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Doctoral thesis

Assessing the role of copy number variations, mitochondrial DNA and microRNAs in neurodegenerative disorders

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Certificate of Direction

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Certify:

That the work entitled "**Assessing the role of copy number variations, mitochondrial DNA and microRNAs in neurodegenerative disorders**" presented by Laura Cervera Carles, candidate of the PhD program in Neurosciences, has been done under our direction and meets all the requirements to be defended in front of the corresponding Thesis Committe.

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Au milieu de l'hiver, j'apprenais enfin qu'il y avait en moi un été invincible.
Albert Camus "Retour à Tipasa" (1952)

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List of contents

Chapter 1. Introduction

Abbreviations

Ago2 = argonaute-2

AD = Alzheimer's disease

ALS = amyotrophic lateral sclerosis

APOE = apolipoprotein E

APP = amyloid precursor protein

Aβ = amyloid-β

bvFTD = behavioral variant frontotemporal dementia

CBD = corticobasal degeneration

CBS = corticobasal syndrome

CHMP2B = charged multivesicular body protein 2B

CNV = copy number variation

CSF = cerebrospinal fluid

ddPCR = droplet digital polymerase chain reaction

DLB = dementia with Lewy bodies

ESCRT = endosomal sorting complexes required for transport

EV = extracellular vesicle

FTD = frontotemporal dementia

FTLD = fronotemporal lobar degeneration

FUS = fused-in-sarcoma

gDNA = genomic DNA

GRN = granulin

GWAS = genome-wide association study

ILV = intraluminal vesicles

LB = Lewy body

LN = Lewy neurite

LNA = Locked Nucleic Acid

lvPPA = logopenic variant primary progressive aphasia

MAPT = microtubule associated protein tau

MCI = mild cognitive impairment

miRNA = microRNA

MSA = multiple system atrophy

MTBD = microtubule-binding domain

mtDNA = mitochondrial DNA

MVB = multivesicular bodies

ncRNA = non-coding RNA

nfvPPA = non-fluent variant primary progressive aphasia

NFT = neurofibrillary tangle

PD = Parkinson's disease

PiD = Pick's disease

PSP = progressive supranuclear palsy

qPCR = quantitative real-time polymerase chain reaction

RT = reverse transcription

SNpc = substantia nigra pars compacta

SNP = single nucleotide polymorphism

svPPA = semantic variant primary progressive aphasia

TDP43 = TAR-DNA binding protein 43

TOMM40 = translocase of outer mitochondrial membrane 40

UPS = ubiquitin proteasome system

List of publications included in this thesis

The main content of this thesis consists of a compliation of the following publications:

Study 1.

Cervera-Carles L, Pagonabarraga J, Pascual-Sedano B, Pastor P, Campolongo A, Fortea J, Blesa R, Alcolea D, Morenas-Rodríguez E, Sala I, Lleó A, Kulisevsky J, Clarimón J. **Copy number variation analysis of the 17q21.31 region and its role in neurodegenerative diseases**. American Journal of Medical Genetics Part B: Neuropsychiatric genetics. 2016; 171B(2):175-180. doi: 10.1002/ajmg.b.32390

Study 2.

Cervera-Carles L, Alcolea D, Estanga A, Ecay-Torres M, Izaguirre A, Clerigué M, García-Sebastián M, Villanúa J, Escalas C, Blesa R, Martínez-Lage P, Lleó A, Fortea J, Clarimón J. **Cerebrospinal fluid mitochondrial DNA in the Alzheimer's disease continuum**. *Neurobiol Aging*. 2017; 53:192.e1-192.e4. doi: 10.1016/j.neurobiolaging.2016.12.009

Study 3.

Cervera-Carles L, Clarimón J *et al*. **Altered microRNAs in extracellular vesicles from CSF as biomarkers for 4R-tauopathies**. 2019. [In preparation]

Summary

Neurodegenerative diseases are complex and progressive disorders that affect millions of people worldwide. Among them, Alzheimer's disease (AD), Parkinson's disease (PD) and frontotemporal dementia (FTD) are three of the most prevalent. In spite of extensive research, the molecular events triggering these pathologies remain elusive.

This thesis aims at understanding the role of certain genetic and epigenetic factors in neurodegenerative diseases through the study of structural genetic rearrangements, and the measurement of circulating mitochondrial DNA and non-coding RNA species.

We first analyzed the structural variation pattern of the chromosome 17q21.31, one of the most complex and dynamic regions of the human genome, and evaluated its contribution to the well-established *MAPT* H1 haplotype relationship with PD, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and dementia with Lewy bodies (DLB). Our results suggest that copy number polymorphisms within this region are not responsible for the H1 effect. However, we found a significant overrepresentation of H1 carriers in DLB patients, thus expanding the biological relevance of the haplotype in neurodegenerative disorders.

We also examined the levels of circulating cell-free mitochondrial DNA in cerebrospinal fluid (CSF) and its utility as an indicator of mitochondrial dysfunction in the AD continuum. Although its measurement is reliable, the considerable inter-individual variability within groups limits its accuracy and usefulness as a diagnostic biomarker.

Finally, we investigated the expression profile of microRNAs, a class of non-coding RNAs involved in the post-transcriptional modulation of gene expression, contained in extracellular vesicles (EVs) from CSF in FTD and other related syndromes. Numerous microRNAs can be detected within EVs from CSF. Moreover, we identified four microRNAs with a specific expression pattern in patients diagnosed with 4R-tau FTD syndromes.

Chapter 1

Introduction

Neurodegenerative disorders

Neurodegenerative disorders represent a substantial medical and public health burden worldwide. Among them, Alzheimer's disease (AD), Parkinson's disease (PD) and frontotemporal dementia (FTD) are three of the most prevalent disorders. The number of people surviving into their 80s and beyond is growing dramatically, and the load of neurodegenerative disorders is expected to almost double in the next 20 years[1]. In Spain, with an actual life expectancy of 85.8 in women and 80.2 in men [**Fig. 1**], AD is now the second cause of death and is the disorder that has experienced the most important percent change since 2007 (Global Burden of Disease Study, http://www.healthdata.org/spain)[2].

Many neurodegenerative diseases have a partial overlapping of cellular and molecular mechanisms, characterized by the formation of distinct pathological alterations in the brain. These features include selective neuronal vulnerability with degeneration in specific brain regions, extracellular aggregates of abnormal proteins,

Figure 1. **Global and national life expectancy** (from Global Burden of Disease Study 2017)
A. In 2017, global life expectancy was 70.5 years in men and 75.6 years for women. Data shown in the figure represent life expectancy at birth for both sexes. **B.** Life expectancy in Spain from 1990 to 2017. Women (in red) tend to live longer than and men (in blue).

Figure 2. **Overview of anatomical location, microscopic and macroscopic changes characteristic of several neurodegenerative disorders** (from Bertram and Tanzi, 2005)

The full neuropathological spectrum of these disorders is more complex than represented here. When more than one characteristic histopathological feature is present, these are depicted from left to right, as indicated in the labels listing microscopic changes.

protein inclusions in brain cells, and changes in brain morphology[3,4] [**Fig. 2**]. An increased protein aggregation rate could be due to different factors, including: covalent alterations of proteins[5] (such as oxidative modifications, phosphorylation or ubiquitination), decreased protein clearance, proteolytic cleavage processes[6], and higher protein concentrations[7] (which can be caused by genetic dosage alterations, polymorphisms in promoter sites or mutations in protein-coding regions).

The molecular events leading to these neurodegenerative pathologies remain elusive, and only a reduced number of pathways have been associated with their etiology. It includes protein misfolding, oxidative damage, mitochondrial dysfunction, defective axonal transport, channel dynamics alterations and impaired autophagy[8–12].

Genetic architecture

Familial aggregation is a common characteristic of these disorders. Studies in familial cases have led to the discovery of autosomal dominant mutations for AD, PD and FTD, which has been crucial to unveil the pathophysiological bases of these diseases. These findings have pointed at certain proteins and pathways now considered essential for the pathogenesis of these diseases, such as amyloid-β in AD, α-synuclein in PD, and Tau or TDP-43 in FTD. However, causal mutations in these genes only account for a small percentage of cases, regardless of the disease.

In fact, these are considered complex disorders, which imply the interplay between environmental and genetic factors. During the last decades numerous risk genes and loci have been reported[13]. Together with the advances in genome analysis, this has prompted the researchers to consider the implications of common and rare variants in genetic architecture, as well as the involvement of epigenetics -including RNA species- in the disease susceptibility and etiology.

Genome-wide association studies (GWAS) are based on the "common disease, common variant" hypothesis, where common diseases can be partly attributable to allelic variants present in more than 5% of the population[14] [**Fig. 3**]. GWAS aim is to reveal the genomic location of common genetic variants that confer a risk for an observable trait or disease. In order to achieve this, many hundreds of thousands of single nucleotide polymorphisms (SNPs) are examined in large series of disease cases and disease-free controls. Cases and controls are compared in order to detect over-represented SNPs in one group or another. GWAS are able to identify loci, but not specific genes. Statistically significant signals are not always within known functional unit of genes, thus the closest gene or a nearby one with biological sense is associated with the observed risk[15]. This methodology is useful to detect common low-risk variants[14,16], which usually exert their effect through direct or indirect regulation of gene expression. However, it is inefficient for finding rare genetic variants, as their effects on disease risk are not large enough.

Most known genetic loci related to neurodegenerative disorders come from GWAS. However, they cannot explain the entire heritability in complex diseases, leaving the possibility that a substantial proportion of genetic risk in these diseases may result from rare alleles. This has led to the "common disease, rare variant" paradigm, where low frequency and rare genetic variants in humans (allele frequencies lower than 5 and 0.5%, respectively) could have substantial effect sizes [**Fig. 3**]. These variants are predicted to be enriched for functional alleles which could consequently have larger phenotypic effects than common alleles[17]. Exome and whole genome sequencing in large series of patients could allow the identification of low frequency risk alleles, including loss-of-function alleles (deletions, missense mutations and splice-site alterations). Loss-of-function alleles result in reduced or even no function genes, being the most easily recognized those that disrupt protein production[15].

Structural genetic variation can also account for some unexplained heritability, and may contribute to genetic basis of human disease. Structural rearrangements could be classified into copy number variations (CNVs; such as insertions and deletions) and copy neutral variations (such as inversions and translocations). Disease-associated CNVs include rare, highly penetrant variants (usually 600kb–3Mb, affecting many genes) with large associated effect sizes, and common variants (200–

Figure 3. **Feasibility of identifying genetic variants by risk allele frequency and strength of genetic effect** (from Manolio, 2009)
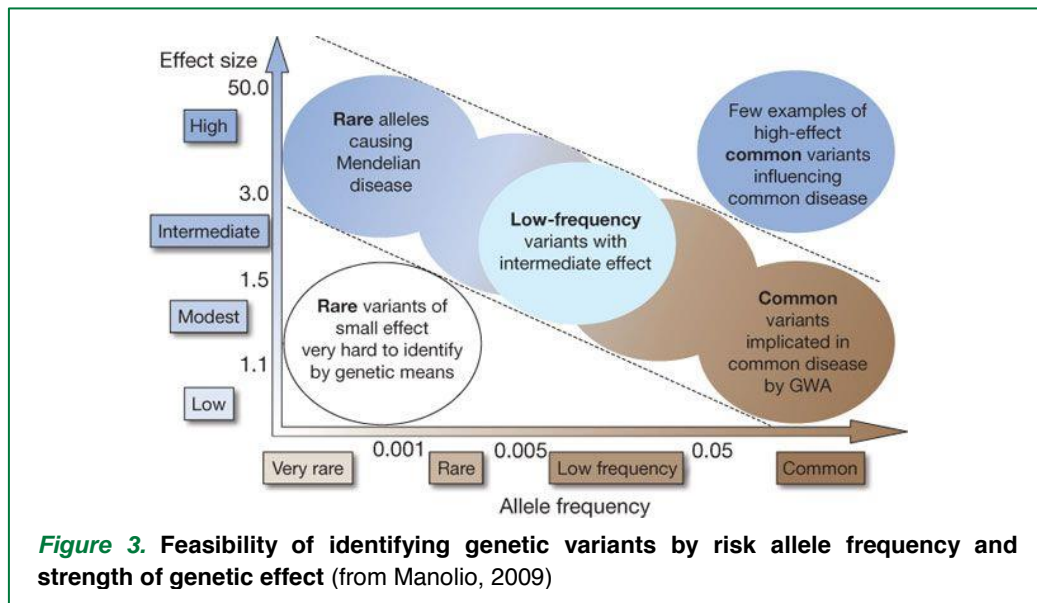
45kb) with more modest effects but present in a greater proportion of the population[14,18].

Apart from the protein-coding genes, which only account for ~2% of the human genome, the vast majority is transcribed to produce large numbers of non-coding RNAs[19–21]. Among them, microRNAs (miRNAs) are small non-coding RNA species that regulate gene expression post-transcriptionally, thus becoming key regulators of cellular homeostasis in health and disease. MiRNA expression is temporally and spatially regulated in various cell types and at different developmental stages. Thus, abnormalities in their expression levels have been implicated in numerous pathologies, including neurodegenerative disorders[22]. Over the last years, several approaches have been used to repress or overexpress proteins involved in their biogenesis, to analyze in detail their role in the brain[23,24]. Some of these experiments resulted in phenotypes that included neuronal death, memory loss, tremors and spontaneous seizures[25–28].

Moreover, these RNA species are stable and present in biofluids (such as plasma, serum, cerebrospinal fluid and urine). Circulation miRNAs are usually associated with protein complexes or encapsulated within vesicles (microvesicles, exosomes or apoptotic bodies), which protects them from degradation by RNases in the extracellular space. These characteristics qualify miRNAs as potential diagnostic tools in neurodegenerative diseases.

Use of fluid biomarkers

A biomarker is a biological factor that can be measured *in vivo* and is associated with the presence or absence of a symptom or disease. In neurodegenerative disorders, some of the most well characterized biomarkers are amyloid-β and tau levels – which are currently determined through PET imaging and CSF analyses– as indicators of specific changes that define AD *in vivo*[29]. CSF, mainly produced by the choroid plexus of the ventricular system, is considered the optimal fluid to determine brain metabolism in health and disease, due to its proximity to the brain parenchyma and the relatively low concentration of proteins in comparison with other biofluids[30]. CSF is collected by lumbar puncture, a procedure that can be safely performed without major complications[31].

Accurate diagnosis of patients in life is important both in clinic and research. It is required for prognosis, clinical care and management; and it is essential for clinical trials. Advances in research, including genetic studies, depend on accurate and refined characterization of the patients, as heterogeneous cohorts may dilute potentially significant findings. However, some syndromes, such as certain types of frontotemporal dementia and AD, may present clinical similarities, especially at early stages. Conversely, patients sharing a common pathology are not clinically homogeneous, which causes a lack of clinical confidence[32]. It has become clear during the past years, that neurodegenerative disorders have different neuroimaging and CSF profiles. The development of both biochemical and imaging biomarkers has allowed the monitorization of normal and pathophysiological events in the brain, and can contribute to the prediction of the underlying pathology in the clinical practice. Thus, CSF biomarkers have become an important tool in diagnosis, prognosis and staging of neurodegenerative diseases.

Moreover, growing evidence indicates that the majority of neurodegenerative disorders (such as AD, PD and FTD) have preclinical and prodromal phases, where the pathophysiological changes are present but the clinical symptoms has not yet appeared. The use of biomarkers would also be essential to identify individuals at these early stages, which could improve their clinical care when disease-modifying treatments are available. However, correlation between biomarkers, neuropathology and clinical symptoms is still imperfect. Therefore, finding novel biomarkers could allow a more accurate clinical diagnosis and help elucidate the underlying neuropathology *in vivo*.

Parkinson's disease

Parkinson's disease (PD) is the most common movement disorder and represents the second most common cause of neurodegeneration, affecting 1-2% of the population over the age of 65 years.

The first complete clinical description of the disease was made by James Parkinson in 1817[33,34]. Phenotypically, it is characterized by parkinsonism, which encompasses the combination of three clinical manifestations: bradykinesia, resting tremor and rigidity. These motor symptoms usually improve with L-DOPA treatment at early stages. Additional non-motor features frequently appear, having a greater impact on the quality of life than motor symptoms. The most common cause of parkinsonism is PD, but other disorders can lead to its appearance, such as progressive supranuclear palsy (PSP), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP17 or FTD-Tau) or multiple system atrophy (MSA).

PD is considered a multisystemic synucleinopathy, a term that covers several progressive neurodegenerative disorders with the commonality of abnormal α-synuclein insoluble inclusions in neuronal or glial cells[35]. The two other main types of synucleinopathy are dementia with Lewy bodies (DLB) and MSA. The neuropathological hallmarks of PD are the presence of intracellular protein aggregates in the form of Lewy bodies and neurites, and the dysfunction or loss of the dopaminergic neurons in the *substantia nigra pars compacta*.

Several familial forms of PD exist, accounting only for 5-10% of the cases; the remaining 90-95% of cases are apparently sporadic, with both genetic and environmental risk factors playing an important role.

Epidemiology

In general, in industrialized countries, the prevalence of PD is estimated at 0.3% in people over the age of 60, increasing with age up to 3% in people over 80 years of age[36,37]. Epidemiology studies in Spanish population estimated the overall prevalence in 1.5%; reaching 3.2% among 80-year-old individuals[38].

Onset of PD occurs rarely before 50 years, and a sharp incidence increase is seen after the sixth decade[36]. Standardized incidence rates of PD range between 8 and 19 per 100,000 person-years[39–41]. In Spain, the annual incidence rate was estimated to be 186.8, adjusted to the standard European population[42].

Several epidemiological studies also reported a 1.37-3.7 times higher prevalence and incidence in men, proposing male sex as a prominent risk factor for PD across ages

and nationalities. These gender variations could be related to biological differences in the affected neural pathways, but also to differences in the levels of circulating estrogens, which seem to protect females against neuronal loss in experimental PD models[43,44].

Clinical symptoms

Clinically, PD is characterized by parkinsonism, which is defined as bradykinesia in combination with rest tremor and/or rigidity. Bradykinesia is described as slowness and decreased amplitude or speed of movement that affects all voluntary and involuntary movements. Although it may also occur in voice, face and axial/gait domains, limb bradykinesia must be present to establish a diagnosis of PD. Rest tremor is defined by 4- to 6-Hz tremor in the fully resting position. It mostly occurs in upper limbs, but also in legs and head, and disappears during voluntary movement initiation. Rigidity refers to a constant increased resistance of a joint to passive movement, resulting in stiffness and failure to relax.

Apart from these three cardinal motor features, non-motor manifestations are present in the majority of patients, and can dominate the clinical presentation at some stages. Many of these non-motor symptoms were included in the last diagnostic criteria for PD published by the Movement Disorders Society[45].

Neuropathological traits

The definitive diagnosis of PD requires histopathological confirmation, namely the presence of Lewy bodies, a specific type of protein inclusions, together with neuronal loss in the *substantia nigra*[46].

The formation of α-synuclein soluble aggregates in the form of Lewy bodies (LBs) and Lewy neurites (LNs) is the main neuropathological hallmark[47–51] [**Fig. 4A**]. Classical LBs are defined as large, round and highly eosinophilic intracytoplasmic aggregates in the dopaminergic neurons of the *substantia nigra pars compacta* (SNpc) and *locus coeruleus*. Cortical LBs have a smaller size, and are predominantly present in limbic and neocortical regions[52]. LNs are curvilinear or dot-like processes detected in regions with high density of LBs[53].

α-synuclein is a small neuronal protein that, under physiological conditions, is mainly located near the synaptic vesicles of the neuronal presynaptic terminals. Abnormal aggregation of α-synuclein occurs early in the disease process and it seems to progress sequentially from brainstem to cortical areas. Thus, a staging system has been proposed for sporadic PD, based on LB number and location[54].
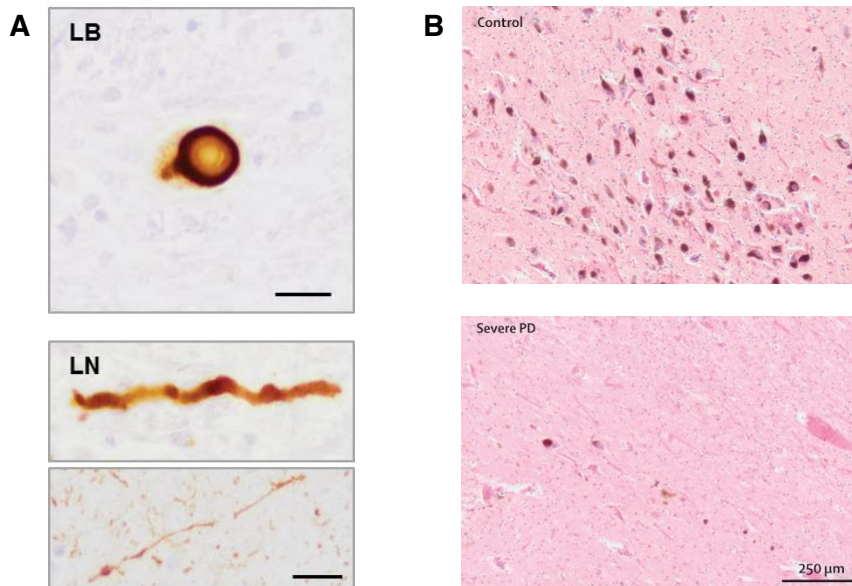
Figure 4. Neuropathological hallmarks of PD

A. Immunoreactivity pattern of α-synuclein in PD, forming Lewy bodies (LB) and Lewy neurites (LN). Scale bar=20μm. Images courtesy of Dr. M. Colom-Cadena[51]. **B.** Density of pigmented neurons in the SNpc of healthy control (top image) and PD patients with severe loss of pigmented neurons (bottom image). Transverse hematoxylin and eosin-stained sections. Scale bar=250μm (adapted from Dickson, 2009).

Another neuropathological feature is the degeneration of dopaminergic neurons in the SNpc [**Fig. 4B**]. The extensive neuronal death in this region results in the decrease of dopaminergic projections to the caudate and putamen (nigro-striatal pathway), which leads to the manifestation of motor clinical symptoms. However, these symptoms only appear when the ~50% of the dopaminergic neurons are lost and the striatum has been depleted of 80% of the dopamine[46].

Some PD cases also present aggregates of abnormal tau protein, encoded by *MAPT* gene[55–57]. The interaction between α-synuclein and tau may promote their fibrillization and drive the formation of pathological inclusions[58].

Genetics of idiopathic PD

Early investigations into PD genetics focused on the identification of rare mutations causing familial forms of the disease[7,59]; however, over the past decades there has been a growing interest on the contribution of genetics in sporadic cases[60,61].

Figure 5. **Manhattan plot** (adapted from Nalls, 2018)

The nearest gene to each of the significant variants is labelled in green for previously identified loci, and blue for novel loci. Variant points are color coded orange for significan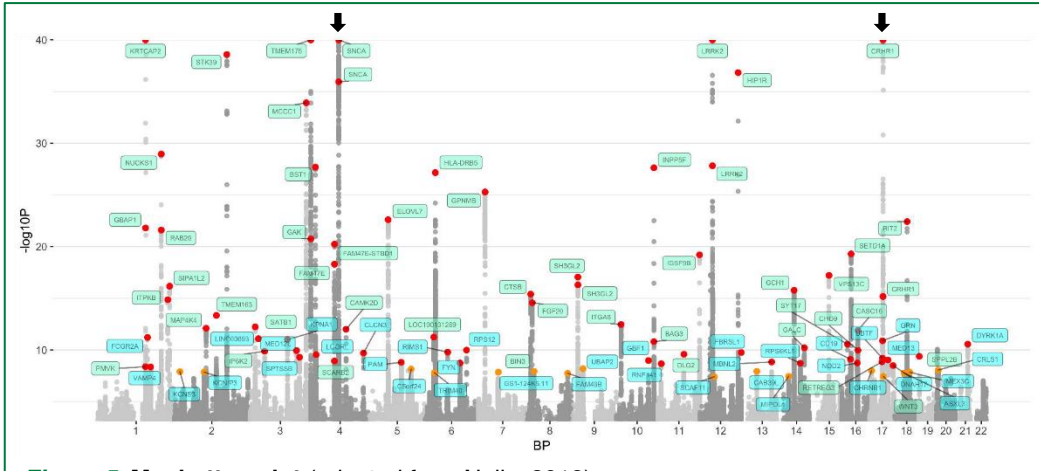ce at $p = 5\text{x}10^{-8}$ and $5\text{x}10^{-9}$, and red for significance $p < 5\text{x}10^{-9}$. The x axis represents the base pair position of variants on chromosomes. Black arrows show hits for *SNCA* (on chromosome 4) and *CRHR1* (within the *MAPT* region, on chromosome 17).

GWAS over the years identified numerous genetic risk variants for PD, highlighting the role of common variants in the etiology of this disease. Two of the strongest association signals observed are located on chromosome 4 and 17, comprising several SNPs within *SNCA* gene (encoding α-synuclein) and the *MAPT* region, respectively[62,63] [**Fig. 5**].

Risk alleles in the *MAPT* locus are also associated with the expression levels of both *MAPT* and *LRRC37A* in the human brain[62].

The most recent and largest-to-date GWAS for PD involved the analysis of almost 8M SNPs in 37.7K cases and 1.4M control individuals[64]. A total of 90 independent genome-wide significant signals were identified across 78 loci, including 38 new signals. Genes with PD risk variants showed enriched expression in several brain regions, especially in the *substantia nigra*, and occurring exclusively in neuronal cell types. Overall, they were able to explain up to 36% of PD heritability, while these yet-to-be defined risk variants will have relatively small effects, cumulatively they may improve the ability to predict PD.

The *MAPT* region

A large proportion of genetic variability in humans results from large-scale genomic structural changes; including deletions, insertions, inversions and copy number variations (CNVs). Copy number polymorphisms are widely distributed throughout

the genome, and frequently located near other types of chromosomal rearrangements. Some of them have been found within or near genomic regions associated with human genetic syndromes. As these variants exist in the genome of phenotypically normal individuals, they may not be directly implicated in these disorders, but may reflect the instability of these genomic regions, which could lead to chromosomal rearrangements that result in disease or influence expression of specific genes[18,65].

The *MAPT* 17q21.31 region is one of the most structurally complex and evolutionarily dynamic regions of the genome[66]. Located in the long arm of the chromosome 17, it is characterized by the presence of a ~900 kilobases (Kb) inversion polymorphism originated about 3 million years ago. This inversion results

Figure 6. **Schematic representation of the 17q21.31 chromosomal region in humans** (adapted from Cervera-Carles, 2015)

A. Distribution of the segments located in the 17q21.31 region; as well as the genes encoded within. **B.** Common subhaplotypes derived from the segregation of α, β and γ structural polymorphisms in H1 and H2 haplotypes. The thick black arrows indicate the orientation of the inverted region within the 17q21 locus. Allele frequencies in European population are shown in brackets next to each subhaplotype.

in an extended (~1.7 megabases) region of linkage disequilibrium; and produces two different haplotype blocks, H1 (direct) and H2 (inverse)[67–70].

H2 haplotype, considered the ancestral state, occurs predominantly in European populations (at a frequency of 20%), but it is rare in African and absent in East Asian populations[66,67,71]. Conversely, H1 haplotype, which was estimated to diverge from H2 approximately 2.3 million years ago[72], is widely present in all populations.

Recently, two studies aimed at disentangling the genetic architecture of this complex region, described large CNVs within each clade[72,73]. These independent evolutive events, without evidence of recombination between them, segregate as nine different subhaplotypes[73,74] [**Fig. 6**]. Three partially overlapping duplications unequally distributed along each haplotype were defined; named α segment (a 150 Kb duplication in the H2 haplotype), β segment (a 300 Kb duplication in the H1 haplotype) and γ segment (a highly multiallelic duplication of 218 Kb present in both H1 and H2 haplotypes). Moreover, these CNVs contain several coding genes, whose expression can be affected depending on the inversion status[75].

Interestingly, the two main haplotype clades have been associated with the development of some degenerative disorders, including PD, DLB and PSP[62,76–79]. H1 correlates with higher risk, while H2 is thought to be a protective haplotype.

In the first work of the thesis, we intended to piece together these previous findings, by studying the involvement of *MAPT* region CNVs in the genetic risk of PD and other neurodegenerative diseases [**Chapter 3**].

Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder, and the leading cause of dementia among the elderly, accounting for 60-80% of the cases. It affects up to 5% of population over 65 years old.

Although it was first described by Alois Alzheimer in 1907[80,81], it was not recognized as a common cause of dementia and a major cause of death until 70 years later[82]. AD is clinically manifested as progressive cognitive decline, amnestic memory deterioration, neuropsychiatric and behavioral disturbances, with consequent impairment of the daily living activities[83].

Accumulation of amyloid-β peptide in plaques and aggregation of hyperphosphorylated tau protein in neurofibrillary tangles constitute the main neuropathological hallmarks of the disease.

Except for rare familial cases, the onset of cognitive decline occurs generally within the sixth decade of life. The vast majority of cases (>95%) are sporadic. Advancing age is the greatest known risk factor, but over the years many genetic variants, such as *APOE*, have been described to contribute to the risk of AD.

Epidemiology

Age is the greater risk factor for AD, with the vast majority of people affected being older than 65. In 2006, 26.6 million people worldwide were living with AD, and it was estimated to quadruple by 2050 as a result of the aging of world's population, reaching 106.2 million cases[84].

The general prevalence of AD in Europe was estimated at 5.05% in people over 60 years of age. It increases progressively with age, from 0.97% in patients aged 65-74 to 22.53% in those older than 85 years[85]. In Spain, the crude prevalence of AD was estimated to be 6.6%[86].

Studies conducted in Europe also reported an average incidence of 11.08 cases per 1000 person-years[85]. The overall incidence rate in Spanish population is 5.4 per 1000 person-years, which increases markedly with age and continued to rise after 90 years of age[87].

Several studies showed about 2 times higher prevalence of AD in women[85,88], especially in those with lower education levels[89], accounting for 62% of worldwide cases[84]. Prevalence differences by gender may reflect the lower background mortality rates among women[84]. Incidence rates were also significantly higher in women than men (~1.9 times)[87].

Clinical symptoms

Classic clinical features include amnesic memory impairment, language deterioration and visuospatial deficits. Motor and sensory abnormalities are uncommon until late stages of the disease[83]. These symptoms reflect the degree of damage to neurons in different brain networks.

Neuropathological changes of AD are thought to occur at least 20 years before the onset of the symptoms, supporting the concept of AD as a continuum[90–92]. The brain is able to compensate the initial pathological changes; but as the neuronal damage increases, individuals start showing subtle cognitive deficits. Clear cognitive decline –memory loss and time/place confusion– is observed when neuronal and synaptic damage is significant; ultimately, even basic body functions are impaired[93].

Current 2011 diagnostic criteria include three stages in the AD continuum, from mild to severe: preclinical AD, mild cognitive impairment due to AD, and dementia due to AD.

In the preclinical AD stage, individuals show changes in biomarkers that indicate earliest signs of disease, but cognitive deficits are absent[94]. Additional research is aimed at validating this proposed early phase of AD, its extent and characteristic traits, which would help identify individuals earlier.

Mild cognitive impairment (MCI) is designated when mild changes in cognitive abilities can be measured and are noticeable to the affected person or family members, but everyday activities remain unaltered[95]. Around 15-20% of people ≥65 years fulfill the criteria of MCI[96]. People presenting memory issues are more likely to develop AD and other dementias, with a conversion rate of 30-40%[97,98]. When MCI is due to AD, it is usually accompanied by increased levels of amyloid-β.

Dementia due to AD is diagnosed when memory, cognitive and behavioral symptoms are noticeable and impair daily life activities, along with evidence of an AD related biomarker change[99]. Within this stage patients can be classified on a severity scale, depending on the impact of dementia in daily living activities[100]. Neuropathological features consistent with AD are required for definite diagnosis, as even highly accurate diagnosis is still probabilistic[101].

Available pharmacological treatments for AD cannot slow or stop the neuronal damage that causes the symptoms, making this disease fatal. Current drugs increase the levels of certain neurotransmitters, temporarily improving the symptomatology. Many researchers believe that future treatments to stop or slow progression and preserve brain function will be more effective if administered earlier (MCI or even

preclinical phases). Biomarkers would be essential to identify individuals at early stages that could receive disease-modifying treatments when available[102].

Neuropathological traits

AD is pathologically defined by the presence of extracellular amyloid plaques, considered central to initiating AD pathogenesis[103], and intracellular neurofibrillary tangles (NFTs), which strongly correlate with neuronal dysfunction and clinical progression of AD. AD pathology is also marked by synaptic and neurotransmitter loss, neuronal death and neuroinflammation[104].

Amyloid plaques are insoluble extracellular deposits, formed when amyloid-β (Aβ) peptides that cannot be degraded are accumulated in the brain[105]. Aβ peptides are cleaved from the amyloid precursor protein (APP) by β- and γ-secretase enzyme complexes. The most abundant species found in plaques are Aβ peptides ending at amino acid 42 ($A\beta_{1-42}$), being the most investigated variant in patients. These plaques are morphologically diverse and complex, with different predominance for specific brain regions. Neuritic plaques are a type of Aβ deposits characterized by the occurrence of dystrophic neurites, great synaptic loss and glial activation[101,106]. Among the different forms of deposits, neuritic plaques are more closely associated with neuronal injury [**Fig. 7**].

In AD, abnormal tau protein forms intracellular aggregates named NFTs [**Fig. 7**]. They are largely composed by paired helical filaments from hyperphosphorylated microtubule associated tau protein. NFTs are commonly observed in limbic regions
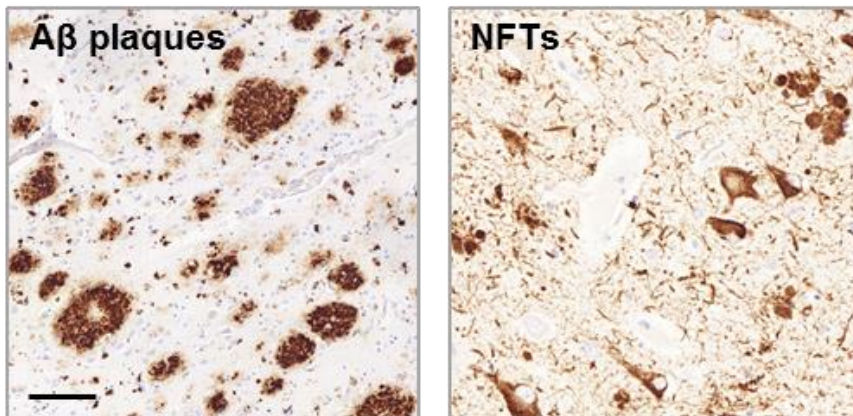

Figure 7. **Neuropathological hallmarks of Alzheimer's disease**
Pathological aggregates observed in AD brains: different types of amyloid-β (Aβ) plaques, from mature to diffuse forms (left) and neurofibrillary tangles (NFTs; right). Scale bar=100μm. Images courtesy of M. Querol-Vilaseca.

at early stages but progresses to other regions as disease advances[107]

The current neuropathological criteria[106] include three staging methods for AD lesions: the Thal Aβ phase for Aβ plaques, the Braak system for NFTs, and the Consortium to Establish Registry for Alzheimer's Disease (CERAD) for neuritic plaques.

Other neuropathological features of AD include neuronal death, associated with gross cerebral atrophy; synaptic loss, reported to occur early in the neurodegenerative process and correlate with cognitive decline; and neuroinflammatory changes.

Current fluid biomarkers

A specific CSF biomarker signature has been identified for AD, which is increasingly being used in clinical practice: decrease of amyloid-β_{1-42} (Aβ_{1-42}), correlating with the amyloid pathology; and increase of total-tau (t-tau) and phospho-tau (p-tau), indicating the presence of neurofibrillary tangles and neuronal damage[29,94,108]. Together with neuroimaging, CSF biomarkers are currently present in the diagnostic criteria as a biochemical indication of the disease. When used in combination, these CSF biomarkers have a good predictive value, but diagnosis of AD still requires the presence of clinical symptoms. The mechanistic relationship between alteration of these biomarkers and the appearance of clinical AD signs remains elusive. Postmortem studies show a considerable proportion of cases without dementia present Aβ deposits. Thus, brain amyloidosis appears to be necessary but not sufficient to cause AD symptomatology[109].

Genetics of sporadic AD

AD, like other common chronic diseases, develops as a result of multiple factors. Less than 5% of the cases are linked to autosomal dominant mutations with early onset, typically before 65 years of age. Nonetheless, the study of this small proportion of patients and the discovery of autosomal dominant mutations in *amyloid beta precursor protein* (*APP*), *presenilin1* (*PS1*) and *presenilin2* (*PS2*) genes has been crucial to understand the genetic and pathophysiological bases of AD.

The majority of AD cases have a late onset of symptoms and do not have causal mutations. However, there is a high genetic component underlying this sporadic forms of the disease, with 58-79% heritability[110]. This has prompted the researchers to consider the implications of common and rare variants in AD genetic architecture.

Most known genetic loci related to AD come from GWAS and, therefore are based on common-variant common-disease hypothesis, including *APOEε4*, the major genetic risk factor. Additionally, several low and rare frequency variants increase AD risk. Many efforts aimed at disentangling the genetic architecture underlying AD pointed at certain biological pathways with probably important roles in the AD pathophysiology. These genes can be broadly classified into five major pathways: amyloid-β clearance (*APOE*, *CLU*, *CR1*), endocytosis (*PICALM*, *BIN1*, *SORL1*, *CD2AP*, *EPHA1*, *CD33*), lipid processing (*APOE*, *CLU*, *SORL1*, *ABCA7*), immune response (*CR1*, *CLU*, *ABCA7*, *CD33*, *EPHA1*, *MS4A* cluster, HLA complex members, *INPP5D*, *TREM2*) and tau phosphorylation (*MAPT*, *GSK3β-CDK5-P53*, *TTBK1*, *CASS4*, *FERMT2*) [**Fig. 8**]. A more detailed review on AD genetics can be found in **Annex 1**[111].

Some proposed susceptibility variants are still under debate, such as the *TOMM40* poly-T repeat. In this case, some studies argue that its genetic association with AD

Figure 8. Schematic representation of the genetic architecture underlying Alzheimer's dementia (from Cervera-Carles and Clarimón, 2016)

The Y axis indicates the strength of genetic effects, the X axis shows the allele frequency, and the Z axis represents the age at onset of disease. Novel genes that have not yet been replicated are presented in grey; and *AKAP9* has a dotted outline, indicating that associated variants are only present in African American population. Genes in bold (*SORL1* and *ABCA7*) have been identified in both GWA and case-control studies.

is due to linkage disequilibrium[112], whereas others claim that this association is maintained after adjusting for APOE status[113]. This gene encodes the translocase of outer mitochondrial membrane 40 homolog (TOMM40), which interacts with voltage-dependent channels at the same membrane, therefore introducing mitochondrial dysfunction and bioenergetics as another potential pathogenic pathway for AD.

Mitochondrial DNA

Despite the fact that AD has been studied for decades, the underlying mechanisms that trigger this neuropathology remain unresolved. Defects in brain metabolism and increased oxidative stress may be observed at early AD stages, even before the hallmarks of the disease[114–116]. Mitochondria are involved in these phenomena by orchestrating energy metabolism, redox homeostasis and apoptotic pathways[117]. These organelles are distributed along axons, nerve terminals and dendrite spines, and contain several copies of mitochondrial DNA (small, organelle-specific, circular DNA that differs from genomic DNA). Impaired mitochondrial function can lead to a pathological state, ranging from subtle alterations in neuronal function to cell

Figure 9. **Mitochondrial dysfunction and AD pathogenic processes** (from Godoy, 2014)

Multiple pathogenic mechanisms contributing to the hallmarks of AD: increase of ROS production; Aβ-induced inhibition of mitochondrial protein import; APP-caused Ca^{2+} homeostasis alteration and apoptosis; mitochondrial DNA damage associated with synaptic and neuronal loss, amyloid plaques and NFT formation; perturbation of cerebral energy metabolism. Abbreviations: Ca^{2+}, calcium; Mptp, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; ROS, reactive oxygen species.

death and neurodegeneration [118,119] [**Fig. 9**].

First proposed by Swerdlow and Khan (2004), the "AD mitochondrial cascade hypothesis" considers mitochondria as central pieces of degenerative mechanisms. It postulates that mitochondrial function may affect APP expression and processing, as well as Aβ deposition, which triggers the amyloid cascade[120]. Recent studies proposed that individuals have certain mitochondrial function that declines at a particular rate[121,122], which may be caused to increased reactive oxidative species or TOMM40 variants among others[109,123]. As a consequence of mitochondria turn-over, later studies suggested that mitochondrial DNA (mtDNA) could be released from the cells. The covalently closed circular structure confers mtDNA resistance to degradation of DNases present in the extracellular space; therefore, it could be detectable in CSF. Contrary to what would be expected, these studies reported decreased mtDNA levels in CSF, suggesting that in the absence of cell damage the amount of cell-free circulating mtDNA would reflect basal mitochondrial impairment[109,124,125].

These previous studies were the bases for the second work of the thesis, focused on the assessment of circulating mtDNA levels in the continuum of AD.

Frontotemporal dementia and other related syndromes

Frontotemporal lobar degeneration (FTLD) is a pathological term that encompasses a clinically, pathologically and genetically heterogeneous group of disorders, with relatively selective degeneration of the frontal and temporal lobes and distinct neuronal and glial protein inclusions[126,127]. FTLD was firstly described by Arnold Pick, when he reported a number of patients with delimited cortical atrophy in 1892[128]. In 1911, Alois Alzheimer described and illustrated argyrophilic globular neuronal cytoplasmic inclusions, which he named "Pick bodies"[129].

FTLD usually presents as frontotemporal dementia (FTD), the second most common dementing disorder in individuals under the age of 65 years[130]. FTD is an umbrella term that covers several clinical syndromes characterized by the degeneration of the frontal and temporal lobes and their subcortical connections, which causes behavioral and/or language deficits[131–134]. Over the last decade, there has been an increasing recognition of the overlapping of different FTD variants, including those with movement disorders (PSP and CBD) and those with motor neuron degeneration (ALS). These co-occurring presentations are now reflected in the latest clinical criteria for these diseases[135–138].

FTLD is a highly heritable disorder with a positive family history in 30-60% of cases, indicating a strong genetic component[139,140]. At a neuropathological level, most of the cases (90-95%) present intracellular aggregates of either protein tau or TDP43[141,142], whereas the remaining 5-10% of them have FUS inclusions[142,143].

Epidemiology

Study of FTD distribution in the population is challenging, due to the heterogeneity of FTD variants, which may lead to misdiagnosis. The limited array of FTLD syndromes considered in some epidemiological studies also results in variable prevalence and incidence rates[134,144].

Overall the prevalence percentage of FTD ranges from 3% to 26.6% in individuals between 45-65 years old, according to a recent meta-analysis on early onset dementias[145]. Estimates of FTD incidence oscillate between 2.7-4.1/100,000 in individuals <70 years of age, showing little variability compared to the prevalence data[146]. Men and women seem to be equally affected by FTD.

The only epidemiological study in Spanish population including FTD patients, reported incidence rates of 1.3 (0.7-2.2, 95% CI) in the early onset subgroup, and 16.7 (12.6-21.7, 95% CI) in individuals over the age of 65[147]. Very high estimates in

people over 65 years may not reflect neuropathological FTLD, and could represent an overdiagnosis of a subtype of FTD[144].

Clinical features
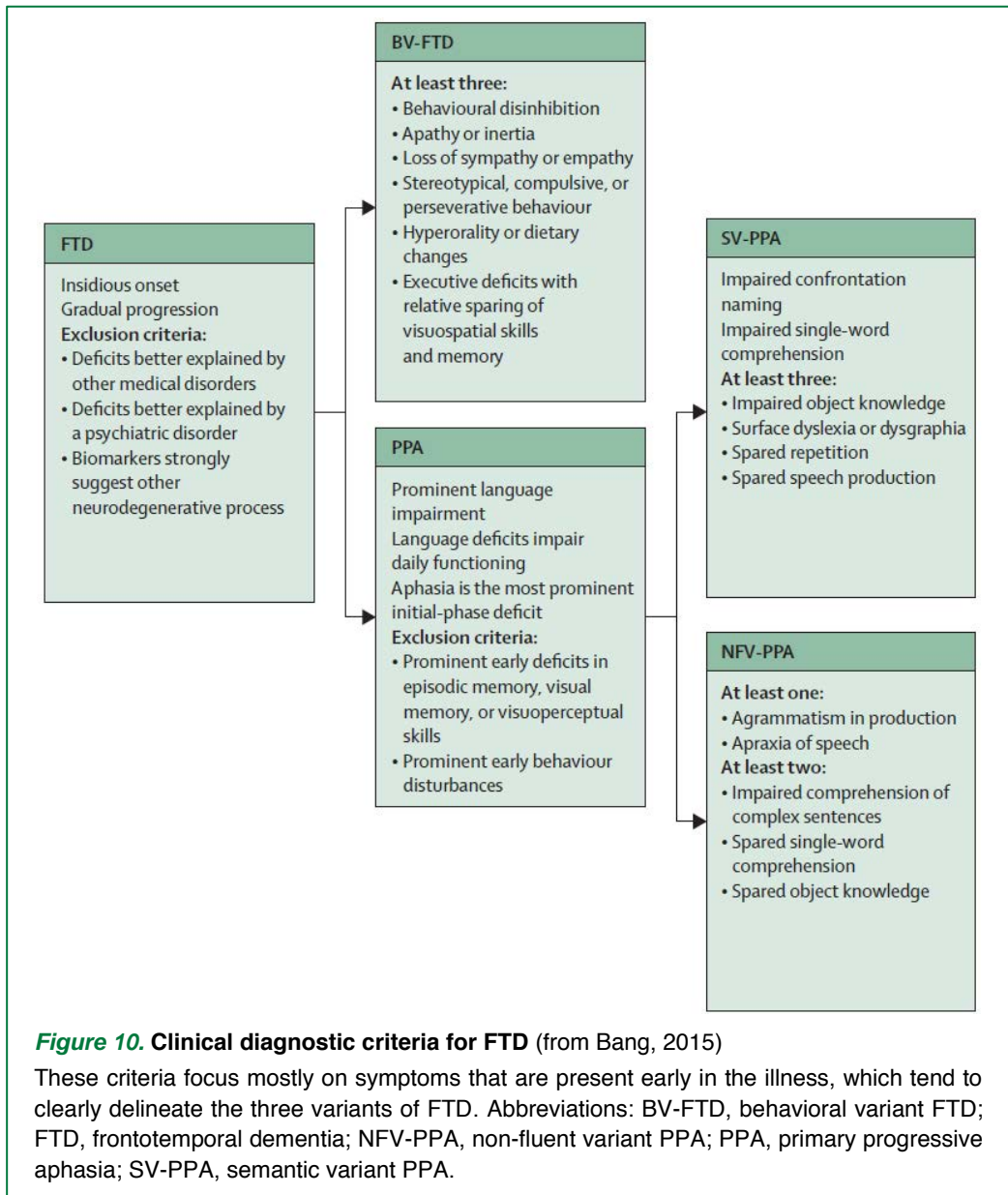
Clinical variants of FTD

Clinically, FTD is classified into three main variants, each characterized by distinct clinical features at onset [**Fig. 10**]: the behavioral variant (bvFTD) and two forms of primary progressive aphasia (PPA), the semantic variant (svPPA) and the non-fluent variant (nfvPPA).

The behavioral variant is the most common type of FTD, accounting for roughly 60% of the cases[146]. Onset of symptoms is around the age of 60, although a significant proportion of late onset cases (after 65 years of age) have been reported in European cohorts[134,148]. Early symptoms of bvFTD include behavioral abnormalities (personality changes, disinhibition, apathy, loss of empathy, compulsive behavior or hyperorality) and a distinct cognitive profile (insensitivity to errors, but relatively preserved memory domains and visuospatial functions)[134,149]. This variant is the most likely to be misdiagnosed as a psychiatric disorder, as some behavioral changes are hardy identified as a part of a neurodegenerative disorder[134]. Current diagnostic criteria for bvFTD[149] incorporate genetic and neuroimaging findings to increase diagnostic certainty.

The language presentations of FTD include two primary progressive aphasias (PPA), which are characterized by prominent language deficits as the primary cause of functional impairment[150]: the non-fluent variant (nfvPPA) and the semantic variant (svPPA)[133,151].

NfvPPA is a rare, heterogeneous syndrome with a mean age of onset of approximately 60 years[152]. The hallmark clinical features included in the current criteria[151] are effortful speech (slow labored speech production due to speech motor planning deficits) and agrammatism (use of short simple phrases and omission of grammatical phonemes). In some cases, the language impairment can be early accompanied by extrapyramidal symptoms, with a higher impact on daily activities; therefore, these patients can be diagnosed as PSP or CBS[133,135,136].

SvPPA is defined by a progressive and multimodal loss of semantic knowledge, with a variable onset age between 55 and 77 years[153]. Progressive anomia (inability to recall words, names and numbers) and markedly impaired comprehension of single words are the core features according to the current diagnostic criteria[151].

FTD

Insidious onset
Gradual progression
Exclusion criteria:
• Deficits better explained by other medical disorders
• Deficits better explained by a psychiatric disorder
• Biomarkers strongly suggest other neurodegenerative process

BV-FTD

At least three:
• Behavioural disinhibition
• Apathy or inertia
• Loss of sympathy or empathy
• Stereotypical, compulsive, or perseverative behaviour
• Hyperorality or dietary changes
• Executive deficits with relative sparing of visuospatial skills and memory

PPA

Prominent language impairment
Language deficits impair daily functioning
Aphasia is the most prominent initial-phase deficit
Exclusion criteria:
• Prominent early deficits in episodic memory, visual memory, or visuoperceptual skills
• Prominent early behaviour disturbances

SV-PPA

Impaired confrontation naming
Impaired single-word comprehension
At least three:
• Impaired object knowledge
• Surface dyslexia or dysgraphia
• Spared repetition
• Spared speech production

NFV-PPA

At least one:
• Agrammatism in production
• Apraxia of speech
At least two:
• Impaired comprehension of complex sentences
• Spared single-word comprehension
• Spared object knowledge

Figure 10. **Clinical diagnostic criteria for FTD** (from Bang, 2015)

These criteria focus mostly on symptoms that are present early in the illness, which tend to clearly delineate the three variants of FTD. Abbreviations: BV-FTD, behavioral variant FTD; FTD, frontotemporal dementia; NFV-PPA, non-fluent variant PPA; PPA, primary progressive aphasia; SV-PPA, semantic variant PPA.

A third variant, the logopenic variant (lvPPA), is included in the PPA consensus classification. It shows a highly consistent clinicopathological association with underlying AD pathology; thus, it will not be considered within the spectrum of FTLD syndromes[154].

Motor syndromes related to FTLD

As the disease progresses and degeneration spreads to affect larger regions, the symptoms of the three clinical variants can converge and result in global cognitive impairment. Over time, some patients can also develop motor deficits, including motor neuron disorders (ALS) and atypical extrapyramidal syndromes (PSP and CBS)[134,155] [**Fig. 11**].

Clinical overlap between ALS and FTD was first observed in the twentieth century[156], and their co-occurrence is now reflected in the latest criteria for ALS-FTD spectrum disorders[138]. Cognitive or behavioral impairment is present in up to 50% of ALS patients, with 15% having FTD. Likewise, approximately 30% of FTD patients show features of motor neuron disease, with ~15% of cases fulfilling the diagnosis criteria of ALS[157,158].

Some FTLD clinical presentations (nfvPPA and bvFTD) can also overlap with two motor phenotypes, namely PSP and CBS. PSP-related clinical features include
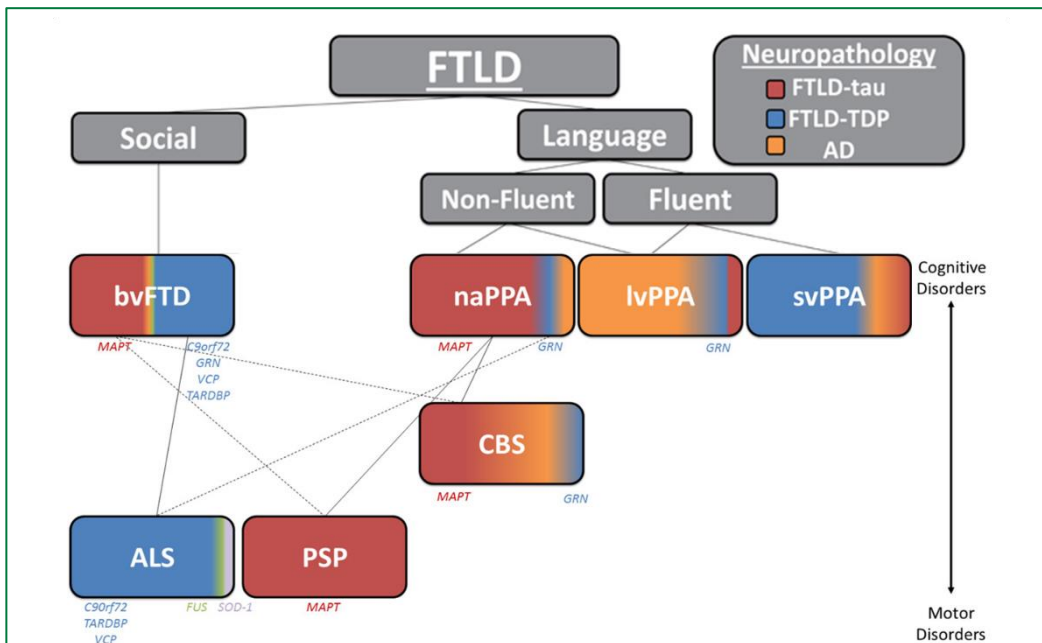

Figure 11. **Clinicopathological classification of frontotemporal dementia and other related syndromes** (adapted from Irwin, 2013, 2015)

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; bvFTD, behavioral variant frontotemporal dementia; CBS, corticobasal syndrome; FTLD, frontotemporal lobar degeneration; lvPPA, logopenic variant primary progressive aphasia; naPPA, non-fluent agrammatic variant PPA; PSP, primary progressive palsy; svPPA, semantic variant PPA.

postural instability, supranuclear oculomotor palsy and freezing of gait[135]. From a clinical perspective, salient aspects of CBS are ideomotor apraxia, myoclonus, dystonia, cortical signs and visuospatial symptoms[136].

Neuropathological classification

FTLD is characterized by neuronal loss, gliosis, and microvacuolar changes of frontal lobes, anterior temporal lobes, anterior cingulated cortex and insular cortex[134]. The neuropathology of FTLD is heterogeneous and most cases can be classified based on the patterns of abnormal protein deposited in the brain[159,160]. The current neuropathological classification of FTLD recognizes four major subtypes: FTLD-Tau, FTLD-TDP, FTLD-FET and FTLD-UPS[126,127].

The abnormal accumulation of tau protein is characteristic of ~40% of cases (FTLD-Tau) [**Fig. 11**]. Tau, a microtubule associated protein coded by *MAPT* gene, promotes microtubule assembly and stabilization, which are essential for maintaining neuronal integrity and exoplasmic transport[161]. In the adult human brain, several tau isoforms are expressed as a result of alternative splicing [**Fig. 12B**]. Exons 9-12 of the *MAPT* gene encode four microtubule-binding domains (MTBD). The exclusion or inclusion of exon 10 produces tau isoforms with either three or four repeat MTBD (3R- and 4R-tau, respectively)[126]. The neurodegenerative disorders that accumulate intracellular hyperphosphorylated tau in the absence of Aβ deposits, are named tauopathies. For each tauopathy, Tau aggregates have different phosphorylation degrees and isoform content[127] [**Fig. 12A**]. Therefore, FTLD-Tau cases can be subdivided into disorders with inclusions containing predominantly 3R-tau (Pick disease, PiD), 4R-tau (progressive supranuclear palsy, PSP; corticobasal degeneration, CBD; argyrophilic grain disease, AGD; globular glial tauopathy, GGT), or an admixture of both isoforms (patients with *MAPT* gene mutations, FTLD-17)[126,127,161].

The FTLD-TDP subgroup is characterized by the accumulation of protein TDP43 in the form of ubiquitin-positive inclusions, and account for ~50% of FTLD cases[162,163] [**Fig. 12A**]. TDP43 is a highly conserved ribonucleoprotein, encoded by the *TARDBP* gene, whose cellular functions include regulation of RNA splicing, translation, transport and stabilization, as well as, miRNA biogenesis[164]. Its expression is tightly controlled by autoregulatory mechanisms and has a predominantly nuclear localization[165]. TDP43 abnormal aggregates can accumulate in both the cytoplasm and nuclei of neurons and glia, resulting in cellular dysfunction. Differences in the morphology and distribution of the inclusions allowed the recognition of five different histopathological subtypes of FTLD-TDP (from type A to E)[166].

About 10% of the FTLD cases, without Tau or TDP pathology, have inclusions containing FET proteins[167]. The FET protein family is comprised by Fused-in-sarcoma (FUS), Ewing's sarcoma (EWS), and TATA-binding protein-associated factor 15 (TAF15)[168]. These proteins are ubiquitously expressed and involved in DNA and RNA metabolism[169,170]. To date, three pathological conditions are included in this FTLD-FET subgroup: neuronal intermediate filament inclusion disease (NIFID)[171], basophilic inclusion body disease (BIBD)[172], and atypical FTLD with ubiquitin-positive inclusions (aFTLD-U)[143].

Few rare cases show ubiquitin/p62 immunoreactive inclusions, which are tau, TDP-43 and FUS negative. This FTLD subgroup, termed ubiquitin proteasome system (FTLD-UPS), mostly contains individuals with mutations in the charged multivesicular body protein 2B (*CHMP2B*) gene[127,173,174].

Genetics of frontotemporal lobar degeneration

Since *MAPT* gene (encoding tau protein) was identified in 1998 as the first gene unequivocally related to FTD[175,176], several other genes have been described associated with familial and sporadic forms of FTD. These include: *C9orf72*[177,178], *GRN*[179,180], *CHMP2B*[173], *VCP*[181], *SQSTM1*[182–184] and *TREM2*[185–187]. Moreover, *FUS* and *TARDBP* genes, typically associated with ALS, have also been related to FTD[188–191]. Finally, GWAS have shown the existence of minor effect genes that could be involved in the genetic architecture of this dementia. A case-control study based on pathogenic FTD forms (TDP-43 positive), revealed the role of *TMEM106B* gene in the risk of developing FTD[192]. Recently an international multicenter study reported two possible loci (HLA locus in chromosome 6p21.3 and locus 11q14, which includes *RAB38* and *CTSC* genes) as possible genetic factors associated with FTD[193,194]

Interestingly, many of the genes associated with FTD, such as *TARDBP*, *FUS*, *C9orf72*, *GRN*, *SQSTM1*, and *CHMP2B*, have been involved in the regulation of coding and non-coding RNA processes [**Fig. 13B**], strongly suggesting that dysregulation of transcriptome homeostasis may be an underlying pathogenic mechanism of FTD.
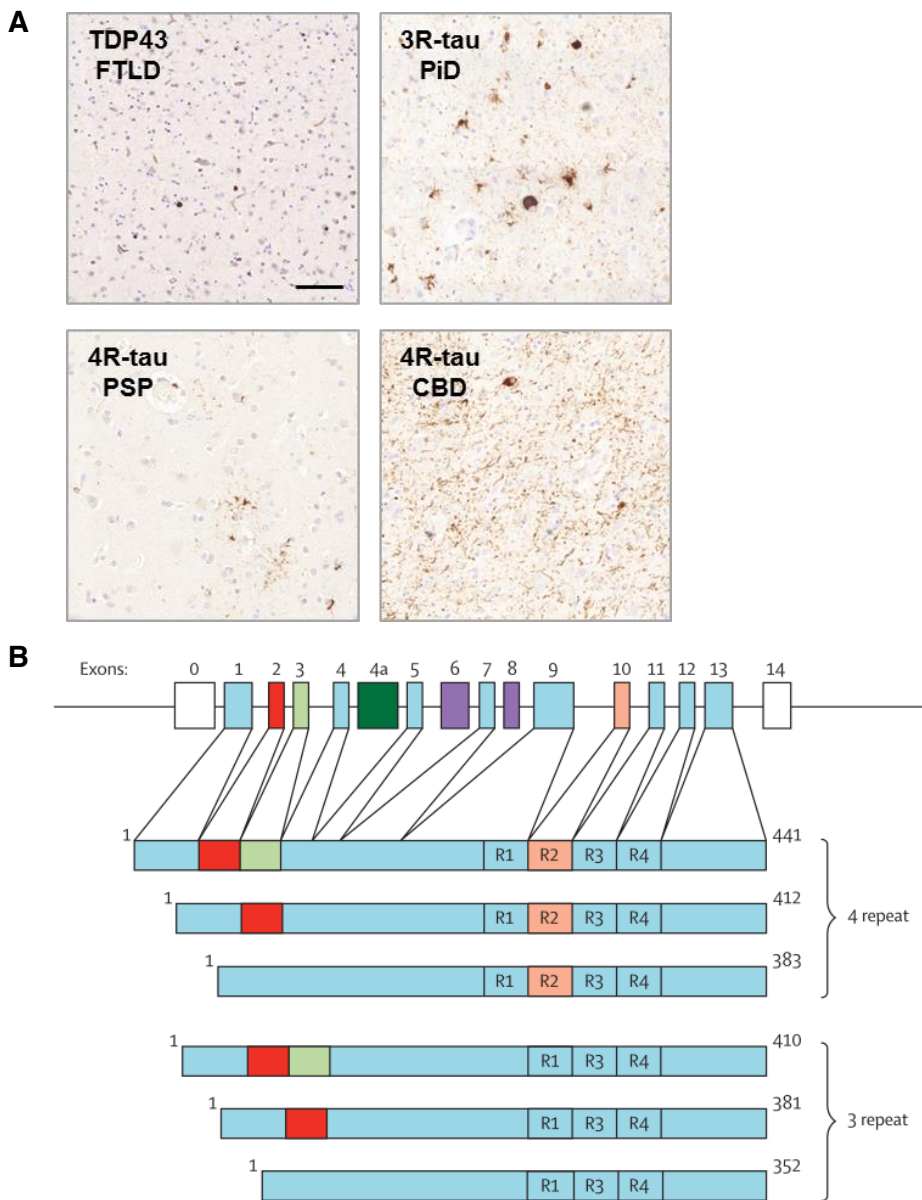
Figure 12. Neuropathological hallmarks of FTLD and other related syndromes

A. Pathological inclusions characteristic of FTLD and other related pathologies. Aggregates of TDP-43 in FTLD (top left), 3R-tau in Pick disease (top right), and 4R-tau in PSP and CBD (bottom images). Abbreviations: CBD, corticobasal degeneration; FTLD, frontotemporal lobar degeneration; PiD, Pick's disease; PSP, progressive supranuclear palsy. Images courtesy of M. Querol-Vilaseca and Dr. S. Sirisi. **B.** Human tau isoforms. *MAPT* gene consists of 16 exons (E). Alternative mRNA splicing of E2 (red), E3 (light green), and E10 (orange) generates the six tau isoforms. The microtubule tau repeats are labeled as R1-R4. (from Spillantini and Goedert, 2013)

MicroRNA metabolism and exosomes

High throughput genomic studies have estimated that only ~2% of the human genome encodes proteins; however the vast majority is transcribed, mainly into non-coding RNAs (ncRNAs)[19–21]. Although the functionality of these ncRNA species is still controversial, they are emerging as a new research field in health and disease. They are also being explored in neurodegenerative disorders, as there is now growing recognition that these epigenetic mechanisms, among others, are likely to participate in the etiology of these diseases[195–197].

MicroRNAs (miRNAs) are small non-coding RNA species of 18-25 nucleotides involved in the regulation of gene expression at a post-transcriptional level[198–200]. They were first discovered in *C. elegans*[201,202] but they recently gained interest in human biomedical research, as shown by the 2654 mature human miRNA sequences already annotated in the miRBase database [http://www.mirbase.org][203]. They play an important role in many biological processes; including neurodevelopment, neurogenesis, neurological diseases and synaptic plasticity[204–208].
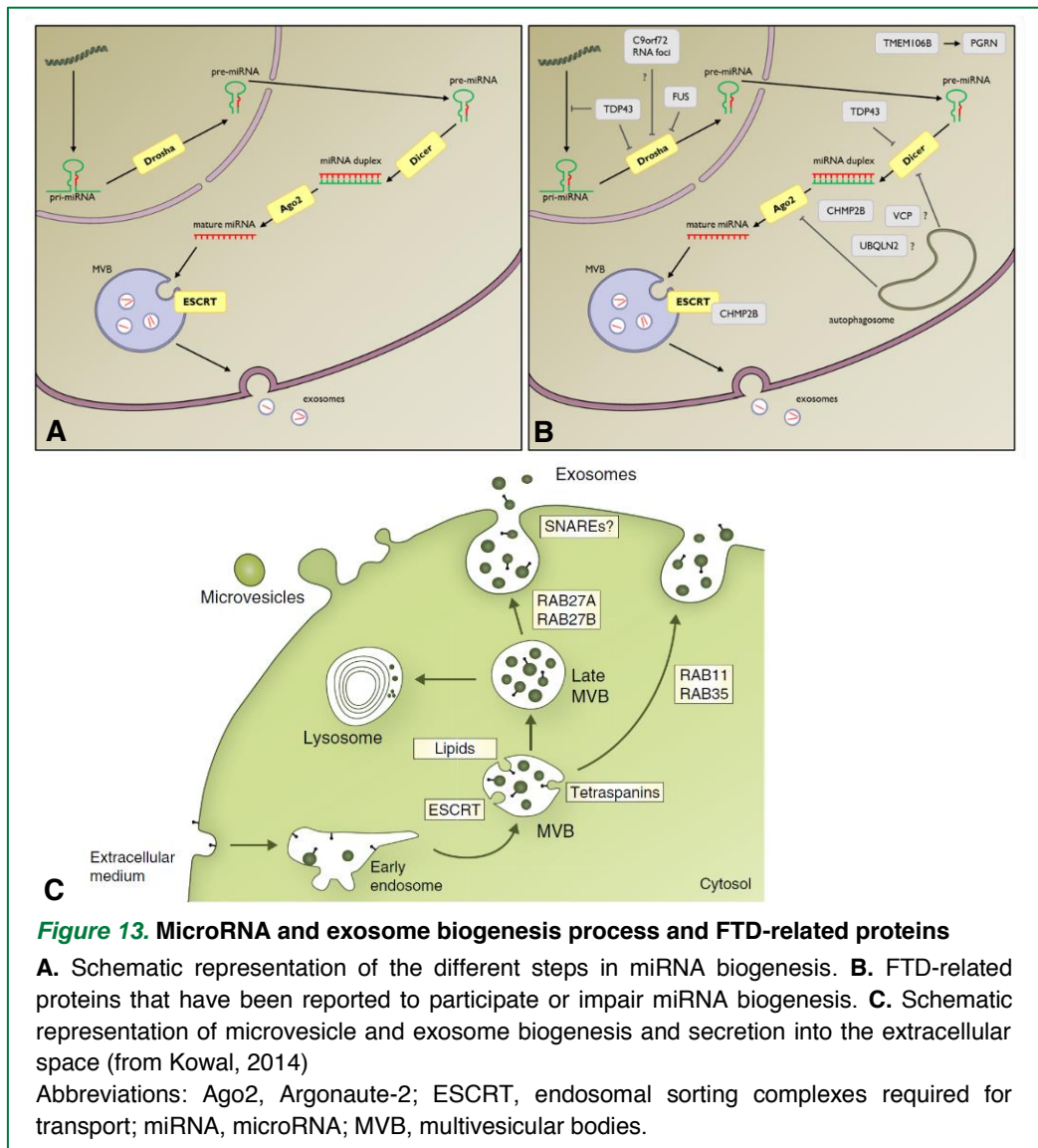
MiRNA-containing genes are distributed along all human chromosomes (except for the Y chromosome[209]), and generally clustered in transcriptional units, which may indicate their functional relationship[210,211]. Due to the imperfect complementarity recognition of mRNA, miRNAs are also characterized by their pleiotropic regulatory function and their enormous versatility to regulate different genes and pathways simultaneously[212,213]. Likewise, a given mRNA may be regulated by more than one miRNA species[204,214].

The vast majority of miRNAs are produced through a canonical pathway[215] [**Fig. 13A**]. Firstly, pri-miRNAs are transcribed from genomic DNA by polymerase II and then cleaved by Drosha/DGCR8 complex[216], producing pre-miRNA molecules. Exportin/RAN-GTP complex participates in pre-miRNAs exportation to the cytoplasm[217], where they are cleaved by Dicer enzyme to produce miRNA duplexes. The Dicer-TRBP complex recruits Argonaute-2 (Ago2), which separates the two chains generating mature miRNAs[218,219]. Mature miRNAs exert their regulatory function through the miRNA-induced silencing complex (miRISC), constituted by Dicer, TRBP and Ago2[220]. Based on their complementarity with target mRNA, miRNAs can induce either destabilization and degradation of the target mRNA, or translational repression[221,222]. Mature miRNAs can also be selectively exported from cells through lipoprotein binding, association with ribonucleoproteins or transported within extracellular vesicles[223].

Many genes and proteins identified in different presentations of FTLD have proven to be involved in miRNA biogenesis. Interestingly, mutations in *TARBP* decrease

the levels of miR-9 in both iPSC from FTD patients and a *Drosophila* model[224,225]. Moreover, levels of progranulin (encoded by *GRN* gene) decrease when miR-29 and miR-107 expression is altered, and this reduction has been previously associated with FTD[226]. Furthermore, miRNAs have also been implicated in the regulation of Tau phosphorylation and alternative splicing[227]. Other genes, such as *FUS*[228], *TMEM106b*[229] and *CHMP2B*[230] have been related to changes in the expression of particular miRNAs.

Extracellular vesicle (EV) term refers to all membrane-derived vesicles, secreted by many cell types, including brain cells[231]. They are detected in most biological fluids

Figure 13. MicroRNA and exosome biogenesis process and FTD-related proteins
A. Schematic representation of the different steps in miRNA biogenesis. **B.** FTD-related proteins that have been reported to participate or impair miRNA biogenesis. **C.** Schematic representation of microvesicle and exosome biogenesis and secretion into the extracellular space (from Kowal, 2014)
Abbreviations: Ago2, Argonaute-2; ESCRT, endosomal sorting complexes required for transport; miRNA, microRNA; MVB, multivesicular bodies.

–such as serum, plasma and CSF[232,233]. The EV notion appeared in 1983 when two independent groups reported that multivesicular late endosomes of reticulocytes released vesicles into the extracellular space[234,235]. Currently, EVs can be divided into two main categories based on their biogenesis: microvesicles and exosomes [**Fig. 13C**].

Microvesicles, ranging from 50 to 1000nm in diameter, are generated via direct outward budding of the plasma membrane and release of vesicles into the extracellular space[236]. Exosomes range from 50 to 150nm in size, and derive from the endosomal network. They originate during endosome maturation, when late endosome membrane bud inward, leading to the formation of multivesicular bodies (MVBs) that contain intraluminal vesicles (ILVs). From there, MVBs can fuse with lysosomes for cargo degradation or with the plasma membrane to release the ILVs to the extracellular space as exosomes[236–238]. The study of EV content revealed they can carry various cargos, including lipids, proteins and nucleic acids. Their composition varies widely between cells and conditions, affecting their specific fate and function.

EVs show overlapping morphology, size and composition; and specific markers to differentiate vesicle subtypes have not been proposed yet[239]. Nonetheless, several membrane-associated (i.e. tetraspanins CD9, CD63, CD81) and luminal (i.e. ALIX, TSG101) proteins have been widely used as "classical exosome markers"[240,241].

In 2007, exosomes were found to contain miRNAs[242]; since then, increasing evidences highlight the significant contribution of exosomes to genetic intercellular communication[241]. This transmission mechanism requires three essential processes: miRNAs have to be selectively and actively secreted and packaged; ought to be protected from circulating RNases and transferred to recipient cells; and must retain their ability to recognize mRNA targets within the recipient cells[223].

Therefore, EVs ability to transfer information may have a significant impact on physiological processes, but also facilitate the spreading of diseases through the delivery of genetic material or proteins[243]. In fact, several studies have shown that neurons can release exosomes and transfer pathogenic proteins, such as Aβ peptides, tau or α-synuclein, to other brain cells[244–246].

Both miRNAs and EVs have been detected in plasma and CSF among other biofluids, and may be important players in the etiology of neurodegenerative disorders. Together with the increasing evidence that FTLD-related genes and proteins are involved in miRNA metabolism, this has prompted us to investigate their potential as biomarkers for FTD and other related disorders, as shown in the third work of the thesis.

PCR-based techniques for genomic quantification

Droplet digital PCR

Droplet digital polymerase chain reaction (ddPCR) appeared in 2011[247] as a new high-throughput molecular method to determine accurately copy number variations (CNVs), as well as gene expression. It is an end-point based PCR performed on partitioned samples, in order to increase the precision and sensitivity in samples with low target concentrations. The use of microfluidic systems allows the generation of ~20,000 water-in-oil droplets, containing both the genetic material and the appropriate reagents for PCR amplification. After end-point amplification, a droplet flow-cytometer reads droplets as positive or negative for each fluorophore, and the outcome lecture correlates to the target concentration by Poisson statistics [**Fig. 14A-C**].

These particularities allow a direct and precise quantification of DNA. Apparently, ddPCR is also more resilient to quality differences between samples. Its end-point approach renders more tolerance to PCR inhibitors, which affect reaction efficiency[248,249]. The published digital MIQE (Minimum Information for Publication of Quantitative Digital PCR Experiments) guidelines[250] would help standardize experimental protocols, maximize reproducibility of data, and enhance the impact of this technology in research.

This approach was used in two of the research lines within this thesis [**Chapter 3**], the determination of CNVs within the chromosomal region 17q21.31, and the assessment of cell-free mitochondrial DNA (mtDNA) copies in CSF. However, due to differences on the genetic input material, and the chemistries used for PCR product detection, processes varied slightly. As represented in **Fig. 14A**, genomic DNA (gDNA) has to be cut because it is too large to be encapsulated within the 1nm water-in-oil droplets. Therefore, specific restriction enzymes (RE) must be used, ensuring that the target regions are completely preserved. On the other hand, the small dimensions of mtDNA (~17Kb) allow its proper encapsulation without the use of RE. Two different chemistries are currently used for ddPCR, fluorogenic probes (labeled with 6-FAM, VIC or HEX dyes) and EvaGreen reagent, depending on the purpose, abundance of the target and specificity of the study [**Fig. 14A**].

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) is a fluorescent- and PCR-based technique capable to detect small amounts of nucleic acids in a variety of sample types. Over the years, it has become the gold standard for nucleic acid quantification, due to its
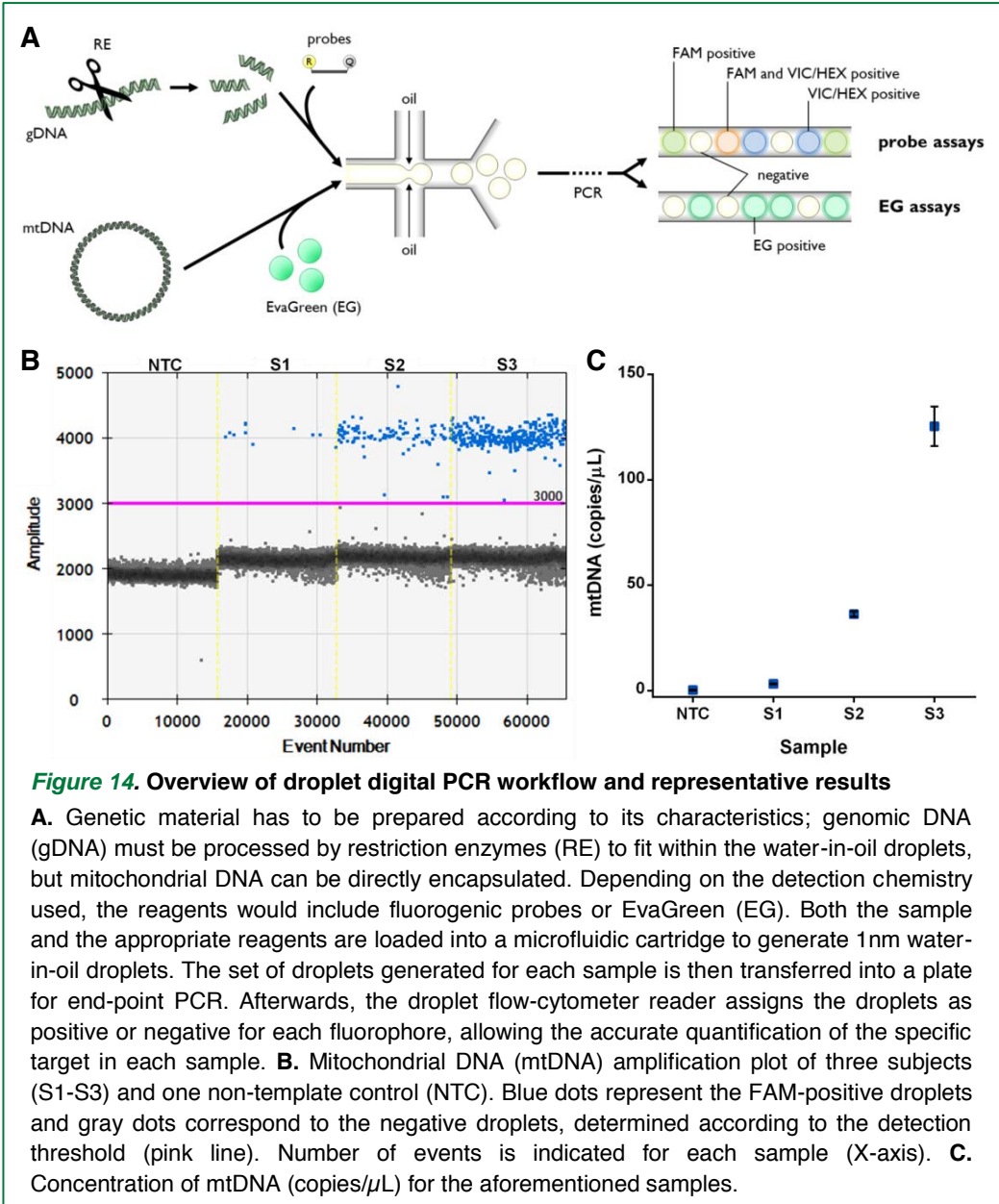
***Figure 14.* Overview of droplet digital PCR workflow and representative results**

A. Genetic material has to be prepared according to its characteristics; genomic DNA (gDNA) must be processed by restriction enzymes (RE) to fit within the water-in-oil droplets, but mitochondrial DNA can be directly encapsulated. Depending on the detection chemistry used, the reagents would include fluorogenic probes or EvaGreen (EG). Both the sample and the appropriate reagents are loaded into a microfluidic cartridge to generate 1nm water-in-oil droplets. The set of droplets generated for each sample is then transferred into a plate for end-point PCR. Afterwards, the droplet flow-cytometer reader assigns the droplets as positive or negative for each fluorophore, allowing the accurate quantification of the specific target in each sample. **B.** Mitochondrial DNA (mtDNA) amplification plot of three subjects (S1-S3) and one non-template control (NTC). Blue dots represent the FAM-positive droplets and gray dots correspond to the negative droplets, determined according to the detection threshold (pink line). Number of events is indicated for each sample (X-axis). **C.** Concentration of mtDNA (copies/μL) for the aforementioned samples.

simplicity, sensitivity and specificity. This technique was developed by Higuchi in 1992 as a refinement of the conventional PCR[251], because the classic PCR method had the limitation that generated the same amount of product independently of the initial concentration of DNA present in the samples.

In qPCR, the amplification process is monitored every cycle by measuring the fluorescent reporter emission, which reflects the amount of product formed. Signal

in the initial cycles is weak and undistinguishable from the background. However, as the reaction product accumulates, the signal increases exponentially (exponential phase), until it saturates (plateau phase). Amplification curves appear separated in the exponential phase, reflecting differences in the initial amounts of the target. For quantification, a particular fluorescence signal threshold is established. The number of cycles required to reach the threshold is named Cq (quantification cycle)[252]. This value is then used to calculate relative changes in gene expression[253]. Optimization is a critical step in qPCR experiments, as reaction efficiency can be affected by PCR inhibitors in the sample[248]. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were published in order to ensure the reliability and reproducibility of results, as well as promote experimental consistency[252].

This methodology was used in the last work of the thesis, for the study of miRNA expression in EVs of CSF [**Chapter 3**]. After purification and isolation of the EV-contained miRNAs, a reverse transcription (RT) step is required to convert RNA into cDNA. For our purposes, RT was performed by tailing a poly(A) sequence to the 3'end of miRNAs, and then using a universal poly(T) primer for transcription. This step could also be done individually for each miRNA, but at risk of introducing more variability to the results[254].

The use of Locked Nucleic Acid (LNA) technology increases the sensitivity of miRNA detection, and it represents one of the more widely used synthetic nucleotide chemistries. Originally developed in the late 1990s, it is characterized by the incorporation of a methylene bridge in the furanose ring of a nucleotide. When some of these modified LNA-bases are introduced into a growing oligonucleotide chain, the nucleotide conformation remains locked, enhancing the hybridization properties of the compound[255].

Although qPCR is one of the most commonly used techniques, finding appropriate endogenous reference miRNAs for normalization is essential, especially in biofluid studies. The measurement of circulating miRNAs requires a highly reliable method and, in this regard, qPCR-based platforms are some of the most sensitive ones[254].

Chapter 2

Hypotheses and aims
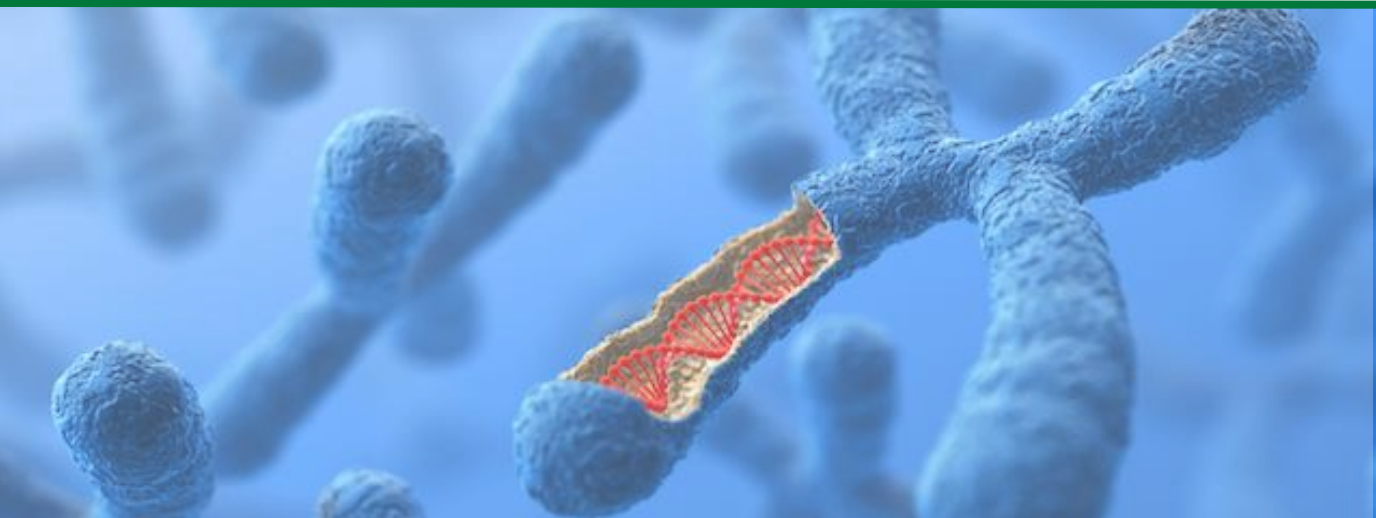
The better understanding of the human genome architecture and the study of familial aggregation in neurodegenerative diseases pointed at relevant genes, proteins and biological pathways implicated in their pathogenesis. Hence, it is important to investigate the role of different classes of genetic and epigenetic material, along with structural genetic rearrangements, in the susceptibility and etiology of neurodegenerative diseases.

Aim 1. Characterize the pattern of structural variation in the 17q21.31 inversion and determine the relationship of copy number polymorphisms present in the *H1* haplotype and the risk of neurodegenerative disorders.

Evidences of large copy number variations (CNVs) in the *MAPT* 17q21.31 region prompted us to study their association with some neurodegenerative diseases. These CNVs segregate as different subhaplotypes, and certain segments are enriched in European population. Thus, we hypothesized that CNVs of the H1 clade could explain the well-established link between this haplotype and the risk of developing Parkinson's disease (PD), progressive supranuclear palsy and corticobasal syndrome. Moreover, as these genomic segments contain several coding genes, we considered that gene dose variation could also be implicated in the pathogenesis. Finally, taking into account that H1 haplotype has been related to a higher burden of Tau pathology in PD, which could ultimately promote α-synuclein accumulation, we speculated that this haplotype might also be overrepresented in dementia with Lewy bodies.

We used a droplet-based PCR approach to accurately assess the two polymorphic duplications characteristic of the H1 clade in a study cohort comprising patients and controls, homozygous for this haplotype.

Aim 2. Assess the changes of cerebrospinal fluid mitochondrial DNA at different stages of the Alzheimer's disease continuum and evaluate its accuracy as diagnostic biomarker.

Previous investigations reported decreased levels of cell-free circulating mitochondrial DNA (mtDNA) in cerebrospinal fluid (CSF) of sporadic Alzheimer's disease (AD) patients, as well as in genetic and presymptomatic forms. CSF mtDNA was proposed to reflect underlying mitochondrial dysfunction prior to the onset of symptoms and suggested as a novel biomarker specific for the disease. We hypothesized that these changes in CSF mtDNA should be gradually observed in the whole AD continuum.

We examined the load of mtDNA through a droplet-based digital PCR, in a comprehensive series of patients at different stages of the AD continuum.

Aim 3. Identify microRNAs within extracellular vesicles derived from CSF that could be used as diagnostic biomarkers in frontotemporal dementia and other related syndromes.

Several causal genes and proteins related to frontotemporal dementia (FTD) are involved in microRNA biogenesis and extracellular vesicle (EV) formation. Certain microRNAs seem to participate in the metabolism of disease-related proteins. We hypothesized that microRNAs contained in EVs from CSF could reflect the underlying pathology and be potentially used as biomarkers for FTD and other related syndromes. Thus, we expected to detect differential microRNA profiles characteristic of each disorder.

We analyzed the microRNA expression levels using quantitative PCR panels containing highly-specific primers, in control individuals, AD patients, and patients diagnosed with FTD and other related syndromes.

Chapter 3

Publications

Copy Number Variation Analysis of the 17q21.31 Region and Its Role in Neurodegenerative Diseases

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The H1 haplotype of the 17q21.31 inversion polymorphism has been consistently associated with progressive supranuclear palsy, corticobasal degeneration, and Parkinson's disease in Caucasians. Recently, large polymorphic segmental duplications resulting into complex rearrangements at this locus with a high diversity range in human populations have been revealed. We sought to explore whether the two multi-allelic copy number variants that are present in the H1 clade (with segmental duplications of 300 and 218 kilo-bases in length) could be responsible for the known H1-related risk of developing these neurodegenerative disorders. A total of 857 Spanish subjects including 330 patients with Parkinson's disease, 96 with progressive supranuclear palsy, 55 with corticobasal degeneration, 51 dementia with Lewy bodies, and 325 neurologically healthy controls, were genotyped for the H1/H2 haplotype. Subsequently, the two copy number variants that are characteristic of the H1 haplotype were evaluated through a high-resolution approach based on droplet digital polymerase chain reaction, in all H1 homozygous subjects. The H1 allele was significantly overrepresented in all diagnostic groups compared with controls (Parkinson's disease, $P=0.0001$; progressive supranuclear palsy, $P=1.22 \times 10^{-6}$; corticobasal degeneration, $P=0.0002$; and dementia with Lewy bodies, $P=0.032$). However, no dosage differences were found for any of the two copy number variants analyzed. The H1 haplotype is associated with the risk of several neurodegenerative disorders, including dementia with Lewy bodies. However, common structural diversity within the 17q21.31-H1 clade does not explain this genetic association.

Introduction

Brain accumulation of hyperphosphorylated tau protein, encoded by the *MAPT* gene, is the main pathological hallmark of tauopathies, such as progressive supranuclear palsy (PSP) or corticobasal degeneration (CBD) [Sergeant et al., 1999]. These abnormal tau aggregates can also exist in other pathologies, such as dementia with Lewy bodies (DLB) and Parkinson's disease (PD); wherein the presence of α-synuclein soluble aggregates, mainly Lewy bodies and Lewy neurites, are the characteristic trait [Schneider et al., 2006; Moussaud et al., 2014; Ferencz and Gerritsen, 2015]. The human *MAPT* gene is located in the 17q21.31 chromosomal region, characterized by a 900 kilobases (Kb) inversion polymorphism originated about 3 million years ago. This inversion leads to the presence of two different haplotype blocks, H1 (direct) and H2 (inverse) [Stefansson et al., 2005; Zody et al., 2008] with no recombination between them and consequently giving rise to an extended (~1.7 megabases) region of linkage disequilibrium with independent evolutive events for the two haplotypes [Oliveira et al., 2004; Skipper et al., 2004; Pittman et al., 2005]. The H1 haplotype has been consistently associated with the risk of developing PSP, CBD, and PD in individuals with European ancestry [Baker et al., 1999; Houlden et al.,

2001; Maraganore et al., 2001; Martin et al., 2001; Farrer et al., 2002; Pastor et al., 2002, 2004; Healy et al., 2004; Webb et al., 2008; Goris et al., 2007; Zabetian et al., 2007; Simon-Sanchez et al., 2009; Ezquerra et al., 2011; Seto-Salvia et al., 2011; Spencer et al., 2011; Charlesworth et al., 2012; Desikan et al., 2015].

Recently, two studies aiming at disentangling the genetic architecture of the 17q21.31 non-recombining chromosomal region disclosed large copy number variants (CNV) with a high diversity range in humans [Boettger et al., 2012; Steinberg et al., 2012]. Specifically, three overlapping polymorphic duplications, distributed unequally in the H1 and H2 clades, were found: a 150 Kb duplication presented on the H2 haplotype (named α segment), a 300 Kb duplication on the H1 haplotype (named β), and a highly multi-allelic 218 Kb duplication (named γ) (**Fig. 1A**). Importantly, these CNVs contain several coding genes and segregate as nine common haplotypes with a high degree of population diversity [Boettger et al., 2012].

In order to determine whether chromosomal rearrangements within the H1 clade could have a role in the well-established association between this haplotype and the risk of PD, PSP, and CBD in Spanish population, we used a droplet-based approach to digital PCR (ddPCR) to accurately
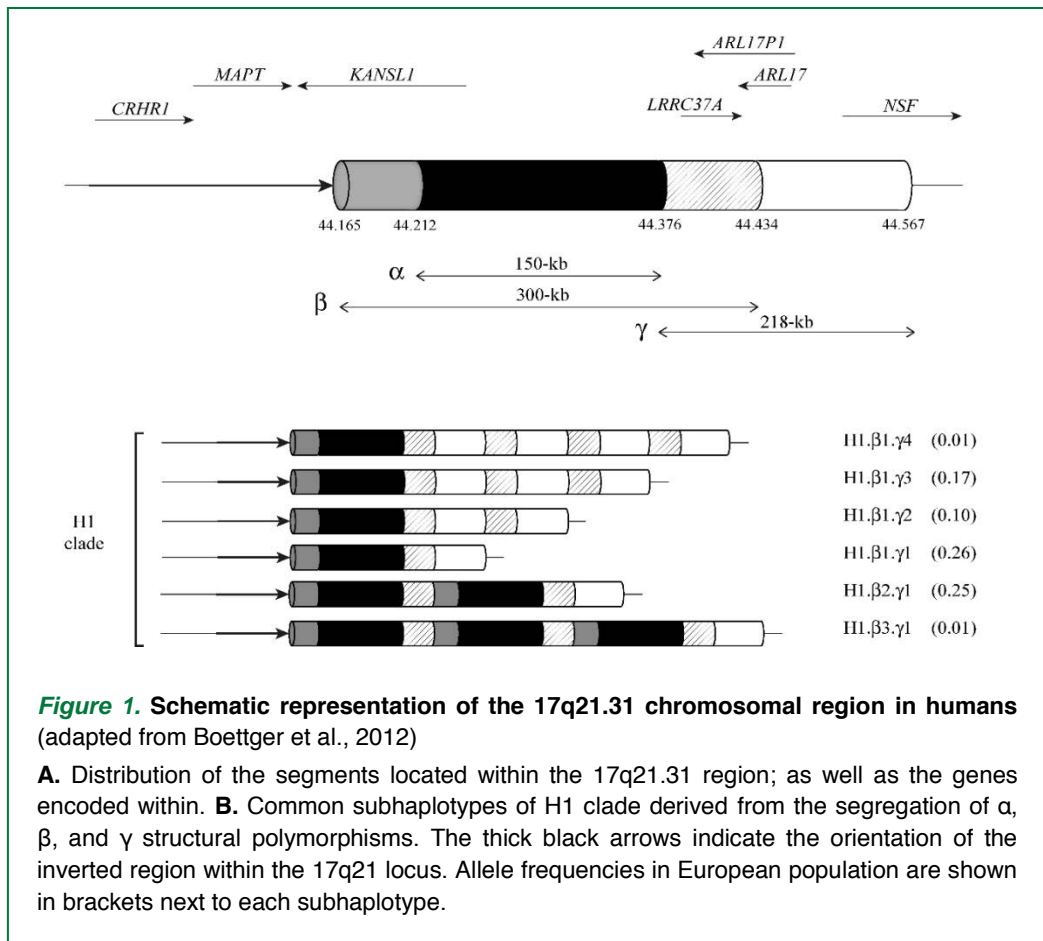
assess the absolute number of duplicated β and γ polymorphic segments that are characteristic of the H1 clade, in a subgroup of Spanish patients and controls homozygous for this haplotype. We also included DLB patients in order to expand the biologic relevance of the 17q21.31 inversion in this closely related neurodegenerative disorder.

Materials and methods

Subjects

A total of 857 Spanish subjects were recruited from the outpatient Movement Disorders and Memory Units at the Hospital Sant Pau, Barcelona, Spain; and the *Clínica Universidad de Navarra*, Pamplona, Spain. The patients were distributed within the following diagnostic groups: 330 with Parkinson's disease (PD, 55.4% males; mean age of disease onset = 69.4 ± 9.2 years), 96 with progressive supranuclear palsy (PSP, 45.8% males; mean age of disease onset = 66.8 ± 9 years), 55 with corticobasal degeneration (CBD, 47.3% males; mean age of disease onset = 67.0 ± 10 years), 51 with dementia with Lewy bodies (DLB, 55.8% males; mean age of disease onset = 72.4 ± 8 years), and 325 cognitively healthy unrelated controls (55.4% males; average

Figure 1. **Schematic representation of the 17q21.31 chromosomal region in humans** (adapted from Boettger et al., 2012)

A. Distribution of the segments located within the 17q21.31 region; as well as the genes encoded within. **B.** Common subhaplotypes of H1 clade derived from the segregation of α, β, and γ structural polymorphisms. The thick black arrows indicate the orientation of the inverted region within the 17q21 locus. Allele frequencies in European population are shown in brackets next to each subhaplotype.

age on the last clinical visit = 69.4 ± 9.2 years). All the subjects were examined by neurologists with expertise in neurodegenerative diseases, and fulfilled the diagnostic criteria described by Hughes et al. [1992] for idiopathic PD and the criteria by Litvan et al. [1996], Watts et al. [1997], and McKeith et al. [2005] for PSP, CBD, and DLB, respectively.

Among the patients with DLB, 11 of them (21.5%) had a neuropathological confirmation of the clinical diagnosis, in accordance with McKeith's recommendations [McKeith et al., 2005]. The average age at death of the postmortem cohort was 74.1 ± 6.2 years. Brain tissue specimens were provided by the Neurological Tissue Bank of the Biobanc-Hospital Clínic-IDIBAPS, Barcelona, Spain.

Control individuals had complete neurologic and medical examinations to exclude relevant illnesses. They underwent an extensive neuropsychological evaluation according to Sala et al. [2008], obtaining normal scores. All participants and their families provided written informed consent, and the study was approved by the respective ethics committees.

Genetic Analysis

Genomic DNA was isolated from whole blood samples using Flexigene DNA kit (Qiagen, Valencia, CA) and DNA from frozen brain tissue was isolated with QIAamp DNA Mini kit (Qiagen, Valencia, CA) following manufacturer's instructions.

All subjects were genotyped for the H1/H2 *MAPT* haplotype by testing for the presence of an intronic 238-base pair

deletion between exons 9 and 10, which is characteristic of the H2 haplotype [Baker et al., 1999; Setó-Salvia et al., 2011].

Gene dosage analyses of the β and γ segments were performed by means of Bio-Rad's QX100™ Droplet Digital™ PCR system [Hindson et al., 2011]. Previously to the PCR reaction, genomic DNA of each sample was digested with *HindIII-HF*, *BamHI-HF*, and *XbaI* enzymes (New England BioLabs, Ipswich, MA) to fit into the droplets. The digested product and primers and probes for the reference gene and the target DNA segments (β and γ) were first partitioned into ~20,000 water-in-oil droplets, using a disposable microfluidic cartridge and a vacuum source (Droplet Generator; Bio-Rad Laboratories, Hercules, CA). The resulting droplets were transferred into a 96-well PCR plate. After end-point amplification, the plate was loaded on the QX100™ Droplet Digital™ PCR System Droplet Reader (Bio-Rad Laboratories, Hercules, CA), which automatically aspirates the emulsion of each well and assigns droplets as positive or negative for each fluorophor according to the presence or absence of template [Hindson et al., 2011; Pinheiro et al., 2012]. The β and γ segments and the reference gene (*β-globin*) were detected using 6FAM™ and VIC® dye labeled gene-specific probes (Life Technologies, Carlsbad, CA) with the following sequences: 6FAM-AAAGCAA AAGGCCTGCCTATGCC-MGBNFQ (β segment); 6FAM-CACATGTGTTCTGG AATGCC-MGBNFQ (γ segment) [Boettger et al., 2012], and VIC-CTCATGGCAAGAAAGTGCTCGGTG C-MGBNFQ (*β-globin*). Absolute quantification of target DNA segments (β

and γ) as well as *β-globin* was performed with the Quantasoft software analysis (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

H1/H2 genotype and allele frequencies were estimated by direct counting and compared between patients, and controls by means of χ^2 analysis with one degree of freedom. The Kruskal–Wallis test was used to compare β and α CNV means between groups, as these variables did not follow a normal distribution. All data were analyzed using the Statistical Package for the Social Sciences Version 19 (SPSS Inc, Chicago, IL). Statistical significance was set at 5% (α = 0.05).

Results

A total of 532 patients diagnosed with PD, PSP, CBD or DLB, and 325 cognitively healthy controls were analyzed. Neither age nor gender frequencies differed between phenotypes. There was a significant association between the H1 allele and the risk of developing one of the neurodegenerative diseases: PD (OR, 1.666; *P*=0.0001); PSP (OR, 3.302; *P*=1.22 x 10^{-6}); CBD (OR, 3.326; *P*=0.0002), and DLB (OR, 1.813; *P*=0.032) groups (**Table I**). Homozygous H1H1 subjects were also overrepresented in all subgroups of patients compared to healthy controls (**Table I**).

	Controls (n=325)	PD (n=330)	PSP (n=96)	CBD (n=55)	DLB (n=51)
H1H1 (%)	181 (55.7)	227 (68.8)	83 (86.5)	45 (81.8)	39 (76.5)
H1H2 (%)	115 (35.4)	88 (26.7)	7 (7.3)	9 (16.4)	7 (13.7)
H2H2 (%)	29 (8.9)	15 (4.5)	6 (6.2)	1 (1.8)	5 (9.8)
Odds ratio [95% CI]	NA	1.753 [1.27-2.41]	5.079 [2.72-9.48]	3.580 [1.74-7.35]	2.586 [1.31-5.12]
P-value[a]	NA	0.001	4.33 x 10^{-8}	0.0003	0.005
H1 allele (%)	477 (73.4)	542 (82.1)	173 (90.1)	99 (90.0)	85 (83.3)
H2 allele (%)	173 (26.6)	118 (17.9)	19 (9.9)	11 (10.0)	17 (16.7)
Odds ratio [95% CI]	NA	1.666 [1.28-2.17]	3.302 [1.99-5.47]	3.264 [1.71-6.23]	1.813 [1.05-3.14]
P-value	NA	0.0001	1.22 x 10^{-6}	0.0002	0.032

PD, Parkinson's disease; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; DLB, dementia with Lewy bodies.
[a] Comparison between H1H1 and H1H2+H2H2 individuals.

Table I. **Demographic data and MAPT allele frequencies for the different diagnostic groups**

Figure 2. **Relative distribution of copy number variations (CNVs) for segments β and γ in H1H1 individuals across the five different diagnostic groups.**

Controls, n=181; Parkinson's disease (PD), n=227; progressive supranuclear palsy (PSP), n=83; corticobasal degeneration (CBD), n=45; and dementia with Lewy bodies (DLB), n=39.
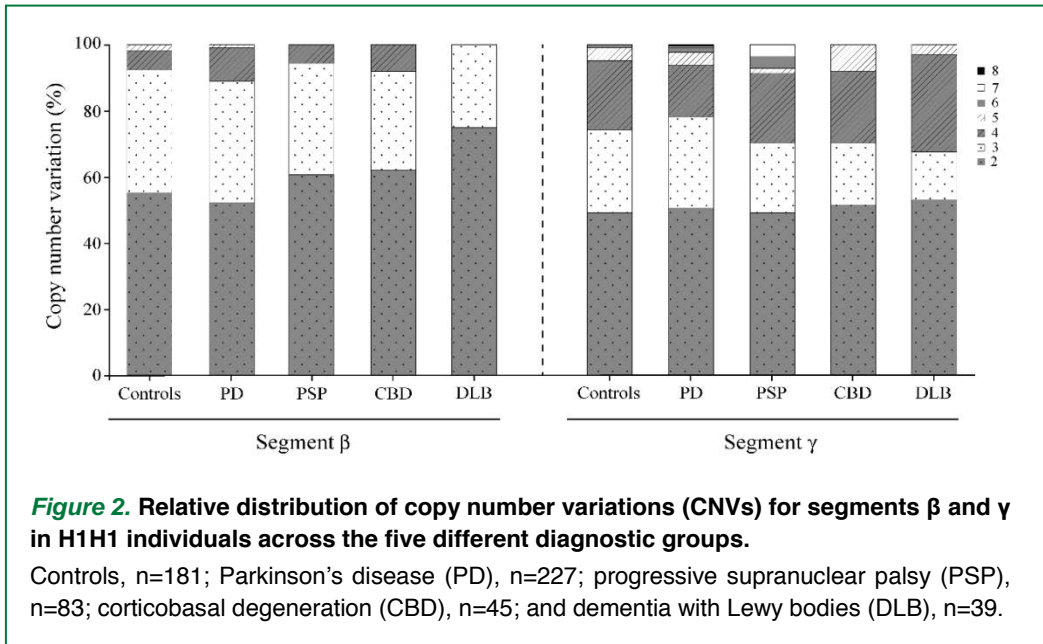
In order to determine whether structural features within the H1 clade could be underlying this robust association, we selected all H1 homozygous individuals for further analysis (PD, n = 227; PSP, n = 83; CBD, n = 45; DLB, n = 39; and Controls, n = 181). Droplet-Digital PCR-based absolute quantification of genetic dosages for the β and γ polymorphic segmental duplications in control individuals disclosed an average of 2.5 copies of the γ segment per subject (ranging from 2 to 5) and an average of 2.7 copies of the γ segment (ranging from 2 to 6). All patients from the four diagnostic groups presented similar CNV distributions. Interestingly, the highest numbers of γ segments were observed in patients with PD and PSP, with up

to seven and eight copies harbored by three subjects (**Fig. 2**).

Discussion

Although there is substantial data demonstrating a robust association between the 17q21.31 H1 haplotype and the risk of developing PD, PSP, and CBD in Europeans [Baker et al., 1999; Houlden et al., 2001; Maraganore et al., 2001; Martin et al., 2001; Farrer et al., 2002; Pastor et al., 2002, 2004; Healy et al., 2004; Pittman et al., 2005; Webb et al., 2008; Goris et al., 2007; Zabetian et al., 2007; Simon-Sanchez et al., 2009; Ezquerra et al., 2011; Setó-Salvia et al., 2011; Spencer et al., 2011; Charlesworth et al., 2012; Desikan et al., 2015], it is still unknown which genetic component within this megabase-long inversion

polymorphism is responsible for the H1 effect. The recent discovery of multiple chromosomal rearrangements within this complex dynamic region of the human genome prompted us to evaluate the possible contribution of the two well characterized segmental duplications that are present in the H1 haplotype (β and γ) in these neurodegenerative diseases, as well as in DLB. The fact that the European population presents a pronounced enrichment of duplicated β segments compared to any other human population group [Boettger et al., 2012] reinforced the possibility that gene dose variation within this unique genomic segment could influence the risk of these neurodegenerative diseases. Our results, however, showed similar copy number values for both β and γ regions between phenotypes, even though the individual values were distributed heterogeneously throughout the range of copies. Thus, a few PD patients and controls had up to five copies of the β segment; and some PSP and PD patients harbored up to seven or even eight copies of the g segment, with no controls presenting more than six copies (**Fig. 2**). These data suggest that gross copy number variation polymorphisms within the 17q21.31 inversion are not related to the H1 effect in PD, PSP, CBD, or DLB.

Interestingly, the H1 polymorphism was not only associated with PD, PSP, and CBD risk -as expected based on previous studies- but also with DLB, with an increased risk of 1.8-fold on average. Two previous studies, including ours, have been unable to disclose significant association between H1 *MAPT* haplotype and the risk of DLB [Setó-Salvia et al., 2011; Bras et al., 2014], despite *MAPT* gene has been found to play a role in the α-synuclein expression in different brain regions [Wider et al., 2012; Colom-Cadena et al., 2013]. In the present study, we have doubled the number of DLB patients compared to our previous analysis (51 vs. 24 patients), thus, increasing our power to detect possible associations between *MAPT* H1 allele and DLB risk. In the case of Bras et al. study (2014), in which 788 DLB cases and 2,624 controls were genotyped at 79,152 markers, none of the SNPs flanking the MAPT region surpassed the overall significance threshold of 3.7×10^{-5}. However, 315 of the 391 biallelic polymorphisms within and surrounding the *MAPT* gene presented a *P*-value below 0.05, and up to 295 SNPs (75%) showed a *P*-value <0.01. These data, together with our results, suggest that the H1 haplotype could influence the risk of DLB and warrants further studies in well-characterized DLB patients, and controls from the same European ancestry to assess the role of *MAPT* H1 in DLB risk.

The association of the H1 haplotype with either tauopathies like PSP and CBD, and α-

synucleinopathies like PD and DLB is intriguing. Previous studies have shown synergistic effects between amyloid, Tau and α-synuclein deposition in different neurodegenerative diseases [Pittman et al., 2006; Colom-Cadena et al., 2013; Moussaud et al., 2014]. *MAPT* H1 haplotype is associated with a higher burden of hyperphosphorylated Tau in cortical and subcortical regions [Colom-Cadena et al., 2013], and both PD and DLB are characterized also by an increased deposition of Tau in the same areas. The association of PD and DLB could be then explained by a reciprocal synergistic effect, so that *MAPT* H1 mutations, by increasing Tau pathology, could promote α-synuclein accumulation, leading to PD and/or DLB.

In summary, we observe an association between the H1 allele and the risk of PD, PSP, CBD, and DLB, thus, expanding the biological relevance of the H1 haplotype in neurodegenerative disorders. Notwithstanding, since the number of case series for the DLB phenotype is limited, caution should be taken when interpreting the results and further studies are warranted to disentangle the role of *MAPT* H1 allele in this neurodegenerative disorder. Our data also suggest that gross structural polymorphisms within the 17q21.31 are not related to these phenotypes.

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Cerebrospinal fluid mitochondrial DNA in the Alzheimer's disease continuum

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Low levels of cell-free mitochondrial DNA (mtDNA) in the cerebrospinal fluid (CSF) of Alzheimer's disease (AD) patients have been identified and proposed as a novel biomarker for the disease. The lack of validation studies of previous results prompted us to replicate this finding in a comprehensive series of patients and controls. We applied droplet digital polymerase chain reaction in CSF specimens from 124 patients representing the AD spectrum and 140 neurologically healthy controls. The following pre-analytical and analytical parameters were evaluated: the effect of freeze-thaw cycles on mtDNA, the linearity of mtDNA load across serial dilutions, and the mtDNA levels in the diagnostic groups. We found a wide range of mtDNA copies, which resulted in a high degree of overlap between groups. Although the AD group presented significantly higher mtDNA counts, the receiver-operating characteristic analysis disclosed an area under the curve of 0.715 to distinguish AD patients from controls. MtDNA was highly stable with low analytical variability. In conclusion, mtDNA levels in CSF show a high interindividual variability, with great overlap within phenotypes and presents low sensitivity for AD.

Introduction

Cerebrospinal fluid (CSF) biomarkers reflect normal and pathophysiological events in the brain. They have become an important tool in diagnosis, prognosis, and staging of neurodegenerative disorders. In Alzheimer's disease (AD), 3 well-established biomarkers are currently used: amyloid-$\beta_{1\text{-}42}$ ($A\beta_{42}$), correlating with the amyloid pathology; total-tau (t-tau), and phospho-tau (p-tau), indicating the presence of neurofibrillary tangles and neuronal damage [Lleó et al., 2015].

Recently, a study comprising 48 sporadic AD patients and 36 controls described a decrease of cell-free circulating mitochondrial DNA (mtDNA) content in CSF in the AD group [Podlesniy et al., 2013]. Reduced mtDNA levels were also found in the same study in subjects at risk to develop AD (without cognitive deficits but low CSF $A\beta_{42}$ levels) and presymptomatic subjects carrying pathogenic mutations in the *Presenilin 1* gene, thus suggesting that the decrease of mtDNA content is an early phenomenon in AD. This reduction was not detected in individuals with frontotemporal dementia and has not been found in an independent study by the same group involving sporadic Creutzfeldt-Jakob disease patients [Podlesniy et al., 2016a]. Altogether, these studies suggested that CSF mtDNA content alterations in CSF could be specific to AD and proposed

mtDNA as a novel biomarker for the disease. However, a recent investigation has revealed a decreased content of mtDNA in Parkinson's disease patients compared with controls [Pyle et al., 2015]. Hence, it is still unclear whether changes in mtDNA are a characteristic feature in AD or a common hallmark in several neurodegenerative disorders.

The aim of the present study was to assess the changes of CSF mtDNA in a comprehensive series of patients at different stages of the AD continuum through a highly sensitive, absolute quantification of mtDNA molecules on a droplet-based digital polymerase chain reaction (ddPCR). Moreover, we sought to evaluate its accuracy as a diagnostic biomarker in the AD continuum.

Materials and methods

Subjects

A total of 264 individuals were recruited from the Memory Unit at the Hospital Sant Pau (Barcelona) and the CITA Alzheimer center (San Sebastián). All subjects were examined by neurologists with expertize in neurodegenerative disorders and had an extensive neuropsychological evaluation [Sala et al., 2008]. Lumbar puncture was performed to obtain CSF samples, following international recommendations [Alcolea et al., 2014; Del Campo et al., 2012; Mattsson et al., 2013]; and $A\beta_{42}$, t-tau, and p-tau levels were determined using ELISA as previously described [Alcolea et al., 2015].

Based on the previously published cut-offs for $A\beta_{42}$, t-tau, and p-tau [Alcolea et al.,

2015], participants were classified according to the National Institute on Aging-Alzheimer's Association (NIA-AA) criteria [Albert et al., 2011; McKhann et al., 2011; Sperling et al., 2011] into the following groups (**Table 1**): 140 controls (cognitively healthy; Aβ42 550 pg/mL, t-tau 350 pg/mL, and p-tau 61 pg/mL); 20 preclinical AD (14 at stage 1 [cognitively healthy; Aβ42 <550 pg/mL, t-tau 350 pg/mL, and p-tau 61 pg/mL] and 6 at stage 2 [cognitively healthy; Aβ42 <550 pg/mL and either t-tau >350 pg/mL or p-tau >61 pg/mL]); 43 mild cognitive impairment (MCI) due to AD with high likelihood; and 59 subjects with

AD dementia with evidence of the AD pathophysiological process.

The respective ethics committees approved the study and all participants or their relatives provided written informed consent.

Genetic Analysis

Genomic DNA was isolated from whole blood samples with FlexiGene DNA Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. APOE genotype was determined as previously described [Calero et al., 2009; Guardia-Laguarta et al., 2010]. *SUCLG2*

	Controls (n=140)	Preclinical AD (n=20)	MCI (n=43)	AD (n=59)
Age[a], y	56.2 ± 4.6	62.7 ± 6.1	71.7 ± 4.4	72.5 ± 6.2
Female, %	60.7	60.0	67.4	52.5
MMSE[a]	29 ± 1.0	29 ± 1.0	27 ± 2.0	22 ± 2.0
APOE-ε4 allele %	20.7	65.0	70.7	54.2
CSF Aβ42 (pg/mL)[a]	841.8 ± 132.4	487.3 ± 45.7	463.5 ± 37.5	398.0 ± 84.0
CSF t-tau (pg/mL)[a]	207.8 ± 46.8	181.5 ± 98.0	631.5 ± 151.0	759.0 ± 284.0
CSF p-tau (pg/mL)[a]	39.8 ± 7.7	40.0 ± 17.8	90.5 ± 19.0	96.0 ± 19.5
CSF t-tau/Aβ42 ratio[a]	0.2 ± 0.0	0.4 ± 0.3	1.3 ± 0.3	2.0 ± 0.8
CSF mtDNA[a]	12.0 ± 5.2	17.3 ± 8.4	18.2 ± 8.6	26.1 ± 17.5

[a]Data expressed as median ± median absolute deviation.

AD, Alzheimer's disease; MCI, mild cognitive impairment; MMSE, mini-mental state examination; CSF, cerebrospinal fluid; Aβ42, amyloid-β1-42; t-tau, total-tau; p-tau, phospho-tau; mtDNA, mitochondrial DNA.

Table 1. Demographic and clinical data of the different diagnostic groups

(rs62256978) genotype was determined by Sanger sequencing in a subset of 178 DNA samples, including all diagnostic groups. The following primers were used: 5'-AGAGTCTGAAGGGCAGTTGG-3' (forward primer) and 5'-GCCCATCTTGTT TCCATCAC-3' (reverse primer).

Mitochondrial DNA analysis

Absolute quantification of mtDNA copies was performed by means of Bio-Rad's QX200 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, the CSF samples together with the reagents were partitioned into ~20,000 water-in-oil droplets, using a disposable microfluidic cartridge and the Droplet Generator as a vacuum source. The droplets were transferred into a plate, followed by end-point amplification. Afterwards, the plate was loaded into the Droplet Reader, which quantifies the positive and negatives droplets for the specific fluorophore, depending on the presence or absence of template [Hindson et al., 2011].

An 85 base-pairs region of the human mtDNA was detected using a 6FAM dye-labeled probe (Sigma-Aldrich, Saint Louis, MO, USA) and the corresponding pair of primers (Isogen, De Meern, Netherlands), as previously described [Podlesniy et al., 2013]. To identify the presence of blood cells in our CSF samples, which could result in altered values of mtDNA copies, we targeted the apoptosis-related BAX gene with an HEX dye-labeled probe (Sigma-Aldrich, Saint Louis, MO, USA) [Oltvai et al., 1993], and the respective primers (Isogen, De Meern, Netherlands) [Podlesniy et al., 2016a,b]. Samples with 1

copy of BAX gene were excluded from further analysis, as the presence of one cell would be a source of potential mtDNA contamination. Probes and primer sequences are detailed in **Supplementary Methods**.

The absolute number of copies of mtDNA in each CSF sample was quantified using the Quantasoft software analysis (Bio-Rad Laboratories, Hercules, CA).

Moreover, freeze-thaw cycles and dilution assays were per-formed to evaluate sample stability and linearity. Further details are provided in **Supplementary Methods**.

The CSF samples were stored at 80°C and new aliquots were used in all the proceedings, except the freeze-thaw cycles assay.

Statistical Analysis

All statistical analyses were performed using the statistical software GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). As CSF mtDNA levels did not follow normal distribution, we used nonparametric tests in all analyses. Kruskal-Wallis test with Dunn's multiple comparisons was applied for comparisons between diagnostic groups and the evaluation of possible family history influence. The Mann-Whitney test was used to compare mtDNA values between genders and APOE-ε4 statuses. Statistical significance was set at 5% ($\alpha = 0.05$). Receiver-operating characteristic (ROC) curve analysis was performed to determine the discriminative ability of mtDNA as a biomarker.

Results

Among the initial cohort of 264 CSF samples, 2 specimens (1 from a patient with MCI and another from a patient with AD) presented more than 1 copy of BAX gene and were therefore excluded from further analyses.

First, we characterized the sample stability and linearity of the ddPCR-based assay on cell-free mtDNA from a randomized subset of CSF samples. Freeze-thaw cycles and dilution assays showed that the assay was not affected by up to 3 successive freeze-thaw cycles, and that the decrease in mtDNA counts followed a linear trend across 4 2-fold serial dilutions (**Supplementary Fig. 1**). Inter- and intra-assay variations were

below 7%. These data indicate that the assay is fairly stable and reliable.

Next, we used CSF specimens from controls to assess whether mtDNA loads could be affected by sex, age, family history of dementia, or APOE-ε4 status (**Supplementary Fig. 2**). Our analyses proved that none of these factors influenced mtDNA levels. In addition, Ab_{42}, t-tau, and p-tau levels did not correlate with mtDNA counts (**Supplementary Fig. 3**). These same results were found across the 3 groups representing the AD continuum (**Supplementary Fig. 2**). The presence of rs62256378-A variant within *SUCLG2* was also analyzed as a potential modifier of mtDNA levels (Ramirez et al., 2014); however,
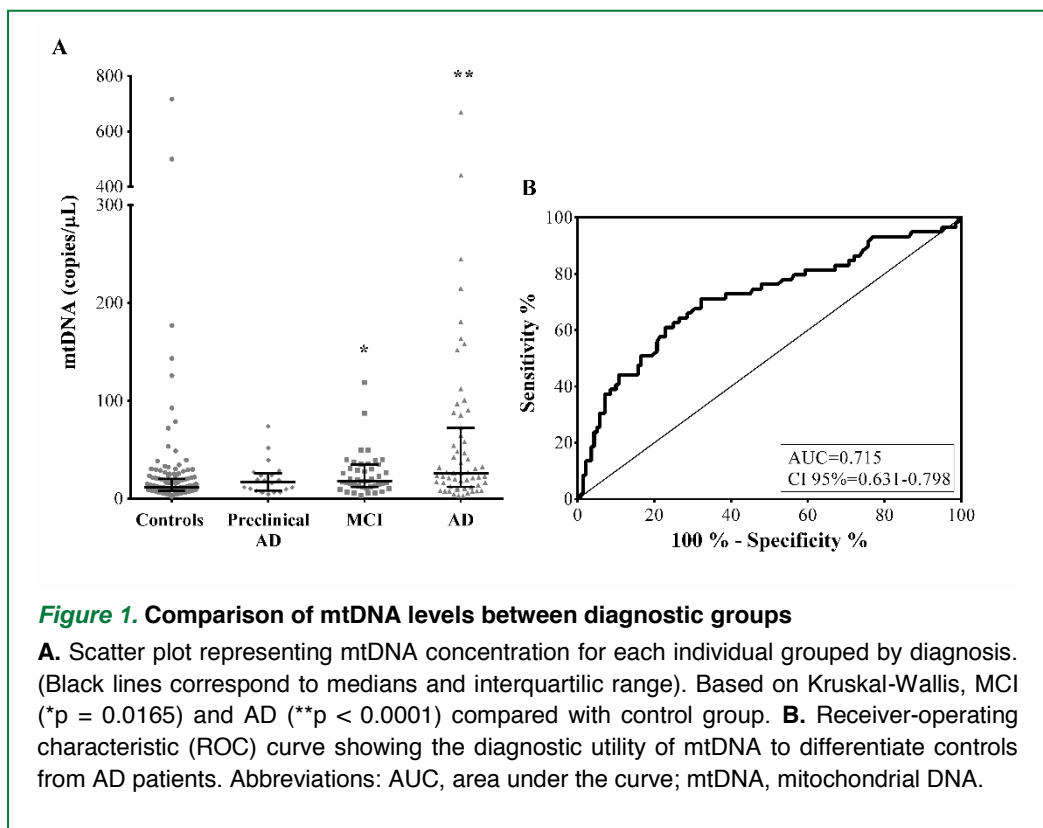

A. Scatter plot representing mtDNA concentration for each individual grouped by diagnosis. (Black lines correspond to medians and interquartilic range). Based on Kruskal-Wallis, MCI (*p = 0.0165) and AD (**p < 0.0001) compared with control group. **B.** Receiver-operating characteristic (ROC) curve showing the diagnostic utility of mtDNA to differentiate controls from AD patients. Abbreviations: AUC, area under the curve; mtDNA, mitochondrial DNA.

Figure 1. **Comparison of mtDNA levels between diagnostic groups**

SUCLG2 genotype did not influence mtDNA in our cohort.

The comparison of mtDNA levels between patients and controls disclosed an average of 12.0 copies of mtDNA/mL in controls, 17.3 in preclinical AD individuals, 18.2 in patients with MCI and 26.1 in AD patients (**Table 1**). Importantly, mtDNA counts ranged widely within groups leading to a high degree of overlap between them (**Fig. 1A**). Finally, the ability of mtDNA to discriminate controls from AD patients was evaluated with a ROC curve analysis, showing an area under the curve (AUC) of 0.715 (**Fig. 1B**).

Discussion

Recent studies reporting a decreased amount of cell-free circulating mtDNA in the CSF of AD patients have recovered the view of mitochondrial perturbations as a relevant player of the disease and have suggested an underlying mitochondrial impairment that precedes the symptomatic stages of this dementing illness (Podlesniy et al., 2013, 2016a). Making use of a large series of well-characterized participants with core AD biomarkers; we investigated the changes of mtDNA load alongside the AD spec-trum. Unexpectedly, we failed to replicate previous studies. In fact, we found an increase of mtDNA levels in AD patients compared with healthy control subjects. The high-throughput ddPCR approach allowed us to quantify with high precision the absolute amount of mtDNA present in a sample. Despite low intra- and interassay coefficients of variation, mtDNA levels showed a wide dispersion among individuals of the same diagnostic group, ranging from 2.3 to 717.4 copies/mL of CSF. This high degree of interindividual variability led to a great overlap in mtDNA levels between controls and patients. Since low sample sizes are prone to show differences between groups in variables with widely dispersed values, large sample sizes are needed to overcome this effect. Therefore, a possible reason to explain the discrepancies between our and preceding results could be related to the smaller sample sizes used in previous studies.

The *APOE*-ε4 allele is a well-known genetic risk factor in AD (Strittmatter et al., 1993), and it has been suggested to influence AD-related biomarkers levels (Alcolea et al., 2015; Toledo et al., 2015). Family history of dementia is also an important risk factor for sporadic AD (Scarabino et al., 2016), and it has been suggested that direct maternal lineage has a stronger influence than paternal (Honea et al., 2012), indicating that changes in mtDNA (which is inherited from the mother only) could be associated with this fac-tor. Our data suggest that neither the *APOE* genotype nor a positive family history of dementia (regardless of the maternal or paternal lineage) have any effect on mtDNA levels. Furthermore, pre-analytical variables, assessed through a series of freeze-thaw cycles and serial dilutions, showed no effect on mtDNA values.

A recent genome-wide association study on CSF biomarkers reported an association of a single-nucleotide polymorphism (rs62256378) in the *SUCLG2* gene with Ab_{42} levels (Ramirez et al., 2014). Besides, mutations in this gene have been previously related to mtDNA depletion syndrome (Miller et al., 2011; Ostergaard, 2008). To explore whether this variant could explain some of the interin-dividual variability that is present in our study, we genotyped this polymorphism in a subset of samples (n=178). Our analyses do not show any strong effect of the *SUCLG2*-A variant in mtDNA levels. However, caution should be taken when interpreting these data since the overall number of samples assessed is limited.

In summary, our analyses indicate that mtDNA in CSF is a stable and highly reproducible biomarker that can be evaluated rapidly and repetitively. However, we failed to replicate previous data. Importantly, our results show a significant degree of interindividual variability within and between phenotypes, which could explain the observed differences between studies. Although the development of a biomarker able to detect mitochondrial dysfunction is very attractive, individual differences of mtDNA limit its use as a diagnostic biomarker, as reflected in our ROC analysis. Nonetheless, more studies in independent cohorts are needed to assess the possible role of CSF mtDNA levels as a biomarker of the mitochondrial mechanisms involved in the etiology of AD and other neurodegenerative disorders.

Acknowledgments

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Supplementary methods

1. Primer sequences

The probes and primers designed to detect the human mtDNA were: FAM-CCTCCAAATCACCACAGGACTATTCCTAGCCATGCA-BHQ1 (probe), 5'-CTCACTCCTTGGCGCCTGCC-3' (forward primer) and 5'-GGCGGTTGAGGCGTCTGGTG-3' (reverse primer) (Podlesniy et al., 2013).

The BAX gene was targeted using the following oligos: HEX-CCCGAGCTGGCCCTGGACCCGGT-BHQ1 (probe), 5'-TTCATCCAGGATCGAGCAGG-3' (forward primer) and 5'-TGAGACACTCGCTCAGCTTC-3' (reverse primer) (Podlesniy et al., 2016).

2. Freeze-thaw cycles

A randomized subset of samples with different diagnosis (n=5) was used to characterize the mtDNA profile. We performed a four-day experiment where each sample was thawed, measured and frozen once a day, up to four times. All samples were quantified following the protocol described in Methods (Section 2.3.).

3. Dilution assay

New aliquots of the previous subset samples underwent a dilution test, ranging from 1:1 to 1:256. The quantification was performed as previously described (Methods, Section 2.3.).

Supplementary data

Supplementary figure 1. **Characterization of mtDNA biological profile**

Unless otherwise specified, data are expressed as median and interquartile range. **A.**Quantification of mtDNA variation after 1 to 4 freeze-thaw cycles. A subset of 5 random samples was selected for the profiling analysis. Each line corresponds to 1 different sample. Mitochondrial DNA showed no significant variability up to the third freeze-thaw cycle. However, some samples presented significant differences between the fourth and the previous cycles. ∗p < 0.05, based on Kruskal-Wallis. **B.** Example of one of the serial dilution assays, showing a linear trend up to the fourth 2-fold serial dilution. Abbreviation: mtDNA, mitochondrial DNA.

***Supplementary figure 2.* Relationship of mtDNA levels with the clinical and genetic characteristics of the cohort**

Unless otherwise specified, data are expressed as median and interquartile range. **A.**Correlation between mtDNA levels (copies/µL) and age in control subjects. **B.** Comparison of mtDNA levels (copies/µL) in males (M) and females (F) of the different study groups. **C.**Analysis of APOE-ε4 influence in mtDNA levels. For each diagnostic group, individuals were classified in APOE-ε4 carriers (ε4+) and noncarriers (ε4–). **D.** Influence of the family history in the mtDNA values. Within the 4 diagnostics, subjects were classified in different subgroups according to the presence and origin of family history of neurodegenerative dementia. ‡Only 1 individual with these characteristics, excluded from the statistical analysis. Abbreviations: AD, Alzheimer disease; MCI, mild cognitive impairment; mtDNA, mitochondrial DNA.

A

B

C

Supplementary figure 3. **Relationship between mtDNA and core AD CSF biomarkers**
Correlation of mtDNA levels and amyloid-β_{1-42} ($A\beta_{42}$), total-tau (t-tau), and phospho-tau (p-tau) in healthy control subjects. Abbreviations: AD, Alzheimer disease; CSF, cerebrospinal fluid; mtDNA, mitochondrial DNA.

Altered microRNAs in extracellular vesicles from CSF as biomarkers for 4R-tauopathies

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Recent studies highlight the importance of microRNAs, involved in the modulation of gene expression, in the pathophysiology of neurodegenerative disorders. Extracellular vesicles (EVs), present in biofluids and containing microRNAs, could act as intermediates in intracellular communication and target signaling pathways related to these diseases.

We hypothesize that microRNAs within EVs are altered in frontotemporal dementia and other related syndromes, and could be detected in cerebrospinal fluid (CSF). This study aims at identifying microRNAs in EVs derived from CSF that could be useful as diagnostic biomarkers for this spectrum of disorders.

We examined the microRNA expression profile, using panels containing microRNA LNA primers, in a total of 144 individuals – including cognitively healthy controls, AD patients and patients with frontotemporal dementia or other related tauopathies.

The use of highly sensitive techniques allows the reliable detection of microRNAs in EVs purified from CSF. We found four microRNAs (miR15b-5p, miR-146a-5p, miR-361-5p and miR-708-3p) differentially expressed in patients diagnosed with 4R-tauopathies (PSP and CBS). This specific molecular signature reflects their potential use as biomarkers for these disorders.

Introduction

Frontotemporal lobar degeneration (FTLD) is a pathological term that encompasses a clinically, pathologically and genetically heterogeneous group of disorders, with frontal and temporal lobe degeneration. FTLD usually manifests as frontotemporal dementia (FTD), the second most common cause of dementia before 65 years of age, which is mainly characterized by behavior and/or language impairment (McKhann et al., 2001; Rosso et al., 2003). Some variants of FTD overlap with parkinsonian (progressive supranuclear palsy – PSP or corticobasal syndrome – CBS) or motor neuron (amyotrophic lateral sclerosis – ALS) disorders (Lomen-Hoerth et al., 2002; Strong et al., 2003; Irwin, 2016; Kovacs, 2016; Höglinger et al., 2018). Many efforts are focused on finding reliable CSF biomarkers to support early and differential diagnosis for FTD and other related syndromes (Oeckl et al., 2015; Abu-Rumeileh et al., 2018; Illán-Gala et al., 2018; Steinacker et al., 2018; Alcolea et al., 2019; Foiani et al., 2019), but they are not yet available for use in clinical settings.

Several causal genes and proteins linked to FTD pathology appear to be involved in microRNA (miRNA) metabolism and formation of extracellular vesicles (EVs) (Kawahara and Mieda-Sato, 2012; Morlando et al., 2012; Zhang et al., 2013; Gascon et al., 2014; Piscopo et al., 2016). Conversely, miRNAs have been implicated in the metabolism regulation of disease-related proteins, such as tau or progranulin (Hébert et al., 2012; Piscopo et al., 2016; El Fatimy et al., 2018). MiRNAs represent a group of small non-coding RNAs (18-25 nucleotides in length) that modulate translation and stability of messenger RNA, ultimately regulating gene expression (Griffiths-Jones et al., 2006). They can be detected in peripheral fluids, such as cerebrospinal fluid (CSF), in either cell-free form or encapsulated within EVs. Exosomes are a class of EVs of endosomal origin, ranging from 50-150nm (Van Niel et al., 2018). The exosome cargoes include proteins, DNA and miRNAs among others. They are now considered a mechanism of intercellular communication, as they can be released to the extracellular milieu and are present in biofluids, allowing cells to exchange proteins, lipids and genetic material (Van Niel et al., 2018). Hence, they could become an ideal starting material to study alterations in the expression of miRNAs in neurodegenerative disorders.

In light of this previous knowledge, we hypothesized that expression of miRNAs may be altered in the central nervous system, and participate in the pathophysiological processes of FTD and other related syndromes. The aim of the present study was to identify miRNAs within EVs derived from CSF that could indicate the underlying pathology and serve as potential biomarkers for these disorders.

Materials and methods

Study samples

A total of 144 individuals were recruited from the Memory and Neuromuscular Units at the Hospital Sant Pau (Barcelona) and the Movement

Disorders Unit at the Hospital Clínic (Barcelona). Participants were thoroughly examined by neurologists with expertise in neurodegenerative disorders, and lumbar puncture was performed to obtain cerebrospinal fluid (CSF) samples (Del Campo et al., 2012; Mattsson et al., 2013; Alcolea et al., 2014). Participants were classified according to their clinical diagnosis into the following study groups [**Table 1**]: 30 cognitively healthy controls, without evidence of pathophysiological changes related to Alzheimer's disease (AD); 20 patients diagnosed with dementia due to AD, with pathophysiological evidences (McKhann et al., 2011); 28 subjects with the behavioral variant of frontotemporal dementia (Rascovsky et al., 2011), and therefore with a high likelihood of either TDP43 or Tau pathology (TDP43/Tau group); 32 subjects with high likelihood of TDP43 pathology (TDP43 group; including 9 patients with the semantic variant primary progressive aphasia (Gorno-Tempini et al., 2011), 12 with amyotrophic lateral sclerosis (Brooks et al., 2000), and 11 with amyotrophic

lateral sclerosis and concomitant FTD (Strong et al., 2017)) and 34 subjects with high likelihood of Tau pathology (Tau group; including 31 patients with either progressive supranuclear palsy (Höglinger et al., 2017) or corticobasal syndrome (Armstrong et al., 2013), and 3 with non-fluent variant primary progressive aphasia (Gorno-Tempini et al., 2011)). $A\beta_{1-42}$, total tau (T-tau) and phosphorylated tau (P-tau) levels were determined using the LUMIPULSE G system as previously described (Alcolea et al., 2018).

Participants were genetically screened for the presence of *C9orf72* hexanucleoride expansion, as well as other mutations in disease-related genes such as *GRN* or *MAPT*. None of the patients included in the study carried these mutations. Diagnostic groups showed no significant differences in gender or age.

Extracellular vesicle and miRNA isolation

Extracellular vesicle fraction was enriched using ExoQuick-TC reagent

	Controls	AD	TDP43/Tau	TDP43	Tau
N	30	20	28	32	34
Clinical diagnoses included	Cognitively healthy subjects	AD	bvFTD	svPPA ALS ALD-FTD	PSP CBS nfvPPA
Age at CSF sampling, y[a]	68.4 ± 6.3	72.2 ± 8.4	67.5 ± 10.6	70.2 ± 7.8	70.8 ± 8.6
Female, %	40.0	35.0	25.0	34.4	55.9
AD biomarkers[b]	Negative	Positive	Negative	Negative	Negative

[a] Data expressed as mean ± SD. [b] AD biomarkers: $A\beta_{1-42}$; T-tau and P-tau. Positive or negative status was given according to reported cut-offs (Alcolea, 2018).
Abbreviations: ALS, amyotrophic lateral sclerosis; bvFTD, behavioral variant FTD; CBS, corticobasal syndrome; non-fluent variant primary progressive aphasia; PSP, progressive supranuclear palsy; svPPA, semantic variant primary progressive aphasia.

***Table 1.* Demographic and clinical data of the different diagnostic groups**

(System Biosciences, Palo Alto, CA, USA) and microRNAs isolated with the SeraMir Exosome RNA Purification Column kit (System Biosciences, Palo Alto, CA, USA). The first steps of the protocol were optimized for small input volumes: 250μL of CSF were diluted with DEPC-treated PBS to a total volume of 1mL; then 333μL of ExoQuick-TC were added and mixed by inversion. The mixture was incubated overnight (~16h) at 4°C. EV precipitation and RNA isolation were thereafter completed following the manufacturer's instructions. In a subset of samples, three synthetic "spike-ins" (Unisp2, Unisp4 and Unisp5) were introduced during RNA extraction, to later monitor their performance by qPCR.

Detection and analysis of miRNAs

RNA was reverse transcribed using the miRCURY LNA RT kit (Qiagen, Germantown, MD, USA), and the resulting cDNA was diluted according to the manufacturer's instructions. Synthetic RNA sequences (Unisp6 spike-in and cel-miR-39) were added prior to RNA retrotranscription to cDNA, in order to assess the reaction efficiency.

The obtained cDNA was assayed in a 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA), using miRCURY LNA SYBR Green reagent (Qiagen, Germantown, MD, USA) and oligonucleotides containing Locked Nucleic Acids (LNA). The human panel I+II (miRCURY LNA miRNA miRNome PCR panel; Qiagen, Germantown, MD, USA), containing 752 human pre-aliquoted primer sets, was used in a subset of samples, to profile the miRNAs present in EVs from CSF. A custom panel with 13 selected miRNA primer sets was used for further analyses.

Prior to the analysis, we established a detection threshold for qPCR of 38 cycles. For miRNA expression, normalized relative quantities (NRQs) were calculated as previously described (Marabita et al., 2016). A synthetic RNA (cel-miR-39) was used for technical normalization: Relative quantities (RQ) = $2^{-\Delta(Cq\ miRNA\ -\ Cq\ cel-miR-39)}$. Since there are no universal normalizers for miRNAs contained in the EV-enriched fraction of CSF the normalization factor (NF) was obtained from the geometric mean of the three more stable endogenous miRNAs (miR-30b-5p, miR-30e-5p and miR-320a, **Suppl. Fig.2b**) by three different methods: geNorm, Normfinder, and CV score. NRQs were then calculated for each miRNA and sample using the following formula: NRQs = RQ/NF.

Bead-based flow cytometry

The precipitated fraction of our samples, containing EVs, was analyzed using bead-based flow cytometry as previously described (Lozano-Ramos et al., 2015; Gámez-Valero et al., 2016). Briefly, 50μL of each fraction were mixed with 0.2μL of aldehyde/sulphate 4μm latex beads (Invitrogen, Carlsbad, CA, USA) and incubated for 15 min. This mix was then re-suspended in 1mL of bead-coupling buffer (BCB; PBS supplemented with 0.1% BSA and 0.01% NaN_3; Sigma Aldrich, Saint Louis, MO, USA) and incubated overnight on rotation. EV-coated beads were centrifuged 10 min at 2,000g, washed with BCB and centrifuged again with the same settings. EV-coated beads were labelled at 4°C for 30 minutes with four specific antibodies: anti-CD9 (Clone VJ1/20), anti-CD63 (Clone TEA 3/18), and anti-CD81 (Clone #G0709; Santa Cruz Biotechnology, Dallas, TX, USA) or polyclonal IgG isotype (Abcam, Cambridge, UK). After a BCB wash, EV-coated beads were incubated for 30 minutes with FITC-conjugated secondary goat anti-mouse (Southern Biotech, Birmingham, AL, USA) and washed twice with BCB. Samples were then analyzed by flow cytometry (FacsVerse; BD Biosciences, San Jose, CA, USA) using FloJo Software (TreeStar, Ashand, OR,

USA). For each sample and marker, a total of 10,000 beads were measured and median fluorescence intensity (MFI) was calculated.

Cryo Transmission Electron Microscopy

Cryo Transmission Electron Microscopy (Cryo-TEM) was performed on EV-enriched fractions of a subset of samples. Specimens were vitrified by placing 3μL of each sample on a Quantifoil® 1.2/1.3 TEM grid (Quantifoil, Großlöbichau, Germany), blotted to a thin film and plunged into liquid ethane-nitrogen in the Leica EM CPC cryo-workstation. Grids were transferred to a 626 Gatan cryoholder maintained at -179°C, and then analyzed with a Jeol 2011 TEM, operating at an accelerating voltage of 200kV. Images were obtained with Gatan Ultrascan 2000 cooled charge-coupled device camera and Digital micrograph software package (Gatan, Pleasanton, CA, USA).

Statistical analyses

Differences in gender and age between diagnostic groups and controls were assessed using Fisher's exact test and unpaired two-sample t-test, respectively. Determination of the most stable endogenous miRNAs for normalization was performed using "NormqPCR" package in R statistical software (v 3.3.3). All statistical analyses post-normalization were performed using the statistical software GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). The non-parametric Kruskal-Wallis test with Dunn's post-hoc comparisons was applied to assess differences between diagnostic groups. Adjusted statistical significance was set at 5% ($\alpha = 0.05$). We performed receiver operating characteristic (ROC) analysis for significant miRNAs combinations to calculate areas under the curve (AUC).

Results

Characterization of Extracellular Vesicles

A subset of CSF samples was used for characterization of EVs by flow cytometry and electron microscopy.

Bead-based flow cytometry allowed us to determine the presence in our samples of three classical exosome markers: tetraspanins CD9, CD63 and CD81 (Van Niel et al., 2018). They could all be detected in the EV enriched fraction but neither in the remaining supernatant of the centrifugation nor in the bead control (**Suppl. Fig 1A**).

Cryo-transmission electron microscopy was used to examine the morphology and size of our vesicles. Our samples contained mostly round-shaped vesicles with an average size of 101.2 nm (**Suppl. Fig 1B**).

Therefore, both surface markers and morphology analyses showed an enrichment of exosomes in our EV fractions purified from CSF. However, we will still refer to them as extracellular vesicles (EVs), due to the imperfection of the purification methods, which could also co-precipitate certain amounts of other vesicles, such as microvesicles.

MiRNAs expressed in EVs derived from CSF

In order to determine the reliability of our method we used the human panel I+II to evaluate the expression profile of 752 miRNAs in a subset of 10 specimens from cognitively healthy subjects. This panel includes spike-ins to monitor the isolation and

retrotranscription processes, as well as specific miRNAs (miR-23a and miR-451) to assess hemolysis. Quality control analyses showed a good performance of the used methodology (**Suppl. Fig. 2A**); Unisp2 = 17.82 ± 0.51, Unisp4 = 24.67 ± 0.52, Unisp5 = 32.72 ± 2.14, and Unisp6 = 18.78 ±
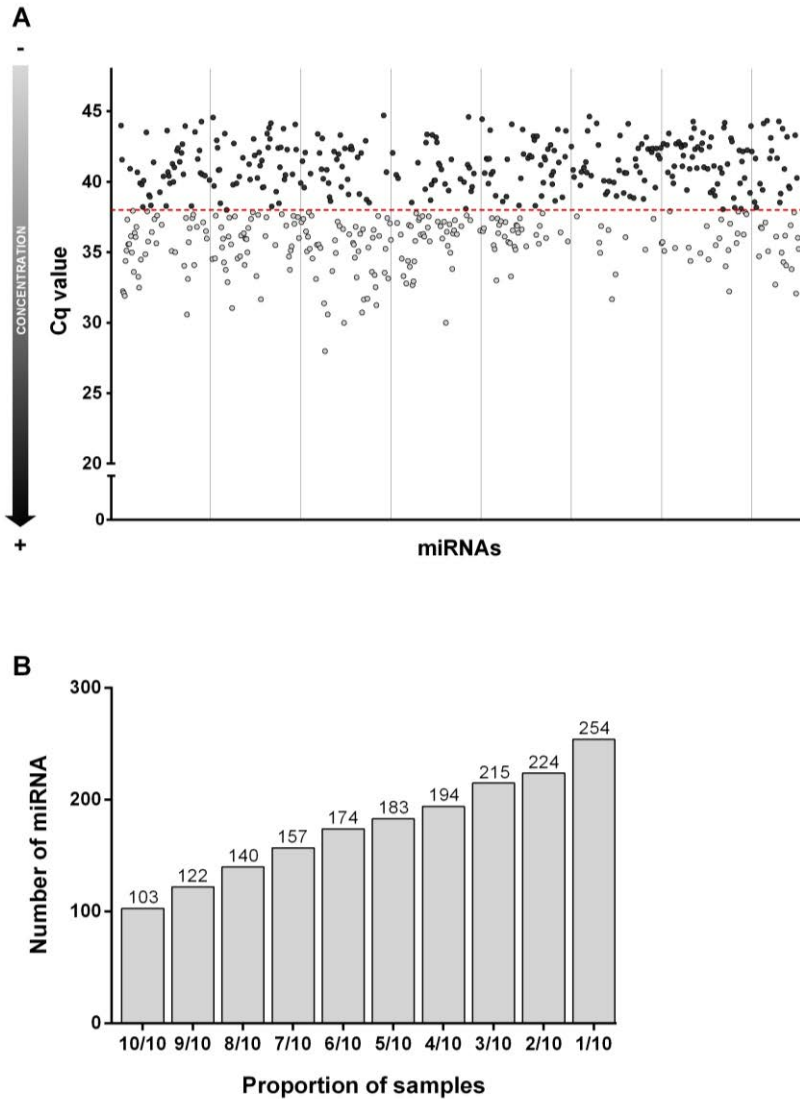

Figure 1. Detectability of miRNAs in EVs from CSF of a cognitively healthy subgroup (n=10)

A) Median raw Cq values for each of the 752-miRNA analyzed. Detection threshold was established at Cq 38 (red dashed line); only gray samples below the threshold are considered as reliable. **B)** Cumulative number of miRNAs present in a proportion of samples, indicating the sensibility and the stability of each miRNA. The remaining 372 miRNAs contained in the panel were absent in EVs from CSF.

0.20 (expressed as median ± IQR). According to the hemolysis ratio (Shah et al., 2016) (ΔCq(miR-23a-3p − miR-451a)), all samples had a miRNA ratio <5, thus discarding the presence of hemolysis (**Suppl. Fig. 2C**). We detected a total of 103 miRNA signals below the detection threshold (Cq 38) that were present in all 10 control samples (**Fig. 1**).

Among those miRNAs detected with a high-moderate level of confidence, 8 candidates were selected for further analyses, based on their detectability in EVs from CSF and their potential relationship with neurodegenerative-related mechanisms. We considered previous investigations showing the relationship between particular miRNAs and neurodegenerative diseases (including FTLD syndromes) (Delay et al., 2012; Zhang et al., 2013; Gascon et al., 2014), as well as pathways typically affected in these disorders (such autophagy processes (Alvarez-Erviti et al., 2013; Ling et al., 2013; Kim et al., 2015) or tau metabolism (Hébert et al., 2012)). We selected the following candidate miRNAs: miR-7-5p, miR-9-5p, miR-15b-5p, miR-124-3p, miR-146a-5p, miR-361-5p, miR-604 and miR-708-3p. Additionally, we included in the analyses the three endogenous normalizers, hemolysis miRNAs, and spike-in synthetic sequences.

After normalizing our data using the geometric mean of the three most constant endogenous miRNAs, we observed significant differences in four miRNAs when comparing the Tau group (patients with PSP or CBS) to other diagnostic groups (**Fig. 2A**). When compared with the control group, two miRNAs were upregulated in Tau-related syndromes (miR-146a-5p, p-value = 0.0191; miR-361-5p, p-value = 0.0008); whereas miR-15b-5p was downregulated (p-value = 0.0002). Interestingly, one of these four miRNAs (miR-708-3p) was absent in all subjects from the Tau group, whereas its levels were detectable and stable in the other study groups.
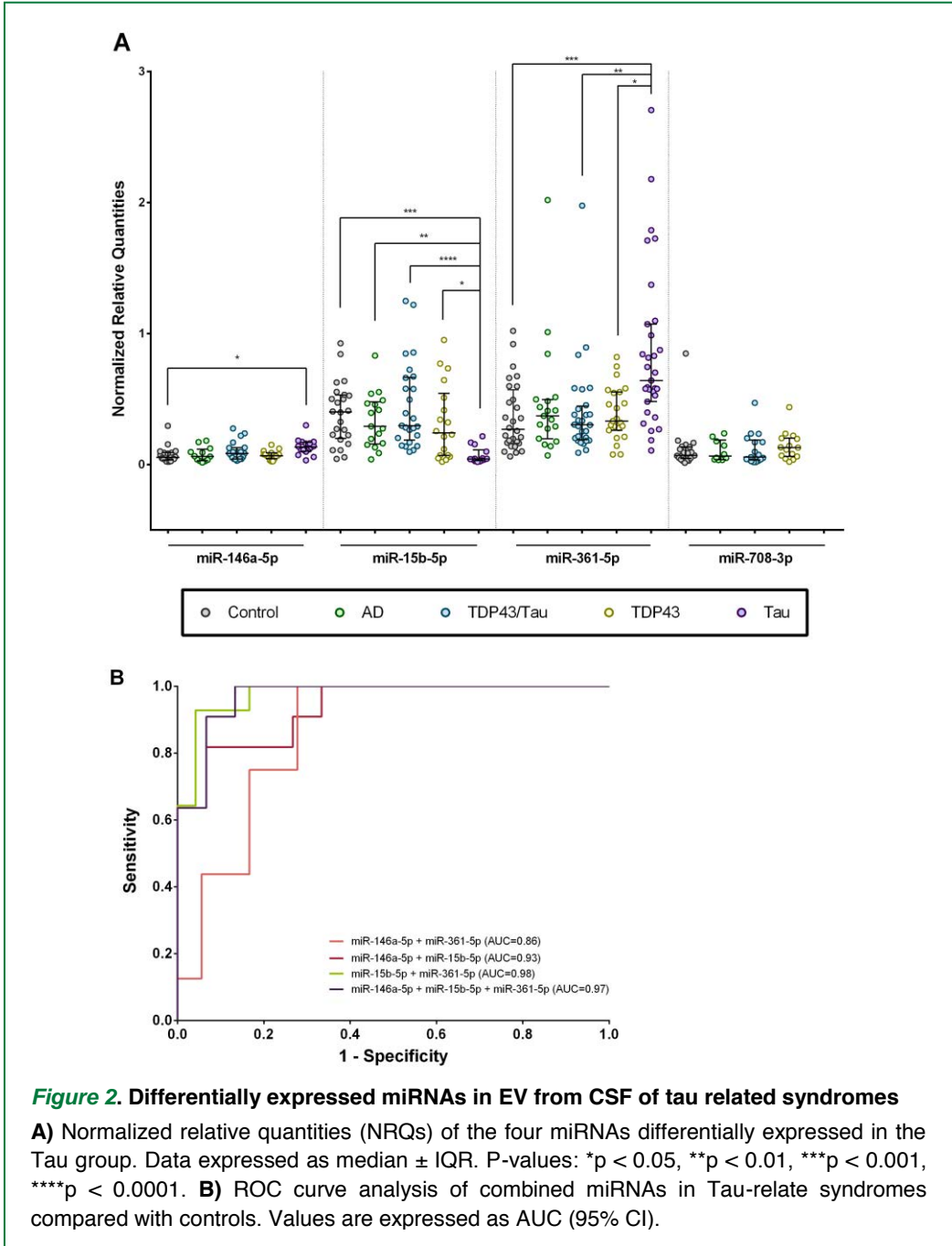
In order to assess the diagnostic utility of these miRNAs, we calculated the receiver operating characteristic (ROC) curves, combining both the upregulated and downregulated miRNAs (**Fig. 2B**). The combination of miR-15b-5p and miR-361-5p had the highest accuracy and showed an AUC of 0.98 (95% CI 0.94-1.02, p<0.0001) to distinguish patients with a presumable Tau proteinopathy and healthy controls.

Discussion

Biomarkers are becoming essential tools for diagnosis, prognosis and staging of neurodegenerative diseases. Finding novel biomarkers could allow a more accurate clinical diagnosis and help elucidate the underlying neuropathology *in vivo*, providing great benefits to future clinical trials. In this regard, miRNAs and EVs (such as exosomes) have gained increasing attention during the last decade. Because CSF is considered the optimal fluid to determine brain metabolism in health and disease (Mattsson, 2011); several reports have profiled miRNA expression in CSF and identified circulating miRNA altered in neurodegenerative disorders (Kiko et al., 2014; Lusardi et al., 2016; Müller et al., 2016; Starhof et al., 2019; Wiedrick

et al., 2019). However, fewer studies have been devoted to the study of miRNAs contained in EVs from CSF (Gui et al., 2015; Riancho et al., 2017; Yagi et al., 2017; Schneider et al., 2018). Moreover, the use of different isolation and quantification techniques hampers the comparison of results between studies, leading to a clear lack of reproducibility. In this scenario, our

Figure 2. **Differentially expressed miRNAs in EV from CSF of tau related syndromes**
A) Normalized relative quantities (NRQs) of the four miRNAs differentially expressed in the Tau group. Data expressed as median ± IQR. P-values: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. **B)** ROC curve analysis of combined miRNAs in Tau-relate syndromes compared with controls. Values are expressed as AUC (95% CI).

study was designed to assess the detectability of miRNAs in EVs from CSF in heathy subjects and identify possible alterations associated to FTD and other related syndromes.

The miRNome expression analysis revealed the presence of at least 103 miRNAs in exosome-like EVs purified from CSF. These miRNAs were expressed in all our samples from cognitively healthy controls. Moreover, up to 140 were present in at least 80% of the samples, and 194 in ≥40% of them, which is fairly similar to the previous work by Riancho *et al* (Riancho et al., 2017).

Our results showed an upregulation of miR-146a-5p and miR-361-3p in 4R-Tau syndromes (Tau group) compared with healthy controls. In previous studies circulating levels of miR-146a-5p in CSF were higher in FTD than AD patients, but they did not report any differences when compared to healthy control (Müller et al., 2016). These discrepancies can be partially due to heterogeneity in the diagnostic groups, while we classified our samples according to their probability of a specific underlying pathology, they only included the three prototypic syndromes of FTD (Neary et al., 1998). Dysregulation of miR-146a levels has also been related with other dementias (Kiko et al., 2014; Dangla-Valls et al., 2016; Marchegiani et al., 2019). This miRNA also seems to be involved in the modulation of inflammatory processes (Olivieri et al., 2013). Expression of miR-361-5p was not previously assessed in FTD syndromes. However, an increase was observed in Huntington's disease (Díez-Planelles et al., 2016; Mendes-

Silva et al., 2016), in which tau pathology has been extensively shown in patients' brain (Vuono et al., 2015). The fact that this miRNA is involved in many biological pathways related to neurodegeneration (Mendes-Silva et al., 2016) (i.e. synapse assembling, vesicle mediated transport, regulation of protein posttranscriptional modifications, protein transport and homooligomerization) reinforces the mechanistic link between miR-361-5p dysregulation and tauopathies.

In contrast, miR-15b-5p appeared downregulated and miR-708-3p was absent in all Tau samples. Decreased levels of miR-15b-5p have been reported in CSF (Cogswell et al., 2008) and brains (Wang et al., 2011) from AD patients. Although we have seen this trend in our study, our data suggests a critical reduction of this miRNA in patients with a high likelihood of 4R tauopathy. Interestingly, the miRNA family containing miR-15b-5p is involved in the regulation of Tau phosphorylation and metabolism (Hébert et al., 2012). Few studies have linked alterations in miR-708 and its binding targets to psychiatric disorders (Forstner et al., 2015; Fiorentino et al., 2016), and a miRNA profiling study in CSF of AD and controls showed differential expression of this miRNA (Denk et al., 2015).

It is important to note that, in the case of miR-15b-5p and miR-361-3p, significant changes were also observed in comparison to other diagnostic groups. This could indicate some degree of diagnostic specificity related to these two miRNA species.

In summary, our results study suggests the presence of a miRNA signature characteristic of certain tauopathies, and expands the possibilities of EV-miRNAs as potential biomarkers. Nonetheless, further investigations would be needed to validate these promising results in other cohorts and to assess whether these changes could be also detected in other peripheral biofluids. Likewise, functional studies of the pathways affected by these four miRNAs would provide in-depth insight and understanding of the underlying neuropathological events in neurodegenerative disorders.

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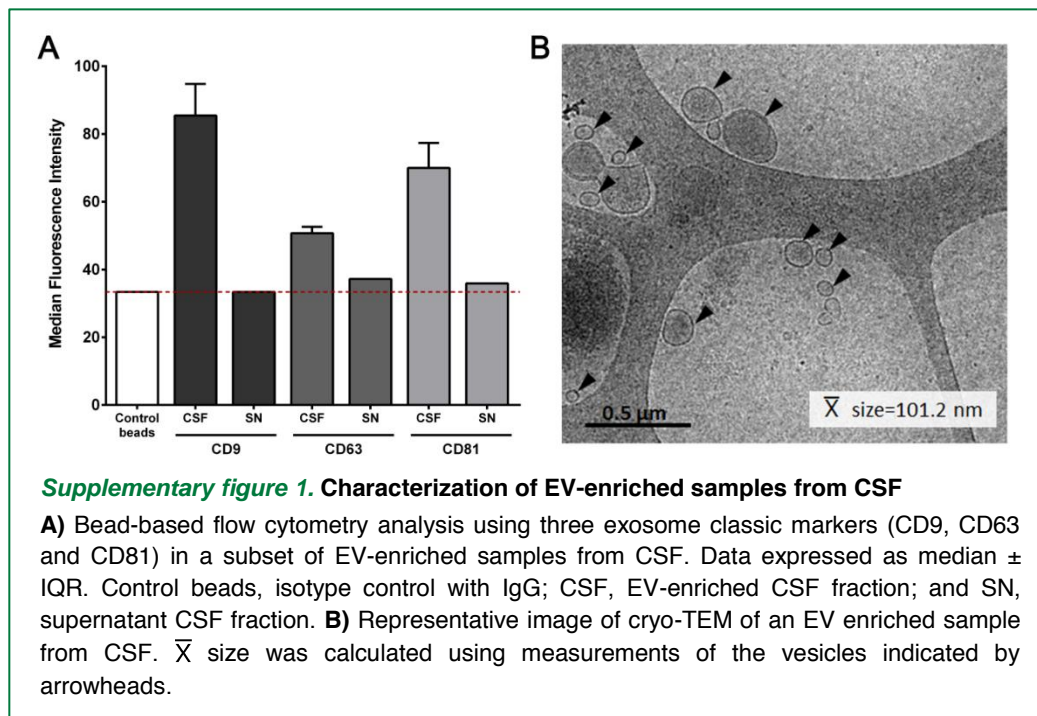
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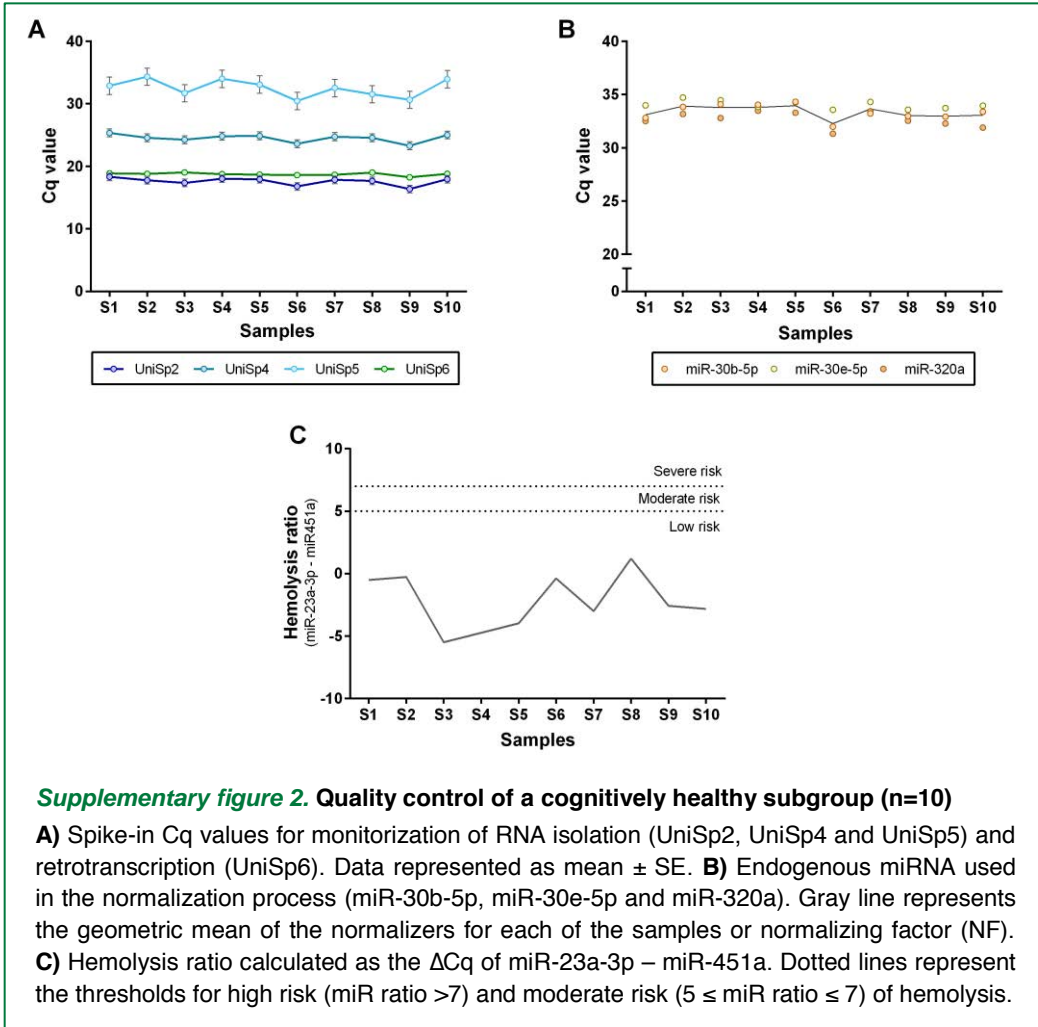
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Supplementary data

Supplementary figure 1. **Characterization of EV-enriched samples from CSF**

A) Bead-based flow cytometry analysis using three exosome classic markers (CD9, CD63 and CD81) in a subset of EV-enriched samples from CSF. Data expressed as median ± IQR. Control beads, isotype control with IgG; CSF, EV-enriched CSF fraction; and SN, supernatant CSF fraction. **B)** Representative image of cryo-TEM of an EV enriched sample from CSF. $\overline{X}$ size was calculated using measurements of the vesicles indicated by arrowheads.

Supplementary figure 2. **Quality control of a cognitively healthy subgroup (n=10)**
A) Spike-in Cq values for monitorization of RNA isolation (UniSp2, UniSp4 and UniSp5) and retrotranscription (UniSp6). Data represented as mean ± SE. **B)** Endogenous miRNA used in the normalization process (miR-30b-5p, miR-30e-5p and miR-320a). Gray line represents the geometric mean of the normalizers for each of the samples or normalizing factor (NF). **C)** Hemolysis ratio calculated as the ΔCq of miR-23a-3p – miR-451a. Dotted lines represent the thresholds for high risk (miR ratio >7) and moderate risk (5 ≤ miR ratio ≤ 7) of hemolysis.

Chapter 4
Discussion

In spite of extensive research, the molecular mechanisms underlying neurodegenerative disorders remain elusive. Although familial aggregation is a common trait among these complex diseases, neither causal mutations nor genetic risk variants can explain their entire heritability.

In this context, the present doctoral thesis aimed at investigating certain genetic and epigenetic aspects of neurodegenerative diseases through highly sensitive PCR-based techniques, focusing on the study of structural genetic rearrangements, and the measurement of mitochondrial DNA loads and non-coding RNA species.

Copy number structural variations in the 17q21.31 region and their relationship with neurodegenerative disorders

In <u>Study 1</u>, we analyzed the structural variation pattern of the chromosome 17q21.31, one of the most complex and dynamic regions of the human genome, and evaluated their contribution to the PD, PSP and CBD genetic risk associated to the H1 haplotype.

Accumulation of predominantly 4R tau isoforms is the main neuropathological hallmark of PSP and CBD. Several rare mutations in MAPT gene, which is located on chromosome 17q21.31, have been described in these two tauopathies[256–260]. These pathogenic mutations mostly affect residues in the microtubule-binding domains (including exon 10); resulting in reduced tau binding avidity, increased fibrillization and neuropathological alterations[261,262]. The predominance of a specific isoform in these disorders pointed at the dysregulation of alternative splicing events as a potential mechanism of pathology.

Common polymorphic variability within MAPT gene, coding for tau protein, was first reported in primary tauopathies. A series of SNPs have been identified within MAPT, usually in complete disequilibrium with each other and mainly associated with the two commonly known haplotypes[263,264]. Surprisingly H1 haplotype was also found to be overrepresented in PD [265–267], despite being a synucleinopathy, which expanded the implications of the MAPT region in neurodegenerative diseases. Nowadays, this association between the MAPT H1 haplotype and the risk of developing PD, PSP and CBD in European populations is sustained by many studies [62,76–79]. In our study cohort, we observed the expected overrepresentation of H1 haplotype in the diagnostic groups of PD, PSP and CBD.

Interestingly, the H1 polymorphism was also related to a 1.8-fold increased disease risk in our DLB group. Compared with a previous study, unable to disclose this

association[76], we doubled the number of DLB patients. This increase in sample size, might probably explain our higher statistical power to detect the effect of H1 in the DLB group. In two recent GWAS, none of the SNPs flanking the MAPT region surpassed the adjusted threshold for significance, even though some polymorphic markers showed nominal (p-value < 0.05) association levels[268,269].

DLB and PD are closely related α-synucleinopathies that share clinical and pathological features. Both are characterized by the presence of α-synuclein aggregates in the brain, and their clinical manifestations include a range of movement and cognitive symptoms[35,270]. Given these similarities, the association of H1 haplotype and DLB would be consistent with previous findings in PD cohorts. Nonetheless, the relationship between MAPT H1 haplotype and synucleinopathies is intriguing. The most accepted hypothesis is that tau and α-synuclein proteins have reciprocal synergistic effects, which contribute to the underlying pathogenic mechanism[271]. MAPT gene seems to play a role in α-synuclein expression and deposition in different brain regions[272–274]. Specifically, the H1 haplotype seems to increase the burden of hyperphosphorylated tau[273], which could promote α-synuclein accumulation and ultimately lead to PD and DLB.

Despite this well-known association in the case of PD, PSP and CBD, the precise genetic component responsible for H1 effect is still unknown. The H1 haplotype has been classically divided into ~20 subhaplotypes[70], based on the combination of SNPs present in the MAPT gene. Different subhaplotypes have been related to different neurodegenerative pathologies, including PSP and DLB[76,275–279]. However, these SNPs only cover the MAPT gene from upstream of the promoter to beyond exon 13. Given that the 17q21.31 chromosomal region (with an extended 1.7Mb block of linkage disequilibrium) contains several other genes, including CRHR1, KANSL1, NSF and STH[73,280], alterations in the expression of genes contained in this genomic segment could be presumably responsible for the H1 effect.

Copy number variations (CNVs), among other large scale genomic structural changes, are far more frequent in the human genome than previously assumed[281]. They could reflect the instability of a genomic region and influence the expression of the genes contained therein. Genome-wide scans have revealed rare and de novo CNV occurring at a higher rate in several neuropsychiatric and neurodevelopmental disorders, including schizophrenia, intellectual disability, ADHD and Tourette syndrome[282].

The recent identification of multiple chromosomal rearrangements in the 17q21.31 region, and their segregation into nine subhaplotypes, prompted us at examining in detail their possible contribution in PD, PSP, CBD and DLB. These newly defined

forms are different from the classical subhaplotypes, as they arise from the combination of four highly polymorphic structural features (three overlapping duplications and the inversion)[73]. As far as we know, no other studies have evaluated the association of CNVs within H1 haplotype and assessed their role in neurodegenerative disorders. For this purpose, we focused on the H1H1 individuals, as any H1-related effect would be stronger in the homozygous group. The use of a digital PCR-based approach allowed us to quantify two common CNVs (β- and γ-segments) contained in the MAPT 17q21.31 region.

Our data showed similar copies of both β- and γ-segments in all phenotypic groups, even though individuals within each group were heterogeneously distributed throughout the range of copies. Notably, PSP and PD groups had a higher percentage of patients with 6-8 copies of the γ-segment, while none of the control subjects presented more than six copies. This is a highly multi-allelic 210-kb structural polymorphism that covers most of the NSF gene, which is highly expressed in brain tissue. It codes for the Vesicle-Fusing ATPase, a protein that participates in vesicle-mediated transport and catalyzes the fusion of transport vesicles within the Golgi cisterna. Interestingly, NSF has been found as a component of the intranuclear inclusions in neuronal intranuclear inclusion disease, a progressive ataxia characterized by numerous intranuclear inclusion bodies in neurons, similar to those found in polyglutamine repeat diseases[283]. Whether an aberrant expression of this gene could be involved in PD or PSP could be a matter worth pursuing.

The main limitations of our approach were the relatively limited DLB sample size, and the fact that we only focused on homozygous H1 individuals, thus excluding the intermediate-risk H1H2 subjects. Furthermore, the use of different methodologies to characterize the subhaplotypes could explain the discrepancies with previous investigations[275–277]. While these studies focused on several tagging SNPs within and surrounding the MAPT gene[70,284], we examined the polymorphic CNVs on the distal ends of the 17q21.31 inversion[73]. Whether these haplotypes are associated with a particular combination of CNVs is a matter of study that deserves further investigation.

In summary, our first study suggests that gross CNV polymorphisms within the 17q21.31 inversion region are not responsible for the H1 effect in PD, PSP, CBD and DLB. However, our data suggests that H1 haplotype could influence the risk of DLB, hence expanding its biological relevance in neurodegenerative disorders. Despite the negative results, this was the first study that looked into the CNV involvement in the H1 risk association. This type of genomic structural changes

could have a relevant impact in gene expression, and their study is essential to disentangle the genetic underpinnings of neurodegenerative diseases

Cerebrospinal fluid mitochondrial DNA alterations in Alzheimer's disease

In Study 2, we examined the role of circulating cell-free mitochondrial DNA as a possible indicator of underlying mitochondrial dysfunction in the AD continuum, and evaluated its utility as a diagnostic biomarker.

Mitochondria, located along axons and synaptic terminals, are essential to maintain an adequate neuronal function. These organelles orchestrate bioenergetics, redox homeostasis and apoptotic mechanisms. At early stages of disease, AD brains already show signs hypometabolism and increased oxidative stress. Mitochondrial metabolism, including both biogenesis and turnover, also seems to be impaired in AD. Thus, mitochondria dysfunction has been proposed as key pathophysiological event in neurodegenerative diseases[120]. The appearance of these mitochondrial perturbations in disease, led to the study of mtDNA circulating levels. The load of mtDNA detected in CSF was proposed to reflect the mitochondrial-related events occurring in the brain, thus becoming a promising biomarker that precedes the symptomatic stages in dementia.

According to this, expecting increased levels of mtDNA would be plausible due high neuronal death in AD brains. However, several studies described low CSF mtDNA levels in AD patients[109,125]. The decreased mtDNA levels were suggestive of mitochondrial depletion. Thus, it was hypothesized that AD patient could have basal mitochondrial perturbations, which would ultimately affect neuronal function and trigger AD onset.

In light of these findings we sought to investigate the levels of mtDNA in preclinical and prodromal phases of AD. Surprisingly, our results showed a considerable inter-individual variability within and between diagnostic groups. This dispersion in mtDNA measurements led to a substantial overlap between cases and controls. Thus, we were unable to replicate the previous findings. Contrarily to what was expected, we also found a significant increase of mtDNA load in AD patients compared with controls; and a more subtle trend was observed in the MCI group. To date, several groups have studied the levels of mtDNA in different neurodegenerative disorders, reporting differences in other pathologies such as PD [285,286], Creutzfeld-Jakob disease[125], or multiple sclerosis[124,287]. Although cell-free mtDNA could reflect early stages of neurodegeneration, these results evince the lack of disease-specificity.

CSF biomarker concentrations can be affected by several pre-analytical factors, including sampling material and methodology, storage procedures as well as other clinical variables[288]. We ruled out the possibility of mtDNA contamination derived from blood cells in our samples, by assessing the presence of BAX gene, an apoptosis-related gene expressed in all cell types. Only two of them presented more than ≥1 copy of BAX gene, and were therefore excluded from further analyses. Additionally, we evaluated the effect of freeze-thaw cycles on mtDNA levels and the linearity of mtDNA load across serial dilutions. The measurements were not affected by up to three freeze-thaw cycles, and inter- and intra-assay variations were below 7%, indicating that mtDNA analysis by ddPCR is reliable and stable.

Mitochondrial dysfunction has been linked to age-related changes in these organelles. Aging leads to a decrease in mtDNA volume, integrity and functionality due to accumulation of mutations and oxidative damage induced by ROS. Moreover, alterations in mitochondrial dynamics also appear with aging, as mitophagy mechanisms and mitochondrial biogenesis become impaired [115]. According to this, we would expect decreased levels of mtDNA in older subjects, but no correlation was observed in our control group.

It is also becoming increasingly apparent that mitochondrial metabolism and cell death signaling are sexually dimorphic[289]. Moreover, the higher prevalence of AD in women and the nearly exclusive maternal inheritance of mtDNA could be in line with these evidences. However, neither sex, nor positive maternal family history of dementia had any effect on mtDNA levels in our cohort.

The lack of differences related to aging, sex and family history could be due to the type of sample. As we are analyzing cell-free circulating mtDNA -not derived from cell death- in CSF, we might not perceive specific alterations in mitochondrial mechanisms due to cell compensatory mechanisms such as apoptosis.

Accumulation of APOE (the most important locus associated with late-onset AD) has been observed in the mitochondria, affecting its functioning[290]. The APOE ε4 status was also shown to correlate with low mtDNA levels[109], however we could not find this association in our series. The previously observed association could be explained by the fact that all APOE ε4 carriers were part of the AD group, while none of the controls carried the ε4 allele.

The presence of polymorphisms in the SUCLG2 gene, encoding the GDP-dependent isoform of succinil-CoA synthase, was additionally assessed. Few polymorphisms in this gene have been associated with mtDNA depletion syndrome[291,292], and could be an important modulator of mtDNA levels, but our data do not show any strong effects of this variant in mtDNA levels.

A possible reason for the discrepancies between other studies and ours could be the use of small sample sizes in previous studies. When the analyzed variables have a wide dispersion, small diagnostic groups are more prone to show differences between them, while larger cohorts could overcome this effect and help evaluate mtDNA accuracy and usefulness in the clinical practice.

In summary, mtDNA in CSF proved to be a stable and highly reproducible analyte, which can be rapidly and accurately evaluated. However, individual variability within groups limits its usefulness as a diagnostic biomarker. Further studies would be essential to understand the involvement of mitochondrial mechanisms in neurodegenerative diseases, and how this common energetic failure could be used as a possible biomarker for diagnostic and prognostic purposes.

Identification of an EV-derived microRNA signature characteristic of 4R-tauopathies

In <u>Study 3</u>, we sought to investigate the function of miRNAs, a class of non-coding RNAs involved in the post-transcriptional modulation of gene expression, as possible markers of pathophysiological processes in FTD, and hence be used as indicators of the pathological substrate in a clinical setting.

Non-coding RNAs have increasingly gained attention due to their function as key modulators of the cellular homeostasis. Although their mechanisms of action are not fully understood, they play important roles in regulation of different cellular functions, including gene expression. Among these species, miRNAs became particularly interesting in complex diseases, such as neurodegenerative disorders[197]. Different studies pointed at the reciprocal interplay between miRNAs and FTD-related proteins and genes. Furthermore, miRNA can be found within EVs, contributing to the transport of genetic material to other cells. These evidences highlight the importance of miRNAs, among other ncRNAs, in the pathophysiological events of diseases[241].

In light of these findings, we explored the presence of miRNAs contained in EVs from CSF, detecting up to 103 different sequences, stably expressed in healthy subjects. As far as we know, there is only one study aimed at describing the miRNA profile in this biofluid compartment[293], reporting slightly higher numbers (239 miRNAs present in at least 40% of the control samples).

The monitorization of analytical variables is also essential in this type of samples. The lack of standard methodologies for the analysis of miRNAs and the reduced number of studies looking into miRNAs contained in EVs, especially from CSF, increases the necessity of including quality control assays. Our data indicated a good

performance of the isolation and retrotranscription methods, as all spike-ins were stable among samples. We also used the miRNA hemolysis ratio to discard the presence of hemolysis in our samples, which reflects their suitability for miRNA analyses.

The selection of adequate endogenous normalizers is another essential aspect in miRNA studies, which would vary depending on the sample type. Unfortunately, universal miRNA normalizing factors do not exist, and a thorough screening should be performed to find the most stable miRNAs. This is a caveat for many similar studies, which could give rise to the worrisome lack of reproducibility that is present in the field. In order to minimize this effect, we used three different statistical methods (Normfinder, geNorm and CV score) to select the best suited normalizers for our assay.

Our results on candidate miRNA expression revealed an upregulation of miR-146a-5p and miR-361-3p in 4R-Tau syndromes (Tau group) compared with healthy controls. On the other hand, miR-15b-5p appeared downregulated in this diagnostic group and, intriguingly, miR-708-3p was absent in all Tau samples. Circulating levels of some of these altered miRNAs have been already assessed in previous studies. Higher levels of miR-146a-5p were reported in prototypic FTD syndromes, compared with AD patients[294]. Although miR-361-5p expression has not been assessed in FTD patients, its levels were increased in Huntington's disease[295,296], which could be related to the tau pathology burden observed in these individuals[297]. The miRNA family containing miR-15b-5p has been involved in Tau metabolism, including phosphorylation and alternative splicing[227], thus supporting the dysregulation that seems to be present in patients with a high likelihood of a 4R tauopathy. Alterations of miR-708 have only been reported in psychiatric disorders[298]; however, its complete lack of expression in 4R-Tau syndromes is striking and needs further investigation.

Studying EVs still entails certain limitations, mainly due to the absence of standardized protocols for the purification and enrichment of EVs, and for their characterization. To overcome this issue, many efforts are focused at stablishing guidelines for EV studies, as shown in the recent publication by the International Society for Extracellular Vesicles (ISEV)[239]. In the present work we have characterized the enriched vesicular fraction through two independent methods: cryo-TEM to assess their morphology and size and bead-based flow cytometry to evaluate the presence of classical exosome markers (CD9, CD63 and CD81). Although further characterization methods could be included, our thorough evaluation makes us confident that the evaluated vesicles, and their content, derive from the exosomal fraction.

In summary, our results indicate the presence of characteristic miRNA signature in EVs from CSF, seemingly specific of 4R-tauopathies. Further investigations would be needed in order to unveil the functional relevance of these miRNAs in vivo, and understand their effect on specific pathways that may lead to the underlying pathological events of neurodegenerative disorders.

Chapter 5
Conclusions

In this thesis, copy number structural variations, mitochondrial DNA and microRNAs has been assessed through quantitative and qualitative PCR-based techniques, in order to obtain a broader view of their role in neurodegenerative diseases.

The main conclusions of this thesis are:

1. **The *MAPT* H1 haplotype is associated with DLB risk, besides its well-known association with PD, PSP and CBD. However, gross CNV polymorphisms within this region are not responsible for the H1 effect.**

2. **Circulating cell-free mitochondrial DNA can be reliably measured in CSF, but presents a wide variability between individuals regardless of their diagnosis, limiting its use as a diagnostic biomarker.**

3. **Numerous microRNAs are stably present within extracellular vesicles purified from CSF. Four microRNAs have a specific pattern of expression in patients diagnosed with 4R-tau syndromes.**

Chapter 6
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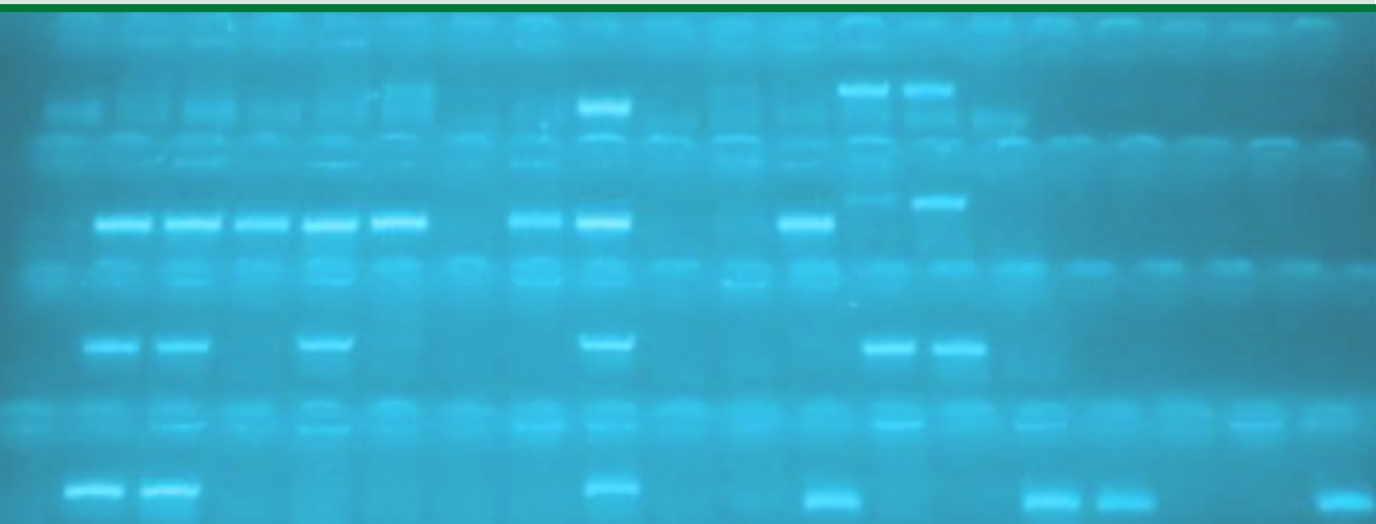
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Chapter 7
Annexes

Genetic and Epigenetic Architecture of Alzheimer's Dementia

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Alzheimer's disease (AD) is the most common neurodegenerative disease, affecting up to 5 % of the population over 65 years old. It is characterized by the accumulation of amyloid-β peptide and aggregation of hyperphosphorylated tau protein. Mutations in pivotal genes (*APP*, *PSEN1*, and *PSEN2*) result in the autosomal dominant form of the disease; however, the genetic cause of the majority of cases remains unexplained. Advances in genomic techniques have allowed the discovery of com-mon variants that influence susceptibility to AD. In addition, low-frequency and rare variants in a small number of genes have emerged as important contributors of disease risk. Recent studies have focused on the epigenetic changes in affected individuals, such as DNA methylation patterns or microRNA levels, highlighting the possible involvement of these mechanisms in the etiology of AD. Here we review the known genetic and epigenetic factors underlying AD, emphasizing the molecular networks that appear to be fundamental players in its etiology.

Introduction

Alzheimer's disease (AD) is the leading cause of dementia, accounting for 50–70% of all cases. In most patients, the symptoms begin to appear after the patient has reached 65 years of age. However, in around 5% of cases, cognitive decline occurs earlier [1]. In 2010, an estimated 35.6 million people worldwide were living with dementia. Because the number of people surviving into their 80s and beyond is expected to grow dramatically, the global burden of disease is expected almost to double in the next 20 years, reaching a predicted 115.4 million by 2050 [2]. As a result, AD represents one of the major health challenges facing modern society with clear implications to associated healthcare costs.

The most predominant hypothesis to explain the process contributing to AD is the biochemical pathway leading to the altered production or clearance of the amyloid-β (Aβ) peptide that aggregates into amyloid plaques, neurofibrillary tangle (NFT) formation, and cell death. Known as the "amyloid cascade hypothesis", it holds that altered processing and aggregation of Aβ due to genetic and environmental influences constitute the key pathogenic factor in AD [3].

Autosomal Dominant Genes in Alzheimer's Disease

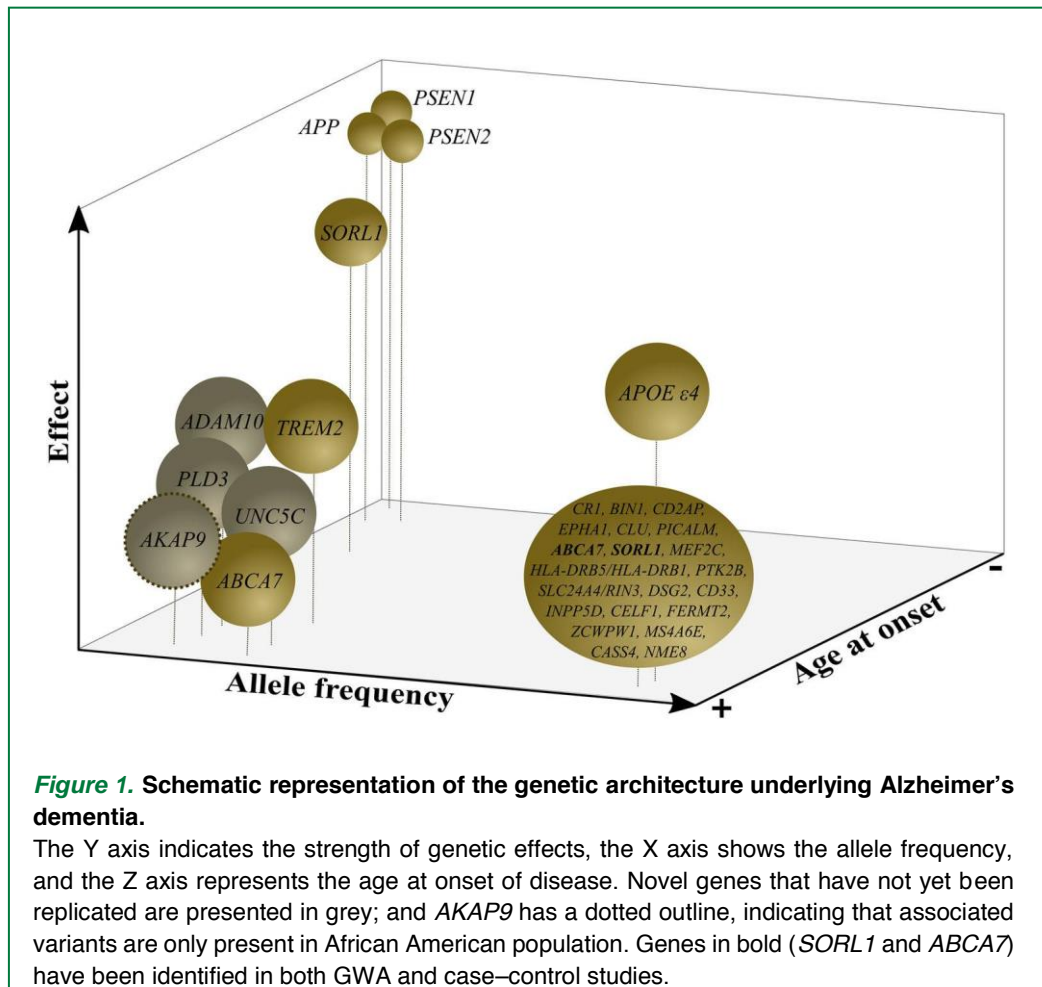In approximately 5% of AD patients, the disease is transmitted as an autosomal dominant Mendelian trait with age-dependent penetrance. Studying this small fraction of patients, who typically present with onset ages younger than 65 years, has been extremely informative to disentangle the genetic bases of this neurodegenerative dementia. To date, only three genes have been shown to cause the disease in some of these families: presenilin-1 (*PSEN1*), accounting for 18–50% of early-onset familial AD cases, presenilin-2 (*PSEN2*), which accounts for less than 1 % of cases, and the gene encoding for the amyloid precursor protein (*APP*), accounting for less than 5% (**Fig. 1**). Interestingly, all three genes are linked to Aβ processing or cleavage, and mutations in any of these genes lead to the altered production of Aβ, thus indicating a shared biochemical pathway. The discovery of these mutations has been of pivotal importance to disentangle the molecular basis of the disease. However, the fact that half of the families with early-onset AD do not show mutations in these genes, strongly suggests that other genes have to be present in early forms of AD.

The *APP* gene is located on chromosome 21q21.3 [4, 5]; it covers a genomic region of 290-kilobases (Kb) and includes 18 exons, of which exons 16 and 17 encode the amyloid-β peptide [6]. Since it was first described in families with autosomal dominant early-onset AD, up to 42 different mutations have been identified in this gene (see AD Mutation database, available at

http://www.molgene.ua.ac.be/ADMut ations/) [7]. *APP* mutations leading to autosomal dominant AD are mostly missense; however, two recessive mutations and several duplications have also been described [7–9]. All these pathogenic mutations are located near the cleavage sites, thus altering its proteolytic processing [10]. Recent findings revealed that *APP* locus duplication could be a somewhat important cause of early-onset AD [9]. Interestingly, a protective variant (p.A673T) in *APP* was recently described in Icelanders. This substitution is adjacent to the β-cleavage site of APP and was proposed to reduce the Aβ formation to ~40 % [11**].

The *PSEN1* gene is located on chromosome 14q24.3; it has 13 exons, spanning in a genomic region of ~84-Kb [12]. The paralogous *PSEN2* gene is otherwise located on chromosome 1q42.13, it extends ~25-Kb and has 12 exons [13]. Through its important function in APP processing, mutations in *PSEN* genes could lead to the altered function of APP processing and/or

Figure 1. Schematic representation of the genetic architecture underlying Alzheimer's dementia.
The Y axis indicates the strength of genetic effects, the X axis shows the allele frequency, and the Z axis represents the age at onset of disease. Novel genes that have not yet been replicated are presented in grey; and *AKAP9* has a dotted outline, indicating that associated variants are only present in African American population. Genes in bold (*SORL1* and *ABCA7*) have been identified in both GWA and case–control studies.

aggregation, resulting in an increased $A\beta_{42}/A\beta_{40}$ ratio [14].

Genes Related to APP Processing

Presenilins represent the catalytic subunit of the γ-secretase, a multi-protein complex in which at least three additional subunits —nicastrin (NCSTN), anterior pharynx-defective 1 (APH1), and presenilin enhancer 2 (PEN2)— are also necessary for the correct endoproteolitic function. Little is known about possible effects of genetic alterations in the genes encoding for proteins that participate in the γ-secretase complex formation. An association study using four genetic variants within *PEN2*, which seems to have an important role in the complex maturation, did not report any association between this gene and the risk of AD [15]. However, most of the sample comprised late-onset AD patients and the study had not enough power to detect risks lower than 3.0. *APH1* gene encodes a seven transmembrane protein that forms, together with NCSTN, an initial complex scaffold to which presenilin binds. Association analysis between two single nucleotide polymorphisms (SNPs) in *APH1* and late onset AD in an Italian case–control series revealed no relationship between any of these genetic variants and late-onset AD [16]. Finally, association analyses between *NCSTN* and AD have been performed with incongruent results. A multicentric

study that compared the allele frequencies of two coding SNPs within *NCSTN* did not find any significant association between these variants and the risk of AD [17]. On the contrary, a significant increase of one of the haplotypes resulting from the analysis of 14 different SNPs was reported in the group of early-onset familial AD from a Dutch population-based sample [18].

The metalloprotease ADAM10 is considered the major a-secretase implicated in APP processing [19]. In a family-based study, two rare non-synonymous mutations were identified (p.Q170H and p.R181G) in its prodomain [20]. Furthermore, these mutations were found to be responsible of a decrease in α-secretase activity, which led to an enhancement of Aβ plaques [21].

Genetic variants in proteins related to Aβ degradation could lead to an abnormal increase of this peptide and its subsequent accumulation. In this sense, Neprilysin (encoded by *NEP* gene) is the major protease involved in the degradation process. To date, *NEP* is one of the few genes that meta-analyses associate with a genetic influence to AD (see AlzGene database at the Alzheimer Research Forum, available at http://www.alzgene.org) [22].

Genetic Risk Factors and Molecular Networks

Although late-onset Alzheimer's disease does not follow a Mendelian

inheritance, it has a heritability of 58–79%, thus indicating a high genetic component [23]. Most of the known genetic loci related to AD come from genome-wide association (GWA) studies and, therefore, are based on the common-variant common-disease hypothesis, which implies that common genetic variants (with allele frequencies greater than 5%) explain a significant fraction of its biological bases. On the other hand, low frequency and rare genetic variants in humans (with allele frequencies lower than 5 or 1%, respectively) are predicted to be enriched for functional alleles which would consequently have larger phenotypic effects than common alleles [24]. This has led to the rare-variant common-disease paradigm, in which genetic variants that are present at very low frequencies in humans may explain the so-called missing heritability that is present in diseases such as AD (**Fig. 1**).

The intense labor aimed at disentangling the genetic architecture underlying AD has led to the awareness of certain biological pathways that seem to have important roles in the AD pathophysiology. Broadly, these genes appear to be enrolled in five major pathways: amyloid-β clearance, endocytosis, lipid processing, immune response, and tau phosphorylation.

Amyloid-β Clearance

The most important locus associated with late-onset AD is the Apolipoprotein E (*APOE*). Located on chromosome 19q13.1, this haplotype has three alleles: ε2, ε3, and ε4. The ε4 allele, which is present in ~14 % [25] of Caucasians, increases the AD risk in fourfold compared to non-carriers [26]. Apolipoprotein E is involved in the metabolism of lipoproteins and cholesterol transport and the e4 allele seems to bind to Aβ peptide with higher avidity, thus modifying its clearance and aggregation [27*].

Apart from APOE, two other players seem to be involved in Aβ clearance, named complement component receptor 1 (CR1) and clusterin/apolipoprotein J (CLU) [28]. The *CR1* gene, on chromosome 1q32.2, encodes the main receptor of the complement 3b, which seems to have a protective role against AD when activated, decreasing the amyloid accumulation through promotion of Aβ clearance. Genetic variants in CR1 (rs6656401, and rs3818361) may influence susceptibility to AD [28–33] as a consequence of impaired removal of Aβ peptide. Clusterin, encoded by *CLU* gene on chromosome 8p21, is a chaperone that binds the soluble amyloid-β present in the cerebrospinal fluid (CSF) and fuels its penetrance through the blood–brain barrier. Clusterin is incorporated into the amyloid plaques and an increase of its expression has been observed in various brain pathological conditions [34]. Its association with AD has been supported by multiple genome-wide studies [28, 32, 33].

Endocytosis Mechanisms

Endocytosis is an important cellular mechanism in amyloid processing. To date, five genes involved in trafficking and endocytosis have shown significant association with AD: *PICALM*, *BIN1*, *SORL1*, *CD2AP*, and *EPHA1* [28, 30, 32, 33, 35**, 36].

The *PICALM* gene, on chromosome 11q14, encodes the phosphatidylinositol binding clathrin assembly protein, which is involved in the clathrin-mediated endocytosis, clearance of apoptotic cells, and synaptic transmission [33]. It colocalizes with APP in AD human brains and changes in its expression appear to influence Aβ accumulation in a transgenic mouse model [37].

BIN1 (bridging integrator 1 or amphiphysin I) gene, on chromosome 2q14, encodes an adaptor protein involved in membrane dynamics, including receptor-mediated endocytosis [38]. A study using *BIN1* knock-out mice revealed that disruption in protein expression results in altered synaptic vesicle recycling [39].

SORL1 is located on chromosome 11q23 and encodes the sortilin-related receptor L1, a type I transmembrane receptor highly expressed in the brain. This receptor binds APP and transfers it to subcellular compartments with low secretase activity, where its retention derives in a decrease of γ-secretase cleavage, thus reducing the amyloid products. Several GWA studies identified genetic variants in *SORL1*

associated with AD [35**, 40, 41]. Furthermore, an enrichment of rare variants in this gene as well as two missense and nonsense mutations has been reported, with an increased risk of both autosomal dominant and sporadic AD [42, 43*].

CD33 also appears as a disease susceptibility locus in some AD genome-wide studies [31, 32, 36]; however, Lambert *et al.* were unable to replicate this association [35**]. At the protein level, CD33 belongs to the sialic acid-binding, immunoglobulin-like lectin family of receptors and promotes endocytosis independent from clathrin. Interestingly, it has been shown that the minor allele of the biallelic polymorphism rs3865444 leads to a reduction of CD33 expression in microglia and also to decreased Aβ$_{42}$ levels in AD brains [44].

The CD2-associated protein (CD2AP) is involved in receptor-mediated endocytosis. Several SNPs at the CD2AP gene, on chromosome 6q12, have been associated with late-onset AD [31, 36]. A relationship between SNPs in *EPHA1* and AD has also been reported [31, 32, 36]. The protein encoded by *EPHA1*, ephrin receptor A1, participates in synaptic plasticity. Additionally, significant signals appeared at SNPs near *MEF2C* —encoding the myocyte-specific enhancer factor 2C— and *PTK2B* — encoding the protein tyrosine kinase 2 beta— in a recent genome-wide study. Although little is known about its

putative function, a role in cell migration and hippocampal synaptic activity has been suggested [35**].

Lipid Processing

The early association of *APOE* with AD pointed towards genes of the lipid metabolism as risk candidates of the disease. Among all the genes involved in this pathway, *CLU* and *SORL1*, as previously mentioned, and *ABCA7* have shown association with AD.

The *ABCA7* gene codifies the ATP-binding cassette transporter A7, a cell membrane transporter that promotes the transport of cholesterol and phospholipids to lipoproteins [45]; other studies propose phagocytosis as its primary function [46]. Through a whole-genome sequencing approach and imputation strategies, rare loss-of-function variants within ABCA7 were found to be associated with AD in the Icelandic population [47]. An independent study performed in a population from northern Belgium also found four-time enrichment of rare loss-of-function variants in *ABCA7* in AD patients compared to controls [48]. Previous GWA studies have already linked a common biallelic variant within *ABCA7* to AD risk [31, 35**, 36].

Immune Response and Inflammation Pathway

Neuroinflammation and impaired immune response are characteristic traits of AD pathology, although it is still under debate if these mechanisms are cause or effect of the pathophysiological events [49]. Many loci that showed association with AD have a presumed involvement in immune response. Apart from the previously mentioned functions, *CR1*, *CLU*, *ABCA7*, *CD33*, and *EPHA1* also have a role in immunity processes. Recent GWA studies have revealed three additional genes that could influence AD risk: *MS4A6E*, *HLA-DRB5/HLA-DRB1*, and *INPP5D* [31, 32, 35**]. Although its specific function in the disease pathology is unclear, they are involved in immune response. The *MS4A6E* is included in the MS4A cluster, which comprises a family of cell-surface proteins. Two members of the major histocompatibility complex, DRβ5 and DRβ1, encoded by *HLA-DRB5/HLA-DRB1* have also been linked to AD [35**]. The inositol polyphosphate-5-phosphatase (INPP5D) is able to bind CD2AP, and it has been proposed as a modulator of the APP metabolism [35**].

In 2013 two independent studies identified a low-frequency non-synonymous variant in *TREM2* (triggering receptor expressed in myeloid cells 2) gene, p.R47H [50, 51], which raised the risk of AD to a threefold increase. One study, performed using whole-genome sequencing in an Icelander population, reported a combined OR of 2.83 [51]; the other group associated p.R47H variant with a 4.5-fold increase of AD risk [50]. These results were replicated

by other groups in populations with European-descent [29, 35**, 52], although at a lower risk degree. Nonetheless, this association was absent in Asian and African American populations, where the p.R47H variant is almost nonexistent. Another variant associated with AD (p.R62H) was recently found in European Americans, as well as two new variants that are only present in African Americans (p.L211P and p.W191X) [53]. TREM2 plays a role in the suppression of inflammatory reactivity and the regulation of the phagocytic activity [54]. Furthermore, a confocal imaging study revealed that TREM2 was differentially expressed in brain tissue with amyloid plaques than in tissue free of Aβ aggregates [55].

Tau Phosphorylation Pathway

Even though AD is characterized by the presence of amyloid plaques, another main pathognomonic sign is the presence of NFTs, with the hyperphosphorylated tau protein as the major component. Although mutations in *MAPT* gene (encoding the tau protein) have been associated with tau-related disorders, such as frontotemporal dementia, corticobasal syndrome, or supranuclear palsy, there is no apparent link between them and AD. Recently, a rare, non-synonymous variant (p.A152T) was shown to increase the risk for AD significantly [56], probably due to its effect in decreasing the stability of microtubules and exacerbating tau oligomerization. This

finding was replicated in two independent studies, whereas the effect was found in non-APOE-ε4 carriers [57, 58].

Glycogen synthase kinase 3β (GSK3β), the cyclin dependent kinase 5 (Cdk5), and the regulatory proteins p35 and p39 have been recently proposed to have a pivotal role in tau phosphorylation. To date, only one extensive genetic analysis has been reported in which mutations in genes encoding Cdk5, p35, and p39 where tested in early-onset AD patients from the Netherlands [59]. Although no pathogenic mutations were found, an association between one single variant of *CDK5* gene and early-onset AD was described.

Genetic variants of *TTBK1* (rs2651206, rs10807287, and rs7764257) have been linked with the late-onset form of AD [60]. The tau-tubulin kinase-1 encoded by this gene is partially responsible of the phosphorylation and subsequent aggregation of tau, its overexpression has been related to axonal neurodegeneration and it has a role in Cdk5/GSK3β activation. Moreover, the protein levels are increased in AD brains [60].

Another locus on chromosome 20, near the gene encoding the Cas scaffolding protein family member 4 (CASS4), reached genome-wide significance [35**]. Little is known about the functional role of this protein; however, it has been related to both

APP pathway [35**] and tau pathology, as its *Drosophila melanogaster* ortholog binds to CD2AP ortholog [61]. The fermitin family member 2 (encoded by *FERMT2* gene) has been associated with AD. The link with tau pathology arises from a study in *Drosophila melanogaster*, where its ortholog was involved in toxicity mediated by tau [61].

Other AD susceptibility loci (*SLC24A4/RIN3*, *DSG2*, *NME8*, *ZCWPW1*, and *CELF1*) were stated in the meta-analysis performed by Lambert *et al.*, but their role in the pathology is still difficult to elucidate [35**].

UNC5C, PLD3, and AKAP9: New Genes Related to Alzheimer's Disease?

The new genomic era has led to the discovery of genes with rare variants having a role in AD pathophysiology (i.e., *TREM2*, *SORL1* and *APP*). This has opened a complete new landscape in which additional loci will probably emerge periodically. Recent examples are *UNC5C*, *PLD3*, and *AKAP9*.

The *UNC5C* (unc-5 netrin receptor C) variant p.T835M has been associated with late-onset AD (OR 2.15) in a whole-exome sequencing study [62]. The p.T835M variant is associated with high levels of extracellular tau, thus suggesting a relationship with cell death in AD.

A rare variant (p.V232 M) in the phospholipase D3 (*PLD3*) gene was related to AD through a whole-exome sequencing approach in late-onset AD families, combined with a genotyping case–control sub-study [63]. Two additional variants (p.M6R and p.A442A) were also over-represented in the affected group [63]. However, recent studies describe a lack of association between these *PLD3* rare variants and AD risk [64–67]. At the protein level, PLD3 has been detected in Aβ plaques and it seems to indirectly interact with APP through SORL1.

Two rare variants (rs144662445 and rs149979685) in *AKAP9* (encoding the A-kinase anchoring protein 9) were identified in familial AD African American population. This gene is located on chromosome 7q21.2, and the main function of the AKAP9 protein is to regulate the activity of NMDA channels [68].

Because of the architecture and evolution of the human genome, with most of its variation being ancient and shared, and the majority of alleles recent and rare, replication of these new findings in different populations from shared ancestry and with well-defined phenotypes will be imperative to better define the role of these new loci in AD risk.

Epigenetic Changes in Alzheimer's Disease

Epigenetics refers to those mechanisms that modulate gene expression in a potentially heritable manner without changes in the primary

DNA sequence. Epigenetic modifications in AD are just beginning to be explored, and there is now growing recognition that epigenetic changes are likely to participate in its etiology [69].

The most widely characterized epigenetic mark is DNA methylation, which only occurs on the fifth carbon of cytosine (5-methylcytosine or 5mC). Predominantly found on CpG dinucleotides, this modification plays an important role in multiple cellular processes involved in the regulation of gene expression, and can result in long-term changes in cellular function [70]. Recent evidence from immunochemical studies show global changes in DNA methylation at brain areas typically affected in AD [71–74, 75**]. However, both hypomethylation and hypermethylation patterns have been reported, and conclusions about the direction of methylation alterations in AD are still unclear [76]. DNA methylation has been associated with aging [71, 77], suggesting that variations observed in brains of AD patients would be a result of accelerated age-related changes [78]. The differences in DNA methylation observed between two monozygotic twins discordant for AD supports the possible implication of this epigenetic mechanism in the pathophysiology of the disease [75**].

Another epigenetic marker is 5-hydroxymethylcytosine (5hmC), the oxidized form of 5mC. Several studies have linked this marker with both gene expression and repression [79] and it is thought to be an intermediate of DNA demethylation process [80].

Mechanisms modulating methylation patterns appear to be impaired in AD patients, prior to clinical manifestations [72]. Disregulation of methylation and hydroxymethylation would lead to a global increment of 5mC and 5hmC, which has been proposed as a cause of cell death [73].

Apart from global changes, alterations in DNA methylation patterns in multiple AD-related loci have also been reported, including genes implicated in both amyloid-β processing and tau pathology. Controversy exists about the involvement of methylation in *APP* gene promoter in AD, although most studies point towards similar methylation patterns in affected and healthy individuals [81–84]. Several Aβ degrading enzymes have also been assessed. Neprilysin levels are apparently decreased in AD brain due to the hypermethylation of its promoter induced by Aβ accumulation [85]. However, differences in *NEP* expression between diagnostic groups are still debated, as other studies show divergent results [86, 87]. A recent methylome study in AD brains showed a significant degree of hypermethylation in three genes (*TBXA2R*, thromboxane A2 receptor; *SORBS3*, sorbin and SH3 domain containing 3; and *SPTBN4*, spectrin β4) all involved in the CREB activation pathway [88]. Lately, two epigenome-wide studies reported alterations in specific loci in association

with AD neuropathology [89, 90]. Both analysis identified variations at *ANK1* (ankyrin 1) gene; and the latter also described a significant association of AD with CpG methylation of *CDH23*, *DIP2A*, *RHBDF2*, *RPL13*, *SER-PINF1*, and *SERPINF2* genes [90].

Epigenetic modifications have also been investigated in genes related to tau pathway. *MAPT* gene presents similar DNA methylation levels in both AD and healthy individuals [82]. Alterations in methylation reactions modify the activity of the protein phosphatase 2A (PP2A) and GSK-3β, two enzymes involved in tau phosphorylation, resulting in an abnormal hyperphosphorylation of the protein [91, 92].

The one-carbon metabolism (OCM) regulates the accessibility of methyltransferases to methyl groups [93]. Some studies have linked AD with OCM and the metabolites involved in this cycle [93, 94]. Nevertheless, conclusions regarding a possible association between altered OCM and AD are still unclear.

Small regulatory RNA species, such as microRNAs, is an emerging field of study in neurodegenerative diseases [95]. MicroRNAs are non-coding RNAs of 18–25 nucleotides that usually regulate gene expression in a post-transcriptional manner. These microRNAs have been detected in plasma and CSF among other biofluids, which points them as potential biomarkers for disease. Both

PCR- and sequencing-based studies have revealed a downregulation of microRNA-132 cluster in AD brains [96, 97]. Recently, the microRNA-29a/b-1 cluster, capable of modulating the β-secretase activity, has been shown to be reduced in AD patients, and this effect correlates with the presence of high β-secretase levels [98]. The microRNA let-7b has also been related to neurotoxicity and neuronal cell death through the activation of Toll-like receptor 7 [99]. Moreover, a decrease in microRNA-27a was observed in a cohort of CSF samples from AD patients, compared to the CSF from healthy patients [100*].

Conclusions

The discovery of autosomal dominant mutations in *APP*, *PSEN1*, and *PSEN2* has been crucial to unveil the pathophysiological bases of AD; however, these mutations only explain a small percentage of cases. This fact, together with the advances in genome analysis, has prompted the researchers to consider the implications of common and rare variants in AD genetic architecture. Many genes involved in different pathways have been associated with AD, including *APOEε4*, the major genetic risk factor. Additionally, several low-frequency and rare genetic variants have been shown to increase the risk of AD and one *APP* variant with potential protective effects has also been described.

Epigenetic modifications have emerged as a new research field in AD.

Differences in methylation patterns have been reported in brains of affected individuals, although the direction of these changes has to be clarified. Moreover, the association of microRNAs with susceptibility to AD is just beginning to be explored, and few studies have already revealed its possible contribution in its etiology.

Undoubtedly, the discovery of epigenetic marks occurring through the entire AD process will help, not only to understand its etiology but also to define new biomarkers of AD progression, prognosis and assessment of therapeutic response to clinical interventions.

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Cerebrospinal fluid mitochondrial DNA levels in patients with multiple sclerosis

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The role of cerebrospinal fluid (CSF) mitochondrial DNA (mtDNA) levels as biomarker in multiple sclerosis (MS) is unknown. We determined CSF mtDNA levels in a cohort of 237 individuals, including patients with MS and clinically isolated syndrome (CIS), inflammatory and non-inflammatory neurological controls, and cognitively healthy controls (HC). mtDNA concentration was measured by droplet digital polymerase chain reaction. CSF mtDNA levels were increased in all pathological conditions compared with HC, though no differences were observed between relapse-onset and progressive MS clinical forms, CIS patients and neurological controls. These findings do not support the determination of CSF mtDNA levels as a useful biomarker in MS clinical practice.

Introduction

Mitochondria are intracellular organelles, critical for energy production and a host of metabolic processes, which contain their own distinct genome. Mitochondrial damage and mitochondrial DNA (mtDNA) copy number within brain tissue have been associated with several neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases.[1,2] More recently, mtDNA levels measured in cerebrospinal fluid (CSF) were proposed as a bio-marker in multiple sclerosis (MS), based on the findings of increased CSF mtDNA levels in MS patients compared to cognitively healthy controls (HC),[3] and the detection of higher levels of mtDNA in the CSF of patients with progressive forms of MS respect to non-inflammatory neurological disease controls (NINDC).[4] Despite these studies, the role of CSF mtDNA as biomarker in MS remains unknown. In this study, we aimed to further investigate its role as a potential diagnostic and disease activity biomarker in MS by measuring CSF cell-free mtDNA levels in a large cohort of individuals with relapse-onset and progressive clinical forms of MS, patients with clinical isolated syndrome (CIS) and control subjects.

Methods

Subjects

This is a collaborative study of 237 individuals recruited from four different centres: Memory Unit at the Hospital Sant Pau (Barcelona, Spain), Neurology Clinic of the University of Belgrade (Serbia), Department of Neurology at the Medical University of Graz (Austria) and Hospital Ramón y Cajal (Madrid, Spain). Approval was received from the local ethical standards committee on human research. Participants provided written informed consent.

The study cohort comprised 125 MS patients; 31 patients with CIS (16 patients who converted to clinically definite MS and 15 who remained as CIS), 41 HC, 20 NINDC and 20 inflammatory neurologic dis-ease controls (INDC). The MS group included 51 patients with relapsing-remitting MS (RRMS), 38 patients with secondary progressive MS (SPMS) and 36 patients with primary progressive MS (PPMS). In four MS patients, CSF was collected at the time of an acute relapse. All MS and CIS patients were untreated at the time of CSF collection. A summary of the demographic and main clinical characteristics is shown in **Table 1**.

mtDNA analysis

The concentration of mtDNA was measured using a digital droplet polymerase chain reaction (ddPCR) technique (QX200TM ddPCR System, Bio-Rad Laboratories, Hercules, CA, USA) in CSF samples collected by lumbar puncture between years 1999 and 2017 for routine CSF diagnostics. Briefly, CSF samples were partitioned into ~20,000 water-in-oil

Table 1. Demographic and baseline clinical characteristics of MS patients and control subjects included in the study.

Baseline characteristics	CIS	MS[b]	RRMS	SPMS	PPMS	INDC[c]	NINDC[d]	HC
N	31	125	51	38	36	20	20	41
Age (years)	36.9 (11.5)	44.7 (12.2)	36.3 (9.8)	51.3 (10.1)	49.3 (10.4)	49.7 (17.5)	68.0 (9.5)	53.3 (13.7)
Female/male (% women)	19/12 (78.7)	76/49 (60.8)	32/19 (62.7)	22/16 (57.9)	22/14 (61.1)	7/13 (35.0)	9/11 (45.0)	20/21 (48.8)
Disease duration (years)	–	8.3 (8.9)	5.3 (6.3)	15.4 (10.9)	5.0 (4.4)	–	–	–
EDSS[a]	–	3.5 (2.0–5.7)	2.0 (1.5–3.0)	5.5 (4.0–6.5)	3.0 (4.8–6.0)	–	–	–

EDSS: Expanded Disability Status Scale; CIS: clinically isolated syndrome; MS: multiple sclerosis; RRMS: relapsing-remitting MS; SPMS: secondary progressive MS; PPMS: primary progressive MS; INDC: inflammatory neurologic disease controls; NINC: non-inflammatory neurologic disease controls; HC: healthy controls.

[a]Data are expressed as mean (standard deviation) unless otherwise stated.

[b]Refers to the whole MS group.

[c]The INDC group included patients with autoimmune encephalitis (*n* = 13), acute disseminated encephalomyelitis (*n* = 3), vasculitis (*n* = 2), Behçet disease (*n* = 1), transient headache and neurologic deficits with CSF lymphocytosis (*n* = 1).

[d]The NINDC included patients with a diagnosis of Alzheimer's disease (*n* = 20).

droplets. The droplets were transferred into a plate, followed by end-point amplification. Afterwards, the plate was loaded into the Droplet Reader, which quantifies the positive and negatives droplets, depending on the presence or absence of template.[5] The absolute number of copies of mtDNA was quantified using the Quantasoft software analysis (Bio-Rad Laboratories). To avoid contamination of CSF with cells that could be a source of cellular mtDNA, a simultaneous detection of mtDNA and the Bcl-2-associated X (*BAX*) gene (a single copy nuclear gene) was used in a multiplex ddPCR reaction.[6] Samples with 1 copy of BAX gene were excluded from further analysis.

Statistical analysis

Statistical analysis was performed using the SPSS 17.0 package (SPSS Inc, Chicago, IL) for MS-Windows. Parametric and non-parametric tests were applied depending on data distribution. Differences were considered statistically significant when p-values were below 0.05.

Results

Cell-free CSF mtDNA levels are increased in different pathological conditions

As shown in **Figure 1(a)**, CSF mtDNA levels were significantly increased in all pathological conditions compared with the HC group ($p = 0.008$ for CIS, $p = 0.004$ for the whole group of MS patients, $p = 0.03$ for NINDC and $p = 0.004$ for INDC). No significant differences were observed between MS patients, CIS patients and
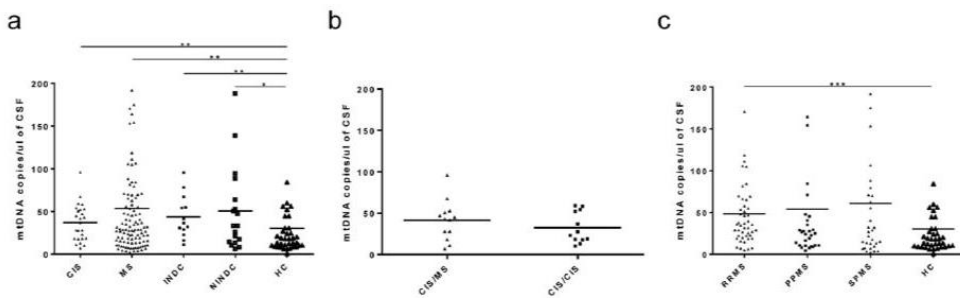
Figure 1. **Dot plots showing cell-free mtDNA levels in CSF samples.**

As described in section 'Methods', CSF levels of mtDNA were determined by ddPCR and represented as mtDNA copies/μl. Graphs show (a) higher levels of mtDNA in all pathological conditions compared with HC, (b) comparable mtDNA levels between CIS patients who converted to MS (CIS/MS) and CIS patients who remained as CIS (CIS/CIS) and (c) higher mtDNA levels in RRMS patients compared with HC. CIS: clinically isolated syndrome. MS: whole group of MS patients. RRMS: relapsing-remitting MS. SPMS: secondary progressive MS. PPMS: primary progressive MS. INDC: inflammatory neurologic disease controls. NINDC: non-inflammatory neurologic disease controls. HC: healthy controls.

Only significant p values are shown in the graphs. For the sake of clarity, outliers (defined as cases that have values more than three times the height of the boxes) were removed from the graphs and atypical values (representing values 1.5 times the height of the box) are represented with dots. *p values < 0.05. **p values < 0.01. ***p values < 0.001.

neurologic disease controls (**Figure 1(a)**). Within the CIS group, CSF mtDNA levels did not significantly differ between CIS patients who converted to MS and those who continued as CIS (**Figure 1(b)**). Within the MS group, CSF mtDNA levels were increased in RRMS patients compared with HC ($p = 0.0004$), though no significant differences were observed between relapsing and progressive forms of MS (**Figure 1(c)**). Within the MS group, CSF mtDNA levels were similar between patients in relapse and remission, patients with and without gadolinium enhancing lesions, and patients with and without progression on the EDSS score during follow-up

(data not shown). Finally, mtDNA levels were not influenced by age or gender.

Discussion

In this study, we found that cell-free mtDNA levels were increased in the CSF of patients with MS and other inflammatory and non-inflammatory neurological conditions compared with HC. Moreover, within the MS group, mtDNA levels were comparable among clinical forms and differences only reached statistical significance in RRMS patients when compared with HC.

In a recent report by Varhaug et al.,[3] the authors showed an increase in the CSF levels of mtDNA in patients with

MS with respect to HC, observing that it is in line with our findings in a larger cohort. In another recent study by Leurs et al.,[4] a direct comparison of CSF mtDNA levels between the whole MS group and the HC group was not provided, though the authors demonstrated increased mtDNA levels in MS patients with a progressive course compared to NINDC,[4] differences that were not observed in our study.

Although it has been suggested that the CSF mtDNA content could be associated with inflammation,[7] in our study mtDNA levels did not help to discriminate between inflammatory and non-inflammatory neurological conditions. In addition, CSF mtDNA levels were not useful to differentiate between CIS patients who eventually converted to MS from patients who remained as CIS.

It should be mentioned that the quantification of CSF mtDNA levels in other neurological conditions have resulted in discrepant results, and whereas some studies showed reduced mtDNA content, for instance, in Alzheimer's and Parkinson's diseases compared to HC,[1,2] other reports were in line with our findings and observed higher mtDNA content in pathological conditions compared to healthy individuals.[3,8] In this regard, it has been discussed that mtDNA levels could reflect alterations in brain metabolism1 or an inflammatory activity within the CNS.[3]

The high-throughput ddPCR approach allowed to quantify with high precision the absolute amount of mtDNA present in a sample. Despite low intra- and inter-assay coefficients of variation, mtDNA levels showed wide dispersion among individuals of the same group. This high degree of interindividual variability led to great overlap in the mtDNA levels between controls and patients, and warrants cautious interpretation of our data. Altogether, our findings as well as results from previous studies do not support the quantification of CSF mtDNA levels as a useful biomarker in MS clinical practice.

Acknowledgments

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