Assessing the genetic overlap in amyotrophic lateral sclerosis and frontotemporal dementia

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Summary

There are overwhelming evidences that amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are part of a clinical, pathological and genetic continuum. The *C9orf72* repeat expansion is the most common genetic defect among ALS/FTD patients. Besides, the use of next generation sequencing technologies has launched a new era in the study of human diseases. These approaches have unraveled an unprecedented amount of genes associated with both ALS and FTD. This work focuses on the study of the genetic overlap in the ALS/FTD spectrum. In this context, we have studied the *C9orf72* expansion mutation in ALS and FTD patients. Also, we have assessed the role of *CHCHD10* and *TUBA4A*, two genes recently found to cause ALS, in Spanish patients with ALS and/or FTD. Finally, we have performed whole-exome sequencing in ALS/FTD patients without the *C9orf72* expansion mutation to study the genetic profile of these cases free from the most common genetic defect related to these neurodegenerative disorders.

Resum

Una gran quantitat d'evidències suggereixen que l'esclerosi lateral amiotròfica (ELA) i la demència frontotemporal (DFT) formen part d'un contínuum clínic, patològic i genètic. L'expansió d'un hexanucleòtid al gen *C9orf72* és l'alteració genètica més freqüent en els pacients amb ELA/DFT. L'ús de les tecnologies d'ultraseqüenciació ha iniciat una nova era en l'estudi de les malalties humanes. Aquests mètodes han permès el descobriment d'una gran quantitat de gens associats a l'ELA i la DFT. Aquesta tesi se centra en estudiar el contínuum genètic en l'espectre ELA/DFT. Així doncs, s'ha estudiat el paper de l'expansió de l'hexanucleòtid a *C9orf72* en pacients amb ELA i DFT. També s'ha estudiat el rol dels gens *CHCHD10* i *TUBA4A*, dos gens que recentment s'ha demostrat que causen ELA, en pacients espanyols amb ELA i/o DFT. Finalment, l'exoma de pacients amb ELA/DFT no portadors de l'expansió a *C9orf72* s'ha seqüenciat per tal d'estudiar el component genètic associat a l'espectre ELA/DFT lliures de l'alteració genètica més comuna.

Preface

Amyotrophic lateral sclerosis (ALS) is a motor neuron disease characterized by the degeneration of upper and lower motor neurons, which leads to muscle weakness and a progressive paralysis[1]. Frontotemporal dementia (FTD) is a neurodegenerative disorder defined by atrophy of the frontal and temporal lobes resulting in behavioral and/or language impairment[2]. Both disorders have been considered two separate entities until 1981, when Hudson reviewed and firmly associated ALS with dementia and other neurological disorders[3]. Notably, cognitive deterioration was still interpreted as an exclusion criterion for the diagnosis of ALS at the ALS diagnostic guidelines defined in 1990 at the Consensus Conference held in El Escorial[4]. These criteria were renewed and updated in 1998 in order to include cognitive and/or behavioral impairment in ALS diagnosis[5], and a definite framework provided in 2009 by Strong and collaborators[6]. At the beginning of the 21th century, the evidence that ALS and FTD were overlapping clinical syndromes was overwhelming[7,8]. Advances in molecular pathology and genetics provided a definite link between both diseases in the following years. Firstly, in 2006, the trans-active response DNA-binding protein of 43 KDa (TDP-43) was identified as the pathological hallmark of ubiquitinated inclusions in the majority of ALS cases (ALS-TDP) and in the most common frontotemporal lobar degeneration (FTLD) subtype, consequently named FTLD-TDP[9,10]. The most important genetic evidence supporting this overlap came from linkage studies in a set of autosomal-dominant families in which affected members suffered from ALS and/or FTD

and showed a linkage to chromosome 9p21[11–13]. Subsequently, genome wide association studies (GWAs) performed in ALS[14–16] and FTLD-TDP patients[17] provided support for this finding. Data from these studies pointed to the identification of the most common genetic cause of the ALS/FTD disease continuum: the hexanucleotide repeat expansion within the chromosome 9 open reading frame 72 (*C9orf72*) gene[18,19]. Recently, next generation sequencing (NGS) technologies have generated huge amount of data, mainly by exome sequencing, and has led to the identification of rare genetic variants (<1% in the general population). By applying exome-wide rare variant analyses, new ALS causing genes have been identified. Of note, some of the ALS patients shown to carry rare pathogenic variants in these genes also suffered from FTD, further supporting the genetic overlap between both disorders[20–22].

The objectives of this thesis were to assess the genetic overlap in ALS and FTD. For this purpose, the prevalence of the hexanucleotide repeat expansion was determined using a repeat-primed PCR (rpPCR) in a series of 936 DNA samples from ALS patients recruited through a collaborative effort across the country (see publication 3.1). As the exact number of hexanucleotide repeats cannot be resolved through the rpPCR method, a Southern blot approach was applied in order to determine the hexanucleotide repeats, compare its length between ALS and FTD cases and seek for correlations with clinical features of both disorders (see publication 3.2). Further, we aimed to estimate the mutation

spectrum and the prevalence of recently identified genes through the study of patients from Spanish ancestry. In this regard, we evaluated the *CHCHD10* and the *TUBA4A* genes in a series of 1224 ALS and/or FTD patients (see publication 3.3) and a series of 814 FTD patients (see publication 3.4), respectively. We finally performed exome-sequencing in a cohort of 54 patients presenting with both ALS and FTD not carrying the *C9orf72* repeat expansion mutation. Our goal was to explore the genetic profile implicated in the ALS/FTD continuum without the hexanucleotide repeat expansion in the *C9orf72* gene (see publication 3.5).

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1. INTRODUCTION

"The more I learn, the more I realize how much I don't know"

Albert Einstein

1.1. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) was first described by the French neurologist Jean-Martin Charcot in 1869[23], and named by its distinctive neuropathology in 1874[24]. ALS is a neurodegenerative disorder and the most common presentation of the motor neuron disease (MND) spectrum. It is characterized by the progressive loss of motor neurons of the primary motor cortex (upper motor neurons (UMN)), brainstem and spinal cord (lower motor neurons (LMN))[1].

a) Clinical phenotypes, prognosis and epidemiology

As stated in the first ALS diagnostic guidelines[4], the evidence of UMN and LMN involvement is required for the diagnosis of ALS. Clinically, UMN degeneration results in spasticity, slowed rapid alternating movements, increased reflexes and gait disorder. Affection of LMN is characterized by severe weakness, reduced reflexes, muscle atrophy and fasciculations. ALS patients may show UMN or LMN predominant signs. Patients with only UMN or LMN

involvement represent two ends of the MND continuum, and are diagnosed with primary lateral sclerosis and primary muscular atrophy, respectively.

The disease phenotype is usually classified by the site of onset. Commonly, ALS starts with asymmetric painless weakness in a limb, referred to as limb-onset, which represents about 70% of ALS cases. Alternatively, patients can present with bulbar-onset (25% of ALS cases), in which the weakness starts in bulbar muscles, resulting in dysarthria, dysphagia and tongue fasciculations. Individuals with bulbar-onset have a worse prognosis than patients with limb-onset, as they are prone to aspiration and nutritional problems. Finally, 5% of patients have respiratory-onset disease, characterized by orthopnoea or dyspnoea, and almost absent limb or bulbar signs. These patients show the worst prognosis among ALS cases[1,25,26].

ALS patients have a mean age at onset of 58-63 years in sporadic cases and between 47-52 years in patients with positive family history. Despite this, ALS patients may have an age at onset before 25 years of age, also known as juvenile ALS[1,26]. Median survival is around 3 years from diagnosis, although about 10% of patients live longer than 10 years after diagnosis. A longer survival from diagnosis is associated with juvenile ALS, UMN-predominant ALS and limb-onset[1,27]. In Europe, the mean incidence of ALS is 2.08 cases per 100.000 people and the prevalence is 5.4/100.000. The

overall lifetime risk of developing the disease is 1:400. The male to female incidence rate ratio is 1.4[25,26,28].

b) Pathology

The first correlations between ALS clinical features and its neuropathological hallmarks were made by Jean-Martin Charcot in 1869[23]. Other important pathological features were subsequently described by other researchers. In 1909, Brodmann included the observation of loss of the giant cells of Betz[29]. Afterwards, the identification of eosinophilic inclusions, now known as Bunina bodies, was made in 1961[30].

Although ubiquitine-immunoreactive deposits in anterior horn cells of MND cases were identified in 1988[31,32], the identity of the major component of the ubiquitinated pathological proteins was not described until 2006, when the trans-active response DNA-binding protein of 43 KDa (TDP-43) was identified as the major constituent of these cytoplasmatic, nuclear and neuritic inclusions[9,10]. TDP-43 pathological inclusions represent 97% of ALS pathological series (ALS-TDP)[33]. The neuropathology of ALS-TDP is characterized by the aberrant cytoplasmatic aggregation of TDP-43 in neurons and glia of the primary motor cortex, brainstem motor nuclei, spinal cord, and the associated white matter tracts (Figure 1)[34].

Two years after the discovery of TDP-43 protein aggregates, the fused in sarcoma (FUS) protein was identified in ubiquitin-positive inclusions without TDP-43 immunoreactivity in few ALS cases (ALS-FUS)[12,35]. This pathological change accounts for 1% of ALS cases[33]. ALS-FUS is associated with mutations in the *FUS* gene and characterized by cytoplasmatic accumulation of FUS in both neurons and glia of motor cortex, spinal cord, brainstem, striatum, thalamus and substantia nigra[36]. Importantly, *FUS* mutations may lead to juvenile ALS onset which has distinct pathological features as compared to mid-adult onset ALS-FUS cases. That is, mid-adult onset ALS-FUS cases show very few basophilic inclusions (BI), filamentous FUS neuronal cytoplasmatic inclusions (Figure 2a and 2b) and several glial cytoplasmatic inclusions (GCI), whereas young onset ALS-FUS cases are pathologically characterized by frequent BI, round FUS cytoplasmatic inclusions (Figure 2c and 2d) and rare GCI[37].

Finally, approximately 2% of ALS patients show Lewy body-like inclusions in the anterior horn motor neurons, which stain positive for the superoxide dismutase 1 (SOD1) protein, ubiquitin, phosphorilated neurofilaments and various chaperone proteins (ALS-SOD)[38,39].

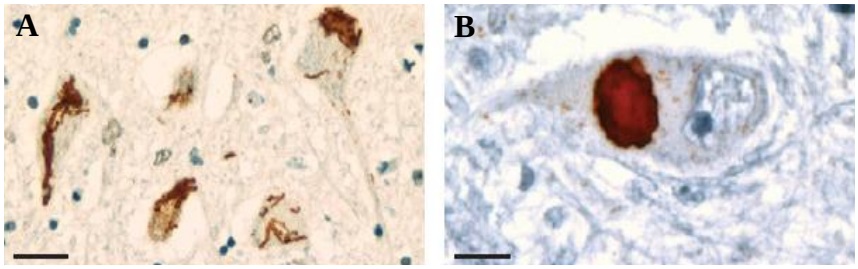

Figure 1. TDP-43 immunoreactive skein-like (a) and round (b) inclusions in motor neurons of ALS-TDP. Scale bars: 20µm (a) and 10µm (b). From Mackenzie et al. *Lancet Neurol.* 2010.

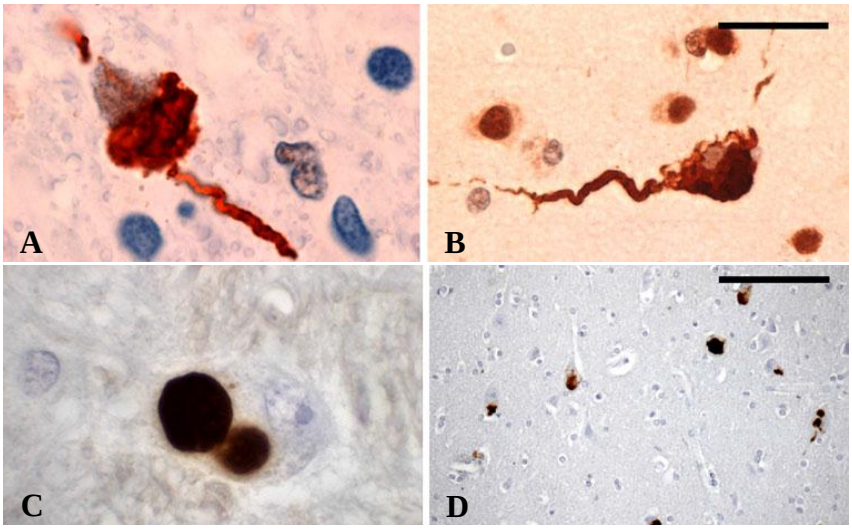

Figure 2. FUS immunoreactive inclusions in LMN (a) and UMN (b) of an adult onset ALS-FUS case, and in LMN (c) and primary motor cortex (d) of a young onset ALS-FUS case. Scale bar: 15µm (a), 25µm (b), 20µm (c) and 100µm (d). From Mackenzie et al. *Acta Neuropathol.* 2011.

c) Genetics

ALS can be inherited in an autosomal dominant, autosomal recessive and X-linked manner. Around 10% of ALS cases are considered familial (fALS), as they have a first-degree affected relative, and 90% are defined as sporadic (sALS)[40].

In 1991, linkage analyses in fALS pedigrees pointed to a locus on chromosome 21q, and the subsequent identification of the first gene responsible for ALS[41,42]: the *SOD1* gene. To date, more than 150 mutations in *SOD1* have been described, which are related to ALS-SOD pathological diagnosis, segregate into dominant and recessive forms, and are rarely associated with juvenile onset forms[43,44].

Following the identification of TDP-43 protein inclusions, mutations in the gene encoding this protein, the trans-active response DNA binding protein (*TARDBP*) gene, were subsequently described in 2008[45,46]. More than 40 mutations in this gene have been shown to cause ALS in an autosomal dominant pattern of inheritance, and are associated with ALS-TDP pathology[47,48].

Shortly after the associations of mutations in *TARDBP* in ALS, the *FUS* gene was cloned in 16p-linked familial ALS[12,35]. More than 50 mutations in this gene have been reported, inherited in autosomal dominant and recessive patterns[40,47,48]. Besides, mutations in *FUS* also cause juvenile onset ALS. Patients with *FUS* mutations have an ALS-FUS diagnosis at autopsy[49].

Optineurin (*OPTN*) was the next ALS causal gene identified. In 2010, mutations in this gene were found to segregate in families from Japan[50]. More than 20 mutations have been found, causing both dominant and recessive traits, although they are very rare and almost absent in European population[40,47,48]. Mutations in this gene result in ALS-TDP pathology[51].

The same year, using exome sequencing, mutations in the valosin containing protein (*VCP*) gene were found to be a cause of autosomal dominant familial ALS[52]. To date, more than 20 mutations have been identified as pathogenic[40,47,48] and associate with TDP-43 positive inclusions[33].

In 2011, mutations in the ubiquilin-2 (*UBQLN2*) gene, although rare, were found to cause X-linked dominant ALS, both in adult and juvenile onset cases. Cases with *UBQLN2* gene mutations show ALS-TDP pathology at autopsy. UBQLN2 positive inclusions are also frequent in these cases and do not colocalize with TDP-43 immunoreactivity [53].

The hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 (*C9orf72)* gene was identified as the cause of autosomal dominant ALS in large families in 2011[19,54]. To date, it is the most important genetic cause of both fALS and sALS[55] (extended in subchapter 1.4).

The sequestosome 1 (*SQSTM1*) gene encodes an ubiquitin-binding protein named p62, which is present in neuronal and glial ubiquitin-positive inclusions of several neurodegenerative diseases[56]. Besides, p62 immunoreactivity has been demonstrated in the spinal anterior horn cells of ALS patients[57]. Using a candidate gene approach, *SQSTM1* mutations were identified as a cause of ALS[58]. Subsequently, larger studies demonstrated that some *SQSTM1* variants are associated with an increased risk of ALS[59]. These cases are pathologically characterized by p62 and TDP-43 positive inclusions[60].

As explained before, ALS patients may have a juvenile onset, which is typically before 25 years old[1]. In addition to *SOD1*, *FUS* and *UBQLN2* mutations, other genes have been related to both autosomal recessive and dominant juvenile onset cases. The spatacsin (*SPG11*) gene was first related to autosomal recessive hereditary spastic paraplegia[61]. The overlap between this disorder and ALS[62], led to the assessment of *SPG11* in juvenile ALS, which was found to be associated with autosomal recessive juvenile onset ALS[63]. Two other genes implicated in juvenile ALS are the senataxin (*SETX*) and alsin (*ALS2*) genes, which through linkage analyses were found to cause autosomal dominant and recessive juvenile ALS, respectively[64–67].

Recently, exome sequencing technologies have unraveled other genes and pathways implicated in the disease[22]. A good example of the value of this approach is the identification of the

serine/threonine protein kinase (*TBK1*) gene. It was suggested as a new ALS-susceptibility gene by Cirulli and colleagues using exome sequencing in 2869 ALS patients and 6405 controls[22]. This finding was subsequently supported by a study which identified an enrichment of loss-of-function *TBK1* mutations in 252 fALS cases and found that *TBK1* haploinsufficiency is associated with ALS[68]. Besides, the use of exome sequencing technologies have disclosed the association of other genes with ALS, such as the heterogeneous nuclear ribonucleoprotein A1 (*HNRNPA1*) or A2/B1 (*HNRNPA2B1*) genes[69], the tubulin alpha 4a (*TUBA4A*) gene[70], the profilin 1 (*PFN1*) gene[71] or the coiled-coil helix coiled-coil helix domain containing 10 (*CHCHD10*) gene[20].

Susceptibility loci or genetic variants influencing the ALS phenotype, mainly age at onset or survival, have largely been studied. Most of them have not been replicated in large cohorts, and others appear to have a more robust signal of association, such as the ataxin 2 (*ATXN2*) gene, the survival of motor neuron 1 (*SMN1*) gene or the unc-13 homolog A (*UNC13A*) gene. CAG-repeat expansions of intermediate length in the *ATXN2* gene have been found to increase the risk of ALS and modify disease survival[72,73]. Besides, *SMN1* gene duplications are associated with ALS susceptibility[74–76]. The *UNC13A* gene has been associated as a susceptibility locus and modifier of survival in ALS[14,77–79]. Recently, a GWAs performed in 6100 ALS patients and 7125 controls identified a new susceptibility locus for ALS, which encompasses the sterile alpha and TIR-containing motif 1 (*SARM1*)

gene. Finally, the largest GWAs performed to date included, for the discovery phase, data derived from whole genome sequencing of 12577 cases and 23475 controls. The study replicated previous associations (*C9orf72*, *UNC13A* and *SARM1* genes), but also identified three new loci which reached genome-wide significance in the discovery and replication cohorts. These signals include the chromosome 21 open reading frame 2 (*C21orf2*), the myelin-associated oligodendrocyte basic protein (*MOBP*) and the sec 1 family domain containing 1 (*SCFD1*) genes. Also, the same authors that proposed *TBK1* as a new susceptibility gene, suggested that loss-of-function variants in the nima-related kinase 1 (*NEK1*) gene constitute a risk for ALS[22]. Two recent studies support the notion that *NEK1* is involved in ALS etiology. Both studies show that rare missense variants in *NEK1* increase the risk of ALS, but also demonstrate heterozygous loss-of-function variants as a cause of fALS[80,81]. The above described disease causing genes together with rarer genetic causes and other susceptibility loci related to ALS pathogenesis are summarized in table 1.

Table 1. Genes involved in ALS etiology.

Gene	Location	Inheritance	fALS (%)	sALS (%)	Onset	Mean AAO	AAO range
C9orf72	9p21.2	AD	20-50	3-20	Adult	57	27-80
SOD1	21q22.11	AD/AR	15-20	2	Adult/Juvenile	48	18-94
TARDBP	1p36.22	AD	4	1	Adult	55	30-77
TBK1	12q14.2	AD	3	1	Adult	60	29-78
FUS	16p11.2	AD/AR	3	1	Adult/Juvenile	46	13-80
SQSTM1	5q35.3	AD/Risk	2	<1	Adult	56	41-78
VCP	9p13.3	AD	1	1	Adult	49	36-68
OPTN	10p13	AD/AR	<1	<1	Adult	51	24-83
UBQLN2	Xp11.21	XL	<1	<1	Adult/Juvenile	44	14-78
CHMP2B	3p11.2	AD	<1	<1	Adult	NA	NA
PFN1	17p13.2	AD	<1	<1	Adult	53	40-70
ANG	14q11.2	AD	<1	<1	Adult	55	27-83
FIG4	6q21	AD	<1	<1	Adult	55	29-77
ALS2	2q33.1	AR	<1	<1	Juvenile	2	1-3
SETX	9q34.13	AD	<1	<1	Juvenile	18	1-42
SPG11	15q21.1	AR	<1	<1	Juvenile	16	7-23
VAPB	20q13.33	AD	<1	<1	Adult	44	25-73

SIGMAR1	9p13.3	AR	<1	<1	Juvenile	1-2	1
SPAST	2p24	AD	<1	<1	Juvenile	24	24
DAO	12q24.1	AD	<1	<1	Adult	44	42-55
CHCHD10	22q11.2	AD	<1	<1	Adult	56	35-73
DCTN1	2p13.1	AD	<1	<1	Adult	55	48-64
TUBA4A	2q35	AD	<1	<1	Adult	60	48-78
HNRNRPA1	12q13.1	AD	<1	<1	Adult	NA	NA
HNRNPA2B1	7p15.2	AD	<1	<1	Adult	NA	NA
ERBB4	2q34	AD	<1	<1	Adult	61	45-70
MATR3	5q31.2	AD	<1	<1	Adult	52	36-64
MAPT	17q21.31	AD	<1	<1	Adutl	50	37-65
DJ1	1p36.23	AR	<1	<1	Adult	32	24-36
CCNF	16p13.3	AD	<1	<1	Adult	55	42-66
NEK1	4q33	AD/Risk	<1	<1	Adult	56	54-59
TAF15	17q12	AD	<1	<1	Adult	60	47-68
EWSR1	22q12.2	AD	<1	<1	Adult	43	36-50
ATXN2	12q24.1	Risk/Disease modifier	-	-	-	-	-
SMN1	5q13.2	Risk	-	-	-	-	-
UNC13A	19p13.12	Risk/Disease modifier	-	-	-	-	-
SARM1	17q11.2	Risk	-	-	-	-	-

C21orf2	21q22.3	Risk	-	-	-	-	-
SCFD1	14q12	Risk	-	-	-	-	-
MOBP	3p22.1	Risk	-	-	-	-	-
ELP3	8p21.1	Risk	-	-	-	-	-

AD: autosomal dominant; AR: autosomal recessive; XL: X-linked; AAO: Age at onset; NA: Not Available.

1.2. Frontotemporal dementia

Frontotemporal dementia (FTD) is a neurodegenerative disease characterized by the degeneration of the frontal and temporal lobes, which clinically results in behavioral and/or language impairment[2]. The first description of a patient with FTD was made by Arnold Pick in 1892, who reported the case of August H. The patient, a 71-year-old male, presented with cognitive decline and a progressive speech disorder[82].

a) Clinical phenotypes, prognosis and epidemiology

FTD is the second most common form of dementia in individuals younger than 65 years. In the 45-65 years age group, the estimated mean prevalence is 15-22 per 100,000 people[83,84], and an incidence between 3,5 and 4,1 new cases per 100,000 people every year[85,86]. Importantly, as FTD is still misdiagnosed mostly in cases older than 65 years old at onset, the majority of estimations probably undervalue its exact prevalence and incidence[87]. A recent report estimated the prevalence of FTD in the 66-75 and over 75 age groups, disclosing a prevalence of 78 and 54 per 100,000 people, respectively[88]. The mean disease duration in FTD is 6-11 years from symptoms onset, ranging from 2 to 20 years[89,90].

The behavioral-variant of frontotemporal dementia (bvFTD) is the most common clinical presentation of FTD and includes personality changes, disinhibition, loss of empathy and apathy. Patients with

bvFTD show heterogeneous phenotypes, with some of them presenting with overactivity and disinhibition, while others showing predominantly apathy and lack of drive. Dietary changes and stereotyped/repetitive behaviors are also common features in the bvFTD. Importantly, psychiatric misdiagnosis might be an important issue in some bvFTD patients[91]. Cognitive deficits in these patients are typically dysexecutive, with problems in judgment, attention and planning, among others. However, memory and visuospacial functions are typically preserved[2,92]. Parkinsonism is the most common movement disorder in bvFTD patients[93].

Primary progressive aphasia (PPA) is the term that comprises the language impairment in FTD[94], and is characterized by an insidious and progressive decline in linguistic skills. PPA encompasses two main forms: semantic dementia (SD or svPPA) and non-fluent progressive aphasia (NFPA or nfvPPA). SD patients have prominent word-finding difficulties in spontaneous speech, severe anomia and impaired word comprehension. Notably, behavioral changes, involving dietary modifications, irritability and/or social withdrawal are also common in SD. Patients diagnosed with NFPA are characterized by a dysfunction in language production and comprehension, with preservation of word meaning. As disease progresses, it eventually results in mutism, although some patients are still able to communicate in writing[2,95].

b) Pathology

The first description of argyrophilic globular neuronal cytoplasmatic inclusions (Pick bodies) and diffusely staining ballooned neurons (Pick cells) was made by Alois Alzheimer in 1911 (Figure 3)[96]. In 1925, Gans named the clinicopathological entity Pick's disease (PiD)[97]. However, these lesions seemed to be present only in approximately 20% of patients, which suggested that frontotemporal lobar atrophy is pathologically heterogeneous[98]. The first consensus on the neuropathological classification of PiD was made in 1974. Authors classified PiD into three major groups: group A consisted of cases with both Pick bodies and Pick cells; cases that only presented swollen neurons were part of group B; and cases with neither Pick bodies nor Pick cells belonged to group C[99].

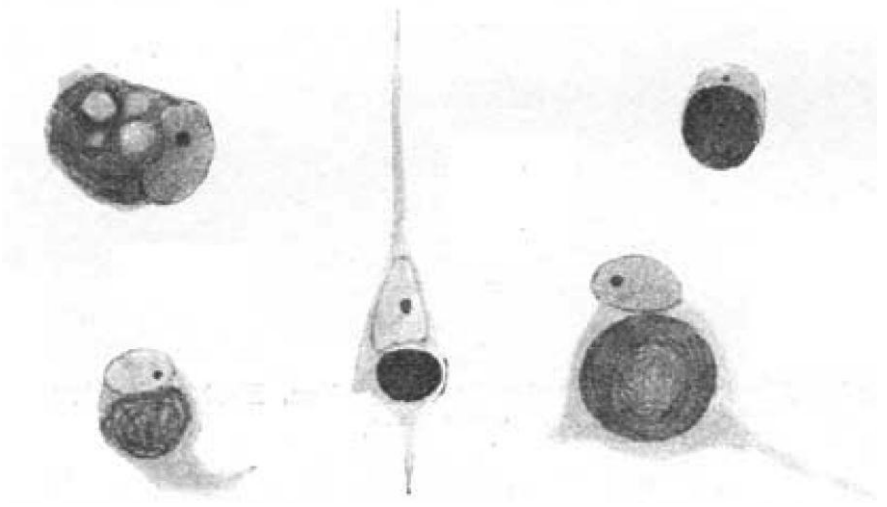

Figure 3. Illustration of Pick bodies by Alois Alzheimer. From Alzheimer *Z Gesamte Neurol Psychiatr* 1911.

Subsequent studies developed in Lund and Manchester Universities emphasized the absence of Pick bodies or Alzheimer type pathology in the majority of frontotemporal lobar degeneration (FTLD) cases. These cases showed loss of neurons and gliosis, and were identified as dementia lacking distinctive histological features (DLDH)[100–102]. In the last decade of the twentieth century, ubiquitin-positive inclusions were described in the majority of cases with DLDH, which replaced the designation of DLDH with FTLD with ubiquitin-positive inclusions (FTLD-U)[103–105].

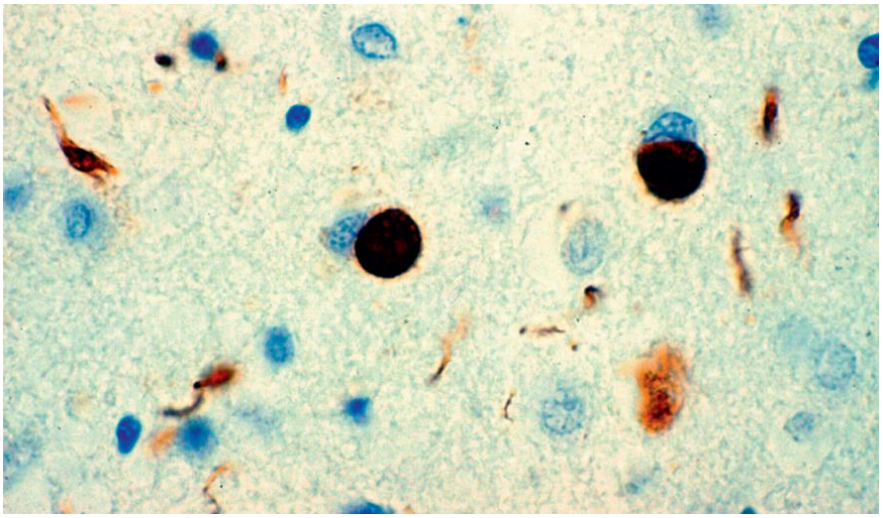

Figure 4. Pick bodies and abnormal neuritic inclusions composed of tau protein. From Spillantini and Goedert, et al. *Lancet Neurol.* 2013.

Within the same decade, the accumulation of abnormal hyperphosphorilated forms of tau protein in neurons and glia (FTLD-tau) was described. The compositions of these abnormal aggregates is a pathological hallmark of PiD (Figure 4), showing a predominance of 3 tandem repeat (3R) with respect to 4 tandem repeat (4R) sequences of the tau protein. The pathological term

FTLD-tau comprises a spectrum which includes progressive nuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease (AGD), globular glial tauopathy (GGT) and PiD[106–109]. In contrast to PiD, PSP, CBD, AGD and GGT present a predominance of the 4R tau isoform. The underlying pathology of about 40% of cases with FTD is FTLD-tau[33].

In 2006, TDP-43 was found to be the major component of the ubiquitin-positive inclusions in most of the FTLD-U cases, consequently reclassifying them as FTLD-TDP[9,10]. Around 45% of FTD cases show FTLD-TDP pathology at autopsy. TDP-43 immunoreactivity is found in neuronal cytoplasmatic inclusions (NCIs), neuronal intranuclear inclusions (NIIs), oligodendroglial inclusions and dystrophic neurites (DNs) in the frontotemporal neocortex and granule cells of the dentate gyrus. The classification of FTLD-TDP includes four different subtypes, based on the predominant type of lesion and their distribution (Figure 5). FTLD-TDP type A is characterized by several NCIs and DNs, and some NIIs in superficial cortical layers (most numerous in layer 2); type B is associated with several NCIs in the superficial and deeper cortical layers, and occasionally DNs; type C pathology is characterized by abundant and long DNs, most of them with a corkscrew appearance in superficial cortical layers, and few NCIs and NIIs; type D is characterized by numerous NIIs and DNs throughout the entire cortical thickness, and few NCIs[110,111].

Despite this discovery, 10% of FTLD-U cases had ubiquitin inclusions negative for tau and TDP-43. Thus, the major component of these ubiquitin-positive inclusions remained unknown until the description of FUS protein aggregates. This finding gave the basis for the nomenclature of the third FTLD subgroup, labeled FTLD-FUS. This group includes three pathological conditions: neuronal intermediate filament inclusion disease (NIFID), basophilic inclusion body disease (BIBD) and atypical FTLD-U (aFTLD-U) depending on inclusion types and their distribution. Besides, severe caudate atrophy and hippocampal sclerosis are consistent features of FTLD-FUS cases[112,113]. Subsequently, two other proteins also belonging to the FET family of proteins (Ewing's sarcoma (EWS) and TATA-binding protein associated factor 15 (TAF15)) and transportin1 (TRN1) were found to be part of these FUS positive pathological inclusions, consequently renamed FTLD-FET[114,115]. FTLD-FET cases appear to be sporadic[36].

Finally, rare cases show ubiquitin/p62 immunoreactivity and TDP-43/FUS negative inclusions at autopsy. These cases belong to the fourth FTLD subgroup, termed FTLD-UPS[111].

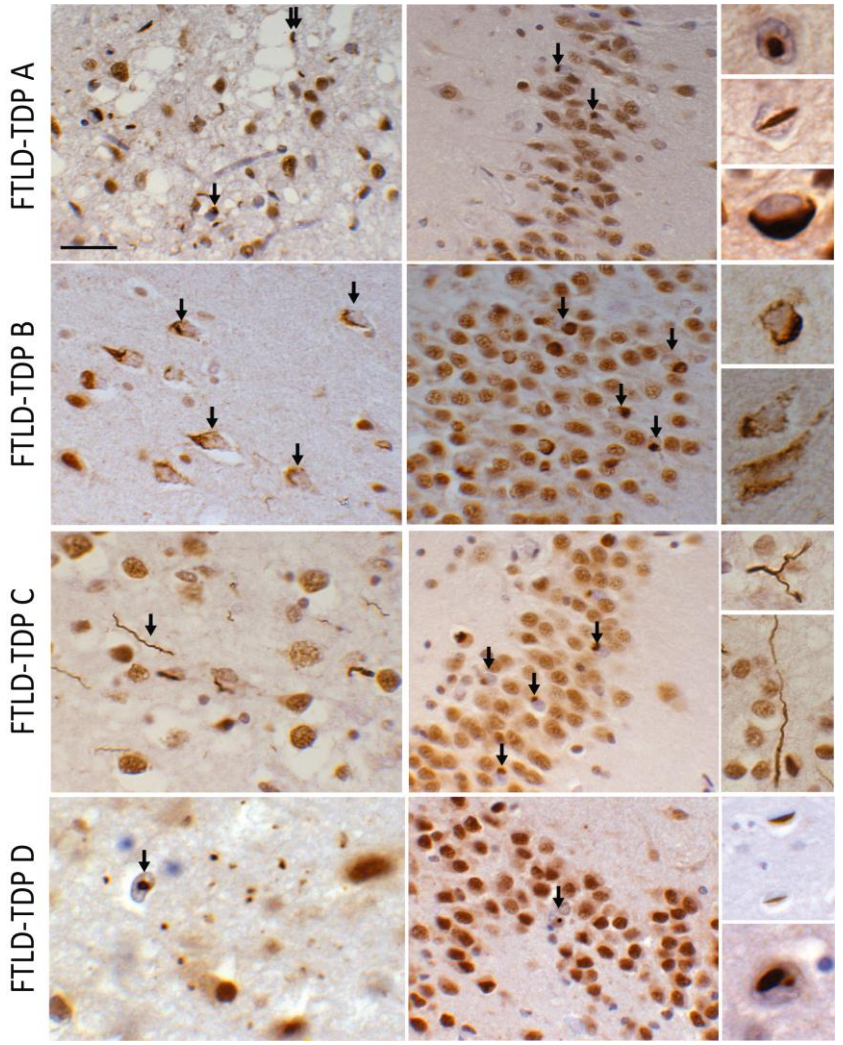

Figure 5. FTLD-TDP subtypes. In FTLD-TDP type A neuronal cytoplasmic inclusions (arrows), short dystrophic neurites and neuronal intranuclear inclusions are characteristic. In FTLD-TDP type B neuronal cytoplasmic inclusions, often with somewhat granular appearances, are common in all cortical layers. FTLD-TDP type C is characterized by unique, long corkscrew-type neurites (arrow). FTLD-TDP type D shows numerous neuronal intranuclear inclusions (arrow) and short neurites while neuronal cytoplasmic inclusions are rare. Scale bar: 40µm. From Lashley et al. *Neuropathology and Applied Neurobiology*. 2015.

c) Genetics

A positive family history is reported in up to 50% of patients with FTD (fFTD), and 10% of cases present a clear autosomal dominant pattern[111,116]. Linkage analyses in FTD families with parkinsonism pointed to a region in chromosome 17 (FTDP-17)[117,118]. In 1998, mutations in the microtubule-associated protein tau (*MAPT*) gene were identified as the susceptibility locus in these families[119,120]. To date, over 50 *MAPT* mutations have been described in more than 130 FTD families. Mutations in the *MAPT* gene are missense variants, which lead to an aminoacid substitution usually in or near to the fourth repeat region of the microtububle-binding domains or mutations that alter the splicing code. Mutations in the *MAPT* gene cause FTLD-tau pathology, which in contrast to sporadic PiD cases, are associated with predominance of either 3R or 4R tau isoforms[111,121].

Studies reporting families with linkage to chromosome 17q21, but not carrying a *MAPT* mutation, pointed to the idea that another disease causing gene was located in the same locus. Notably, individuals belonging to these families did not show tau positive inclusions at autopsy. In fact, they were part of the FTLD-U pathological subgroup[122,123]. In 2006, progranulin (*GRN*) mutations were identified as causative in these FTD families[124,125]. About 70 mutations have been linked to FTD. Most of them are loss-of-function mutations which result in haploinsufficiency[126]. Additionally, one non-synonymous mutation (p.A9D) has also been

demonstrated to cause FTD[127]. Mutations in the *GRN* gene are all associated with FTLD-TDP type A pathology, representing about 40% of this FTLD-TDP subtype[128].

Mutations in the gene encoding the *VCP* gene were first identified in a study that included 13 families suffering from hereditary inclusion body myopathy (IBM) with Paget's disease of bone (PDB) and FTD, also known as IBMPFD. IBMPFD is a rare and adult-onset disorder characterized by distal muscle weakness, early onset PDB and FTD, with some patients presenting with one of these isolated features[129]. To date, more than 20 mutations have been found to be causative of IBMPFD. All Mutations in the *VCP* gene are associated with FTLD-TDP type D pathology[33,130].

Similar to the identification of *VCP* mutations in FTD as a result of their description in IBMPFD, mutations in the gene encoding the triggering receptor expressed on myeloid cells 2 (*TREM2*) protein were associated with FTD. Initially, homozygous *TREM2* mutations were described in patients with Nasu-Hakola disease. The disorder is characterized by cystic-like lesions of the bone which lead to fractures, and brain demyelination which results in early onset dementia[131]. In 2008, three individuals of a family with isolated early onset dementia, without bone cysts, were found to carry a homozygous mutation in this gene[132]. Importantly, the identification of other homozygous mutations in *TREM2* which segregated in an autosomal recessive pattern in two early-onset FTD families supported the role of *TREM2* mutations in FTD[133,134]. Despite that

this early-onset form of FTD was associated with homozygous mutations in *TREM2*, rare heterozygous mutations in this gene were posteriorly demonstrated to cause FTD with typical ages of onset[135,136]. Pathological hallmarks of *TREM2* mutation carriers include loss of myelin and axons, accumulation of axonal spheroids and sudanophilic granules, and severe astrogliosis in frontal and temporal lobes and basal ganglia [137].

A spectrum of neurological disorders is associated with mutations in the DNA methyltransferase 1 (*DNMT1*) gene. Missense variants in the targeting sequence of DNMT1 have been proven to cause: 1) hereditary autonomic sensory neuropathy with dementia and hearing loss and 2) cerebellar ataxia, deafness and narcolepsy. Notably, up to 90% of patients with mutations in *DNMT1* develop behavioral changes by the age of 45, which usually precede the characteristic cognitive decline associated with mutations in *DNMT1*. Interestingly, some of them fulfill the diagnostic criteria of bvFTD[138–140]. Neuropathology of these cases consists of microvascular changes in frontal and temporal lobes, with cerebellar Purkinje cell loss [139].

Mutations in the gene encoding the charged multivesicular body protein 2B (*CHMP2B*) were identified in a Danish FTD pedigree by linkage analysis[141]. CHMP2B C-terminal truncating mutations segregate with FTD and have been described in two families of Danish and Belgian origin[141,143]. These cases are characterized by FTLD-UPS pathology[144]. Missense mutations have also been

described, although confirmed pathogenicity is still pending in these cases[143,145].

TARDBP mutations in FTD were identified as a result of a gene candidate approach derived from the observation that TDP-43 was the major component of ubiquitin-positive inclusions in FTLD-U cases[9,10]. Mutations in *TARDBP* result in TDP-43 positive inclusions. The few cases described show a complex and unclassifiable pathology, thus preventing the assignment of a particular FTLD-TDP type [146–148].

Similarly, after the identification of p62 deposits in neuronal and glial ubiquitin-positive inclusions of several neurodegenerative diseases[56], mutations in *SQSTM1* were shown to segregate with FTD[149]. Importantly, some mutations were later found in control individuals, thus leading to the notion that some of them increase the risk of developing FTD[59]. TDP-43 and p62 immunoreactivity are pathological hallmarks of cases carrying a *SQSTM1* pathogenic variant[150].

As previously described, FUS protein aggregates are found in a small proportion of FTLD cases. The contribution of *FUS* mutations in FTD was first described in 2010, in a patient suffering from bvFTD[151]. However, lack of segregation and absence of any subsequent report, makes the assumption that *FUS* mutations lead to a pure FTD phenotype at least controversial[151–153].

Several mutations in the presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) genes have been described in Alzheimer's disease (AD) since their initial identification in 1995[154–156]. Strikingly, some cases showing at autopsy Pick bodies but not amyloid-β plaques (a pathological hallmark of AD[157]) have been associated with mutations in the *PSEN1* gene[158,159]. Mutations in *PSEN2* have also been described in FTD phenotypes[160].

The hexanucleotide repeat expansion in the *C9orf72* gene was also identified in 2011 as a cause of FTD[18,19]. To date it is the most important genetic cause of both fFTD and sporadic FTD (sFTD)[55,161] (extended in subchapter 1.4).

Few studies have assessed the role of susceptibility loci in FTD through GWAs. The first GWAs included 515 FTLD-TDP cases and found that carriers of the minor allele ("C") of the rs1990622 in the transmembrane protein 106B (*TMEM106B*) gene had a reduced risk of FTLD-TDP. Besides, the study demonstrated that expression of TMEM106B is significantly higher in: 1) carriers of the risk allele of rs1990622 ("T") as compared to non-carriers, 2) in FTLD-TDP cases with respect to healthy controls and, 3) in FTD patients with a *GRN* mutation compared to FTD patients without mutations in the *GRN* gene[17]. Interestingly, the risk allele of *TMEM106B* gene has also been found to decrease the mean age of onset by 13 years in *GRN* mutation carriers[162]. The myelin-associated olygondendrocyte basic protein (*MOBP*) gene has also been described as a clinical modifier in FTD. The study found that

patients carrying the risk allele ("T") of the rs1768208 in *MOBP* had a shorted disease duration as compared to non-carriers [163].

A GWAs including 3526 FTD patients and 9402 healthy controls has been recently published. The study revealed a significant association at the human leucocyte antigen (*HLA*) locus (6p21.3) and a suggestive association with bvFTD at 11q14, which encompasses the ras associated protein RAB38 (*RAB38*) and the cathepsin c (*CTSC*) genes[164]. In addition, a non-synonymous rare variant (p.A152T) in *MAPT* was demonstrated to increase the risk of FTD. Besides, the study demonstrated that this missense mutation decreases the binding of tau to microtubules and forms aggregates which are less efficient in stabilizing microtubules [165]. Other FTD susceptibility loci and rare disease causing genes are summarized in table 2.

Table 2. Genes involved in FTD etiology.

Gene	Location	MI	fFTD (%)	sFTD (%)	Mean AAO	AAO range
C9orf72	9p21.2	AD	20-50	2-18	57	27-80
GRN	21q22.1	AD	15-20	3	60	35-87
MAPT	17q21.31	AD	5-20	2	50	30-70
TBK1	12q14.2	AD	3	1	60	29-78
TARDBP	1p36.2	AD	3	<1	55	30-77
SQSTM1	5q35.3	AD/Risk	2	<1	56	41-78
VCP	9p13.3	AD	1	<1	55	46-79
OPTN	10p13	AD/AR	<1	<1	46	33-64
FUS	16p11.2	AD/AR	<1	<1	52	52
PSEN1	14q24.2	AD	<1	<1	57	52-60
PSEN2	1q42.13	AD	<1	<1	52	52
TREM2	6p21.1	AD/AR	<1	<1	47	20-68
UBQLN2	Xp11	XL	<1	<1	44	14-78
CHMP2B	3p11.2	AD	<1	<1	56	46-65
CHCHD10	22q11.2	AD	<1	<1	56	35-73
DCTN1	2p13.1	AD	<1	<1	55	48-64
DNMT1	19p13.2	AD	<1	<1	45	25-50
TMEM106B	7p21.3	Risk/DM	-	-	-	-
MOBP	3p22.1	DM	-	-	-	-
HLA	6p21.3	Risk	-	-	-	-
RAB8/CTSC	11q14	Risk	-	-	-	-
*MAPT**	17q21.31	Risk	-	-	-	-

MI: Mode of inheritance; AAO: Age at onset; DM: disease modifier; AD: autosomal dominant; AR: autosomal recessive.* A part from disease causing mutations in *MAPT* gene, a rare missense variant (p.A152T) has also been implicated in increasing the risk of FTD[165].

1.3. Overlapping features in amyotrophic lateral sclerosis and frontotemporal dementia

a) Clinical overlap

ALS and FTD have historically been considered two different disorders. During the twentieth century, clinical observations pointed to the idea that ALS and FTD can be presented in the same patient. Cognitive and behavioral complaints in ALS were already reported 30 years after the first description of ALS[166]. Subsequent studies also noted that ALS cases suffered from personality changes or dementia[166–168] . In 1932, the first link between ALS and FTD (at that time known as PiD) was established[169], which was also supported by successive studies by others[170–172]. In 1961, the description of individuals from the island of Guam who presented with ALS, dementia and parkinsonism[173], as well as the report of presenile dementia associated with motor neuron disease in the Kii peninsula of Japan[174] strengthened this view. Despite this, a link between both ALS and dementia was not firmly established until 1982, when Hudson reported a wide revision of the clinical features of ALS patients who suffered from other neurological disorders[3]. Since then, studies exploring the relationship between ALS and FTD have been reported[175–177].

It is now widely accepted that a subgroup of patients with FTD develop features of ALS, and that ALS patients may present also

with behavioral and/or cognitive changes. It is estimated, depending on the series, that cognitive impairment presents in up to 50% of ALS, and 15% of ALS patients fulfill the diagnosis of FTD (ALS/FTD), mostly bvFTD[178–180]. In the case of FTD patients, approximately 30% show features of motor neuron disease and around 15% fulfill the diagnosis of ALS[181,182]. Notably, up to 40% of FTD cases with no clinical evidence of ALS are pathologically confirmed as FTLD with motor neuron disease (FTLD-MND)[183]. A consistent finding across studies is that the presence of ALS and FTD in the same patient shortens survival of these patients as compared to isolated ALS and FTD phenotypes[89,184]. The overlapping features above described have important consequences in terms of defining the family history of ALS and FTD patients. In 2005, Goldman et al. examined the family history of a large cohort of FTD patients and showed that ALS in association with FTD have a strong familial aggregation (59,2%), suggesting that not considering ALS family history in FTD patients and vice-versa, might underestimate the strong heritability of both disorders[185].

b) Pathological overlap

In 1988, ubiquitin-positive inclusions were described in the anterior horn cells of ALS cases by two different reports[31,32]. The observation that these aggregates were also present in the extra-motor cortices of pure clinical ALS cases and ALS cases with dementia,[186,187] and that these inclusions were also seen in FTLD[104],

pointed to the existence of a pathological overlap between ALS and FTLD. In 2006, the discovery of TDP-43 as the major constituent of the ubiquitin-positive inclusions in ALS and FTLD cases was the most important observation supporting the pathological overlap between both disorders[9,10]. Likewise, a study which included clinical and neuropathological data from 102 ALS cases showed that executive dysfunction and FTD-like features correlated with TDP-43 pathology in frontotemporal regions[188]. Also, a recent report provided evidence that the mechanism of TDP-43 pathology progression in cases clinically presenting with bvFTD and classified as FTLD-TDP at autopsy is similar to ALS cases[189], as previously suggested in ALS[188].

Another relevant finding was the discovery of FUS aggregates in both ALS and FTLD cases (ALS-FUS and FTLD-FET, respectively), as a result of the identification of *FUS* mutations in ALS[12,35,36]. Importantly, ALS-FUS is caused by mutations in the proline-tyrosine nuclear localization signal (PY-NLS) domain of the FUS protein and the inclusions contain exclusively the FUS protein[36], whereas FTLD-FET cases, a part from showing FUS protein aggregates, are also characterized by the presence of EWS, TAF15 and TRN1 immunoreactivity in these ubiquitin positive inclusions. In contrast to ALS-FUS, FTLD-FET cases are thought to be sporadic[115]. Besides, while FUS inclusions in ALS-FUS patients are methylated, they are not in FTLD-FET cases, suggesting that both conditions arise from different disease mechanisms[190].

Finally, although very rare, tau pathology has been described in motor neurons of ALS/FTD cases with a mutation in the *MAPT* gene[191,192] (detailed below), but also in an apparently sporadic ALS/FTD patient[193]. Together with the evidences of tau pathology in the ALS/parkinsonism-dementia complex from Guam[173] and the Kii peninsula of Japan[174], it suggests that tau protein aggregates might be involved in the MND pathogenesis.

c) Genetic overlap

As described before, in patients presenting with ALS and/or FTD. the presence of both diseases in their families should be considered prior to exclude a positive family history[185,194]. In this context, several studies have reported large pedigrees of individuals affected by ALS, FTD and/or both diseases. Most of these reports were published before the discovery of the hexanucleotide repeat expansion in *C9orf72*[11,195,196], which to date is the most important genetic cause of ALS and FTD[33,55] (see chapter 1.4).

The presence of TDP-43 aggregates in both ALS and FTLD[9,10] led to the genetic assessment of *TARDBP* in ALS and FTD. Mutations in this gene were initially found in ALS cases[45,46]. Subsequently, pathogenic variants were described in patients with ALS/FTD[197] and in pure FTD[198].

Although its involvement in patients with isolated FTD has not been yet confirmed, mutations in the *FUS* gene are associated with ALS and a few cases with ALS/FTD[12,35,151].

Before assessing and confirming the role of *SQSTM1* mutations in ALS, mutations in this gene were reported in patients with PDB[199]. Soon afterwards, a study also demonstrated co-segregation with FTD[149] and to be a causal gene in the ALS/FTD continuum, with some variants showing high to intermediate penetrance in both diseases or described as risk factors[59].

Mutations in *VCP* were initially associated with IBMPFD, with some patients presenting with isolated FTD[129]. *VCP* mutations were posteriorly described in ALS[52].

Disease-causing variants in *UBQLN2* gene are also rare causes of ALS/FTD, as well as pure FTD syndromes[53,200]. Also, *UBQLN2* mutations are associated with a wide range of phenotypes, such as spastic paraplegia or multiple sclerosis[201].

In addition to the confirmed pathogenic role of truncating variants in *CHMP2B* associated with FTD, missense mutations have been reported in ALS, FTD and ALS/FTD patients. Segregation analyses and/or functional studies are still pending for these nonsynonimous variants in order to support their pathogenic role[143,145,202,203].

OPTN mutations were first identified as a cause of ALS[50].

Following reports of ALS patients carrying *OPTN* mutations described some patients with concomitant FTD[51] and also pure FTD[204]. Remarkably, OPTN is phosphorylated by TBK1[205]. Mutations in the gene encoding TBK1 have recently proven to cause ALS and FTD[68,204]. Disease-causing mutations in *TBK1* are usually loss-of-function variants which result in haploinsufficiency, but some missense mutations affecting the binding domain with OPTN have been reported as pathogenic[204]. Importantly, after the hexanucleotide repeat expansion mutation, *TBK1* mutations are the second most prevalent genetic alteration in ALS/FTD patients[68,206,207].

Two rare pathogenic mutations within *MAPT* have been demonstrated to cause ALS. In fact, the first *MAPT* mutation (p.K317M) described segregated with parkinsonism, MND and FTD[191]. The second (p.D348G) was recently shown to segregate with lower motor neuron disease, without signs of cognitive impairment[192]. Importantly, both mutations show tau protein inclusions in spinal motor neurons[191,192].

Rare mutations in genes associated with ALS etiology have been demonstrated to be causal in a few ALS cases with concomitant FTD. To date, three *SOD1* mutations (p.G41S, p.I113T and p.L144F) have been related to ALS/FTD[208–211]. Besides, a mutation (p.F115C) in the matrin 3 (*MATR3*) gene was associated with ALS and cognitive impairment in two individuals of a large ALS pedigree[212]. The involvement of angiogenin (*ANG*) mutations (such

as p.K17I) in ALS/FTD remains controversial[213]. The dynactin 1 (*DCTN1*) represents another example of a gene with mutations associated to multiple phenotypes. Mutations in *DCTN1* are associated with autosomal dominant Perry syndrome, characterized by parkinsonism, depression, disinhibition, weight loss and central hypoventilation[214], but also with bvFTD, MND and PSP-like phenotypes[215–218]. Finally, a homozygous mutation (p.E163K) in the *DJ1* gene, also known as *PARK7*, was demonstrated to segregate in a family with early-onset parkinsonism, ALS and FTD[219]. All genes contributing to the ALS and/or FTD etiology are represented in figure 6.

Susceptibility loci or modifiers of disease progression of both diseases have not yet been described, except for the *TMEM106B* locus which has been associated with the risk of FTLD-TDP. The same risk allele of rs1990622 was later associated with cognitive impairment in ALS, although it did not influence the risk of developing ALS[220].

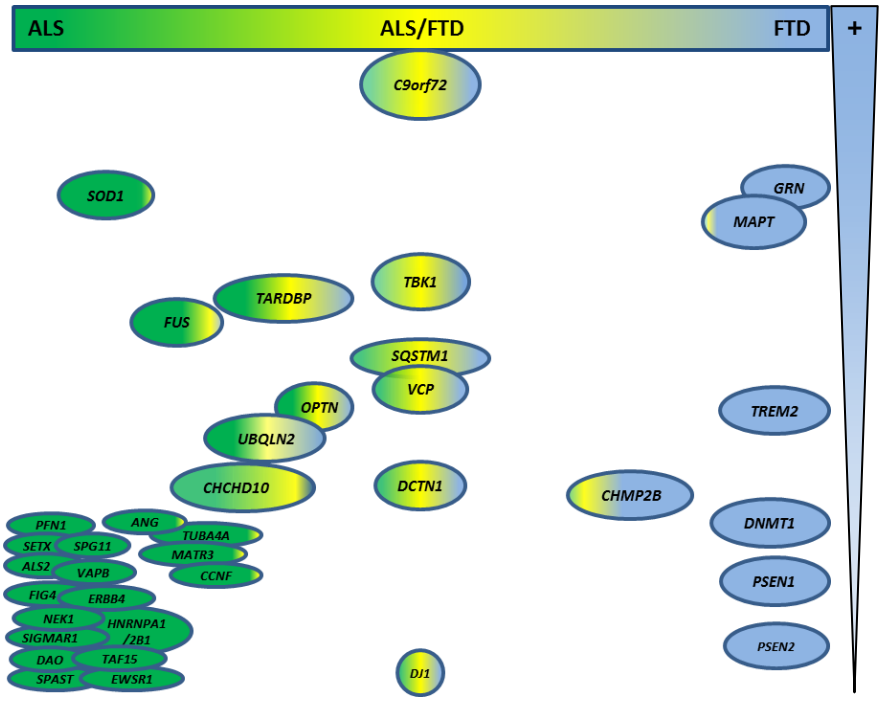

Figure 6. ALS and FTD disease-causing genes plotted to show associated phenotypes (ALS in green, ALS/FTD in yellow and FTD in blue). Y axis represents a gradient from most commonly mutated genes (top) and rare disease causing genes (bottom). All data are based on evidence from peer-reviewed publications.

1.4. *C9orf72* in the ALS/FTD continuum

a) The discovery

Since the discovery in 2011 of the hexanucleotide repeat expansion mutation in the *C9orf72* gene, it has been proven that it is the most prevalent genetic defect across the ALS/FTD spectrum[18,19]. The human *C9orf72* gene is predicted to encode two protein isoforms and, through an alternative splicing mechanism, generates three transcripts. The hexanucleotide repeat is located between alternatively spliced non-coding first exons (termed 1a and 1b). If exon 1a is included, the hexanucleotide repeat is transcribed (transcript variant 1, NM_145005.5 and transcript variant 3, NM_001256054.1), whereas if exon 1b is the one included, the repeat is located in the promoter region (transcript variant 2, NM_018325.3). Several reports describing large pedigrees of individuals presenting with ALS, FTD and/or ALS/FTD were linked to a region on chromosome 9q21-22 (figure 7)[11,195,196]. Subsequently, genome wide association studies (GWAs) of large series of ALS patients showed a significant association of the susceptibility locus previously identified through linkage analyses (figure 8)[14,15,221]. Interestingly, a GWAs performed in 515 FTLD-TDP proven cases found a trend for association of the same locus on chromosome 9 and helped refining the chromosome region to a 7.7 megabase interval[17]. This region was narrowed to a 232 kilobase block of linkage disequilibrium which unraveled a common founder

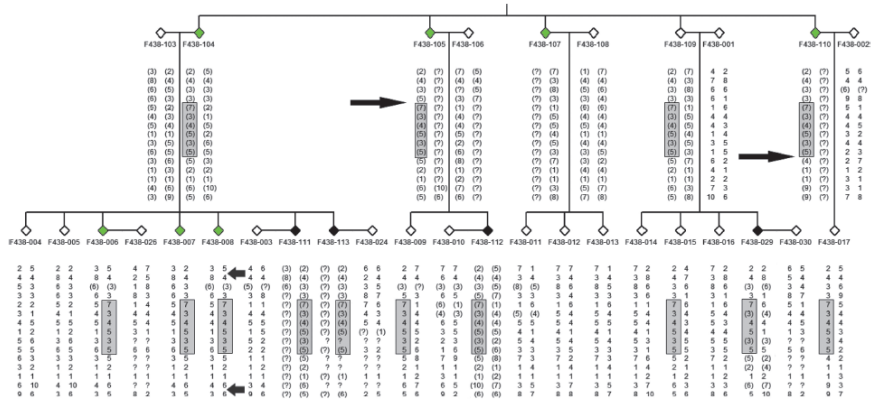

Figure 7. A representative family linked to chromosome 9q21-q22. FTD patients are symbolized in green whereas ALS patients in black. From Morita et al. *Neurology*, 2006.

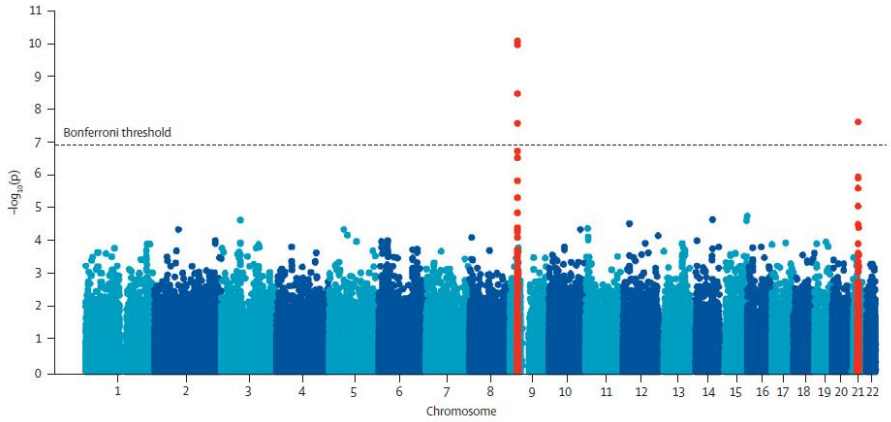

Figure 8. A representative manhattan plot from a GWAs performed in Finnish ALS cases showing the strongest signal within the chromosome 9q21 region. The peak in chromosome 21 corresponds to the p.D90A mutation in *SOD1*. From Laaksovirta et al., *Lancet Neurol.*, 2010.

haplotype in all families with linkage to this locus[222].

Finally, a hexanucleotide (GGGGCC) repeat expansion within the *C9orf72* gene was found to segregate in all family members affected with ALS and/or FTD. Importantly, the two pivotal studies reporting this association described that the maximum hexanucleotide repeat number within the control population was of 23 units, with two repeats being the most common allele. Further analyses in ALS and FTD patients (sporadic and familial) using a repeat primed PCR (rpPCR) approach demonstrated that this massive hexanucleotide repeat expansion is the most frequent cause of ALS and FTD[18,19,55,223–227]. All mutation carriers also carried the founder haplotype previously described by Mok et al. in the Finnish population, broadly defined by the "A" allele of the single nucleotide polymorphism (SNP) (rs3849942)[222]. Within Europe, a clear north to south cline of this expansion mutation suggests that this alteration spread from northern Europe (Figure 9 and 10)[55,161]. Importantly, the frequency of *C9orf72* expansion carriers in ALS/FTD patients is higher than those with isolated ALS or FTD. In two studies which included a large European cohort, the prevalence of the hexanucleotide repeat expansion in ALS/FTD patients was found to be higher than 30%[161,223], reaching almost 50% in those with positive family history[161]. The penetrance of this mutation was also assessed in a large study that included 4448 ALS and 1425 FTD patients from 17 regions. The authors showed that the hexanucleotide repeat expansion was almost fully penetrant by 80 years of age, whereas penetrance was of 50% by 58 years old[55].

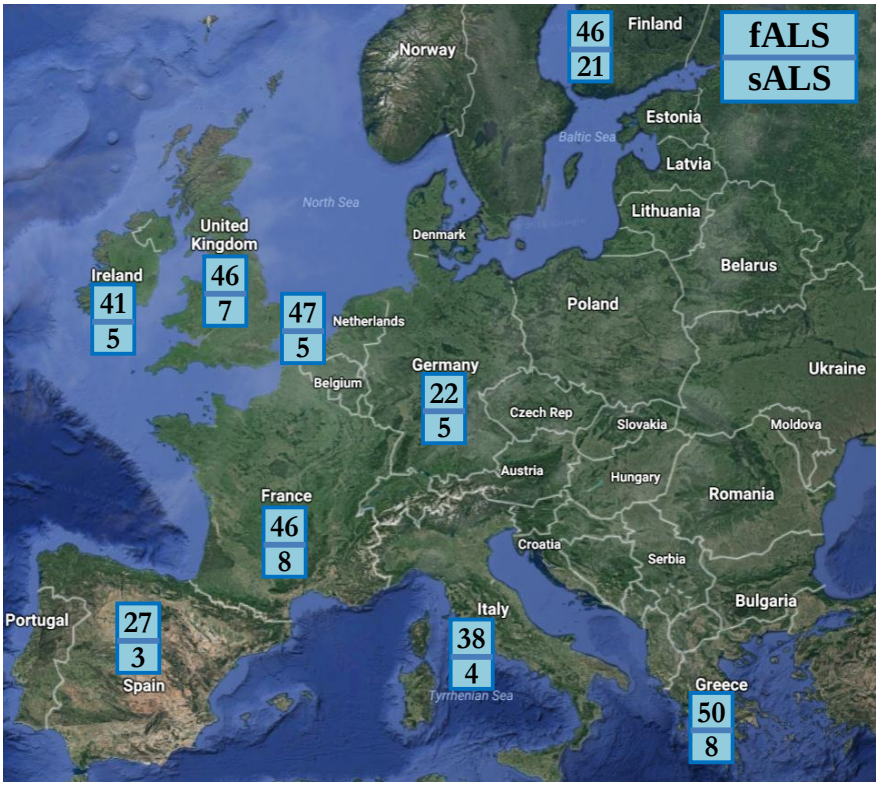

Figure 9. Prevalence (expressed as a percentage) of the *C9orf72* hexanucleotide repeat expansion in Europe. fALS: familial ALS; sALS: sporadic ALS.

It is important to note that the rpPCR method is not able to determine the exact number of hexanucleotide repeat units in expansion mutation carriers. In the two first published studies, an approximation to estimate the repeat expansion size was performed through two different techniques. A fluorescent in situ hybridization method sized the repeat expansion of at least 1.5 kilobases[18] and a southern blot approach showed that it ranged from 700 to 1600 repeat units[19]. The size of the repeat expansion has also been assessed in subsequent reports. The goal of these studies was to associate the repeat expansion length with the clinical phenotype or with clinical traits in both ALS and FTD cohorts, but no conclusive results have been obtained[228–231]. Despite this, longer hexanucleotide repeat expansions lengths have recently been correlated in ALS and FTD patients with a younger age at onset and increased methylation patterns of the CpG island in the *C9orf72* promoter region, which in turns lead to reduced transcriptional activity of the promoter[232]. In contrast, a recent study reported that shorter hexanucleotide repeat expansions are correlated with increased methylation patterns, leading to a longer disease duration[233]. Additionally, hypermethylation of the *C9orf72* promoter region has been linked to reduced RNA foci and dipeptide repeat protein aggregates[234], which are pathological hallmarks of the *C9orf72* expansion (described below).

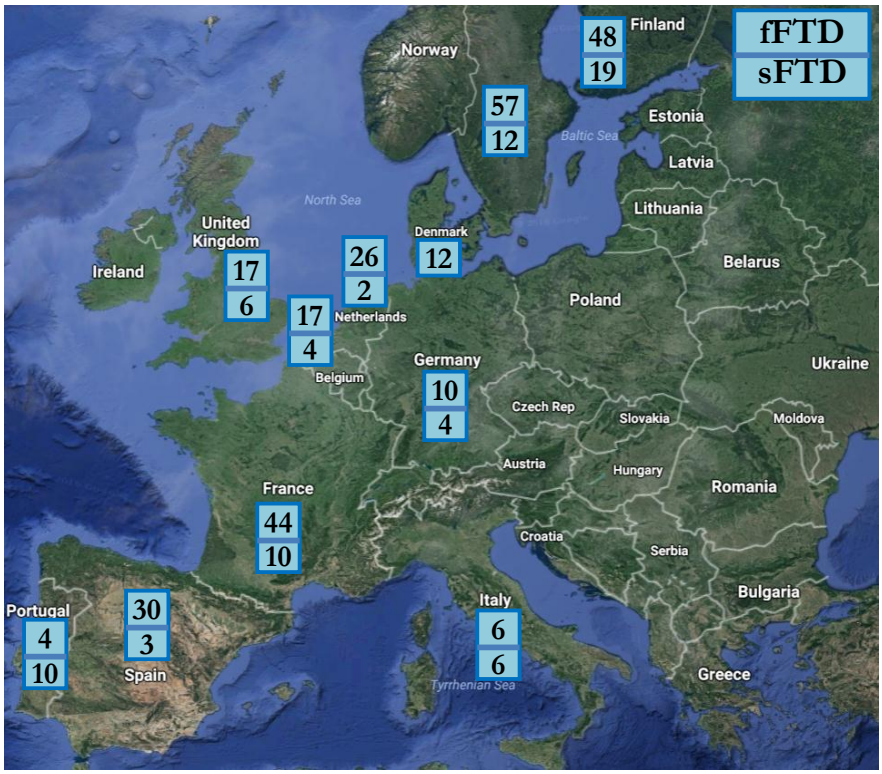

Figure 10. Prevalence (expressed as a percentage) of the *C9orf72* hexanucleotide repeat expansion in Europe. Note than in Denmark no information about familial or sporadic cases was available, thus the overall prevalence is depicted. fFTD: familial FTD; sFTD: sporadic FTD.

b) Clinical manifestations

ALS patients carrying the hexanucleotide repeat expansion have more common cognitive and/or behavioral impairment than those without the *C9orf72* expansion[235]. In addition, a more frequent bulbar onset has been observed in several,[55,236–238] but not all studies[239]. On the other hand, the bvFTD is the most common presentation of FTD patients with the repeat expansion mutation, and the diagnosis of concomitant ALS is more common than in non-expansion carriers[55]. Importantly, these patients also present with hallucinations and delusions more frequently than those not carrying the hexanucleotide repeat expansion[224,240,241]. The expansion mutation also explains a small proportion of patients clinically diagnosed with AD (<1%)[242–244]. Besides, parkinsonism, cerebellar ataxia, multiple system atrophy, corticobasal syndrome, dementia with Lewy bodies and Huntington disease phenocopies, although rare, have been reported in *C9orf72* expansion carriers[245,246].

c) Pathological mechanisms

ALS and FTD cases carrying the hexanucleotide repeat expansion in *C9orf72* show TDP-43 positive inclusions at autopsy. Most patients with clinical FTD have a pathological diagnosis of FTLD-TDP type B[224], while the rest are classified as FTLD-TDP type A[247]

and rarely as FTLD-TDP type C[189]. In addition, p62 positive inclusions in the cerebellum, hippocampus and neocortex are pathological hallmarks of brains from patients with the *C9orf72* repeat expansion. These inclusions are characteristically TDP-43 negative and have a star-like morphology[224,248,249].

RNA foci composed of sense (GGGGCC) and antisense (CCCCGG) transcripts have frequently been observed in different brain and spinal cord regions of expansion carriers[250–252], which have led to the hypothesis that these transcripts are able to sequester and deregulate RNA binding proteins[253] and interfere with the nuclear pore complex[254].

Repeat-associated non-ATG (RAN) translation of expanded repeat tracts has been found to occur in patients with the repeat expansion mutation in *C9orf72*[255]. RAN translation of sense and antisense transcripts trigger the formation of dipeptide repeat (DPR) proteins; poly(GA), poly(GP) and poly(GR), and poly(PR), poly(PG) and poly(PA), respectively. DPR proteins are present in brains of FTLD cases carrying the expansion mutation, and, although less frequently, are also found in spinal cord of ALS repeat expansion carriers[251,256]. These inclusions do not correlate with neurodegeneration or TDP-43 aggregation[254,256]. Additionally, DPR proteins have been shown to alter the nuclear pore complex and the ubiquitin proteasome system[254]. RAN translation and RNA foci formation support the gain-of-function mechanism for the hexanucleotide repeat expansion mutation.

On the other hand, a loss-of-function hypothesis as the cause of the C9orf72-related pathophysiological process has also been suggested, since decreased expression of all *C9orf72* transcript variants have been found in frontal and motor cortices, cerebellum and cervical spinal cord of repeat expansion carriers[223,257].

d) Genetic modifiers

Few reports have investigated the role of genetic variants that might modify disease progression in *C9orf72* expansion mutation carriers. The most important and robust association has been found in the *TMEM106B* locus, previously associated with increased risk of FTLD-TDP[17]. Intriguingly, the major allele of rs1990622 in *TMEM106B* (previously found to advance the age at onset of FTD in *GRN* mutation carriers and increase the risk for developing FTLD-TDP) has been found to delay the age at onset and death in FTD with the *C9orf72* hexanucleotide repeat expansion mutation[258].

2. OBJECTIVES

The goal of this thesis is assessing the genetic overlap between ALS and FTD. For this purpose, the objectives of this work are:

1. To study the prevalence of the hexanucleotide repeat expansion in the *C9orf72* gene in Spanish ALS patients through a repeat primed PCR approach and find clinical features that differentiate repeat expansion carriers from non-carriers.

2. To size the hexanucleotide repeat in ALS and FTD repeat expansion carriers through a southern blot method and correlate with clinical traits.

3. To evaluate the role of *CHCHD10* in ALS and FTD.

4. To assess the mutation spectrum of *TUBA4A* in FTD patients.

5. To estimate the mutation burden that is present in patients with concomitant ALS and FTD (not carrying the *C9orf72* repeat expansion) through a whole-exome sequencing approach.

3. PUBLICATIONS

3.1. Analysis of the *C9orf72* gene in patients with amyotrophic lateral sclerosis from Spain and different populations worldwide

García-Redondo A, Dols-Icardo O, Rojas-García R, Esteban-Pérez J, Cordero-Vázquez P, Muñoz-Blanco JL, et al. Analysis of the C9orf72 Gene in Patients with Amyotrophic Lateral Sclerosis in Spain and Different Populations Worldwide. Hum Mutat. 2013 Jan;34(1):79–82. DOI: 10.1002/humu.22211

3.2. Characterization of the repeat expansion size in *C9orf72* in amyotrophic lateral sclerosis and frontotemporal dementia

Dols-Icardo O, Garcia-Redondo A, Rojas-Garcia R, Sanchez-Valle R, Noguera A, Gomez-Tortosa E, et al. Characterization of the repeat expansion size in C9orf72 in amyotrophic lateral sclerosis and frontotemporal dementia. Hum Mol Genet. 2014 Feb 1;23(3):749–54. DOI: 10.1093/hmg/ddt460

3.3. Analysis of *CHCHD10* in patients with frontotemporal dementia and amyotrophic lateral sclerosis from Spain

Dols-Icardo O, Nebot I, Gorostidi A, Ortega-Cubero S, Hernández I, Rojas-García R, et al. Analysis of the CHCHD10 gene in patients with frontotemporal dementia and amyotrophic lateral sclerosis from Spain. Brain. 2015 Dec;138(12):e400–e400. DOI: 10.1093/brain/awv175

3.4. Assessing the role of *TUBA4A* gene in frontotemporal degeneration

Dols-Icardo O, Iborra O, Valdivia J, Pastor P, Ruiz A, López de Munain A, et al. Assessing the role of TUBA4A gene in frontotemporal degeneration. Neurobiol Aging. 2016 Feb;38:215.e13-215.e14. DOI: 10.1016/ j.neurobiolaging.2015.10.030

3.5. Exome sequencing reveals a high genetic component in patients with amyotrophic lateral sclerosis and concomitant frontotemporal dementia without the *C9orf72* expansion mutation

Oriol Dols-Icardo, Alberto García-Redondo, Ricard Rojas, Alexandra Juárez-Rufián, Laura Cervera-Carles, José Luís Muñoz-Blanco, Lucía Galán, Juan Fortea, Ignacio Illán-Gala, Rafael Blesa, Oriol Grau-Rivera, Alberto Lleó, Jesús Esteban-Pérez, Ellen Gelpí, Jordi Clarimón. Exome sequencing reveals a high genetic component in patients with amyotrophic lateral sclerosis and concomitant frontotemporal dementia without the C9orf72 expansion mutation. 2016 (*in preparation*).

EXOME SEQUENCING REVEALS A HIGH GENETIC COMPONENT IN PATIENTS WITH AMYOTROPHIC LATERAL SCLEROSIS AND CONCOMITANT FRONTOTEMPORAL DEMENTIA WITHOUT THE *C9ORF72* EXPANSION MUTATION

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Abstract

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are part of a clinical, pathological and genetic continuum. The purpose of the present study was to assess the mutation burden that is present in patients with concurrent ALS and FTD (ALS/FTD) not-carrying the *C9orf72* hexanucleotide repeat expansion, the most important genetic cause in both diseases. We used data from whole-exome sequencing performed in 54 patients with ALS/FTD (13 with postmortem neuropathological confirmation and 41 with a clinical diagnosis), to screen for mutations in genes associated with ALS and/or FTD. We identified 11 patients carrying pathogenic mutations, representing an overall mutation frequency of 20.4%. The tank binding kinase 1 (*TBK1*) gene was the most important genetic cause of ALS/FTD, with five

patients (9.3%) harboring a heterozygous mutation. The second most common mutated gene was *SQSTM1*, with three mutation carriers (one of them also harbored a *TBK1* mutation). We also detected genetic alterations in *VCP*, *TARDBP*, *TAF15*, and *ERBB4*. Five mutations represent novel genetic variants. Our results indicate a high genetic background underlying the co-occurrence of ALS and FTD, and suggest that other genetic loci should be tested in those ALS/FTD patients without the *C9orf72* expansion mutation, regardless of family history of disease.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the degeneration of upper and lower motor neurons, which results in death usually within 3-5 years from symptoms onset (1). Frontotemporal dementia (FTD) is associated with the degeneration of the frontal and/or temporal lobes, which leads to a progressive deterioration in behavior and/or language, with relative preservation of memory (2). To date, there is overwhelming evidence that ALS and FTD are part of a disease

continuum with genetic, pathological and clinical overlapping features (3). The hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 (*C9orf72*) gene is the most prevalent genetic cause of both conditions. Within the European population, it accounts for up to 8% of sporadic and 40% of familial ALS, and 6% of sporadic and 25% of familial FTD (4, 5). Importantly, the prevalence of this genetic alteration increases to 14% of sporadic and 57% of familial patients with concomitant clinical features of ALS and FTD (ALS/FTD), thus suggesting enriched genetic factors underlying ALS/FTD co-occurrence (4).

A systematic evaluation of genes previously related to ALS and/or FTD in patients manifesting both phenotypes who are free of the *C9orf72* genetic alteration has not yet been performed, probably as a consequence of the low prevalence of individuals with concomitant ALS/FTD (which makes the identification and recruitment of patients laborious), the large number of genes that should be evaluated (there are more than thirty genes reported to date), or the high incidence of the *C9orf72* expansion mutation that is present in this rare condition (3, 6, 7).

In order to evaluate the overall genetic contribution of genes previously related to ALS or FTD in this complex phenotype, and to uncover possible novel disease causing variants in these genes, we have performed an in-depth genetic analysis through a whole exome sequencing approach in a series of patients with concomitant ALS/FTD not carrying the *C9orf72* hexanucleotide repeat expansion mutation.

MATERIAL AND METHODS

Case series

Our study population included a total of 54 patients (44 males and 10 females), all Caucasian from Spanish ancestry. DNA from 13 postmortem brain specimens of patients fulfilling the pathological diagnosis of motor neuron disease (MND) and frontotemporal lobar degeneration (FTLD) was obtained from the Neurological Tissue Bank of the Biobanc-Hospital Clinic-IDIBAPS (Barcelona). Clinical series was composed of 41 patients who were evaluated by neurologists with expertise in neuromuscular disorders as well as in neurodegenerative dementias. Patients with possible, probable or

definite ALS were included and diagnosed according to the El Escorial revised criteria (8), and those with signs of cognitive impairment underwent a structured neurological examination. FTD diagnosis was performed following the international criteria for the behavioral variant of FTD (9) or the international consensus criteria for primary progressive aphasias (10). Mean age at onset was 63 years old, ranging from 36 to 82. Mean age at death of brain donors was 71 years old, ranging from 59 to 84. Among all patients, 16,7% presented a positive family history of disease, which was defined as having a first or second-degree relative diagnosed with ALS and/or FTD.

Genetic analyses

The C*9orf72* hexanucleotide expansion mutation was evaluated as previously described (11), and discarded in all samples that underwent whole-exome sequencing. Genomic regions corresponding to all annotated human exons were enriched by hybridization using the Agilent SureSelect Human All-Exons V5 kit (Agilent Technologies, Santa Clara, CA, USA). Final libraries were

sequenced on a HiSeq 2500 (Illumina, San Diego, CA, USA), with paired end 100bp reads. Resulting raw sequencing data was processed following GATK 3.4-46 best practices (12). Reads were mapped to the human reference GRCh37 build using the Burrows-Wheeler Aligner 0.7.10 (13). Duplicate reads were flagged using Picard Tools 1.119 (http://picard.sourceforge.net) and realignment of insertions and deletions (Indels) as well as base quality score recalibration was performed with GATK. Variant calling was executed with the Haplotype Caller tool of GATK. High quality single nucleotide variants and Indels (defined in our final data set as having a Variant Quality Score VQSLOD above 0.002 and 0.175, respectively) were kept in the final vcf file which was annotated using Annovar (14) and dbNSFP v3.0 (15). The GERP score ranges from -12.3 (not conserved) to 6.17 (highly conserved). All coding regions of genes previously associated with ALS or FTD (Suppl. Table 1) were examined. Mean coverage of all the exome targeted region was of 79 reads per base and, on average, 94% of the targeted region was covered with more than 10 reads in each sequenced sample.

RESULTS

All likely pathogenic variants identified in this study are summarized in Table 1. Overall, 11 out of 54 ALS/FTD patients (20.4%) harbored a disease causing variant which potentially explains the associated phenotype. Five patients (9.3%) harbored a mutation in the TANK-binding kinase 1 (*TBK1*), thus representing the most frequently mutated gene. All of them were diagnosed with a limb onset probable ALS and a probable behavioral variant FTD (bvFTD), except one patient who presented with non-fluent progressive aphasia. This patient carried the p.N254fs *TBK1* mutation and also harbored a missense variant (p.P392L) in the sequestosome 1 (*SQSTM1*) gene. Family history was negative in this patient. Among *TBK1* variants, three of them (p.K30_E76del, p.T79del and p.N254fs) have not been previously described.

Two mutations (p.P392L and p. A33V) in *SQSTM1*, which have been previously reported as pathogenic (16, 17), were identified in three patients with a presumable sporadic disease, thus representing the second most important genetic cause of ALS/FTD in our series. Neuropathological examination was achievable in two of them

(patients CS1505 and CS1480) and disclosed a definite FTLD-TDP with upper and lower motor neuron involvement associated with TDP-43 aggregates. Both cases showed hippocampal sclerosis. Strikingly, CS1480, a case carrying the p.P392L variant, was clinically diagnosed with Paget disease of bone, parkinsonism and FTD. His neuropathological evaluation disclosed a severe degeneration of the basal ganglia and substantia nigra, which was probably related to the clinical manifestation of parkinsonism.

The analysis of the TAR DNA-binding protein (*TARDBP*) gene revealed a mutation (p.A90V) in a patient who presented with bvFTD at the age of 48 and developed ALS fourteen years later, when he was 62 years old. Family history revealed psychiatric symptoms in his father and two sisters. This mutation has been previously reported in a patient with slowly progressive FTD/ALS and a family history of dementia (18).

Genetic evaluation of valosin-containing protein (*VCP*) revealed a mutation carrier of the p.I27V amino acid exchange. This patient was diagnosed with probable bvFTD at the age of 80, after 40 years history of depression with family history of FTD. He deceased at the age of 84 years. Neuropathological examination disclosed a

motor neuron disease with lower motor neuron involvement associated with TDP-43 aggregates. Brain analysis also revealed argyrophilic grain disease (stage I) as well as Alzheimer's disease (AD) neuropatholgical changes: Braak stage II and Thal phase III, corresponding to a A2B1C1 score of AD, according to current guidelines (19, 20). This genetic alteration has been previously described in three independent patients: one with a FTD syndrome, another showing an isolated- slowly-progressive dysarthria (21), and a patient with pure inclusion body myositis (22).

The inclusion in the present genetic screening of the FET protein family members FUS, TAF15 and EWS (three structurally similar RNA-binding proteins that have been linked with ALS and FTLD) disclosed the presence of a novel missense variant (p.G462S) in the C-terminal RGG domain of TAF15 in a patient with a definite neuropathological diagnosis of ALS/FTLD-TDP who died two years after disease onset.

Finally, genetic screening of the erb-b2 receptor tyrosine kinase 4 (*ERBB4*), also known as ALS19 locus, revealed a novel missense mutation (p.I666T) in a patient who presented with a bulbar onset ALS at the age of 36, and was diagnosed with probable bvFTD

shortly afterwards. Interestingly, another missense mutation (p.R1275W) in this gene has been described in a patient who also presented with an early-onset ALS (45 years of age) (23).

We did not find any likely pathogenic genetic alteration among the 29 remaining genes that were included in the study.

DISCUSSION

The last years have witnessed a seed change in our concept of the biological basis of ALS and there is substantial evidence that genetic components play a pivotal role in its etiology (6). Although only a fraction of patients with ALS fulfill the consensus criteria for probable or definite FTD, these patients may exemplify the end results of strong genetic influences leading to these overlapping clinical and pathological entities (3). In order to minimize the phenotypic heterogeneity during sample selection we have included patients with postmortem neuropathological diagnosis and clinical cases that have been carefully evaluated by neurologists with expertise in neuromuscular disorders as well as neurologists from clinical memory units. This has led to a highly homogeneous cohort

with a fairly significant genetic component, with 20.4% of patients carrying a genetic defect that can be detected through a comprehensive analysis of the 35 bona fide genes with prior evidence to play a role in ALS and/or FTD.

Our results indicate that mutations in *TBK1* are the most important cause of ALS/FTD in patients without the *C9orf72* expansion mutation, and may account for up to 9.3% of Caucasian patients from Spanish origin. Recently, mutations in *TBK1* were found in 4.5% of subjects with concomitant ALS and FTD from Belgium (24), and this prevalence reached 10.8% in an independent study performed among French cases (25). Taken together, these data strongly suggest that a full *TBK1* genetic screening should be performed as a first choice in ALS/FTD cases where the *C9orf72* expansion has been discarded.

We have identified three patients carrying a previously reported genetic alteration within the *SQSTM1* gene (one of them was also a *TBK1* mutation carrier). Interestingly, the p.P392L mutation was found in two subjects. This mutation is the most important genetic alteration in PDB in Europeans and is particularly common among Spanish patients, representing 15.6% of PDB in this population

(26). Therefore, it might be expected that this missense mutation could be encountered in a substantial proportion of ALS/FTD patients from Spanish origin.

Nearly all of the missense mutations described so far in *TARDBP* reside in exon 6, which encodes the C-terminal glycine-rich domain of the TDP-43 protein. Notably, the p.A90V missense variant in *TARDBP* encountered herein is located in the exon 3 and placed between the bipartite nuclear localization signal sequence of the protein. This rare mutation has been extensively studied *in vitro* and has shown to negatively influence cell survival in neurons under stress conditions (27). Furthermore, cell expression of the TDP-43-A90V promoted its sequestration with endogenous TDP-43 as insoluble cytoplasmic pathological aggregates (18). However, the disruption of the nuclear localization was only observed in a sub-set of transfected cells, thus suggesting a partial deleterious effect related to this mutation. The fact that p.A90V has been also described in a patient with a similar phenotype consisting of a very slow ALS/FTD progression (18), prompts us to speculate that the p.A90V-driven incomplete disruption of TDP-43 cell localization may give rise to a slower disease course.

Mutations in *VCP* were first linked to a rare syndrome characterized by hereditary inclusion body myopathy (IBM) associated with PDB and early onset FTD (IBMPFD) (28). Subsequently, the phenotypic spectrum has been broadened by the discovery of pathogenic mutations in patients with ALS (29), hereditary spastic paraplegia (30) and Charcot-Marie-Tooth disease type 2 (31). In the present study we have discovered the missense p.I27V variant in a patient with a definitive diagnosis of FTLD accompanied with MND. This mutation has been reported in a case with sporadic, isolated inclusion body myositis (22), a case with a progressive speech disturbance with no evidence of myopathy, and a patient with a typical bvFTD (21). Thus, not only the *VCP* gene might have pleiotropic effects, but also a particular mutation in *VCP* can give rise to distinct phenotypes. It is important to stress, however, that brain assessment of the patient described in the present study did not present the typical FTLD-TDP type D neuropathological pattern, which has been widely reported in *VCP* mutation carriers (32). This finding might argue against a pathogenic role of the p.I27V mutation. However, cell biology studies have demonstrated a disruption of the autophagic properties (a typical consequence related to VCP missfunction) in cells expressing this genetic

alteration (22). Therefore, further studies are necessary to evidence the pathological role of this particular rare nonsynonimous change.

Our genetic screening disclosed a novel mutation in *TAF15* (p.G462S), which is placed within the C-terminal RGG domain. Of note, several missense rare variants in this protein domain (i.e. p.G391D, p.R395Q, p.R408C, p.452E and p.G473E) have been identified in sporadic and familial ALS cases (33, 34). Interestingly, mutations such as the p.G391E, p.R408C and p.G437E have been shown to induce cytoplasmatic foci in spinal cord neurons, accelerate protein aggregation *in vitro*, and exacerbate the deleterious neurodegenerative effect that is present in flies expressing human TAF15 (33). Our findings support a pathogenic role of *TAF15* in ALS and expand its contribution to FTD. Importantly, the mutation carrier identified in our study is the first case with a presumably pathogenic *TAF15* mutation with a neuropathological assessment. Our data suggest that mutations in this gene might be a cause of FTLD-TDP.

Mutations in the *ERBB4* gene have been described in autosomal dominant familial and sporadic (due to a *de novo* mutation) forms of isolated ALS (23). To our knowledge this is the first description

of a novel *ERBB4* mutation in a patient with ALS that also suffered from a concomitant bvFTD. This finding supports the pathogenic role of *ERBB4* in ALS and broadens its phenotypic consequences. The fact that this residue is highly conserved across evolution (GERP score = 5.58), the aminoacid exchange is classified as deleterious by four independent *in silico* analyses, and is present in only one out of 66,716 chromosomes from European origin, strengthens the damaging effect of the p.I666T mutation (table 1). However, the lack of family history of disease prevents any strong conclusion and warrants further analyses of the role of *ERBB4* in ALS and/or FTD.

It is important to note that 9 out of 11 mutation carriers (81.9%) seemed to be sporadic. This data suggest that family history of ALS and/or FTD is not mandatory to conduct a genetic screening in these patients. Nevertheless, since this is a multicenter study in which clinical data has been ascertained retrospectively, there is a lack of a formal, homogeneous evaluation of family history of disease, which could underestimate the presence of disease in familial members of the index patient.

To our knowledge, this is the first comprehensive screening of all genes that have been previously linked to ALS and/or FTD with a high confidence, in a cohort of ALS/FTD patients not carrying the *C9orf72* hexanucleotide repeat expansion. Our results strongly indicate that the mutation landscape in such patients is complex and highly heterogeneous, and suggest that high-throughput sequencing approaches in uniform series of patients with concomitant ALS and FTD might be a strategic design to help disentangle the genetic architecture of ALS and/or FTD.

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Table 1. Demographic description of patients carrying a likely pathogenic genetic alteration and mutation characteristics.

Gene	Case	Gender	AAO	Transcript	cDNA position	Aminoacid change	PolyPhen-2	SIFT	MutationTaster	CADD score	ExAC (NFE_MAF)
TBK1	NM4	M	64	NM_013254	c.228+1G>A[a]	p.K30_E76del	-	-	D	29.4	0
TBK1	NM13	M	55	NM_013254	c.235_237del[a]	p.T79del	-	-	-	-	$1.49x10^{-5}$
TBK1	1066[b]	M	49	NM_013254	c.762_763del[a]	p.N254fs	-	-	-	-	0
TBK1	NM207	F	69	NM_013254	c.992+1_4del	p.G272_T331del	-	-	-	-	0
TBK1	1513	F	56	NM_013254	c.1921_1923del	p.E643del	-	-	-	-	0
SQSTM1	CS1505	M	60	NM_003900	c.98C>T	p.A33V	B	T	N	14.68	0.002
SQSTM1	CS1480	M	69	NM_003900	c.1175C>T	p.P392L	D	D	A	22	0.001
SQSTM1	1066[b]	M	49	NM_003900	c.1175C>T	p.P392L	D	D	A	22	0.001
TARDBP	NM244	M	48	NM_007375	c.269C>T	p.A90V	B	T	D	23	0.0004
VCP	CS1295	M	NA	NM_007126	c.79A>G	p.I27V	B	T	D	16.49	0.0005
ERBB4	438	M	36	NM_005235	c.1997T>C[a]	p.I666T	D	D	D	26	$1.48x10^{-5}$
TAF15	CS0615	M	69	NM_139215	c.1384G>A[a]	p.G462S	B	D	D	17.56	$3.85x10^{-5}$

AOO: Age at onset; ExAC (NFE_MAF): Minor allele frequency in the Non-Finnish European population from the exome aggregation consortium; M: Male; F: Female; Abbreviations in PolyPhen-2 prediction= B: Benign; P: Possibly damaging; D: Probably damaging. Abbreviations in SIFT prediction= T: Tolerated; D: Damaging. Abbreviations in MutationTaster prediction= N: Neutral; D: Disease-causing; A: Automatic disease-causing.[a] Novel mutations. [b] This patient is a double mutation carrier.

Supplementary table 1. Genes analyzed in the present study.

Gene	Location	Inheritance	Phenotype
C9orf72	9p21.2	AD	ALS, FTD, ALS/FTD
TARDBP	1p36.22	AD	ALS, FTD, ALS/FTD
TBK1	12q14.2	AD	ALS, FTD, ALS/FTD
FUS	16p11.2	AD/AR	ALS, FTD, ALS/FTD
SQSTM1	5q35.3	AD/Risk	ALS, FTD, ALS/FTD
VCP	9p13.3	AD	ALS, FTD, ALS/FTD
OPTN	10p13	AD/AR	ALS, FTD, ALS/FTD
UBQLN2	Xp11.21	XL	ALS, FTD, ALS/FTD
DCTN1	2p13.1	AD	ALS, FTD, ALS/FTD
CHCHD10	22q11.2	AD	ALS, FTD, ALS/FTD
CHMP2B	3p11.2	AD	FTD, ALS/FTD
MAPT	17q21.31	AD	FTD, ALS/FTD
MATR3	5q31.2	AD	ALS, ALS/FTD
ANG	14q11.2	AD	ALS, ALS/FTD
TUBA4A	2q35	AD	ALS, ALS/FTD
CCNF	16p13.3	AD	ALS, ALS/FTD
SOD1	21q22.11	AD/AR	ALS, ALS/FTD
PFN1	17p13.2	AD	ALS
ALS2	2q33.1	AR	ALS
SETX	9q34.13	AD	ALS
SPG11	15q21.1	AR	ALS
VAPB	20q13.33	AD	ALS
TAF15	17q12	AD	ALS
EWSR1	22q12	AD	ALS
FIG4	6q21	AD	ALS
SIGMAR1	9p13.3	AR	ALS
SPAST	2p24	AD	ALS
DAO	12q24.1	AD	ALS
HNRNPA2B1	7p15.2	AD	ALS
ERBB4	2q34	AD	ALS
NEK1	4q33	AD/Risk	ALS
GRN	21q22.1	AD	FTD
PSEN1	14q24.2	AD	FTD

TREM2	6p21.1	AD/AR	FTD
PSEN2	1q42.13	AD	FTD

AD: autosomic dominant inheritance; AR: autosomic recessive inheritance; XL: X-linked inheritance.

4. DISCUSSION

"The only true wisdom is in knowing you know nothing"

Socrates

Molecular genetics have provided and is still adding major evidences which support the overlap between ALS and FTD[18,19,21,259]. Mutations in the *TARDBP* gene emerged as the first genetic link between both disorders[12,45], but the most important genetic cause in the ALS/FTD continuum is the hexanucleotide repeat expansion in the *C9orf72* gene[18,19]. In this context, we aimed at assessing the prevalence of this expansion mutation in a large series (n=936) of ALS patients from Spain (publication 3.1; García-Redondo, A., et al. *Hum. Mutat*. 2013). Also, clinical features of patients carrying the pathogenic hexanucleotide repeat expansion were compared with those of non-carriers in order to find specific signs that could be attributed to this pathogenic alteration. In this work, a high prevalence of ALS patients harboring the expansion mutation was demonstrated, as it explained 27.1% and 3% of fALS and sALS patients, respectively. This result also reinforced the north to south cline previously suggested across Europe. In agreement with the literature, clinical features of ALS expansion carriers in our cohort which significantly differed from ALS patients not carrying the expansion mutation were: a younger age at onset, reduced disease duration, a more frequent positive family

history and a higher co-occurrence of FTD. Observation of age at onset among ALS patients carrying the repeat expansion mutation also revealed a wide range of onset ages (26 to 71 years), which is in accordance with the fact that complete penetrance is observed by 80 years of age and that by 56 years of age the penetrance is only about to 50%[55].

In order to complement these findings, as the hexanucleotide repeat expansion size cannot be determined through the rpPCR approach, in publication 3.2 (Dols-Icardo, O., et al. *Hum. Mol. Genetics* 2014), the hexanucleotide repeat in ALS and FTD expansion mutation carriers was characterized with the hypothesis that it might influence clinical features or the disease phenotype. To achieve this goal, a non-radioactive southern blot protocol which allowed the detection from 2 to approximately 4500 hexanucleotide repeat units was developed. Significant differences in repeat expansion sizes (maximum, median and modal metrics) were found between ALS and FTD patients, suggesting that patients with larger repeat expansions were more prone to develop ALS. However, the huge overlap in the repeat length between both disorders precluded any firm conclusion in this respect. No correlation was found between the expansion length and any of the clinical features analyzed, including age of clinical onset or disease duration. A possible explanation for this lack of correlation point to disease modifiers which might contribute to the pleiotropy observed in *C9orf72* expansion carriers. In this context, two monozygotic twins discordant for ALS were also reported in this study to harbor

different repeat expansion lengths. Our work also presented evidence of stable and unstable hexanucleotide repeat number transference through generations, as published in other reports[260,261]. Interestingly, different repeat expansion sizes were identified in blood and cerebellar tissue from the same individuals, which suggested that stochastic expansion events are taking place during cell division. The last observation suggests that there is genetic mosaicism in the length of this expansion. These could have consequences in final conclusions substracted from data that has been obtained from the analysis of a particular tissue. A study which evaluated the repeat expansion length in different tissues from the same individuals supports this notion. The authors demonstrated that, independent of the phenotype analyzed, the repeat lengths were larger in the frontal lobe and spinal cord than in cerebellum, and differed across brain regions[229].

Since the discovery of the hexanucleotide repeat expansion in *C9orf72*, next generation sequencing technologies have provided an unprecedented huge amount of genetic data, mainly by exome sequencing, which has proven to be a powerful strategy in order to unravel other genes that might contribute to the ALS/FTD spectrum[21,70,259]. Elucidating the role of these new genes in specific populations is of major interest in terms of future genetic analyses and counseling. In this context, mutations in genes first demonstrated to cause ALS, such as *CHCHD10*, were also found in ALS patients with concomitant FTD [21,70,259,262]. This prompted us to re-sequence the *CHCHD10* gene in a large FTD (n=709) and ALS

(n=423) cohort, and in 92 patients diagnosed with ALS/FTD, in order to evaluate its role in ALS and FTD within the Spanish population. In publication 3.3 (Dols-Icardo, O., et al. *Brain* 2015), we described two mutations in *CHCHD10* gene; a rare missense variant in an ALS patient who had a long disease duration, as previously reported[263,264], and a nonsense mutation in an atypical FTD patient presenting with NFPA and parkinsonism. This data provided further evidence of the genetic overlap in ALS/FTD continuum and supported the mitochondrial functional impairment in neurodegenerative disorders[265].

Following the same strategy, the description of *TUBA4A* mutations in ALS patients, and, in two ALS patients who also developed FTD[70,262], led us to study its role in FTD (publication 3.4; Dols-Icardo, O., et al. *Neurobiol. Aging* 2016). This study included 814 FTD patients, 31 of them with concomitant ALS. *TUBA4A* gene re-sequencing did not reveal any potentially damaging variant. Hence, our study did not support a role of *TUBA4A* mutations in FTD patients from Spain.

In publication 3.5 (Dols-Icardo, O., et al. *in preparation*), we took advantage of exome sequencing technologies to analyze the DNA material from 54 patients with concomitant ALS and FTD. Importantly, the expansion mutation in *C9orf72* (which is the most important genetic alteration in ALS/FTD, as demonstrated in this thesis) was discarded prior to whole-exome sequencing. Thirty-five bona fide genes with prior evidence to contribute to the ALS or

FTD etiology were analyzed. A total of 11 patients (20.4%) carried a disease causing mutation, which suggests a significant genetic component in patients with ALS/FTD. Among them, five patients carried a mutation in the *TBK1* gene, representing 9.3% of this cohort. Thus, in the Spanish population, mutations in *TBK1* are the most common cause of ALS/FTD cases not carrying the hexanucleotide repeat expansion in *C9orf72*. This is in agreement with recent data which reported that *TBK1* mutations are present in 4.5% of ALS/FTD patients from Belgium[206], and this prevalence reached 10.8% in an independent study performed among French cases [207]. The second most common gene mutated in our cohort was *SQSTM1*. Interestingly, the p.P392L mutation, which is particularly common among PDB patients from Spain (15.6%) [199], was found in two cases and supports the high frequency of this variant in this population. Our data reinforces the role of *SQSTM1* mutations in the etiology of ALS and FTD [59]. Our study disclosed a *VCP* mutation (p.I27V) and a *TARDBP* missense variant (p.A90V), both of them already reported in the literature[266,267]. Our data also revealed a likely pathogenic mutation in *ERBB4* (p.I666T), which is located in the transmembrane domain of ERBB4 and might affect protein localization [268]. In addition, a novel mutation in *TAF15* (p.G462S) was also discovered. This variant is placed within the C-terminal RGG domain of TAF15, were missense mutations have been described in sALS and fALS patients and demonstrated to induce cytoplasmatic foci in spinal cord neurons, accelerates protein aggregation *in vitro,* and exacerbate the deleterious neurodegenerative effect that is present in flies expressing human

TAF15 [269]. Thus, the finding of a likely pathogenic mutation in *ERBB4* and *TAF15* expands its contribution to FTD beyond the initially phenotype restricted to ALS. Taking together, our data strongly indicate that the mutation landscape in patients lacking the *C9orf72* expansion is complex and highly heterogeneous. Thus, it suggests that high-throughput sequencing applied in large and uniform series composed of patients with concomitant ALS and FTD might be a strategic approach to disentangle novel genetic factors contributing to the genetic architecture of ALS and/or FTD.

In this thesis, the hexanucleotide repeat expansion (the most common genetic cause of the ALS/FTD disease spectrum) has been investigated and proven to account for a high frequency of cases with ALS and/or FTD. Importantly, this thesis reinforces the idea that ALS and FTD have a significant genetic overlap. Our data suggest a high and heterogeneous burden of rare genetic mutations in patients diagnosed with ALS and concomitant FTD not carrying the *C9orf72* expansion mutation. Genetic data derived from next generation sequencing of several neurological diseases demonstrate that there are increasing evidences that mutations in some genes are involved in other neurodegenerative disorders beyond their initial association to a single disease. This is the case of some genes studied herein, such as the *C9orf72* repeat expansion [228,242,246,270], but also of mutations in the *CHCHD10* gene, involved in myopathy and cerebellar ataxia[259], ALS[271], FTD[271] and CMT2[272]; or in *VCP*, related to IBMPFD, FTD[129], ALS[52], and CMT2[31]. In addition, in contrast to the substantial role of mutations in *SOD1* in ALS, in

MAPT and *GRN* in FTD, and *C9orf72* and *TBK1* in both ALS and FTD, novel disease-causing genes will probably explain few ALS/FTD cases or single families linked to this spectrum of disorders. Thus, whole-exome sequencing is the most straightforward approach for an in deep genetic testing. Next generation sequencing technologies will improve the capability of screening an expanded number of genes beyond the most important and plausible candidates. Hence, they will lead to the identification of rare disease causing mutations and modifiers, and even decipher which rare genetic variants influence the disease phenotype at onset. Hence, it will help in clinical diagnoses and have a potential impact in the development of therapeutic strategies and in the discovery of disease-associated pathways.

5. CONCLUSIONS

In conclusion, ALS and FTD constitute a genetic disease continuum. In this thesis, the genetic overlap of this spectrum of disorders has been assessed through different strategies, including a rpPCR approach, southern blot methodology, Sanger sequencing and whole-exome sequencing in order to obtain a broader view of the genetic causes related to the ALS/FTD spectrum.

The main conclusions of this thesis are:

1. **The hexanucleotide repeat expansion in the *C9orf72* gene is the most common genetic cause of ALS in Europeans from Spanish origin.**
    a. Shorter disease duration, younger age at onset and a more prominent family history and concomitant FTD are features which differentiate ALS expansion carriers to non-expansion carriers.

2. **The size of the hexanucleotide repeat in *C9orf72* expansion carriers is larger in ALS than in FTD patients.**
    a. There is a significant overlap of repeat expansion sizes between both diseases, thereby precluding any firm conclusion.
    b. The number of hexanucleotide repeat units does not correlate with clinical features in neither ALS nor FTD.

3. **Mutations in the *CHCHD10* gene are a cause of both ALS and FTD.**
    a. Mutations in this gene may give raise to a slow disease progression in ALS patients.
    b. An atypical FTD, comprising PNFA and parkinsonism, was presented in a case carrying a nonsense mutation in this gene, with no motor neuron involvement. This broadens the phenotypic effect related to *CHCHD10* mutations.

4. **Mutations in the *TUBA4A* gene do not play a major role in FTD.**

5. **Exome sequencing in ALS/FTD patients not carrying the *C9orf72* expansion mutation reveals a high genetic burden of mutations.**
    a. 20% of ALS/FTD patients harbor a likely pathogenic mutation in genes previously shown to play a role in ALS or FTD.
    b. Mutations in *TBK1* are the second most important genetic cause in ALS/FTD, after the hexanucleotide repeat expansion in *C9orf72*.

c. Mutations in the *SQSTM1* may be frequent in ALS/FTD patients from Spain.
d. Mutations in *ERBB4* and *TAF15* might be also a cause of ALS with concomitant FTD.

6. LIST OF PUBLICATIONS

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