabolismo e acumulação cerebral do Aβ. Consequentemente, estes indivíduos podem ter uma maior suscetibilidade para o desenvolvimento de DA no futuro. Deste modo, os nossos dados corroboram a ideia de que o alelo ε4 é um forte fator de risco para a DA e que deve ser considerado como um teste genético relevante que pode contribuir para o diagnóstico clínico da demência.

keywords

Apolipoprotein E, dementia, alzheimer, amyloid pathology, neurochemical diagnosis, genetic testing.

abstract

Dementia is one of the leading causes of disability among the elderly, affecting over 36 million people worldwide. It is characterized by progressive deterioration in cognitive functions that impair the successful performance of daily living activities. Most forms of dementia are progressive in nature, increasing in severity over time, with the rate of symptoms progression and the age at onset differing among the major dementing disorders. The most frequent types of dementia include Alzheimer´s Disease (AD), Vascular Dementia, Dementia of Lewy bodies and Frontotemporal Dementia. Differential diagnosis of dementia is typically done by neuropsychological testing (for exclusion of other dementias) and neuroimaging investigations. However, many of the clinical symptoms typical of dementia may overlap across dementia subtypes, which may constitute a diagnosis problem leading to lack of specificity and misdiagnosis. Understanding the environmental and genetic factors that modulate risks and outcomes of diseases can open new perspectives on the management of these neurological disorders.

Apolipoprotein E gene (*ApoE*) is recognized as the major genetic risk factor in dementia, in particular playing a central role in AD development, being that *ApoE* allele ε4 carriers are more susceptible to disease. Furthermore, putative associations have also been proposed between this gene and other neurological disorders, nonetheless controversial data have been reported regarding these aspect. Hence, the aim of this work was to determine *ApoE* genotypic and allelic frequencies in a pilot study group of dementing patients from the catchment area of the "Hospital de São Sebastião" in "Santa Maria da Feira". This group was subdivided according to their neurochemical-based diagnosis of dementia, in which $A\beta_{1-42}$, phospho-Tau 181 and total-Tau levels in patients' cerebrospinal fluid were evaluated. In this study, we could observe that allele ε3 was the most frequent in the total study group, independent of the type of pathology, and that allele ε2 was the less frequent. Allele ε4 was in fact more frequent in AD patients than in other neurological disease patients, in agreement with the proposed link established by other authors. Additionally, we also find that allele ε4 frequency in patients suffering from amyloid pathology is similar to that observed in AD group, suggesting a role for *ApoE* in Aβ metabolism and brain accumulation. Potentially, these patients have a higher susceptibility to develop AD pathology in the future. Thus, our data supports the idea that *ApoE* allele ε4 is a strong risk factor for AD development and that it may be considered as a relevant genetic test that may assist in the clinical diagnosis of dementia.

ABBREVIATIONS

AD	Alzheimer´s Disease
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
ARD	Alcohol Related Dementia
Aβ	Amyloid-β peptide
BBB	Blood-brain Barrier
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukencephalopathy
CHMP2B	Chromatin Modifying Protein 2B
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computerized Tomography
DLB	Dementia of Lewy Bodies
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded DNA
EDTA	Ethylenediamine Tetra-acetic Acid
ELISA	Enzyme-linked Immunoabsorbent Assay
ER	Enzymatic Restriction
EtBr	Ethidium Bromide
FTD	Frontotemporal Dementia
FTLD	Frontotemporal Lobar Degeneration
FTLD-FUS	FTLD with FUS-positive inclusions
FTLD-τ	FTLD with Tau-positive inclusions
FTLD-TDP	FTLD with TDP-43 positive inclusions
FTLD-U	FTLD with Ubiquitin-positive inclusions
FTLD-UPS	FTLD with UPS components positive inclusions
FUS	Fused in Sarcoma protein
HD	Huntington's Disease

HTT	Huntingtin
gDNA	Genomic DNA
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LBs	Lewy Bodies
LB	Loading Buffer
LRRK2	Leucine-rich Repeat Kinase 2
MAPT	Microtubule-associated Protein Tau
MCI	Mild Cognitive Impairment
MD	Mixed Dementia
MID	Multi-infarct Dementia
MMSE	Mini-mental State Examination
MRI	Magnetic Resonance Imaging
MTHFR	Methylenetetrahydrofolate Reductase
NFL	Neurofilament Light protein
NFTs	Neurofibrillary Tangles
NOTCH3	Neurogenic locus notch homolog protein 3
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PET	Positron Emission Tomography
PGRN	Progranulin
PSEN1	Presenilin 1
PSEN2	Presenilin 2
P-Tau 181	Phospho-Tau 181
SNCA	α-Synuclein
SPECT	Single Photon Emission Computerized Tomography
SPs	Senile Plaques
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TMEM106B	Transmembrane protein 106B

TDP-43	Transactive response-DNA binding protein-43
T-Tau	Total-Tau
UPS	Ubiquitin-proteasome System
VaD	Vascular Dementia
VCP	Valosin-containing Protein

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1. INTRODUCTION

1.1. DEMENTIA, RECOGNIZED AS A MULTIFACTORIAL SYNDROME

Dementia, a syndrome usually associated with many causes, is characterized by a progressive loss of intellectual and cognitive functions that impairs the successful performance of daily living activities. It is most frequent in the developed world and is becoming even more so as a consequence of life span increase, thus contributing to an augmented risk of the elderly population suffering from dementia. Among the clinical symptoms, memory is the most common cognitive ability lost with dementia, affecting 10% of persons aged over 70 years and 20-40% of individuals aged over 85 years. In addition to memory, other mental faculties are also affected, such as language, visuospatial ability, calculation, judgment and problem solving. Neuropsychiatric and behavioral alterations are also present in many cases of dementia, resulting in depression, agitation, insomnia, hallucinations and disinhibition (Bird and Miller, 2010). Treatment is generally supportive or directed at relieving symptoms, and is usually far from perfect. Dementia is now an area of intense scientific study, which brings the perspective of more effective therapies and adequate treatments for the different dementias types in the future.

Recognizing dementia is easy if clinical symptoms are severe, which are normally associated with the late stages of disease. However, it is much harder to distinguish early dementia from the forgetfulness due to anxiety or from the mild cognitive impairment (MCI), that often accompanies ageing (usually affecting memory for names and recent events), and does not necessarily progress to more severe disability (Wilkinson and Lennox, 2005). Also, alterations of multiple capacities usually distinguish dementia from other disorders, such as amnesia and aphasia, which affect a single functional domain (memory and language, respectively).

Most forms of dementia are progressive in nature, increasing in severity over time. The age of onset and the progression rate of symptoms differ among the major dementing disorders. Most have an insidious onset and develop slowly, sometimes over a period of many years, even before clinical manifestation of the symptoms. These include pathologies such as Alzheimer's disease (AD), Huntington's disease (HD) and frontotemporal dementia (FTD). Vascular dementia (VaD) follows another pattern of

decline: initial cognitive symptoms develop acutely, but in this case the clinical course typically proceeds in a stepwise fashion over many years, with periods of relative stability punctuated by abrupt deterioration (Squire et al., 2008).

In Europe, 7,3 million citizens suffer from dementing disorders and in Portugal over 153.000 people are affected. As life span is increasing, specialists predict that this value will duplicate in 2040 (http://www.alzheimerportugal.org). Presently, estimates indicate that there are nearly 36 million people with dementia worldwide (http://alzheimers.org.uk). Approximately 1% of the population is affected at age of 60-65 years, rising to 10–35% in those over 85 years old. Of the patients with late onset dementia (>65 years), about half have AD, 16% VaD and 30% other forms of dementia, such as dementia with Lewy bodies (DLB) and FTD (Figure 1) (Lobo et al., 2000). In early-onset cases, AD is relatively less common but it still is the most prevalent cause, while FTD, alcohol related dementia and dementia secondary to other diseases (14%), such as multiple sclerosis, are relatively more common (Harvey et al., 2003). In particular, for the Portuguese population, AD and VaD were also the most common forms of dementia in a study realized in rural and urban areas from Northern Portugal (Nunes et al., 2010).

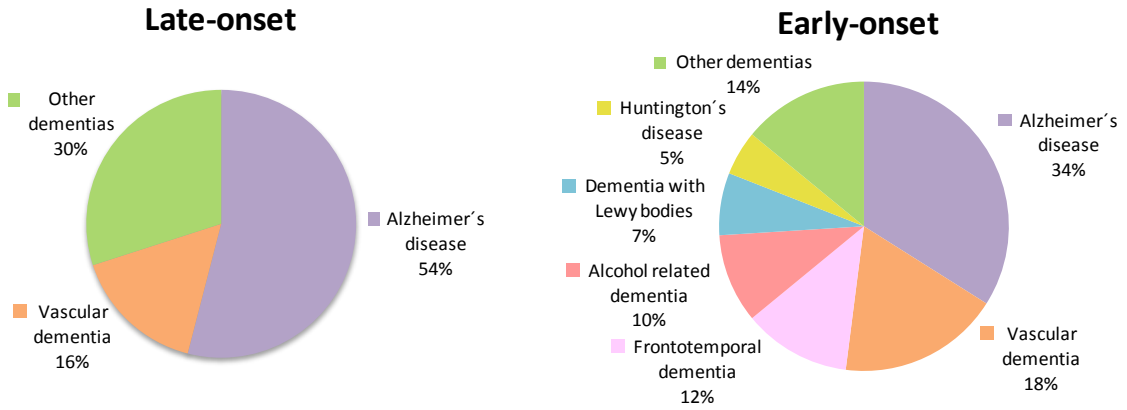


Figure 1: Major types of late-onset (>65 years) and early-onset (<65 years) dementia. Based on Lobo et al. (2000) and Harvey et al. (2003).

In the following sections an overview on the molecular and genetic basis of the most common forms of dementia will be given, which includes AD, VaD, FTD, DLB, HD and alcohol related dementia (ARD). The main clinical characteristics and symptoms will also be addressed, demonstrating the disease manifestations overlap and the challenges that clinicians have to face to overcome misdiagnosis.

1.2. MOLECULAR BASIS OF DEMENTIA

1.2.1. Alzheimer´s Disease

AD is the most common form of age-related dementia and one of the most serious health problems. In Portugal it affects more than 90.000 individuals (http://www.alzheimerportugal.org). In the early-onset form of AD, clinical symptoms start before 65 years, and is usually caused by hereditary genetic factors (about 5% of all AD cases). The most common form is late-onset sporadic AD (>65 years), accounting for more than 95% of the cases, which in time is triggered by normal aging neurodegeneration and diverse genetic and environmental risk factors. In these forms, onset and progression of disease are insidious. Memory is usually affected first, followed by language and spatial abilities. After a few years, all aspects of intellectual function are affected and the patient may become frail and unsteady, requiring, in general, a full-time caregiver.

Neuropathological Hallmarks of AD

The major pathological hallmarks of AD are the presence of extracellular senile plaques and intracellular neurofibrillary tangles, as well as synaptic and neuronal loss, namely in the neocortex and hippocampus (Figure 2) (Allsop et al., 1983; Goedert et al., 1992; Anoop et al., 2010; O'Brien and Wong, 2011), affecting the proper functioning of memory and learning processes.

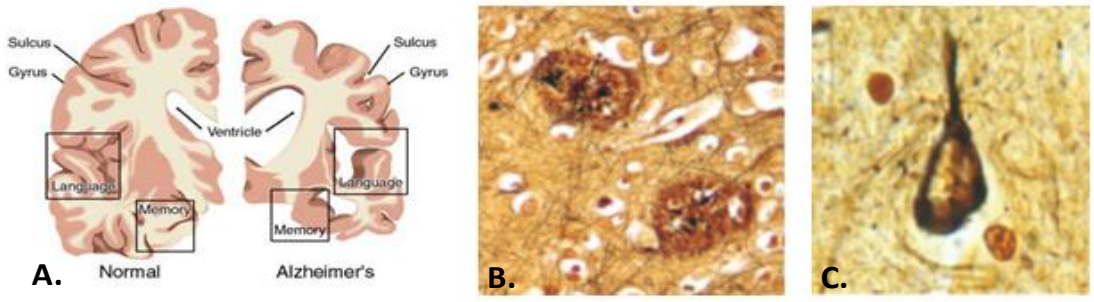


Figure 2: Brain alterations and histopathological hallmarks in AD. A - Brain cross sections of a healthy person (left) and an AD patient (right). From http://wp.stockton.edu/gfb1/tag/alzheimers-disease/. In the latter one, an overall shrinkage of the brain tissue is visible, affecting mostly the cerebral cortex, hippocampus and amygdala, while ventricles enlarge. **B -** Senile plaques mainly composed of aggregates of amyloid-β peptides (silver stained). **C -** Neurofibrillary tangles inside the neurons, resulting from Tau protein hyperphosphorylation (silver stained). From O'Brien and Wong (2011).

Senile Plaques (SPs) are aggregates of amyloid-β peptides (Aβ), which derives from the proteolitic cleavage of the Amyloid Precursor Protein (APP), a protein that is produced in large quantities in neurons and is metabolized very rapidly (Allsop et al., 1983). APP can be cleaved trough the amyloidogenic pathway, producing Aβ, or trough the non-amyloidogenic pathway, which precludes Aβ formation (Figure 3) (O'Brien and Wong, 2011). The most common forms of Aβ are constituted by 40 aminoacids ($A\beta_{1-40}$) or 42 aminoacids ($A\beta_{1-42}$). The longer form, $A\beta_{1-42}$, is less soluble and has a higher propensity to aggregate, leading to a greater neurotoxicity relative to $A\beta_{1-40.}$ All factors that contribute to altered APP processing, such as abnormal phosphorylation, oxidative stress and Aβ itself, can lead to abnormal Aβ production (Lee et al., 2003; Rebelo et al., 2007; Coma et al., 2008; Sodhi et al., 2008; Henriques et al., 2010). Increased Aβ production and accumulation triggers a series of pathogenic processes, such as oxidative stress and inflammation, which result in impaired neuronal functions and, consequently, neuronal death (Masters et al., 2006; Shen and Kelleher, 2007; Chow et al., 2010).

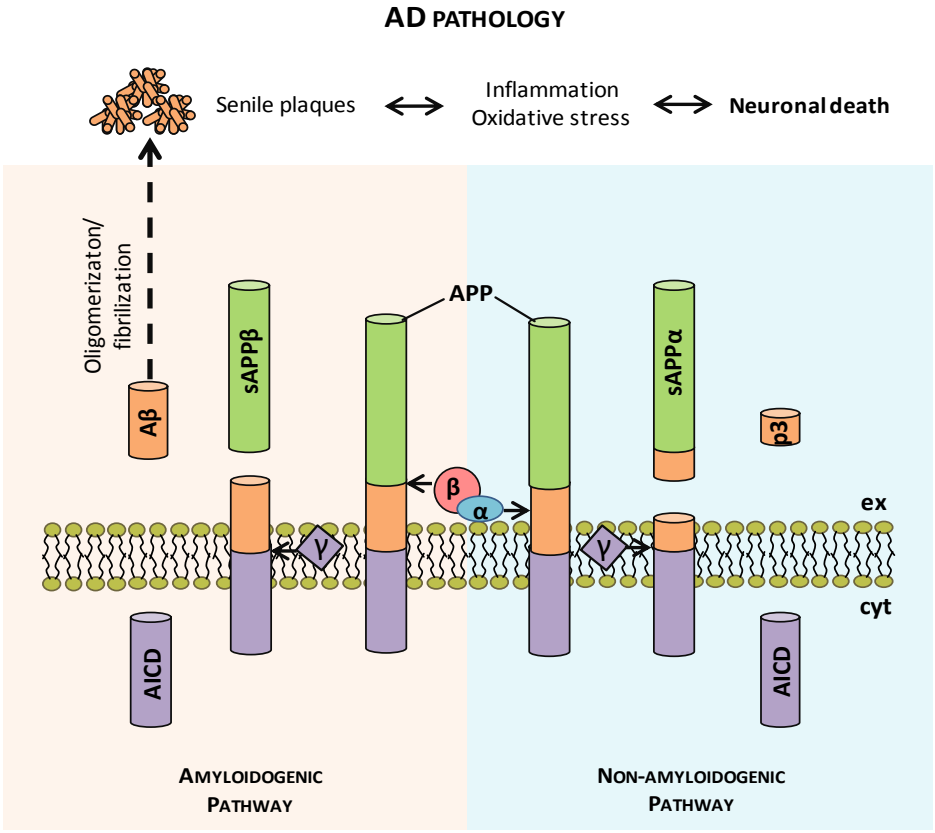


Figure 3: APP processing and cleavage products. The non-amyloidogenic APP processing pathway (right) involves cleavages by α- and ɤ-secretases, resulting in the generation of a long secreted form of APP (sAPPα) and C-terminal fragments (p3 and AICD). The amyloidogenic APP processing pathway (left) involves cleavages by β- and ɤ-secretases resulting in the generation of a long secreted form of APP (sAPPβ) and C-terminal fragments (Aβ and AICD). Aβ fragments oligomerize and fibrillize leading to AD pathology (upper panel). ex- extracellular space, cyt – cytosol. Adapted from Chow et al. (2010).

Neurofibrillary tangles (NFTs) are primarily composed of hyperphosphorylated form of Tau protein (Goedert et al., 1992). Tau is synthesized within the neuron and localized in the axon, where it promotes stability and microtubule assembly, also acting as a regulator of intracellular vesicles and organelle traffic, by interaction with cytoskeletal proteins, such as actin (Drouet et al., 2000). During AD progression, Tau is hyperphosphorylated and subsequently dissociated from microtubules, leading to their breakdown in NFTs and paired helical filaments (PHF), which in turn results in neuronal degeneration (Sorrentino and Bonavita, 2007).

Several studies demonstrated that biochemical and pathological alterations observed in the AD brain result from abnormal cellular processes such as altered APP metabolism, Tau hyperphosphorylation, oxidative stress, inflammation and lipid dysregulation (Irizarry, 2004). Currently, one predominant hypothesis for AD pathogenesis states that Aβ accumulation in the central nervous system is the first event that initiates the pathogenic cascade. According to this hypothesis, increased Aβ production and decreased clearance results into Aβ accumulation and aggregation in SPs, which in turn interferes with synaptic transmission, activates microglia and stimulates an inflammatory response. Consequently, these events lead to neuronal dysfunction, altered kinase/phosphatase activity and NFTs formation (Jakob-Roetne and Jacobsen, 2009; Karran et al., 2011). This cascade of events will culminate in neuronal death and neurodegeneration events.

1.2.2. Vascular Dementia

Following AD, Vascular dementia (VaD) is the most common type of dementia and is associated with problems in blood circulation to the brain, denominated as cerebrovascular disease. The most common type of VaD is multi-infarct dementia (MID), caused by a series of small strokes (which may result in brain tissue death). However, it can also be the result of a single major stroke (single-infarct dementia). Individuals who have had several strokes may develop chronic cognitive deficits, often in a stepwise progression manner, as already mentioned. The occurrence of dementia depends partly on the total area of damaged cortex (Bird and Miller, 2010). Strokes usually involve

several distinct brain regions, so that clinical features will depend on the location and size of vascular lesions. As a consequence, there can be deficits in spatial and executive functions, memory, personality changes and also neuropsychiatric manifestations (Kester and Scheltens, 2009).

Another subtype of vascular dementia is CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukencephalopathy), which has a genetic component associated with mutations in the *NOTCH3* gene, on chromosome 19. This disease tends to affect young patients who lack vascular risk factors and the core symptoms are recurrent strokes and migraine. (Kester and Scheltens, 2009).

Neuropathological Characteristics of VaD

If blood vessels in the brain burst (cerebral hemorrhage) or if arteries are blocked by plaque formation or clots (thrombosis or embolism), there is insufficient blood flow to some parts of the brain and neuronal tissue may die, leading to dementia, depending on the brain area affected. The principal risk factors associated with VaD are the same as those for heart disease: history of heart attack, atherosclerosis, high cholesterol and blood pressure, diabetes and smoking (Pinkston et al., 2009). The more important pathological alterations seen in VaD are multiple areas of infarction and, in some patients, bilateral abnormalities of subcortical white matter, often occurring in association with lacunar infarctions. Examples of such alterations are showed in Figure 4.

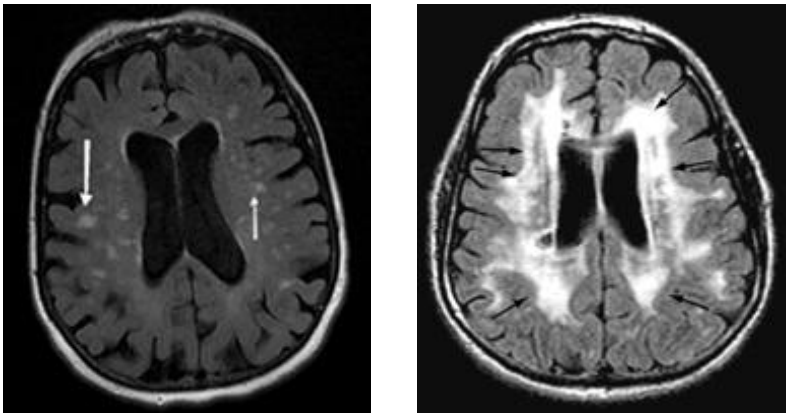


Figure 4: Pathological alterations of VaD. Multi-infarct sites (left); abnormalities in subcortical white matter, with multiple areas of abnormal high signal intensity (right) (MRI images). From http://dementiatypes.org/Vascular_Dementia.htm and Bird and Miller (2010).

1.2.3. Dementia with Lewy Bodies

Dementia with Lewy Bodies (DLB) is a form of dementia that shares clinical and histological characteristics with both AD and Parkinson´s Disease (PD). It is characterized by fluctuating cognitive impairment, persistent visual hallucinations and parkinsonism. Dementia can precede or follow the appearance of parkinsonism. DLB patients are highly susceptible to metabolic perturbations, and in some cases the first manifestation of illness is delirium, often precipitated by an infection or other systemic disturbance. Fluctuations can be marked in DLB patients, with occurrence of episodic confusion mixed with lucid intervals (Bird and Miller, 2010).

Neuropathological Hallmarks of DLB

Pathologically, DLB is characterized by the presence of Lewy Bodies in cortical and subcortical regions of the brain, such as limbic system, brainstem and substantia nigra. SPs are also present, as well as NFTs, although much less commonly than in AD (Spillantini et al., 1998; McKeith et al., 2004; Bird and Miller, 2010).

Lewy Bodies (LBs) are spherical, intracytoplasmic, eosinophilic neuronal inclusions which are mainly constituted by α-synuclein and ubiquitin (Figure 5). Despite major efforts, the identification of factors that promote LBs formation and their function in the pathological process remain unclear. When LBs accumulate in large numbers in the brain, they may interfere with normal cell function and be associated with abnormalities in metabolic processes. The role of intracellular inclusion bodies in neurodegenerative disorders has long been controversial; some claiming that inclusions constitute part of the pathological process, while others suggest a protective role for them against toxicity (Moore et al., 2005; Ross and Poirier, 2005).

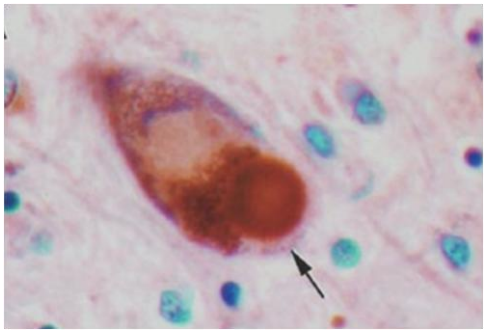


Figure 5: Lewy body inside a neuronal cell. Lewy body was stained for ubiquitin (arrow). From Chu et al. (2000).

1.2.4. Frontotemporal Dementia

FTD is the most common clinical manifestation of Frontotemporal Lobar Degeneration (FTLD). Typically, it is a disease of early onset, and the third most common neurodegenerative cause of dementia in patients under the age of 65. In Portugal there is no information about the prevalence of this type of dementia, possibly being under-diagnosed (Guimarães et al., 2006). Up to 50% of patients with FTD report a family history of dementia, suggesting a strong genetic component associated with this disease (McKhann et al., 2001; Ghidoni et al., 2011). FTD is an umbrella term for a diverse group of disorders which are primarily caused by atrophy or shrinkage of the frontal and temporal lobes of the brain, and so, the clinical symptoms generally associated are behavior and personality changes, cognitive impairment and language disorders of expression and comprehension (McKhann et al., 2001; Goedert et al., 2012).

1.2.4.1. Neuropathological Hallmarks of FTD

As mentioned, the major distinguishing anatomical hallmarks of FTD is the selective atrophy of the frontal and/or temporal lobes due to neuronal loss, also accompanied by gliosis and spongiosis of the superficial layers. The atrophy is sometimes asymmetric and may involve subcortical brain regions, such as the basal ganglia or the hippocampus (Bird and Miller, 2010; Seelaar et al., 2011; Goedert et al., 2012). At the histopathological level, FTD may be characterized as FTLD-τ or FTLD-U if Tau-positive neuronal inclusions (Figure 6A) or if ubiquitin-positive and Tau-negative neuronal inclusions are present, respectively.

For the latter cases, besides ubiquitin, other proteins may be found in these inclusions. Transactive response-DNA binding protein-43 (TDP-43) was identified as the major component of the inclusions in most cases of FTLD-U, which are now called FTLD-TDP (Figure 6B). Most of FTLD-U cases negative for TDP-43 were discovered to be positive for fused in sarcoma (FUS) protein, which made up the FTLD-FUS subtype. Additional rare forms remain to be discovered, because inclusions negative for TDP-43 and FUS, but positive for components of the ubiquitin-proteasome system (UPS), have been described and defined as FTLD-UPS. Around 40% of FTD patients show Tau positive inclusions, 50% show TDP-43 inclusions and less than 10% have FUS inclusions (reviewed in Goedert et al. (2012)). Nonetheless, despite the heterogeneity of the underlying neuropathology, clinical symptoms correlate better with specific patterns of brain atrophy in each FTD case (Goedert et al., 2012).

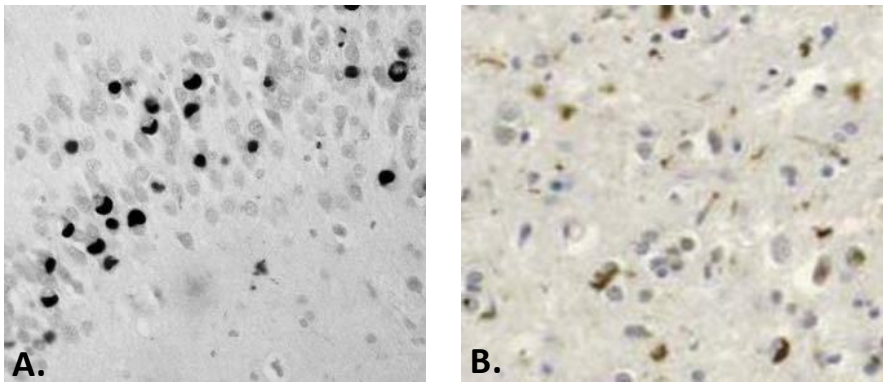


Figure 6: Histopathological hallmarks in FTLD patients. A – FTLD-τ brain. Numerous Tau-immunoreactive inclusions are seen inside the neurons. From Cairns et al. (2007). **B** – FTLD-TDP brain. Abundant TDP-43 immunoreactive compact neuronal cytoplasmic inclusions and short dystrophic neuritis, often with neuronal intranuclear inclusions. From Goedert et al. (2012).

1.2.5. Huntington's Disease

HD is a neurodegenerative disorder that is transmitted within families from generation to generation, caused by mutated huntingtin gene (*HTT*). The mean age at onset is between 30 and 50 years, but it can range from 2 to 85 years, and is characterized by chorea, behavioral and psychiatric disturbances and dementia. Usually, memory is unaffected until late stages of the disease, but attention, judgment, awareness, and executive functions may be seriously impaired even in early stages. The progression of the disease leads to more dependency in daily life and ultimately to patients death. The most common cause of death is pneumonia, followed by suicide (Huntington, 1872; Bird and Miller, 2010).

1.2.5.1. Neuropathological Hallmarks of HD

Distinct topographic and cellular alterations, notably in the striatum and cerebral cortex, are characteristic of HD. Patients suffering from this disease have a total brain volume smaller, when compared to normal individuals, with a marked neuronal loss observed in the striatal area. Also, nuclear or cytoplasmic inclusions in neurons and glial cells are present, which can be detected long before the symptoms onset in otherwise apparently normal brains of asymptomatic gene carriers. These inclusions contain ubiquitin and the mutated huntingtin protein (Figure 7), and contribute to the disease neurodegenerative process (Gomez-Tortosa et al., 2001; Vonsattel, 2008; Zuccato et al., 2010).

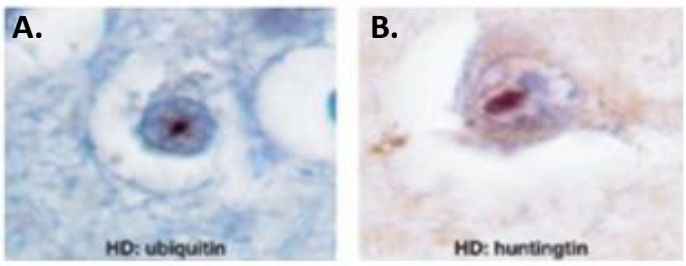


Figure 7: Neurons of a HD patient displaying intranuclear inclusions. Inclusions were stained for ubiquitin and huntingtin**.** From (Crichton et al., 2008).

1.2.6. Alcohol Related Dementia

The influence of alcohol on health varies depending on the amount consumed, drinking duration, type of alcohol and frequency, environmental factors and family history. Frequent heavy drinking or binge drinking is associated with alcohol dependence, alcohol-related cognitive impairment and ARD, which may lead to severe social, economic, physical, and psychological burdens (Oscar-Berman and Marinkovic, 2003; Panza et al., 2009; Fichter et al., 2011). In people diagnosed with ARD, the cognitive deficit manifests as poor working memory, decreased verbal fluency, perseveration, impaired abstraction and decreased behavioral initiation (Saxton et al., 2000).

1.2.6.1. Neuropathological Hallmarks of ARD

Magnetic resonance imaging (MRI) scans of ARD patients show ventricular enlargement and diffuse brain atrophy affecting the prefrontal regions (Figure 8) (Pfefferbaum et al., 1997; Pfefferbaum et al., 1998).

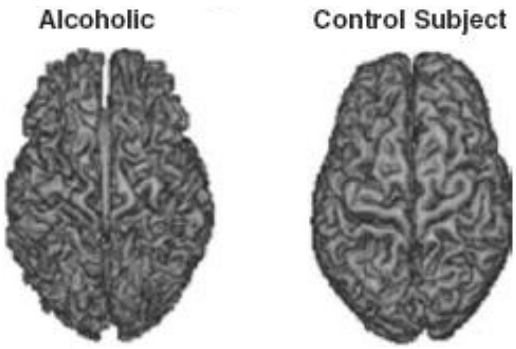


Figure 8: Brains of age-equivalent men with different histories of alcohol use. The image shows clear evidence of brain shrinkage in the alcoholic compared with the control subject. Adapted from Oscar-Berman and Marinkovic (2003).

These brain alterations are due to neurotoxic effects caused by chronic alcohol abuse, leading to cognitive impairment (Verbaten, 2009). The cognitive deficits may be mediated directly through damage in brain structures or indirectly through malnutrition, metabolite toxicity, electrolyte imbalance, or accompanying physical illnesses including liver disease and infection (Neiman, 1998). The direct neurotoxic effect of alcohol is

mediated via its action on the N-Methyl-D-aspartate (NMDA) receptors of glutamatergic neurons. Acute alcohol intake exerts an inhibitory effect on NMDA receptors and, thus, induces receptor up-regulation. However, when alcohol intake ceases, the up-regulated receptors are no longer inhibited, still resulting in excessive stimulation of NMDA receptors and calcium influx, consequently leading to cytotoxic effects. Glutamatergic neurons are densely concentrated in the frontal lobes and subcortical areas such as the hippocampus, and these brain regions are particularly vulnerable to excitotoxic effects produced by alcohol intake (Verbaten, 2009). The neuropsychological deficits and brain damage caused by ARD may be partially reversed with continued abstinence (Shear et al., 1994; Pfefferbaum et al., 1995).

Since no specific genetic basis has been described for this disease, and once ARD is clinically different from AD and VaD (Oslin and Cary, 2003), it will not be addressed in the subsequent sections.

1.3. GENETIC BASIS OF DEMENTIA

Many dementing disorders show an extensive family history, suggesting a substantial contribution of hereditary genetic factors to disease onset and progression. This section will focus on the main genetic factors associated with the most common forms of dementia.

1.3.1. Alzheimer's disease

Familial cases of AD (age of onset <65 years) represent less than 5% of total cases and are caused by mutations in 3 different genes: *APP*, Presenilin 1 (*PSEN1*) and Presenilin 2 (*PSEN 2*). Although AD-causing mutations occur in 3 different genes located in 3 different chromosomes, they all share a common biochemical pathogenic pathway, converging on the Aβ peptide (Table 1). Unrevealing the molecular mechanisms underlying the disease pathogenesis will contribute not only to the understanding of the genetic cases, but also of the sporadic forms, since increased Aβ production and accumulation into SPs is a common feature in both cases. Age, environment, decreased reserve brain capacity and reduced mental and physical activity during life are among the factors associated with sporadic cases (Mortimer et al., 2003; Gatz et al., 2006). Nonetheless, genes that increase the susceptibility to develop the disease have also been linked with these cases. Apolipoprotein E gene (*ApoE*) is such an example, with several studies demonstrating a strong correlation between the presence of *ApoE* allele ε4 and AD development (Noguchi et al., 1993; Roses, 1996; Rocha et al., 1997; Josephs et al., 2004; Kim et al., 2009).

1.3.2. Vascular Dementia

Fewer studies have been done in order to examine the genetic associations of VaD (Jones et al., 2011), partly due to the heterogeneity of this disorder. The particular subtype of VaD, CADASIL, is a hereditary condition resulting from mutations in *NOTCH3* gene (Table 1) (Ungaro et al., 2009). In general, proposed genetic risk factors associated with an increased susceptibility to develop VaD include *MTHFR* gene (Methylenetetrahydrofolate reductase) and also the *ApoE,* like for AD (Jones et al., 2011). Nevertheless, a lack of association between the last gene and VaD development was also reported (Sulkava et al., 1996; Kim et al., 2008).

1.3.3. Dementia with Lewy Bodies

DLB occurs sporadically in most cases, but familial forms also exist, suggesting the importance of genetic determinants. Recent molecular genetic discoveries are now revealing that DLB is etiologically heterogeneous, and the known genetic determinants of DLB overlap substantially with those of PD (Bonifati, 2008). The main mendelian genes associated with DLB are for now *SNCA* (Zarranz et al., 2004) and *LRRK2* (Ross et al., 2006) (Table 1), which are also genetic factors associated with PD. *ApoE* gene has also been proposed as a risk factor for DLB development (Harrington et al., 1994), but this finding is still controversial (Carrillo Garcia et al., 2008).

1.3.4. Frontotemporal Dementia

A strong genetic component is linked to FTD, as FTD patients report a family history of dementia in up to 50% of the cases. Nevertheless, the clinical and neuropathological variability of the syndrome suggests the existence of several distinct genetic factors underlying or modifying disease pathogenesis (Table 1) (Bertram and Tanzi, 2005). In fact, mutations in *MAPT* gene are associated with FTLD-τ, while mutations in progranulin (*PGRN*) gene are associated with FTLD-TDP (Kumar-Singh and Van Broeckhoven, 2007; Goedert et al., 2012). These are the main genes responsible for hereditary forms of FTD, however, mutations in *VCP, FUS* and *CHMP2B* genes can also lead to disease, but at a

much lower frequency. With respect to susceptibility genes, it has been shown that *TMEM106B* variants may increase the risk of FTD development, in particular the FTLD-U subtype (Van Deerlin et al., 2010). As for DLB and VaD, the role of *ApoE* as a risk factor for FTD is not consensual (Geschwind et al., 1998; Borroni et al., 2006), and additional studies should be carried out to establish the association.

1.3.5. Huntington's Disease

HD is an autosomal dominant degenerative brain disorder. A DNA elongated CAG repeat on the short arm of chromosome 4p16.3 in the *HTT* gene is the basis of the pathological process (Table 1). The non mutated gene contains CAG repeats, coding for a polyglutamine stretch in the protein, in the range 6 to 26. HD is associated with 36 CAG repeats or more, that results in the formation of HTT with polyglutamine expansions. Definite clinical manifestation will occur if the number of repeats exceeds 40. The range 36-39 leads to an incomplete penetrance of the disease or to a very late onset. The range between 27 and 35, the so-called intermediate alleles, is unstable, which means that these alleles are prone to changes during reproduction, thus increasing the propensity of the next generation to develop the disease (Trottier et al., 1994; Walker, 2007).

Comparatively to other forms of dementia, HD is easier to diagnose using currently available genetics base diagnostics, thus it will not be further discussed in the work here presented.

Table 1 exhibits the principal inherited genes linked to the dementing disorders discussed above. The main proposed mechanisms in the base of disease pathogenesis caused by the mutated genes were also included.

Table 1: Main mendelian genes associated with AD, FTD, VaD, DLB and HD. (Selkoe, 1998; Brunkan and Goate, 2005; Zuccato et al., 2010; Duering et al., 2011; Lill and Bertram, 2011).

Disease	Gene	Protein	Mechanism of pathogenesis
AD	*APP*	Amyloid precursor protein	Increases $A\beta_{1-42}$ production from APP processing
	PSEN1	Presenilin 1	Promotes cleavage at γ-secretase site and increases $A\beta_{1-42}$ production and accumulation
	PSEN2	Presenilin 2	Promotes cleavage at γ-secretase site and increases $A\beta_{1-42}$ production and accumulation
VaD	*NOTCH3*	Neurogenic locus notch homolog protein 3	Accumulation and deposition of the NOTCH3 (N3) extracellular domain in small blood vessels
DLB	*SNCA*	α-synuclein	Aggregation of α-synuclein; altered neurotransmitter release and vesicle turnover
	LRRK2	Leucine-rich repeat kinase 2	Mishandling of a-synuclein, reduction of neurite outgrowth, alteration of endosomal trafficking
FTD	*PGRN*	Progranulin	Impaired neuronal survival; inflammation
	MAPT	Microtubule-associated protein Tau	Impaired microtubule assembly and axoplasmic transport
	CHMP2B	Chromatin modifying protein 2B	Interference with endosome-lysosome fusion
	VCP	Valosin-containing protein	Impaired proteasomal degradation, altered membrane sorting at endosomes/degradation in lysosomes, impaired endoplasmic reticulum induced stress response
HD	*HTT*	Huntingtin	Accumulation of NH_2 terminal fragments of mutated HTT, impairment of signaling processes and homeostasis, proteasomal and mitochondrial dysfunction, impaired gene transcription, altered vesicular transport

1.4. *APOE* AS A GENETIC RISK FACTOR FOR DEMENTIA

ApoE genotype has been proposed as a risk factor for various types of dementia. However, a strong correlation for this gene with disease development is better established for AD. In particular, it seems clear that APOE isoform 4 plays an important role in the pathogenesis of this disease, while controversial data have been reported for other dementias.

1.4.1. APOE role

Human APOE is a lipoprotein of 317 aminoacids that is expressed in many organs, with the highest expression in the liver, followed by the brain. APOE exists mainly as a component of lipoprotein complexes along with other apolipoproteins and proteins in plasma and cerebrospinal fluid (CSF). It is encoded by the *ApoE* gene (19q13.2), where alleles ε2, ε3 and ε4 give rise to the three major polymorphic forms of APOE: APOE2 (Cys130, Cys176), APOE3 (Cys130, Arg176) and APOE4 (Arg130, Arg176). Aminoacid differences at these positions are crucial as they alter the charge and structural properties of the protein, which may ultimately influence the functional properties of the APOE isoforms, such as stability, folding characteristics, lipoprotein particle preferences and binding affinities to low density lipoprotein receptor (LDLR) and LDLR related protein (LRP) (Davignon et al., 1988).

APOE is one of the key components in lipoprotein complexes that regulate lipid metabolism by directing their transport, delivery and distribution from one tissue or cell type to another (Mahley, 1988). In addition, this lipoprotein may be involved in many other physiological and pathological processes, including immunoregulation, nerve regeneration, activation of lipolytic enzymes, ligand for several cell receptors, neuronal homeostasis and tissue repair (Mahley and Rall, 2000). In the brain, APOE is mainly produced by astrocytes, but also by microglia and neurons (Kim et al., 2009), and plays an essential role in cholesterol and phospholipid transport to neuronal membrane regeneration and remyelination sites (Lane and Farlow, 2005).

1.4.2. *ApoE* and dementia

Not only familial but also sporadic forms of dementia are likely to be substantially controlled by genetic factors, given that possible risk genes may be involved. *ApoE* has been one of the most studied risk factors, mainly associated with sporadic cases. A series of landmark studies identify a strong association between the presence of the isoform APOE4 and an increased risk for AD: the susceptibility to develop the disease is more strongly associated with APOE4 than with APOE3 and in turn more strongly with APOE3 than with APOE2, the latter suggested to have a protective role in the disease (Corder et al., 1994; Panza et al., 2000). The presence of one *ApoE* ε4 copy increases the risk to develop AD by about three times and two copies by about 12 times (Saunders, 2000). Also, the *ApoE* ε4 allele modifies age of disease onset (Meyer et al., 1998; Xiong et al., 2005), with each allele copy lowering the age of onset by almost 10 years, suggesting that APOE4 association with AD may be related to longer disease duration in these cases (Basun et al., 1995).

Although the mechanism by which APOE isoforms affect the risk to develop AD is not entirely understood, relevant evidence have been demonstrated:

(i) there is strong evidence that APOE isoforms differentially modulate Aβ metabolism; *in-vitro* and *in-vivo* studies show that APOE has an important role in determining whether and when Aβ converts from a monomeric, non-toxic molecule into higher-molecular-weight forms such as oligomers and fibrils, certain forms of which probably mediate neurotoxic effects (Wisniewski et al., 1994; Castano et al., 1995);

(ii) APOE isoforms differentially regulate Aβ clearance from the brain: increased Aβ accumulation has been demonstrated in carriers of ε4 allele (Polvikoski et al., 1995; Castellano et al., 2011; Bachmeier et al., 2012);

(iii) Genotype also influences pathological changes in relation to the time course of disease onset by affecting the probability that Aβ begins to deposit, such that the timing of accumulation is shifted to an earlier age depending on APOE isoforms, in particular, APOE4 (Figure 9) (Jack et al., 2010)

(iv) Allele 4 of *ApoE* may be more vulnerable than other isoforms to aberrant degradation thereby limiting lipid mobilization for neuronal repair, neuroplasticity and cognitive reserve in the face of the neurodegenerative process (Finch and Sapolsky, 1999; Acharya et al., 2002);

(v) Enhanced formation of C-terminal-truncated fragments characteristic of E4 isoform stimulates Tau hyperphosphorylation and the formation of NFTs (Harris et al., 2003)

(vi) Ischemic brain insults and hypertension-related white matter lesions, known contributors to the severity of dementia in AD, tend to be more pronounced in ε4-positive patients (de Leeuw et al., 2004)

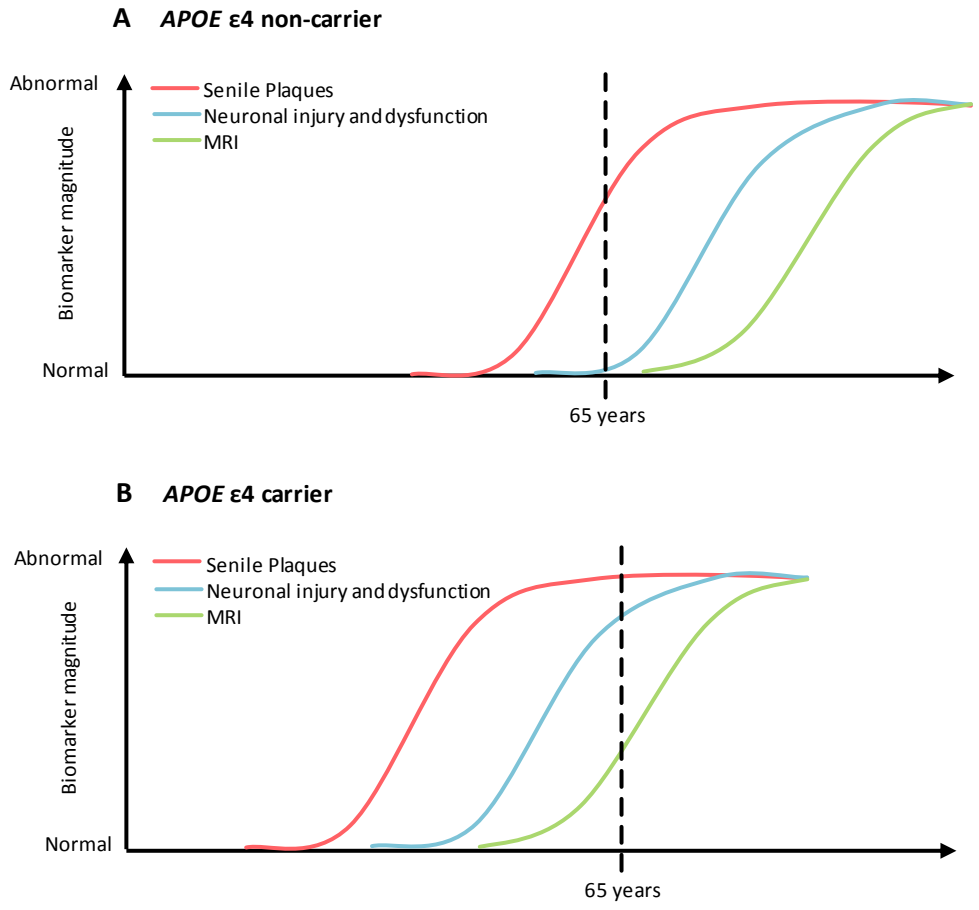


Figure 9: *ApoE* as a genetic risk factor for AD. (A,B) Relative to a fixed age (here, 65 years), the hypothesized effect of *ApoE* ε4 is to shift β-amyloid plaque deposition and the neurodegenerative cascade both to an earlier age compared with ε4 non-carriers, lowering the age of disease onset. MRI – Magnetic resonance imaging. Adapted from Jack et al. (2010).

Numerous studies have investigated putative associations of *ApoE* genotype with risk of progression of various other dementing disorders (Table 2), nevertheless the data reported has been controversial. While some studies reported that the frequency of allele ε4 of *ApoE* is increased in VaD, FTD and DLB (Noguchi et al., 1993; Harrington et al., 1994; Morris et al., 1996; Ingelson et al., 2001; Baum et al., 2006; Borroni et al., 2006; Davidson et al., 2006), other authors have reported that there are no evidence of association between the individual *ApoE* genotype and the development of the diseases mentioned above (Sulkava et al., 1996; Geschwind et al., 1998; Verpillat et al., 2002; Nielsen et al., 2003; Carrillo Garcia et al., 2008; Kim et al., 2008). Hence, the relationship between APOE4 and VaD, FTD or DLB remains uncertain. Interestingly, contrary to what happens in AD, where allele ε2 is a protective factor, some studies have proposed that the expression of this allele can be a risk factor for FTD (Lehmann et al., 2000; Verpillat et al., 2002).

Table 2: Association of *ApoE* genotype with occurrence and progression of neurological disorders. Reviewed in Verghese et al. (2011).

	Disease occurrence (level of evidence)	**Disease progression (level of evidence)**
AD	ε4>ε3>ε2 (sufficient evidence of direct relation)[1]	Inadequate or insufficient evidence[2]
VaD	ε4>non-carriers (suggestive evidence of association)[3]	Inadequate or insufficient evidence[2]
FTD	Inadequate or insufficient evidence[2]	Inadequate or insufficient evidence[2]
DLB	ε4>non-carriers (suggestive evidence of association)[3]	Inadequate or insufficient evidence[2]

Levels of evidence were adapted from the categories established and used by the US Institute of Medicine for association between a factor and a specific health outcome. [1]Evidence fulfils guidelines for sufficient indication of an association, is supported by experimental data in humans and animals, and satisfies several of the guidelines used to assess causality (strength of association, dose–response relation, and consistency of association). [2]Evidence is of insufficient quantity, quality, or consistency to permit a conclusion about the existence of an association between *ApoE* and the neurological disorder in humans. [3]Evidence is suggestive of an association between *ApoE* and the neurological disorder in humans, but the body of evidence is restricted by the inability to exclude chance, bias, and confounding factors with confidence.

1.5. DIAGNOSIS OF DEMENTIA

Many dementing disorders share a number of clinical, pathological and molecular characteristics that complicate diagnosis specificity, due to the high degree of overlap in the associated symptoms across diverse dementia types (Bertram and Tanzi, 2005; Reilly et al., 2010). Differential diagnosis of dementia is typically done by clinical evaluation, that includes cognitive and behavior assessment, neuroimaging exams and a weighted combination of genetic and protein biomarkers. Regarding protein analysis, a recent promising and accurate tool emerged in the neuroscience field based on the evaluation of a panel of CSF biomarkers, whose pattern allows for the distinction among neurodegenerative diseases. This novel CSF-based neurochemical diagnosis is currently used in Europe and is starting to be established in Portugal. It seems a promising tool that will assist clinical diagnosis and hopefully help in overcoming dementia misdiagnosis.

1.5.1. Clinical Diagnosis

Clinical diagnosis requires general physical and neurological examination, behavior and cognitive neurological assessment, including attention, concentration, language, memory and learning ability, as well as visuospatial and executive functions. To confirm cognitive impairment and to follow the progression of dementia, screening tools such as the mini-mental state examination (MMSE) are usually used. MMSE is an easily performed 30-point test of cognitive function that contains orientation, working memory (e.g., spell world backwards), episodic memory (orientation and recall), language comprehension, naming and copying tests. In most patients with MCI and some with clinically apparent AD, the MMSE may be normal and additional neuropsychological tests may be required, such as DemTect or the clock drawing test (Bird and Miller, 2010; Eschweiler et al., 2010). This clinical diagnosis is not 100% conclusive and needs additional exams for neuronal cognitive examination and evaluation. Once the cognitive deficits have been objectively demonstrated, further investigations requires brain imaging and laboratory testing (Kester and Scheltens, 2009). Although head computerized tomography (CT) enables judgments of brain atrophy patterns and of vascular changes, the MRI

affords higher resolution without exposing the patient to ionizing radiation. Furthermore, the techniques of single photon emission computerized tomography (SPECT) and positron emission tomography (PET) can also be used to study cerebral perfusion and to measure brain energy metabolism, or, more recently, to quantify Aβ burden inside the brain (by using a chemical, named Pittsburgh Compound-B) (Klunk et al., 2004; Rowe et al., 2007; Tartaglia et al., 2011). Relative to laboratory tests, analysis of blood biomarkers to assess thyroid, liver and renal functions, as well as complete blood count, electrolytes, vitamin B_{12} and APOE levels, as well as urine toxin screen, should be performed in order to discard other possible causes of dementia such as hypothyroidism, vitamin B_{12} deficiency, chronic infection, brain tumor and drug intoxication (Bird and Miller, 2010). However, the biggest disadvantage of the clinical diagnosis is that in some cases it confirms pathology but only in a late stage, where cognitive impairment is already evident.

The main clinical symptoms and signs associated with the most frequent dementias are presented in Table 3. The overlap of the vast possible clinical symptoms among the various dementing disorders is clearly evident, which may turn difficult the interpretation of the disease stage and the accurate clinical diagnosis of the pathology. Moreover, the occurrence of mixed dementia (MD) cases further complicates the diagnosis. MD is a condition in which abnormalities characteristic of more than one type of dementia occur simultaneously in the brain. In the most common form of MD, the abnormal protein deposits associated with AD coexist with blood vessel problems linked to VaD. However, Alzheimer's brain changes coexistent with LBs are also often observed, or even, in some cases, a person may have brain changes linked to AD, VaD and DLB. MD symptoms may vary, depending on the brain alterations involved and regions affected (http://www.alz.org; Zurad, 2001; Jellinger and Attems, 2007; Dubois et al., 2010).

Table 3: Neuropathological characteristics and symptoms of the most common dementias. (Ritchie and Lovestone, 2002; Armstrong et al., 2005; Bird and Miller, 2010; Reilly et al., 2010; Tartaglia et al., 2011).

Disease	Early Symptoms	Late Symptoms	Neuropsychiatric Symptoms	Pathological Characteristics	Neuroimaging
AD	Memory impairment (verbal and visual), personality changes	Memory and language problems worsen, difficulty performing activities of daily living, visuospatial problems, disorientation	Mild depressive features, social withdrawal, denial of illness, delusions, agitation, apathy, hallucinations, euphoria	Senile Plaques, Neurofibrillary Tangles	Hippocampal and diffuse cortical atrophy, reduced glucose metabolism in the superior/posterior temporal and parietal regions
VaD	Mixture of deficits in executive and spatial functions, memory impairment, mild confusion, personality changes	Difficulties in judgment and orientation, dependence on others for daily activities	Depression, delusions, disinhibition, apathy, hallucinations	Ischemic lesions	Large white-matter abnormalities, cortical and/or subcortical infarctions
DLB	Visual hallucinations, language disturbance, parkinsonian symptoms, memory can be affected	Severe visuospatial symptoms, movement disorders	Delusions related to personal identity, day-to-day fluctuations, depression	Lewy bodies	Posterior parietal atrophy, hippocampus larger than in AD
FTD	Deficit in frontal executive or language functions, personality changes, increased apetite, compulsive or repetitive behavior	Memory loss, problems maintaining normal interactions and following social conventions	Depression, apathy, disinhibition, loss of empathy, anxiety	Tau or ubiquitin inclusions	Focal frontal and/or anterior temporal atrophy, hypometabolism in the frontal and anterior temporal regions

1.5.2. Neurochemical Diagnosis

As mentioned before, sometimes it is complicated to differentiate among dementing syndromes due to the overlap of diverse signals and symptoms, which may in some cases contribute to misdiagnosis. Recently, a reliable approach to differentiate benign cognitive deficits from AD and other dementias has been established, based on patient´s CSF biochemical analysis (Lewczuk et al., 2009; Dubois et al., 2010; Zetterberg et al., 2010). In this neurochemical diagnosis, a set of biomarkers can be evaluated, including:

Aβ$_{1-42}$ – Amyloid pathology assessment is relevant because of its robust association with AD, as this seems to be the earliest biochemical change during the course of the disease (Stomrud et al., 2007; Fagan et al., 2009). Low CSF levels indicate retention of $A\beta_{1-42}$ in the brain parenchyma (Strozyk et al., 2003; Grimmer et al., 2009);

Phospho-Tau – Tangle pathology is the most specific finding suggesting an ongoing AD process in the brain (Hampel et al., 2010). Elevated P-Tau 181 levels in CSF correlates with MCI and with neocortical neurofibrillary pathology in AD (Buerger et al., 2002; Buerger et al., 2006). The reason why P-Tau 181 levels are normal in FTD patients is to date unknown (Bian et al., 2008);

Total-Tau – Increased CSF levels of T-Tau traduce axonal degeneration: the more pronounced increase, the more intense degenerative process and the faster the disease progression (Hampel et al., 2010). The cortical axonal degeneration in AD makes elevated T-Tau an obligatory finding. Very high levels predict a rapid cognitive decline in AD (Blom et al., 2009) and short survival in DLB (Bostrom et al., 2009);

Neurofilament light protein (NFL) – it is the best-established biomarker for sub-cortical axonal degeneration/damage. This kind of degeneration is frequently seen in VaD (Rosengren et al., 1999; Wallin and Sjogren, 2001) and FTD (de Jong et al., 2007). Elevated NFL levels in CSF help in differentiating pure AD from other conditions;

CSF/serum albumin ratio – this ratio is a direct measurement of blood-brain barrier (BBB) integrity. Typically, the CSF/serum albumin ratio is normal in patients with pure AD, while patients with vascular dementia generally present an elevated ratio (Blennow et al., 1990; Wallin et al., 1999);

Inflammation biomarkers – white blood cell count and Immunoglobulin G (IgG) or Immunoglobulin M (IgM) production, within central nervous system (CNS), are in general negative in AD, VaD, FTD and DLB (Blennow et al., 1990). When positive results are obtained it is an indication that other neuroinflammatory conditions may contribute to the cognitive symptoms observed and must be investigated.

Table 4 summarizes the typical changes in relation to different diagnosis entities.

Table 4: CSF-based neurochemical diagnosis of dementia. Adapted from Zetterberg et al. (2010).

Diagnosis	Amyloid pathology ($A\beta_{1-42}$)	Tangle pathology (P-Tau 181)	Cortical axonal damage (T-Tau)	Sub-cortical axonal damage (NFL)	BBB dysfunction (CSF/serum albumin ratio)	Inflammation (CSF cell counts, IgG or IgM production)
AD	Yes	Yes	Yes	No	No	No
VaD	No	No	Yes (especially in relation to new brain infarcts)	Yes	Yes	Yes
DLB	Yes	No	Yes (mild)	No	No	No
FTD	No	No	Yes (mild)	Yes	No	No
Normal Aging	No	No	No	No	No	No

As evident, the evaluation of these CSF biomarkers allows differential diagnosis of dementia and is more precise as more biomarkers are considered. Of note, according to some authors, alterations in CSF levels of $A\beta_{1-42}$, P-Tau 181 and T-Tau can identify AD with dementia and prodromal AD in patients with MCI with 75-95% sensitivity and specificity (Hansson et al., 2006; Mattsson et al., 2009; Shaw et al., 2009; Visser et al., 2009). Thus, the neurochemical diagnosis, in particular the evaluation of $A\beta_{1-42}$ biomarker, allows for the earliest preclinical evidence for AD pathology (Figure 10).

In comparison with other diagnostic tools, the sensitivity of the CSF analysis seems to be higher than, for example, neuroimaging techniques (Weih et al., 2009), which further validates it as a useful diagnostic tool. Nonetheless, the CSF biochemical data should be interpreted with great care and in combination with the entire clinical picture (Zetterberg et al., 2010).

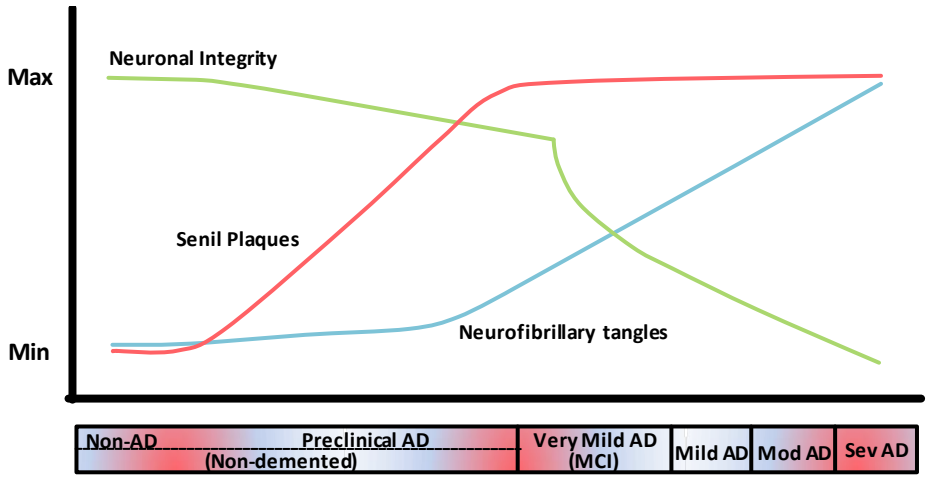


Figure 10: Biomarkers for early diagnosis of AD (proposed changes in biomarkers in relation to time course of pathological and clinical stages). The clinical stages of AD, marked by progressive dementia described as 'very mild/mild cognitive impairment' (MCI), 'mild,' 'moderate,' and 'severe,' are associated with abundant SPs (red line), gradual accumulation of NFTs (blue line) and synaptic and neuronal loss in certain brain regions (green line). In the 'preclinical' stage of AD, $A\beta_{1\text{-}42}$ peptide forms amyloid plaques in the brains of non-demented individuals for approximately 10-15 years, and damages neuronal processes and synapses. AD biomarker research seeks to measure changes in the structure and function of the brain (e.g. atrophy, regional activity changes and hypometabolism, SPs and NFT formation, microgliosis, inflammation, oxidative stress) that might be useful for diagnosis and prognosis during this 'preclinical' phase of AD. Before irreversible neuronal loss occurs, a reduced concentration of CSF $A\beta_{1\text{-}42}$ may provide the earliest definitive evidence of AD pathology in the brain. Adapted from Perrin et al. (2009).

1.5.3. Genetic Diagnosis

Progress in the genetics of dementing disorders and the availability of clinical tests for practicing physicians, increase the need for a better understanding of multifaceted issues associated with genetic testing. Even though the genetic basis of some dementia forms is complex and the fact that genetic testing is associated with ethical concerns (Hedera, 2001), a genetic approach may be of benefit in suspected familial forms of dementia, in particular when a highly penetrant gene mutation is inherited in an autossomal dominant pattern. In this situation, genetic testing may be an advantage since the identification of the specific mutations in affected family members will confirm the dementia diagnosis (Atkins and Panegyres, 2011). Examples of such situations include familial AD triggered by mutated *APP*, *PSEN1* or *PSEN2* genes, and FTD caused by mutations in *MAPT* and *PGRN* genes. However, as these usually represent a minor part of total cases of dementia, genetic testing is not a routinely used diagnostic tool for patient

evaluation and diagnosis. Further, genetic testing of affected patients should be accompanied by competent genetic counseling that focuses on probabilistic implications for at-risk first degree relatives.

Even more controversy lies on the test of genetic susceptibility risk factors, associated with sporadic forms of dementia. Some authors consider that testing using susceptibility genes has only a limited diagnostic value because the potential improvement in diagnostic accuracy does not justify potentially negative consequences for first-degree relatives. Also, predictive testing of unaffected subjects using susceptibility genes should not be recommended because individual risk cannot be quantified and presently there are no therapeutic interventions for dementia in pre-symptomatic patients (Hedera, 2001). On the other hand, some authors consider that this genetic testing may be important, in particular for genetic risk factors already described to be strongly associated with disease, such as *ApoE* for AD pathology. *ApoE* "advocates" claim that testing may be useful when used as a diagnostic adjunct (Roses, 1995; Xiong et al., 2005); if a patient exhibits symptoms of dementia, then the susceptibility testing may help determine what type of dementia the patient is suffering from (Roses, 1997).

2. AIMS OF THE THESIS

Dementia is among the most common and disabling diseases, which represents a major health problem in society, affecting nearly 36 million people worldwide. Disturbingly, numbers of dementia cases tend to rise even more due to increased average of life expectancy. Among the most common types of dementia are Alzheimer´s Disease (AD), Vascular Dementia (VaD), Dementia of Lewy Bodies (DLB) and Frontotemporal Dementia (FTD), whose diagnosis is not always accurate due to the wide possibility of symptoms overlap, in particular in the early stages of the disease. Understanding the environmental and genetic components that contribute to risks and outcomes of neurological diseases, could provide useful information with respect to the development and management of these devastating disorders.

Apolipoprotein E is recognized as a powerful genetic risk factor, in particular for AD pathogenesis. Specifically, *ApoE* allele ε4 carriers have an increased risk of developing the disease, as this protein isoform has been described to play a role in Aβ oligomerization and its accumulation in the brain. Putative associations have also been proposed between this gene and other neurodegenerative disorders, although controversial data have been reported. Hence, the main goal of this thesis was to determine genotypic and allelic frequencies of *ApoE* gene in a pilot study group of neurological disease patients from the catchment area of the "Hospital de São Sebastião" in "Santa Maria da Feira". This group was subdivided according to their neurochemical diagnosis of dementia (by analyzing a triplet of biomarkers, namely $A\beta_{1-42}$, P-Tau 181 and T-Tau), which allowed for the characterization of the study group, in particular in distinguishing AD from other forms of dementia. The specific aims of this work were to:

1. *Isolate genomic DNA from blood samples of the study group patients;*
2. *Quantify and evaluate purity of DNA extracted;*
3. *Amplify the polymorphic regions of ApoE gene by Polymerase Chain Reaction (PCR) and determine the patients' genotype through enzymatic restriction analysis;*
4. *Determine the ApoE genotypic and allelic frequencies in the pilot study group.*

3. METHODS

3.1. STUDY GROUP

Our pilot study group included a population of 32 individuals with neurological disease from the catchment area of the "Hospital de São Sebastião" in "Santa Maria da Feira", herein referred to as "the catchment area", whose age and gender variables are shown in Table 5.

Table 5: Characteristics of age and gender from pilot study group.

Variable	
Age (years)	
Mean (±SD)	68 (±10)
Min-Max	46-85
Gender	
Male n (%)	14 (44%)
Female n (%)	18 (56%)

Sample collection

Blood was collected according to standard procedures in an EDTA tube, to prevent coagulation. Once arrived at the laboratory, samples were immediately aliquoted and frozen at -80°C. CSF samples were collected and stored according to pre-established procedures ("The Alzheimer's Association Quality Control program for cerebrospinal fluid biomarkers").

3.2. CSF-BASED NEUROCHEMICAL ASSAYS

Biochemical CSF analysis using an ELISA based approach for the evaluation of the biomarker triplet ($A\beta_{1-42}$, P-Tau 181 and T-Tau), was carried out accordingly to the manufacturer's instructions (Innogenetics, ELISA Kits). This analyses had been previously performed by colleagues from the Neuroscience Group, Center for Cell Biology. Results are here used to assist in the interpretation of the results.

3.3. *APOE* GENOTYPING

ApoE genotyping was performed as described by Zuo et al., (2006), with minor modifications. Briefly, the genotyping procedure includes the Polymerase Chain Reaction (PCR) to amplify the polymorphic regions of *ApoE* gene, containing the coding portion for amino acid positions 130 and 176, from genomic DNA (gDNA) of each patient. Following amplification, PCR products were submitted to enzymatic restriction (ER) with *HhaI*. Each *ApoE* allele yields distinct fragment sizes, whose pattern was analyzed in an agarose gel electrophoresis system.

3.3.1. Genomic DNA extraction

Genomic DNA (gDNA) was extracted from blood samples using the QIAamp DNA Blood Mini Kit (QIAGEN). In this procedure, DNA is adsorbed onto the silica membrane, under specific salt and pH conditions, and then eluted (Figure 11).

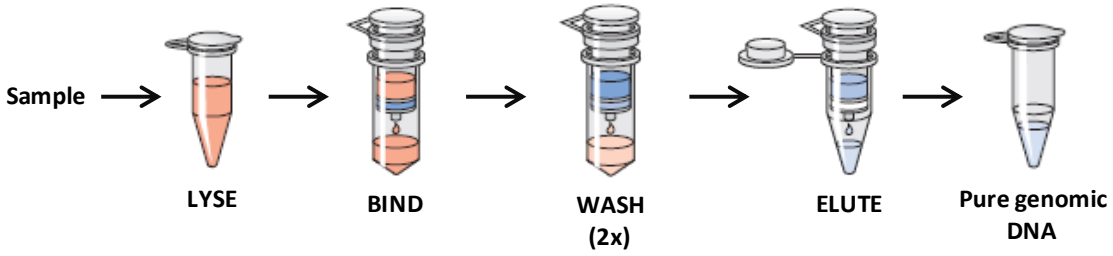


Figure 11: General scheme for genomic DNA extraction.

Samples were equilibrated at room temperature and 200 µL of total blood used for gDNA extraction. Blood was added to 20 µL of protease and after that to 4 µL of RNase A 100 mg/mL, for protein and RNA digestion (as the last can inhibit some downstream enzymatic reactions). Subsequently, 200 µL of lysis buffer were added and tubes mixed by pulse-vortexing for 45sec, to ensure that sample and buffer are mixed thoroughly and yield a homogeneous solution, for an efficient lysis. Additionally, tubes were incubated during 15min at 56°C. After a brief centrifugation step, 200 µL of ethanol (96-100%) were added, further mixed in the vortex for 15sec and briefly centrifuged again. This mixture

was carefully applied on the spin column and centrifuged at 8000 rpm during 1min. DNA bound to the membrane was washed in two centrifugation steps: first at 8000 rpm for 1min and second at 14000 rpm during 3 min, with 500 µL of two different wash buffers. Finally, 100 µL of elution buffer were applied to the columns. In order to enhance DNA recovery, this was done two times (50 µL + 50 µL). After each time, columns were incubated at room temperature during 5 min, followed by centrifugation at 8000 rpm for 1min.

To verify successful DNA extraction, 10 µL of each sample were analyzed by agarose gel electrophoresis.

3.3.1.1. 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 through the porous matrix of agarose, by an electric field, separating according to fragment size, as shorter molecules move faster and migrate further. The percentage of the gel depends on the DNA band size that we intend to analyze.

To visualize gDNA extracted, 1% agarose gels were prepared in 1x TAE Buffer. The solution, heated until the agarose melted, was allowed to cool down to 60°C, and ethidium bromide (EtBr) was added to a final concentration of 0.05 mg/mL. The mixture was poured into the gel tray and left to set for 30-40min. For the gel run, the horizontal electrophoresis tank was filled with 1x TAE buffer. DNA samples were prepared and loading buffer (LB) added in the ratio 1:6, to increase sample density. Also, the presence of bromophenol blue in LB, add color to the sample facilitating loading and allowing track of electrophoresis. DNA ladder 1 kb plus (Invitrogen) was used. Gels were run at 90 V for 40min and visualized under UV light in an AlphaImager HP System (Fisher Scientific).

3.3.1.2. DNA concentration and purity with Nanodrop

In order to evaluate concentration and purity of gDNA extracted, 2 µL of each sample were analyzed, in duplicate, using the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). Purity is given by the Abs260/Abs280 ratio and concentration is expressed in µg/µL.

3.3.2. Amplification of *ApoE* gene by PCR

PCR is a scientific tool to replicate DNA from a template, generating innumerous copies of a specific sequence with respect to the primers used. This technique was used to amplify a specific portion of *ApoE* gene (303 bp) from gDNA of each patient, using KlenTaq LA DNA Polymerase® (Sigma Aldrich) and primers FW - CGGGCACGGCTGTCCAAGGAG and Rev - CACGCGGCCCTGTTCCACCAG, which align with *ApoE* gene (NG_007084.2) as follows:

```
NG_007084.2   GGTGGCGGAGGAGACGCGGGCACGGCTGTCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCT 2880
Primer FW     ----------------CGGGCACGGCTGTCCAAGGAG---------------------- 21
                              ********************

NG_007084.2   GGGCGCGGACATGGAGGACGTGTGCGGCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGC 2940
Primer FW     ------------------------------------------------------------


NG_007084.2   CATGCTCGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCT 3000
Primer FW     ------------------------------------------------------------

NG_007084.2   GCGTAAGCGGCTCCTCCGCGATGCCGATGACCTGCAGAAGCGCCTGGCAGTGTACCAGGC 3060
Primer Rev    ------------------------------------------------------------


NG_007084.2   CGGGGCCCGCGAGGGCGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCT 3120
Primer Rev    ------------------------------------------------------------CT 2
                                                                           **

NG_007084.2   GGTGGAACAGGGCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACA 3180
Primer Rev    GGTGGAACAGGGCCGCGTG---------------------------------------- 21
              ******************
```

Figure 12: Primers alignment with *ApoE* gene sequence.

Each PCR reaction was performed in a DNase free microtube using a mixture of components added according to the order in the following table.

Table 6: PCR amplification mixture components.

COMPONENTS	VOLUME/REACTION (µL)
DNA template (4ng/µL)	5,0
Primer FW 10µM	1,0
Primer Rev 10µM	1,0
Betaine 5M	4,0
dNTPs 10mM	0,4
10x PCR Buffer	2,0
Ultra-pure H_2O	6,4
KlenTaq LA DNA Polymerase Mix (5U/µL)	0,2
Total	**20,0**

The thermocycler used was My Cycler™ (Bio-Rad). Cycle conditions for the denaturing, annealing and polymerization steps are indicated in Table 7.

Table 7: PCR conditions for *ApoE* polymorphic regions amplification.

Nº OF CYCLES	TEMPERATURE	TIME	
1	95°C	5 min	Initial denaturation
35	95°C	30 sec	Denaturation
	64°C	30 sec	Annealing
	68°C	30 sec	Polymerization (extension)
1	68°C	5 min	Final extension
-	4°C	∞	

In order to test if the expected DNA band size of 303 bp was amplified, 3 µL of each PCR product were analyzed in 2% agarose gel electrophoresis in 1xTAE buffer (see section 3.3.1.1). Instead of EtBr, gels were stained with GreenSafe (nzytech), using 4 µL for 100 mL of agarose solution. Samples were loaded with glycerol (1:6) as an alternative to LB, given that the latter can interfere with the visualization of 303 bp band. The ladder used was 1 kb plus (Invitrogen). Gels were run at 90 V for 45min and visualized in AlphaImager HP System (Fisher Scientific).

3.3.3. Digestion with restriction enzyme *HhaI*

Restriction enzymes are a group of endonucleases which recognize specific sequences of DNA. They cleave double-stranded DNA (dsDNA) at specific sites within or adjacent to their recognition sequences, which differ for each restriction enzyme, producing differences in the length and sequence of the fragments resultant from an ER. For *ApoE* genotyping, *HhaI* (New England Biolabs®) was used to cut the 303 bp PCR products, within which there are eight constant and two variant *HhaI* cleavage sites - 5'…GCG|C…3'. According to patients' genotype, different patterns of fragment sizes can be produced (Figure 13).

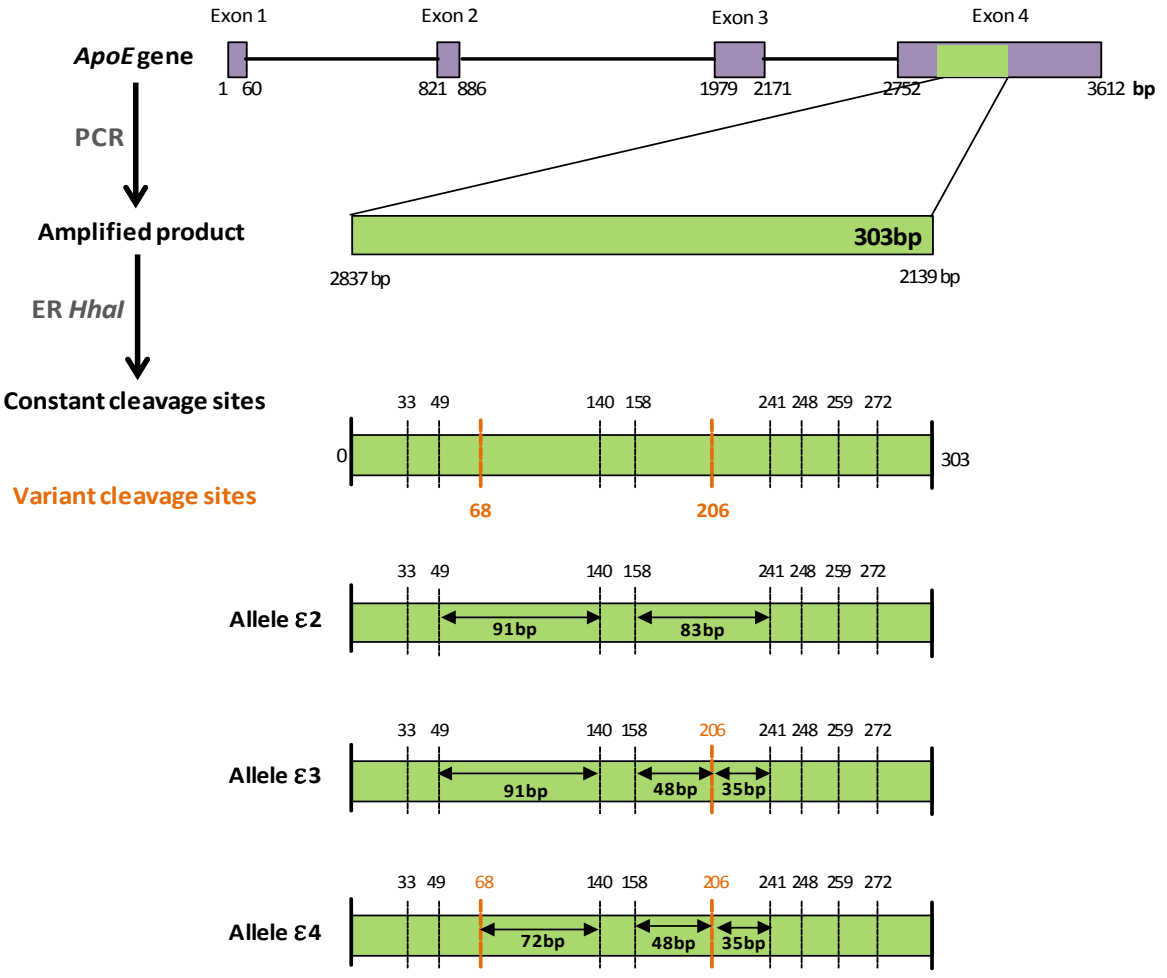


Figure 13: *HhaI* cleavage sites within *ApoE* amplicon. PCR fragments submitted to ER are cleaved in different fragment sizes, according to genotype: ε2 allele loses variant cleavage sites resulting in 91bp+83bp+others (≤33bp) fragments; ε3 allele only has the variant cleavage site at position 206, resulting in 91bp+48bp+35bp+others (≤33bp) fragments; ε4 allele possess the two variant cleavage sites within its sequence, resulting in 72bp+48bp+35bp+19bp+others (≤33bp) fragments.

ER was performed as listed below (Table 8) and reactions were incubated at 37°C for 17h.

Table 8: ER mixture components.

COMPONENTS	VOLUME PER REACTION (µL)
Ultra-pure H_2O	9,4
PCR product	17,0
10x NEBuffer4	3,0
100x BSA	0,3
HhaI 20U/µL	0,3
Total	30,0

3.3.4. Genotyping analysis

ER fragments were analyzed by agarose gel electrophoresis (see section 3.3.1.1), in 4% agarose gels with 1xTBE buffer, stained with GreenSafe (nzytech) (4µL for 100 mL of agarose solution). Glycerol was added to the samples in the ratio 1:6. Two different DNA markers were used: 10 bp DNA ladder (Invitrogen) and 1 kb plus (Invitrogen). Gels were run at 100 V, during 1h30min, and then visualized in AlphaImager HP System (Fisher Scientific).

4. RESULTS

4.1. EXTRACTION AND QUANTIFICATION OF GENOMIC DNA

The main goal of this work was to evaluate the frequency of *ApoE* alleles and genotypes in a pilot study group of neurological disease patients from the catchment area. In order to accomplish this, the first step included gDNA extraction from patients (blood as source), using QIAamp DNA Blood Mini Kit (section 3.2.1).

Following DNA extraction, 10 µL of each sample were analyzed by agarose gel electrophoresis and gDNA bands visualized under UV light (Figure 14).

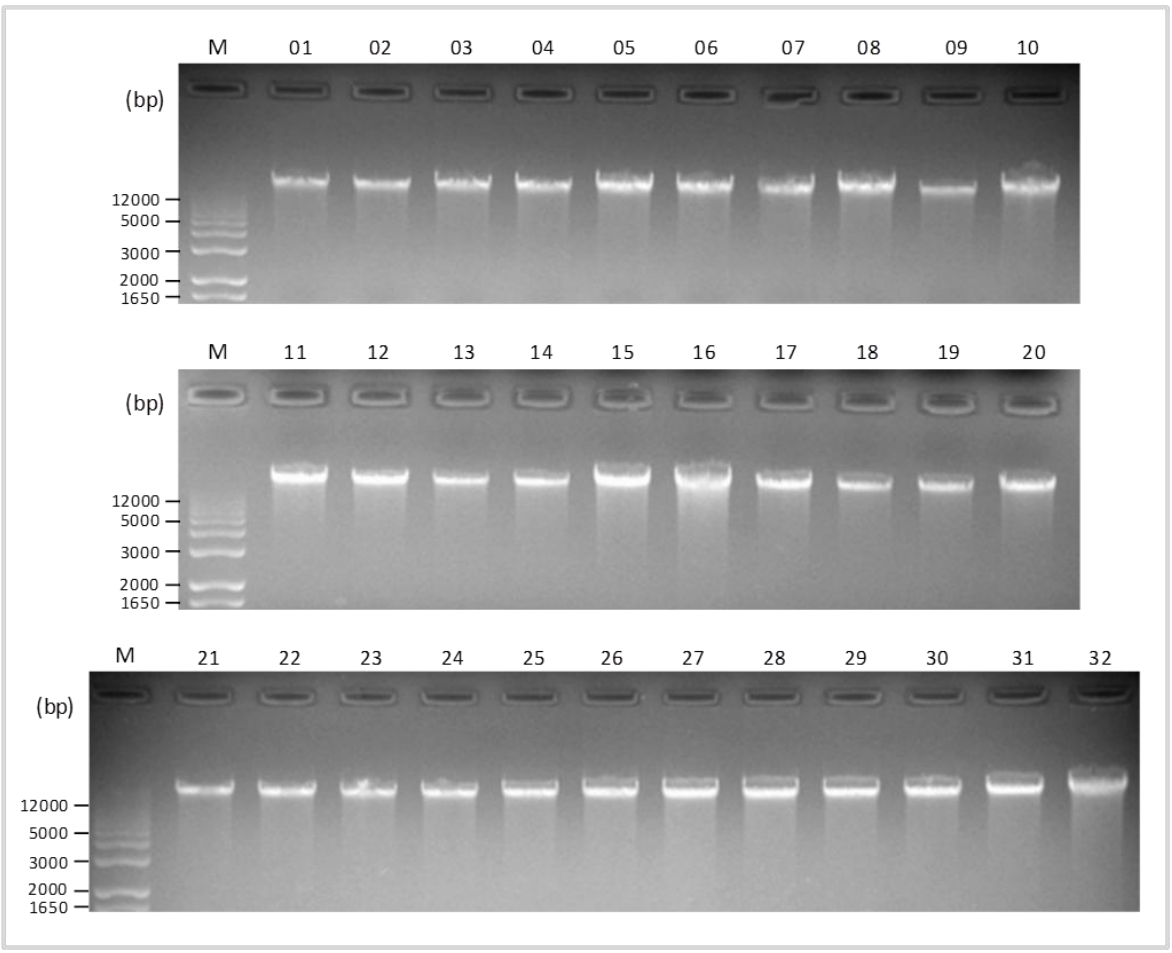


Figure 14: Genomic DNA extracted from blood samples of the study group. QIAamp DNA blood mini kit was used for extraction procedures and DNA samples were analyzed on 1% agarose gel. A band of ≈23kb was obtained in all cases. **M** – 1 Kb plus DNA Marker.

The length of gDNA bands obtained was calculated by comparing the migration distance of the bands obtained with the reference bands of the DNA ladder used (1 kb plus). For that, a standard curve was defined based on the linear relationship between the traveled distance and the base 10 logarithm of fragments' size (bp). Using the equation obtained from the reference points (Table 9 and Figure 15), where y represents Log_{10} [bp] and x represents the distance traveled, the size of the gDNA bands obtained could be extrapolated based on its migration distance. All gDNA bands run approximately 2,4 cm, corresponding to Log_{10}[bp] ≈ 4,36 in the standard curve. Calculating the inverse function of this value, a size of about 23 kb was obtained. Considering the kit information, DNA extracted should be predominantly between 20-30 kb in length; therefore, the length of the gDNA extracted was as expected, for each patient DNA analyzed.

Table 9: Measures of migration distances from DNA ladder fragments and logarithm of fragment sizes.

MIGRATION DISTANCE (CM)	DNA LADDER FRAGMENTS SIZE (BP)	LOG_{10} [BP]
3,4	12000	4,08
3,6	10000	4,00
3,8	9000	3,95
4,0	8000	3,90
4,2	7000	3,85
4,4	6000	3,79

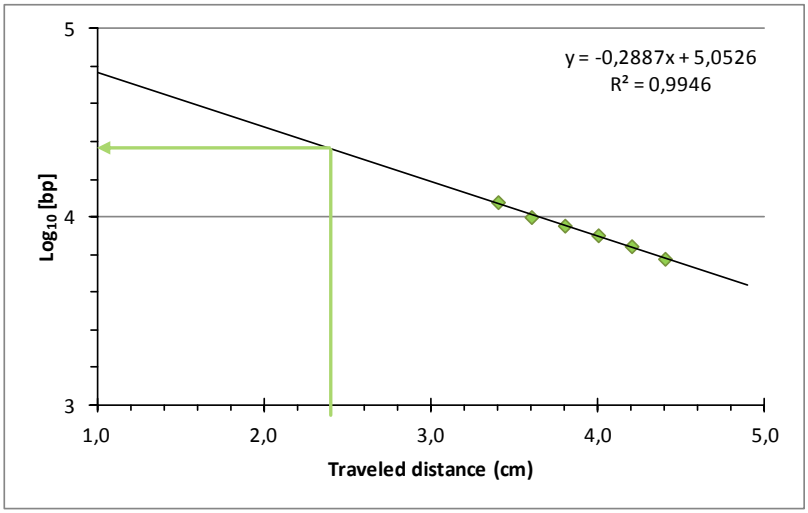


Figure 15: Standard curve obtained for traveled distances of DNA marker fragments relatively to the logarithm of their sizes. In the resultant equation, y represents the Log_{10}[bp], while x represents migration distance.

In order to evaluate the yield, concentration and purity of the DNA (Table 10), all samples were analyzed by spectrophotometry. The ratio of absorbance at 260nm and 280nm is used to assess DNA purity. A value of ≈ 1,80 is considered as 'pure' DNA, while values higher than 1,80 mean contamination with RNA and values appreciably lower may indicate the presence of proteins, phenol or other contaminants that strongly absorb at or near 280 nm. The mean Abs260/Abs280 ratio was 1,77, indicating that, in general, gDNA extracted from blood samples of the study group have a good index of purity, being considered as pure.

Table 10: NanoDrop measures for gDNA.

Sample	Abs260/Abs280*	[DNA]* (ng/µL)	Total DNA extracted* (µg)
01	1,85	24,2	2,4
02	1,86	26,6	2,7
03	1,80	31,2	3,1
04	1,87	35,5	3,6
05	1,77	46,9	4,7
06	1,73	42,0	4,2
07	1,78	38,5	3,8
08	1,74	49,1	4,9
09	1,74	23,0	2,3
10	1,81	57,1	5,7
11	1,77	40,3	4,0
12	1,66	31,9	3,2
13	1,65	25,1	2,5
14	1,64	26,5	2,7
15	1,77	53,2	5,3
16	1,86	80,6	8,1
17	1,84	41,3	4,1
18	1,75	31,9	3,2
19	1,79	30,8	3,1
20	1,80	44,9	4,5
21	1,77	32,0	3,2
22	1,81	44,3	4,4
23	1,74	32,2	3,2
24	1,75	36,1	3,6
25	1,77	34,6	3,5
26	1,77	36,9	3,7
27	1,81	49,1	4,9
28	1,77	42,5	4,2
29	1,76	33,6	3,4
30	1,77	34,1	3,4
31	1,79	41,0	4,1
32	1,82	64,9	6,5

* All values are mean of duplicate measurements

Total gDNA extracted was on average 3,9 µg. Accordingly to the manufacturer's instructions the expected yield ranges from 3-12µg. In general, the results obtained for the study group are within the expected values, except for samples 01, 02, 09, 13 and 14 that had lower total DNA yields. This can be due to a decreased number of white blood cells in patients' blood samples, as this factor can vary greatly between different individuals according to age, sex, immune status and many other factors, thus influencing DNA yield.

4.2. ORGANIZATION OF THE STUDY GROUP ACCORDING TO THE NEUROCHEMICAL DIAGNOSIS

Before genotyping procedures, the pilot study group was organized according to the results obtained for the neurochemical analysis of patients' CSF, which were previously realized in our laboratory. In brief, these clinically validated ELISA-based assays evaluate levels of $A\beta_{1-42}$, T-Tau and P-Tau 181 levels in each patients´ CSF. This triplet of biomarkers was described to be useful in particular to distinguish AD from other forms of dementia (Zetterberg et al., 2010). According to the neurochemical diagnosis, the total neurological disease group (TD group) was subdivided in 3 main subgroups (Table 11): the AD positive group (AD group), that included patients with altered levels of the 3 biomarkers evaluated; the Aβ positive group (Aβ+ group) patients with only $A\beta_{1-42}$ levels altered; and the other neurological disease group (OD group), composed of patients with no alteration in the levels of the triplet of biomarkers analyzed. According to this diagnosis, in our study group we had 8 AD individuals, 10 Aβ+ individuals and 14 individuals with OD.

Subsequent analysis had in consideration these main groups.

Table 11: CSF-based neurochemical diagnosis of the pilot study group[1].

Sample	Sex	Age	Amyloid Pathology ($A\beta_{1-42}$)	Tangle Pathology (P-Tau 181)	Cortical Axonal Damage [2] (T-Tau)	Neurochemical diagnosis
12	M	68	Yes	Yes	Yes	AD +
14	F	72	Yes	Yes	Yes	
06	F	78	Yes	Yes	Yes	
01	M	57	Yes	Yes	Yes	
02	M	85	Yes	Yes	Yes	
23	F	84	Yes	Yes	Yes	
29	F	76	Yes	Yes	Yes	
28	M	56	Yes	Yes	Yes	
30	M	58	Yes	No	No	Aβ +
07	F	85	Yes	No	No	
24	M	66	Yes	No	No	
31	F	78	Yes	No	No	
09	F	79	Yes	No	No	
16	F	79	Yes	No	No	
21	F	81	Yes	No	No	
17	M	68	Yes	No	No	
05	F	57	Yes	No	No	
04	M	46	Yes	No	No	
15	M	69	No	No	No	OD
03	M	60	No	No	No	
08	F	64	No	No	No	
10	M	59	No	No	No	
18	F	72	No	No	No	
25	F	64	No	No	No	
27	M	68	No	No	No	
22	M	69	No	No	No	
20	F	60	No	No	No	
11	M	64	No	No	No	
13	F	56	No	No	No	
26	F	77	No	No	No	
32	F	56	No	No	No	
19	F	60	No	No	No	

[1] Evaluation of $A\beta_{1-42}$, P-Tau 181 and T-Tau levels in patients CSF, to check for amyloid pathology, tangle pathology and cortical axonal damage, respectively. Patients were considered positive or negative comparatively to reference values for control individuals defined in the kit.

[2] Intermediate (mild) stages of cortical axonal damage were not considered here.

The mean age of patients was different between each of the established groups: for the AD group, the mean age was 72 years; for the Aβ+ group was 70 years; and for the OD group it was 64 years (Figure 16). The mean age observed for the AD group is consistent with a higher incidence of the late-onset sporadic forms. However, patients further from the mean could represent early-onset AD and should perhaps be subject to a follow-up study, i.e. subject number 1 and 28.

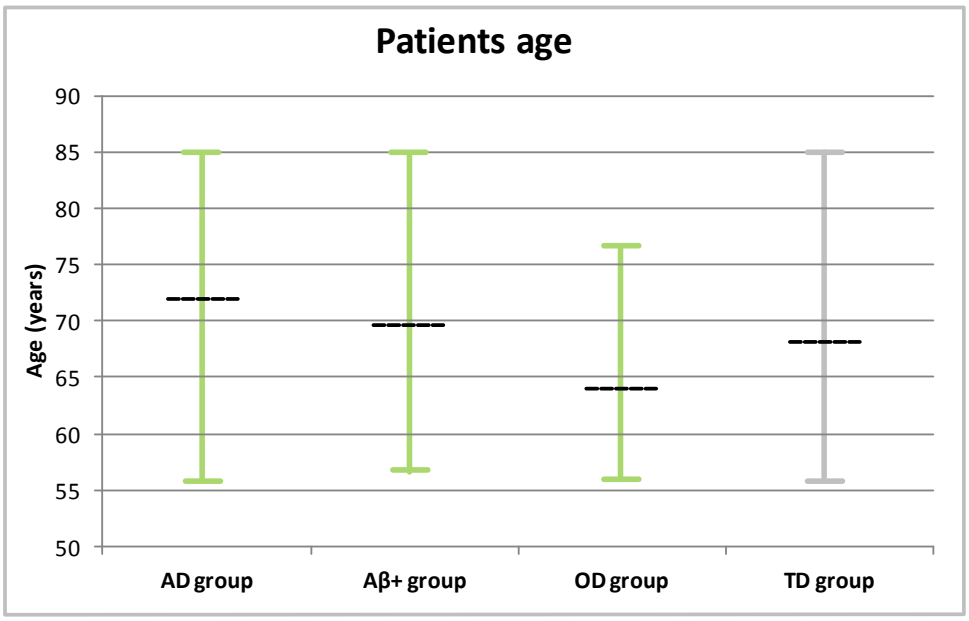


Figure 16: Patients range of ages in each group. Dashed lines represent patients mean ages. Visible differences between the established groups were observed, with AD group having the higher mean age value, while OD group comprises younger patients.

4.3. *APOE* POLYMORPHIC REGIONS AMPLIFICATION BY PCR

In order to perform *ApoE* genotyping, the *ApoE* polymorphic regions were amplified by PCR. Results were visualized in 2% agarose gels and the expected fragment of 303 bp obtained in all cases (Figure 17).

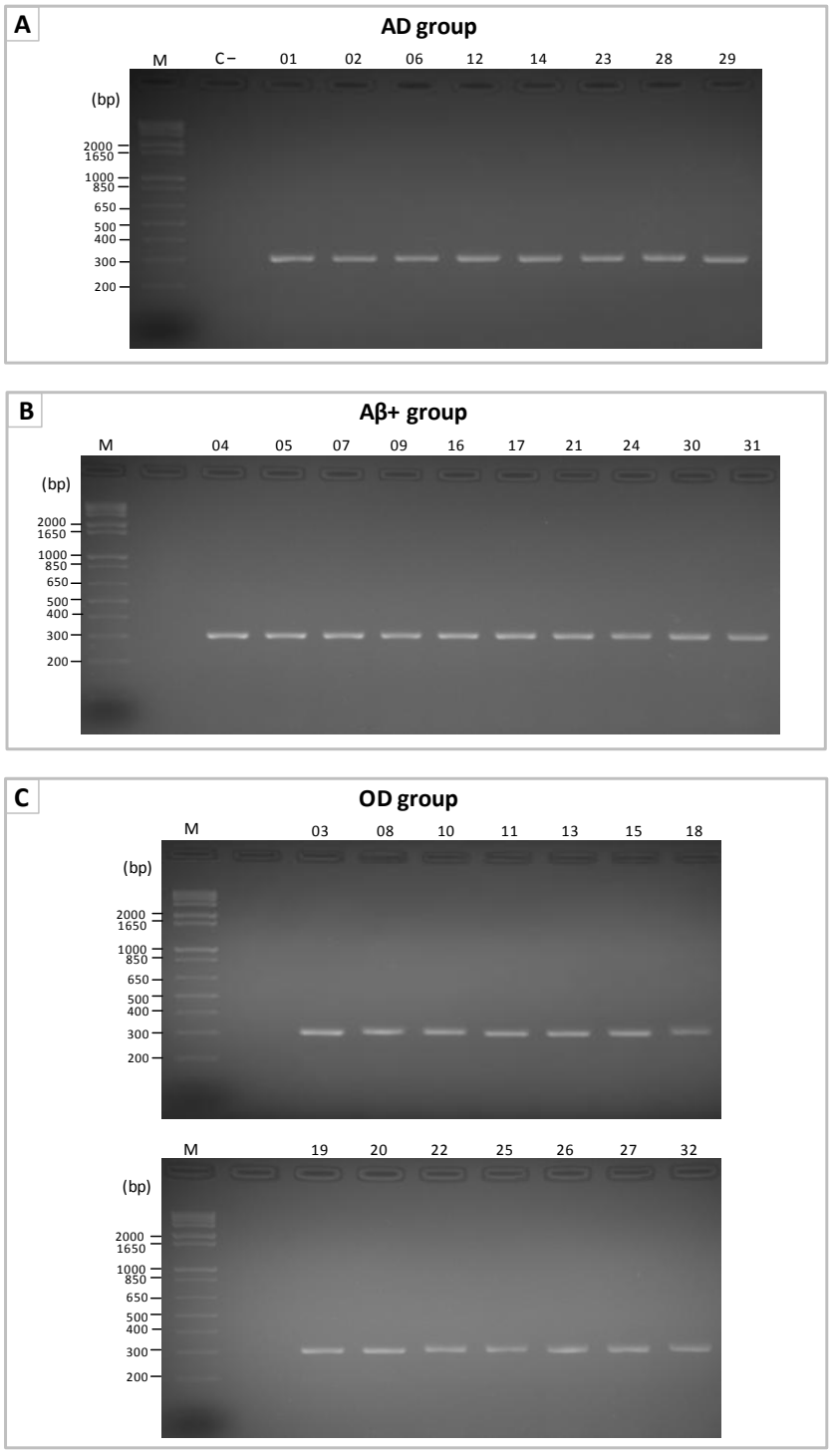
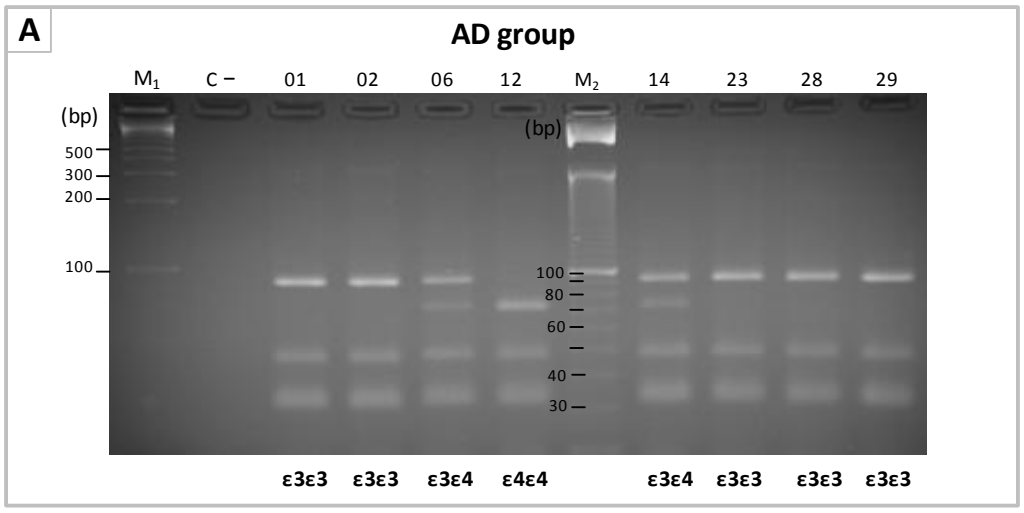


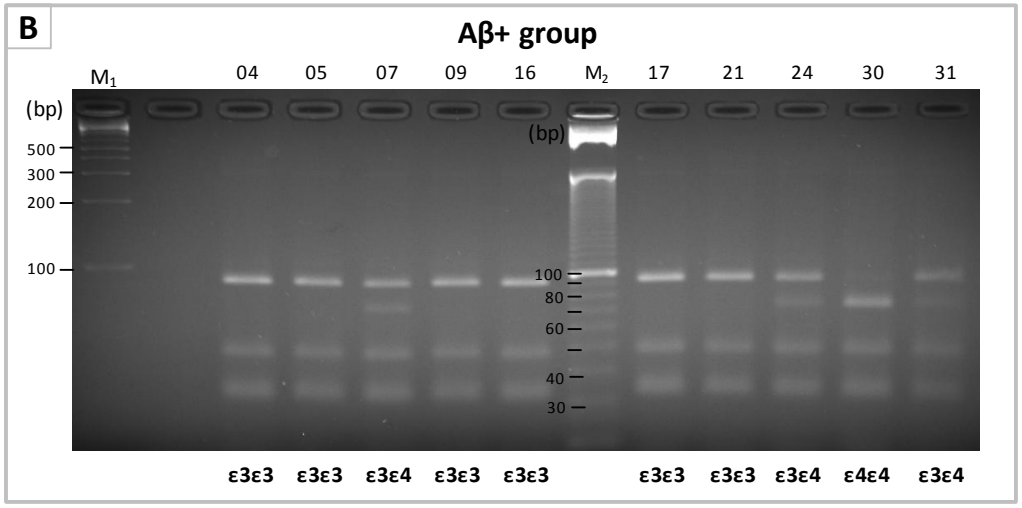
Figure 17: *ApoE* polymorphic regions amplified by PCR. The product was a 303 bp band and was visualized in 2% agarose gel. **A.** Results of PCR amplicon for AD group. **B:** Results of amplicon for Aβ+ group. **C.** Results of PCR amplicon for OD group. M – 1 Kb plus DNA marker; C - – Negative control.

4.4. CORRELATING *APOE* GENOTYPING WITH DEMENTIA

4.4.1. *ApoE* genotyping in the study group

The product obtained by PCR was submitted to ER with *HhaI* to determine *ApoE* genotyping in the study group from the catchment area. Bands obtained were dependent on the genotype of each individual, and resulted from variant cleavage sites of the enzyme, as explained in section 3.3.3. In all cases, other small fragments were also produced, resulting from constant cleavage sites of *HhaI* within PCR fragment. Results obtained from the ER were analyzed in 4% agarose gel electrophoresis (Figure 18).





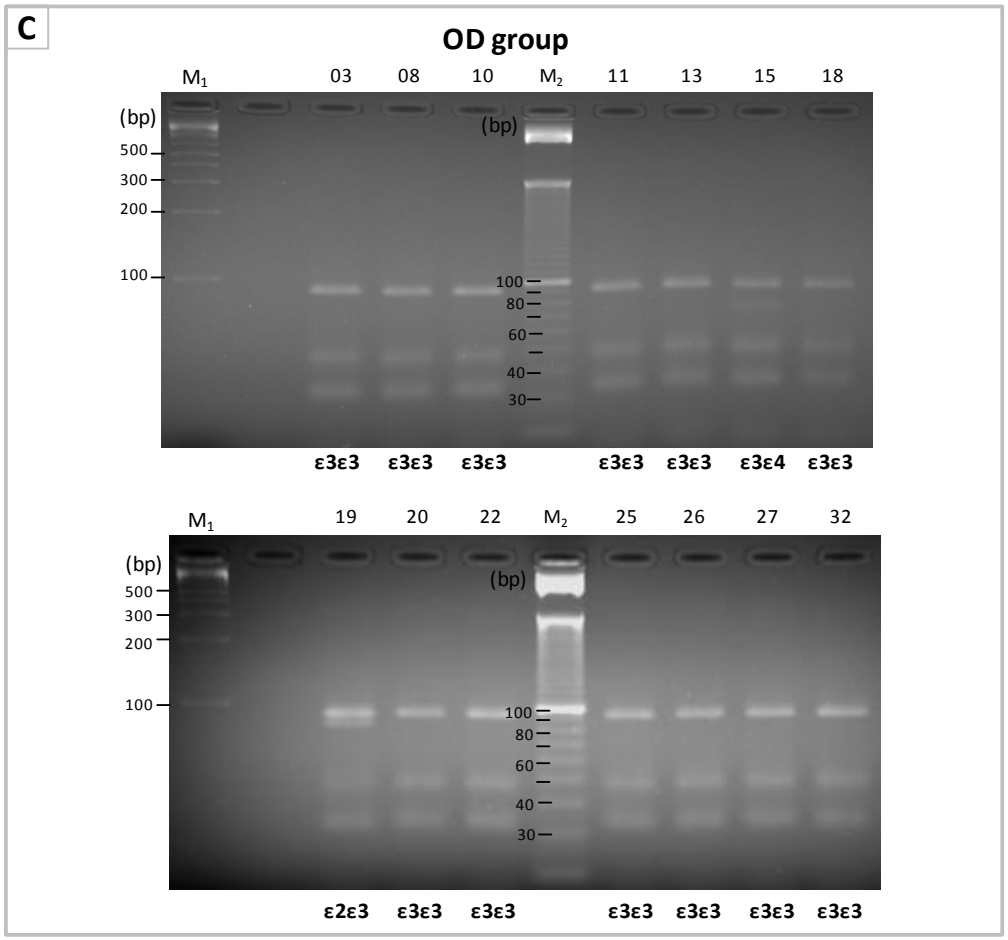


Figure 18: Electrophoretic analysis of *ApoE* genotypes in the neurological disease study group. PCR product submitted to ER was cleaved in different fragment sizes, according to genotype: ε2 allele – 91bp+83bp+others; ε3 allele – 91bp+48bp+35bp+others; ε4 allele – 72bp+48bp+35bp+19bp+others. Fragments were visualized in 4% agarose gel **A.** Genotypes of patients from AD group. **B.** Genotype of patients from Aβ+ group. **C.** Genotype from patients of OD group. M1 – 1 Kb plus DNA Marker; M2 – 10 bp DNA Marker; C - – Negative control.

Analyzing the results from ER, we can conclude that the TD group included 1 individual with genotype ε2ε3, 23 individuals with genotype ε3ε3, 6 individuals with genotype ε3ε4 and 2 individuals with genotype ε4ε4 (Table 12). Our study group comprised 8 individuals carriers of at least one copy of allele ε4 in their *ApoE* genotype, being that these patients mostly belong to the AD and the Aβ+ groups.

Table 12: Total number of individuals with each genotype in the different groups.

Genotype	Nº of individuals			
	AD	Aβ +	OD	TD
ε2ε2	0	0	0	0
ε2ε3	0	0	1	1
ε3ε3	5	6	12	23
ε3ε4	2	3	1	6
ε4ε4	1	1	0	2
ε4ε2	0	0	0	0
Total nº of individuals	**8**	**10**	**14**	**32**

4.4.2. Determination of the *ApoE* genotypic and allelic frequencies in the study group

According to the results obtained from genotyping procedures, the number of individuals with the *ApoE* genotypes and alleles were identified.

The most frequent genotype in all groups was ε3ε3 (with a total of 23 individuals in the TD group), nonetheless, with a higher frequency in the OD group (85,8%), when compared with AD and Aβ+ groups (Table 12 and Figure 19). In the TD group, 8 out of 32 patients were carriers of at least one allele ε4. The ε4ε4 genotype was only present in the AD and Aβ+ groups, corresponding to 12,5% and 10,0% of the patients. Similarly, ε3ε4 genotype was most frequent in the same groups, 25,0% and 30,0% respectively, versus 7,1% for OD group. The genotype ε2ε3 was only obtained in the OD group, corresponding to a frequency of 7,1%. Of note, in the total neurological disease group, no case was found to have genotypes ε2ε4 or ε2ε2, suggesting that allele ε2 is probably rare in the population. Considering all the individuals, the order of genotypes frequencies were: ε3ε3 > ε3ε4 > ε4ε4 > ε2ε3.

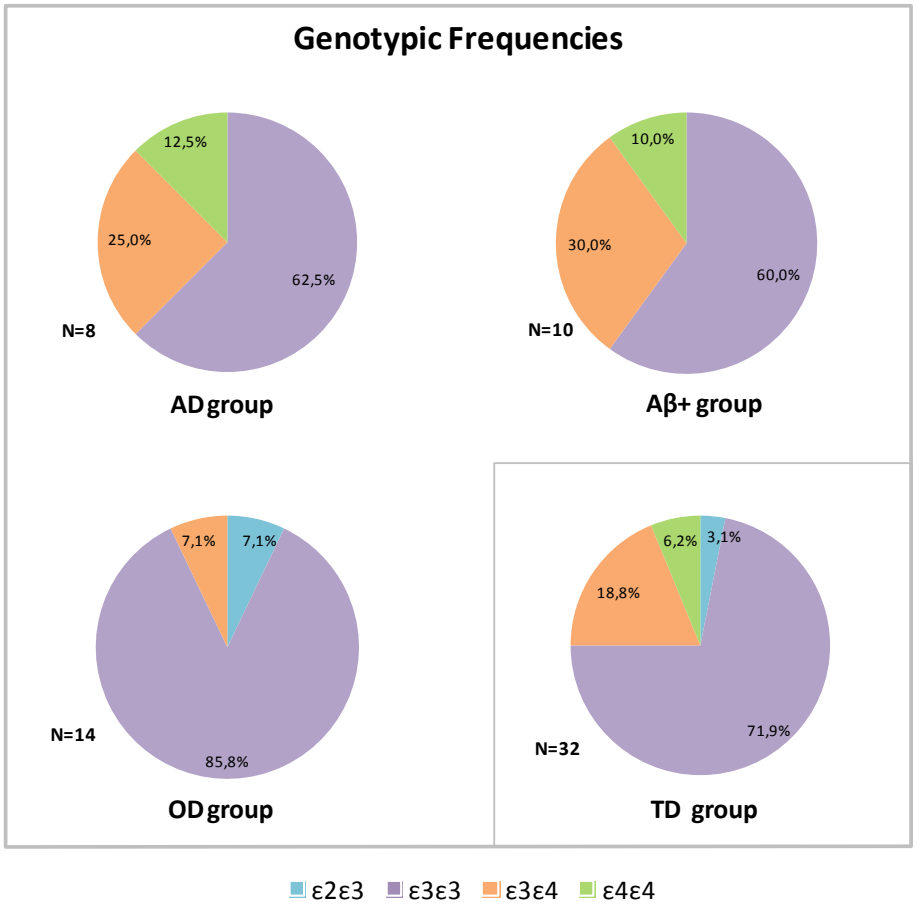


Figure 19: Genotype frequencies in different groups. Genotype frequencies were calculated as a % of the total genotypes in each group.

For the allelic frequencies (Table 13 and Figure 20), in agreement with the genotype frequencies obtained for the TD group, the most frequent allele was ε3 (53 alleles corresponding to 82,8%), followed by ε4 (15,6%) and by ε2 (1,6%).

Table 13: Total number of each allele in the different groups.

Allele	Nº of alleles			
	AD	Aβ +	OD	TD
ε2	0	0	1	1
ε3	12	15	26	53
ε4	4	5	1	10
Total nº of alleles	**16**	**20**	**28**	**64**

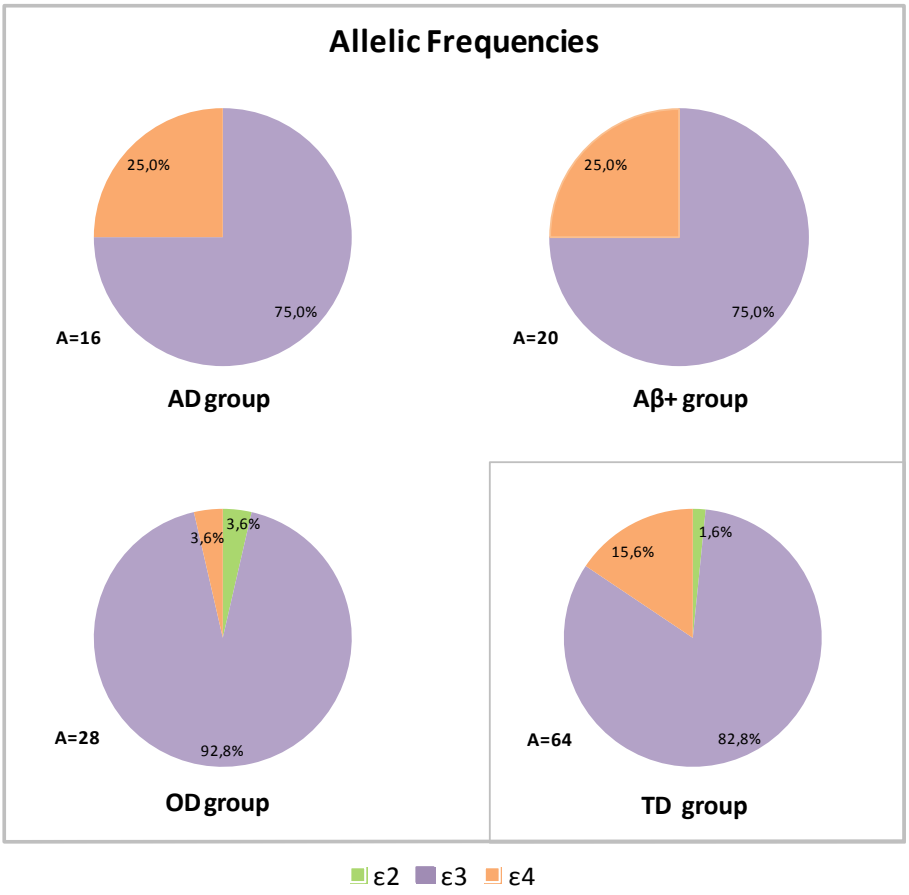


Figure 20: Allelic frequencies in different groups. Each allele frequency was calculated as a % of the total number of alleles in each group.

Allele ε2 was only found in the OD group, with a frequency of 3,6%, the same as for allele ε4 in this group, while ε3 allele was present in 92,8% of the cases. Interestingly, allele frequencies in AD and Aβ+ groups were exactly the same for alleles ε3 and ε4, namely a frequency of 75,0% and 25,0%, respectively (Figure 20).

5. DISCUSSION & CONCLUSIONS

The number of patients suffering from dementia has increased significantly due to extended life spans, resulting in a major health and socio-economic problem worldwide. Considering the limited effectiveness of possible interventions and diagnostic tools for the dementia cases available nowadays, studies have been focusing on understanding the molecular events and factors that may underlie the disease, and contribute to the development of early diagnostic tools and effective disease-modifying therapeutic approaches (Tanaka et al., 2008; Tanimukai et al., 2009). Also, understanding the interaction between environmental and genetic factors that modulate risks and outcomes of neurological disease can provide useful information about management of these devastating disorders (Verghese et al., 2011).

Among the genetic aspects, the *ApoE* gene is recognized as the most powerful risk factor for dementia. It is believed that clinical findings related to *ApoE* should be taken into consideration, in order to reconcile gene and environmental interactions in the pathogenesis of dementia. The various proposed roles of APOE in CNS lipid homeostasis, synaptic activity, response to cellular injury and neuroinflammation, leads to many possible associations between this apolipoprotein and several diseases. However, so far, evidence for a strong association between *ApoE* genotype, specifically for allele ε4, remain the most compelling for AD. For the latter pathology, APOE is believed to be linked to the risk of disease development through isoform dependent modulation of Aβ metabolism and accumulation (Wisniewski et al., 1994; Castano et al., 1995; Castellano et al., 2011; Bachmeier et al., 2012), as well as the decreased efficiency of APOE4 relative to other variants in recycling membrane lipids and neuronal repair (Poirier, 1994; Acharya et al., 2002). Further, ε4 allele has been associated to a lower age of AD onset in patients who are carriers (Xiong et al., 2005; Jack et al., 2010). However, the association between ε4 allele and other types of dementia, such as VaD, DLB and FTD, needs additional research to confirm whether *ApoE* is a relevant risk factor, or not, for those pathologies.

In this perspective, the work here presented aimed to investigate the *ApoE* profile in a pilot study group of 32 patients suffering from neurological diseases. An important aspect of our work is that the pilot study group from the catchment area was divided according to the neurochemical diagnosis, which consisted in the evaluation of a triplet of

biomarkers ($A\beta_{1-42}$, T-Tau and P-Tau 181) in patients' CSF. This approach is a useful tool that helps in the characterization of the study group and, in particular, to distinguish AD from other types of dementia. Biomarker alterations occurring in the CSF are related with pathological processes occurring in the brain, even in the early stages of the disease (Zetterberg et al., 2010). According to the results obtained in neurochemical analyses, patients were organized into 3 main subgroups: AD group (patients with altered $A\beta_{1-42}$, T-Tau and P-Tau 181 levels), Aβ+ group (patients with only altered $A\beta_{1-42}$ levels) and OD group (patients with none of the three biomarkers altered). An interesting aspect regarding the established groups is the difference between the mean ages of patients from each group: for AD group, average age was 72 years; for Aβ+ group it was 70 years; and for OD group it was 64 years. A lower mean age in Aβ+ group comparatively to AD group, is consistent with the idea that Aβ deposition may provide the earliest biochemical change during the course of AD, whereas synaptic loss and neuronal damage, as well as tangle pathology development, are only detected in a more advanced phase of the disease. Relative to the lower mean age in the OD group, we may speculate that these patients have predominantly dementia of early onset types, other than AD or DLB, probably associated to a stronger genetic component, such as FTD, or to lifestyle behaviors, such as alcohol or drug consumption related dementia (Figure 1). However, this would have to be further investigated.

For all patients, gDNA was successfully extracted from blood samples, with a yield and purity level in agreement with the expected values for the kit used. In order to amplify *ApoE* polymorphic regions, PCR reactions were carried out and followed by ER analyses. Genotype and allelic frequencies of *ApoE* gene were determined in the groups subdivided according to the neurochemical diagnosis. Considering the TD group, the genotype frequencies were as followed: ε3ε3 > ε3ε4 > ε4ε4 > ε2ε3, with 71,9%, 18,8%, 6,2% and 3,1%, respectively. However, for the 3 dementia subgroups established, the distribution of ApoE genotypic frequencies were significantly different (Table 12), with ε3ε4 and ε4ε4 genotypes being mainly detected in AD and Aβ+ groups, while ε2ε3 was only detected in OD group. Consistently, *ApoE* ε3ε3 was the most frequent genotype, independent of the group.

Regarding the *ApoE* allelic frequencies and comparing our data with Portuguese studies, we concluded that our data is consistent with prior observations (Table 14).

Table 14: *ApoE* allelic frequencies obtained in previous Portuguese studies vs the study here presented.

	Allele Frequencies			
	ε2	**ε3**	**ε4**	
AD	3,4%	73,6%	23,6%	(Fernandes et al., 1999)
Controls	4,3%	90,0%	5,7%	
AD	0,0%	64,0%	36,0%	(Rocha et al., 1997)
Controls-pw	2,2%	89,0%	8,8%	
Controls	4,3%	87,0%	8,7%	
AD	**0,0%**	**75,0%**	**25,0%**	**This study**
Aβ+	**0,0%**	**75,0%**	**25,0%**	
OD	**3,6%**	**92,8%**	**3,6%**	

AD – Alzheimer's Disease group; **Controls** – Control individuals group; **Control-pw** – Control-pairwise individuals group; **Aβ+** – Aβ+ group; **OD** – Other neurological disease group.

In particular, in our pilot study group, we could observe that *ApoE* allele ε3 was the most common in all groups. However, despite the elevated frequency of this allele in AD and in Aβ+ groups (75,0%), this value is even higher in OD group (92,8%). This is consistent with results obtained in previous studies where *ApoE* ε3 allele, independently of the presence or absence of disease, was the most frequent in the general population (Rocha et al., 1997; Fernandes et al., 1999), strongly represented by ε3ε3 genotype.

ApoE allele ε2 was only found in OD group, with a frequency of 3.6%, being this allele the least represented in TD group. Moreover, the frequency of ε2ε4 and ε2ε2 genotypes in the study group was 0,0%. These data are in agreement with the literature, that points to a very low prevalence of this allele in control individuals, but even more decreased in AD patients (Rocha et al., 1997; Fernandes et al., 1999). Further, the fact that no allele ε2 was found neither in AD nor in Aβ+ groups strengths the link previously proposed between the inheritance of *ApoE* ε2 and the low risk of developing the disease. This allele was reported to have a role as a protective element in sporadic AD (Corder et al., 1994; Panza et al., 2000).

Also, in the studies considered (Table 14), *ApoE* allele ε4 frequency was found to be around 4 times higher in AD groups than in control groups, reflecting the strong association proposed between this allele and AD pathology. In our study, and despite the small size of the sample, this association between *ApoE* allele ε4 and AD was also evident, as it represents 25,0% of total alleles in that group. Interestingly, for the Aβ+ group the frequency of this allele was also 25,0%, which may indicate that individuals from this group have a greater propensity to develop AD pathology. On the other hand, *ApoE* allele ε4 was rarely detected in the OD group (with a frequency of 3,6%), suggesting that this allele strongly correlates with amyloid pathology development and less with other types of dementia.

Our work entirely supports the acquired notion that *ApoE* allele ε4 is a major risk factor for AD, and less for other neurological diseases. Importantly, a very strong correlation was also obtained between ε4 allele and patients with amyloid pathology. As reported, the central role for *ApoE* allele ε4 in the modulation of processes of neurodegeneration, especially in amyloid pathology development, is probably associated with an isoform dependent effect on Aβ metabolism, aggregation and clearance from the brain (Castano et al., 1995; Polvikoski et al., 1995; Castellano et al., 2011; Bachmeier et al., 2012). Of note, according to Zetterberg et al. (2010), patients with altered Aβ levels (Aβ+ group) that progress in neurodegeneration can develop either AD or DLB. However, as *ApoE* ε4 is strongly linked to AD, we speculate that patients with amyloid pathology carrying the allele ε4 will most probably suffer from AD. A follow up study could clarify these aspects.

CONCLUDING REMARKS:

- Of the six possible genotypes for *ApoE* gene, only four of them were observed: ε3ε3 (the more frequent), ε2ε3 (only in OD group), ε3ε4 (more frequent in AD and Aβ+ groups) and ε4ε4 (only in AD and Aβ+ groups).
- Allele ε3 was the most frequent in all groups considered independently of the disease type, consistent with other population studies.
- Allele ε2 was the less frequent allele, being only observed in OD group. This allele is associated with a protective role in AD development.
- Allele ε4 was detected with a significantly higher frequency in the AD group than in the OD group. This supports ε4 allele as a major risk factor for AD development and less so for other neurological pathologies.
- In Aβ+ group, allele ε4 frequency was similar to that of the AD group, suggesting that these individuals have an increased propensity to develop AD pathology.
- In closing, the work carried out supports the notion that *ApoE* genetic testing should be considered as a useful adjunct diagnostic tool in clinical diagnosis of dementia, in particular to predict AD.

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Consulted links:

http://alzheimers.org.uk.
http://dementiatypes.org/Vascular_Dementia.htm.
http://wp.stockton.edu/gfb1/tag/alzheimers-disease/.
http://www.alz.org.
http://www.alzheimerportugal.org.

7. ANNEXES

This section presents the equipment and composition of solutions used for the different techniques applied.

7.1.1. Genomic DNA Extraction

Equipment

- Centrifuge 5417R (Eppendorf)
- Thermomixer comfort (Eppendorf)

Reagents

- QIAamp DNA Blood Mini Kit (Qiagen)
- RNase A (Sigma-Aldrich)

7.1.2. Agarose Gel Electrophoresis

Equipment

- DNA electrophoresis system (Bio-Rad)
- Power PAC300 (Bio-Rad)
- AlphaImager HP System (Fisher Scientific)

Reagents

- Agarose (Invitrogen)
- Bromophenol Blue

  To 7 mL of deionized H_2O add:

  - 0,025 g bromophenol blue (0,25%)
  - 3 ml glycerol (30%)

  Mix and store at 4°C.

- EtBr (10mg/ml)

  To 100 mL of deionized H_2O add:

  - 1g EtBr

  Mix in a magnetic stirrer during several hours to ensure that the dye is totally dissolved. Wrap the tube with aluminum foil to maintain the solution protected from light.

- GreenSafe (nzytech)

- 0,5M Ethylenediamine tetra-acetic acid (EDTA) (pH 8.0)

  To 80 mL of deionized H_2O add:
  - 14,612 g EDTA

  Mix until the solute has dissolved and adjust pH to 8.0 with NaOH. Adjust the volume to 100 ml with deionized H_2O. Store at room temperature.

- 50X TAE (Tris-Acetate-EDTA Buffer)

  To 600 mL of deionized H_2O add:
  - 242g Tris Base
  - 57,1 ml Glacial Acetic Acid
  - 100 ml 0,5M EDTA (pH 8.0)

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

- 10X TBE (Tris-Borate-EDTA Buffer)

  To 600 mL of deionized H_2O add:
  - 108g Tris Base
  - 55g Boric Acid
  - 40ml 0,5M EDTA (pH 8.0)

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

- 1 kb plus DNA Ladder (Invitrogen)

- 10 bp DNA Ladder (Invitrogen)

7.1.3. DNA concentration and purity

Equipment

- NanoDrop Spectrophotometer ND-1000 (Thermo Scientific)

7.1.4. PCR

Equipment

- My Cycler ™ (Bio-Rad)

Reagents

- Primers (nzytech)
  - FW-CGGGCACGGCTGTCCAAGGAG
  - Rev-CTGGTGGAACAGGGCCGCGTG
- Betaine (Sigma-Aldrich)
- dNTPs 10mM (New England Biolabs)
- KlenTaq LA DNA Polymerase Mix (Sigma-Aldrich)
- 10x PCR Buffer (Sigma-Aldrich)

7.1.5. Enzymatic Restriction

Reagents

- *HhaI* (New England Biolabs)
- 10x NEBuffer4 (New England Biolabs)
- 100x BSA (New England Biolabs)