

Title of the article:

Characterization of a FTL-D-PDB family with the coexistence of *SQSTM1* mutation and Hexanucleotide (G₄C₂) Repeat Expansion in *C9orf72* gene

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Up to five keywords:

Frontotemporal dementia; Paget disease of bone, *C9orf72*; *SQSTM1*

ABSTRACT

The *C9orf72* expansion is considered a major genetic cause of familial frontotemporal dementia (FTD) in several patients' cohorts. Interestingly, *C9orf72* expansion carriers, present also abundant neuronal p62- positive inclusions. Although *p62/SQSTM1* mutations were initially associated with Paget disease of bone (PDB), they have been also identified in FTD. We describe a FTD-PDB family in which the proband presented with behavioral FTD phenotype and concomitant Paget disease. The molecular genetic analysis revealed the co-occurrence of two mutations; the pathogenic *C9orf72* expansion and p.P392L heterozygous missense mutation in *SQSTM1* gene. Amongst the six family members analyzed, the p.P392L *SQSTM1* mutation segregated as expected with PDB whereas the *C9orf72* expansion segregated with frontal cognitive impairment or dementia in all but one carrier. The coexistence of these conditions could be underestimated since neither patients with FTD nor patients with PDB undergo bone scintigraphy or cognitive assessment, respectively. The number of cases with double mutations could also be over looked as the molecular strategy adopted in the majority of laboratories ends with the identification of one pathogenic mutation in one of the known causative genes. Therefore, we advocate for further clinical and molecular evaluation in suspect cases.

keywords:

Frontotemporal dementia; Paget disease of bone, *C9orf72*; *SQSTM1*

INTRODUCTION

A pathogenic expansion of hexanucleotide (G₄C₂) repeat upstream of the *C9orf72* coding region in the first intron or the promoter region depending on the transcript variant was identified as a major cause of familial frontotemporal lobar degeneration (FTLD) and/or amyotrophic lateral sclerosis (ALS) in several cohorts from different geographical regions (DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Renton et al., 2011; van der Zee et al., 2013). The repeat units in patients were estimated to be several hundred to several thousand, compared to less than 25 repeat units in healthy controls (Gijselinck et al., 2012; van der Zee et al., 2013). Patients carrying the repeat expansion show a high degree of heterogeneity in clinical phenotypes, even in the same family (Cerami et al., 2013). Atypical symptoms have been reported and include cognitive impairment, psychosis and extrapyramidal dysfunction (Boeve et al., 2012; Galimberti et al., 2013; Snowden et al., 2012). It is of interest to note that, FTLD or ALS patients carrying the *C9orf72* gene expansion, present also abundant neuronal p62- positive inclusions (Al-Sarraj et al., 2011; Murray et al., 2011). The increased p62 immunoreactivity is common to a wide variety of neurodegenerative diseases, such as Alzheimer disease (AD), dementia with Lewy bodies, FTLD, Parkinson disease (PD), and Huntington disease (HD) (Kuusisto et al 2001; Nakaso et al., 2004; Zatloukal et al., 2002). p62 protein, encoded by sequestosome 1 (*SQSTM1*) gene located on chromosome 5q35 is a multifunctional protein that contains several domains, including PB1, ZZ, TRAF6, PEST, and the ubiquitin-binding domain (UBA). Although mutations in this gene were initially identified as a cause of Paget disease of bone (PDB) (Laurin et al., 2002), in 2011 *SQSTM1* mutations were also reported in ALS (Fecto et al., 2011). Recently *SQSTM1* mutations have been identified in patients with FTLD from different geographical regions, suggesting its role also in the pathogenesis of this neurodegenerative disease (Le Ber et al., 2013; Rubino et al., 2012; van der Zee et al 2014). Interestingly, some of these FTLD *SQSTM1* mutation carriers also developed signs and symptoms of PDB (Le Ber et al., 2013). However, due to the low penetrance of Paget disease, the coexistence of these conditions could be underestimated since neither patients with FTLD nor patients with PDB undergo bone scintigraphy or cognitive assessment, respectively. Amongst its multiple cellular functions, the p62 plays a key role in the regulation of osteoclast differentiation as well as their activity and survival. In addition, it has a crucial role in targeting misfolded and/or ubiquitinated proteins for degradation by autophagy or by the ubiquitin–proteasome systems (Bjørkøy et al., 2006; Pankiv et al., 2007; Seibenhener et al., 2004). Interestingly, all mutations identified in the PDB patients were clustered either within or near the UBA domain, impairing the ability of p62 to bind to ubiquitin (Cavey et al., 2006; Najat et al., 2009), probably resulting in aberrant NF- κ B signaling (Vadlamuni et al., 1996). Conversely, the mutations identified thus far in both ALS patients and FTLD were distributed throughout the coding region of the gene. In these cases, p62 mutations may significantly compromise protein–protein interactions and/or the clearance of or promote the aggregation of ubiquitinated protein inclusions found in neurons of both patients with FTLD and ALS (Fecto et al., 2011; Kwok et al 2013; Le Ber et al., 2013; Rubino et al., 2012; Teyssou et al., 2013). The present study aims to describe a FTLD-PDB family with the coexistence of *SQSTM1* mutation and Hexanucleotide (G₄C₂) Repeat Expansion in *C9orf72* gene.

1. MATERIALS AND METHODS

2.1 Subjects

All the seven available family members provided a written consent for their participation in the study. All individuals were in a stable condition, without acute comorbidities and underwent a thorough biochemical and neurological evaluation performed by a behavioural neurologist from the Department of Neurology of Coimbra University Hospital. For all the patients detailed history, clinical neurological examination; psychiatric evaluation, neuropsychological assessment, brain imaging and genetic testing were done. The diagnosis of FTLD was based on the Lund and Manchester clinical criteria (Neary et al., 1998; The Lund and Manchester Groups 1994) revised by the Work Group on Frontotemporal Dementia and Pick's Disease (McKhann et al., 2001) and more recently according to the International Behavioural Variant Frontotemporal Dementia Criteria Consortium for bvFTD (Rascovsky et al 2011). The diagnosis of PDB was defined as monostotic or polyostotic increased bone radionuclide uptake in bone scintigraphy which can be associated with raised levels of serum total alkaline phosphatase.

2.2 Genetic testing

All eight exons of the *SQSTM1* gene were PCR amplified using previously reported primers flanking the intron-exon boundaries (Michou et al., 2012) and were subsequently direct sequenced.

The detection of the *C9orf72* expansion was performed using a two-step PCR based protocol as previously described (van der Zee et al., 2013) and the G₄C₂ repeat sizing was determined using the new short expansion PCR as described (Gijssels et al., 2015). The proband was also screened for *MAPT*, *PGRN* and *VCP* genes.

2. RESULTS

The pedigree is shown in Fig 1. The proband (III3) had 12 years of education, with no consistent job due to personality disorder. He was diagnosed with PDB at the age of 57, presenting a typical polyostotic bone disease in scintigraphy (Fig 2) and elevated levels of serum total alkaline phosphatase (127mg/dL). When he was 58 years of age a neurological evaluation was requested due to the development of weakness in the lower limbs leading to falls with subsequently incapacity to walk unassisted as well as mental/behavior symptoms (desinhibition, puerility and aggressivity). The neurological assessment showed tetraparesis with predominant proximal weakness and general hyporeflexia, suggesting a muscle disease, but he also presented spastic speech, bilateral Babinski signs and ancillary reflexes (glabella, snout and grasping). The mental assessment disclosed psychomotor slowing, paucity of speech, echolalia, palilalia and perseveration. Mental-State Examination-MMSE (Portuguese version) (Folstein et al 1975; Guerreiro et al., 2003a) was abnormal (23/30) and the global score (21) on the Alzheimer's disease Assessment Scale- ADAS-COG (Portuguese version) (Guerreiro 1988b; Mohs et al., 1983) was compatible with mild to moderate cognitive deterioration. A comprehensive cognitive evaluation was performed with the Battery of Lisbon for the Assessment of Dementia (BLAD) (Guerreiro 1988), which includes multiple neuropsychological tests representing key cognitive domains and is validated for the Portuguese population. This evaluation confirmed frontal dysfunction with impairment in tests of motor control (Luria), verbal initiative (Verbal Semantic Fluency), verbal and non-verbal abstraction (Interpretation of Proverbs and the

Raven Progressive Matrices). Language evaluations, as well as tests related to visuo-constructional abilities (Clock Drawing test) were normal, except for the presence of perseverative behaviour. Routine laboratorial tests for treatable dementia including CSF analysis were normal. The EMG was normal and the muscle biopsy did not reveal any abnormal findings. Magnetic resonance imaging (MRI) of the brain showed mild cortical-subcortical atrophy more pronounced in the left hemisphere (Fig 3). The single-photon emission computed tomography (SPECT) revealed cerebral hypoperfusion at the frontal lobe (upper frontal, pre and post central gyrus), and also parietal, predominantly left-sided (Fig 4). According to these results the patient was tested for mutations in valosin-containing protein (*VCP*) gene but no mutations were found. Further genetic studies in the framework of the European Early-Onset Dementia (EU EOD) consortium (van der Zee et al., 2013, 2014), revealed that he harboured both *C9orf72* repeat expansion and p.P392L heterozygous missense mutation in the *SQSTM1* gene. Using the new short expansion PCR technique, it was also demonstrated that this patient carried a full pathogenic long expansion (>80 units). The patient died of lung infection in another hospital at 59 years of age, one year after the dementia diagnosis. The proband had a positive family history for FTLN and PDB. The proband's mother (III1) had behavioral symptoms and developed dementia associated with parkinsonism and also complained of lower limbs pain at 60 years of age. Her DNA was not available. His maternal uncle (II2) had also developed Parkinsonism but without dementia in his nineties, the other uncle (II3) died at young age in the war, still with no clinical signs of FTLN or PDB whereas the youngest uncle (II4) developed dementia at 83 years of age. Although the clinical features initially suggested the diagnosis of Alzheimer Disease, the MRI of the brain showed a severe asymmetrical atrophy involving the hippocampus and PIB-PET was negative for amyloid deposits. He carried the hexanucleotide repeat expansion in *C9orf72* gene.

The five probands' sibs, have been tested for the two mutations previously identified in the family. The subjects III1 and III2 (69 and 65 years of age, respectively) carried only the hexanucleotide repeat expansion in *C9orf72* gene, the III4 (60 years of age) harboured both *C9orf72* expansion and p.P392L mutation; subject III5 (57 years of age) carried only the p.P392L mutation, she had a history of early mental impairment as a result of encephalitis in her childhood and was already diagnosed with PDB; and the remaining sibling (III6) (52 years of age) had none of the mutations identified. Although these subjects had no subjective cognitive complaints and normal neurological examination, they underwent psychiatric and neuropsychological assessment and investigation of possible PDB diagnosis. As expected, all the three *SQSTM1* p.P392L mutation carriers had the clinical imaging diagnosis of PDB, whereas the non carriers presented a negative scintigraphy. Therefore, this mutation segregated with PDB with full penetrance at 60 years of age in this family. Considering neuropsychological and psychiatric evaluation, the subjects III1 and III2 (*C9orf72* expansion carriers) presented mild frontal behavioral and cognitive impairment (impulsivity, perseveration, deficit in attention and mild learning disorder). Patients III5 had multiple deficits congruent with the information of development cognitive impairment. Finally, the subjects III4 (double mutation carrier) and III6 (without mutations) had no mental decline. Considering the seven family members tested in both generations, the *C9orf72* repeat expansion segregated with frontal cognitive impairment in all but one carrier (III4). Curiously, there is only one case (III5) who harbors only the *SQSTM1* p.P392L mutation but unfortunately it is not completely informative in respect of its association with cognitive impairment due to a previous history of mental development

disorder, although there is no insight of cognitive deterioration (The clinical characteristics of the patients and their neuropsychological and psychological evaluations are summarized in Table 1 and Table 2).

3. DISCUSSION

We describe a family with both FTD and PDB carrying the c.1175C>T, p.P392L mutation in exon 8 of the *SQSTM1* gene and a long repeat expansion (>80 units) in *C9orf72* gene. Amongst the six family members analyzed, the *C9orf72* repeat expansion segregated with frontal cognitive impairment or dementia in all but one carrier whereas the p.P392L *SQSTM1* mutation segregated with the PDB in all carrier members. This *SQSTM1* mutation identified was located in the UBA domain of the p62 protein and is considered the most frequent mutation among both familial and sporadic PDB patients (Nakaso et al., 2004). Interestingly, it was also identified in FTLN, ALS patients (with or without concomitant PDB) and in control individuals at low frequency (<1%) (Adzhubei et al., 2010; Fecto et al., 2011; Laurin et al., 2002; Vadlamudi et al., 1996). More recently, it was also found to segregate with dementia in a FTLN-PDB family (Le Ber et al., 2013) and in a familial ALS-PDB (Kumar et al., 2009; Najat et al., 2009). Curiously, we also have previously identified, in the framework of the EU EOD consortium (data not shown), three Portuguese bvFTD patients harbouring this particular mutation, p.P392L, thereby supporting the role for *SQSTM1* as a causative gene in FTLN since they had no clinical symptoms and no familial history of PDB (van der Zee et al., 2014).

In the present FTLN-PDB family (Fig1), the co-occurrence of two pathogenic mutations was identified in two patients (III3 and III4). The III3, was the proband who developed both symptoms of bvFTD and PDB over the course of the disease whereas III4 presented only with PDB signs at 61 years of age, symptoms that are predictable related to the p.P392L mutation. Although this asymptomatic *C9orf72* carrier still presented a preserved performance on executive functions, she might develop cognitive impairment later on due to the age dependent penetrance of the *C9orf72* expansion. Indeed, in accordance with previous studies, approximately 0% of expansion carriers showed symptoms below 35 years, 50% of carriers younger than 58 years were still clinically asymptomatic, and nearly 100% of carriers were symptomatic at an age older than 80 years (Majounie et al., 2012). Actually, within this family, the *C9orf72* carriers showed heterogeneity in clinical presentations and a wide variation in age of onset (ranging from 58 to 80 years). In the other examined relatives, a single *C9orf72* repeat expansion or *SQSTM1* p.P392L mutations was identified and was associated with clinical signs in the spectrum of bvFTD or isolated PDB, respectively. To our knowledge, this is the first kindred that clearly demonstrated a co-segregation FTLN-PDB phenotype due to the occurrence of both mutations. Interestingly, van der zee and colleagues have also previously identified three FTLN patients who carried a *SQSTM1* mutation and the pathogenic *C9orf72* repeat expansion. However, they could not exclude the presence of PDB since this condition often remains asymptomatic and a diagnosis requires confirmatory imaging (van der Zee et al., 2014). Furthermore, our study seems to support previous studies demonstrating the co-occurrence of 2 evidently pathogenic mutations in FTLN patients, in whom the *C9orf72* mutation was one of the two mutations found (Ferrari et al., 2012; Lashley et al., 2014; King et al., 2013; Mignarri et al., 2014; van Blitterswijk et al., 2013).

Despite the fact that each of these mutations independently causes disease, it is tempting to speculate that when they co-occur they could modify the expressivity of the disease. One could hypothesize a synergistic effect of both genetic changes on the disease pathogenesis. However due to the limited number of double mutation carriers in our family we do not have a clear indication that the co-existence of mutations influences clinical expression of disease. Moreover, onset age was no different from those carrying only one of the mutations. We believe that the number of cases with double mutations could be underestimated as the molecular strategy adopted in the vast majority of the laboratories ends with the identification of one pathogenic mutation in one of the known causative genes.

In the future, moving from individual gene testing by conventional methods such as, Sanger sequencing, to deep sequencing methods using a known dementia genes-panel testing, will be critical to explore whether or not the contribution of more than one gene defect is responsible for the clinical heterogeneity seen in patients suffering from neurodegenerative diseases. Thus, in our family, the most recent genes linked to FTL, *C9orf72* and *SQSTM1*, appeared mutated and explain the FTL-PDB phenotype developed. Consequently, these findings have huge implications in genetic counseling of both patients and family members and further elucidating studies involving the respective encoded proteins will be needed to rule out their engagement into the common downstream pathway in the disease process.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICAL STANDARDS

All included family members or responsible caregivers, whichever appropriate, provided informed written consent for their participation in the study.

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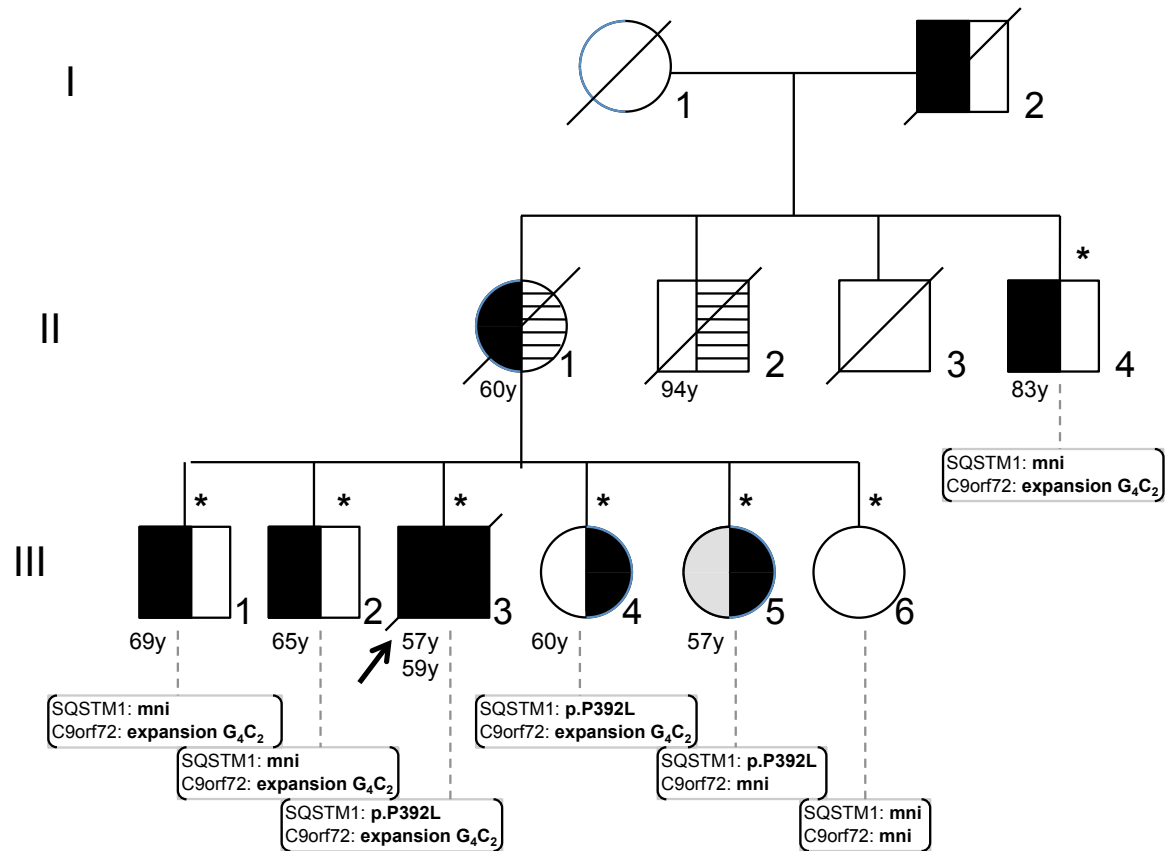
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FIGURES

Fig 1- Pedigree of the Family with FTD-PDB phenotype carrying c.1175C>T, p.P392L, mutation and concomitant long *C9orf72* hexanucleotide expansion (>80 units).

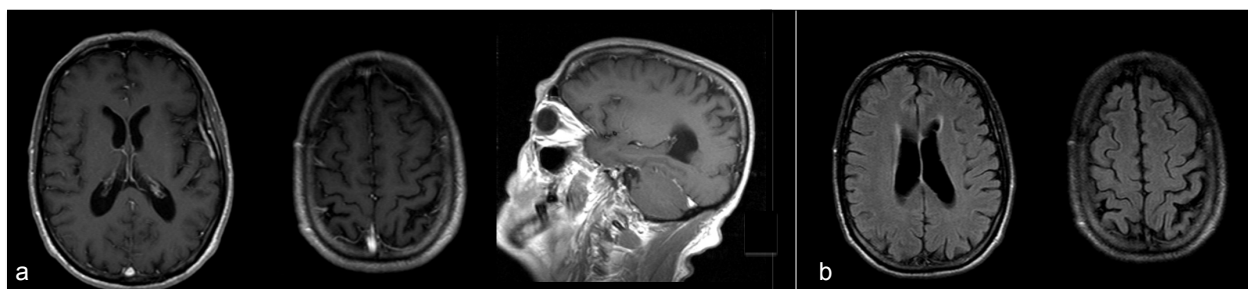


The black symbols represent patients affected with frontal cognitive impairment or dementia (left side filled), Paget disease of bone (right side filled) or both. The grey symbol indicates signs of cognitive impairment resulting from childhood encephalitis (left side filled). The black horizontal stripes (right side filled) indicate Parkinsonism. White symbols represent unaffected individuals. Age of onset in years (y) are shown below symbols (top number) and age of death (bottom number) is also shown for the proband who died. The proband is indicated by an arrow. Individuals with obtainable DNA are shown with an asterisk to the right of the symbol. The Roman numeral to the left of the pedigree denotes the generation; Arabic numbers below the individuals denote individuals. The results of the genetic analysis of *C9orf72* expansion and *SQSTM1* genes appeared inside brackets. mni: no mutation identified; expansion G₄C₂: carrier of the hexanucleotide repeat expansion in *C9orf72*; p.P392L: carrier of the heterozygous p.P392L mutation in exon 8 of the *SQSTM1*.

Fig 2 - Bone scintigraphy with ^{99m}Tc -MDP showing multifocal anomalous hypercaptation, specially in axial skeleton, consistent with Paget's disease. The patient had also Lumbosacral spine CT revealing structural bone deformation.



Fig 3 - (a) Axial and sagittal gadolinium enhanced T1-weighted and (b) axial T2-weighted fluid-attenuated inversion recovery (FLAIR) brain MRI showing cortical-subcortical left parietal atrophy.



Images are displayed according to radiological convention (right side of the brain is shown on the left side of figure).

Fig 4 - Cerebral SPECT (HMPAO single-photon emission computed tomography) revealed hypoperfusion mainly in the frontal lobe including the upper frontal and pre and post central gyrus, predominantly on the left-side.

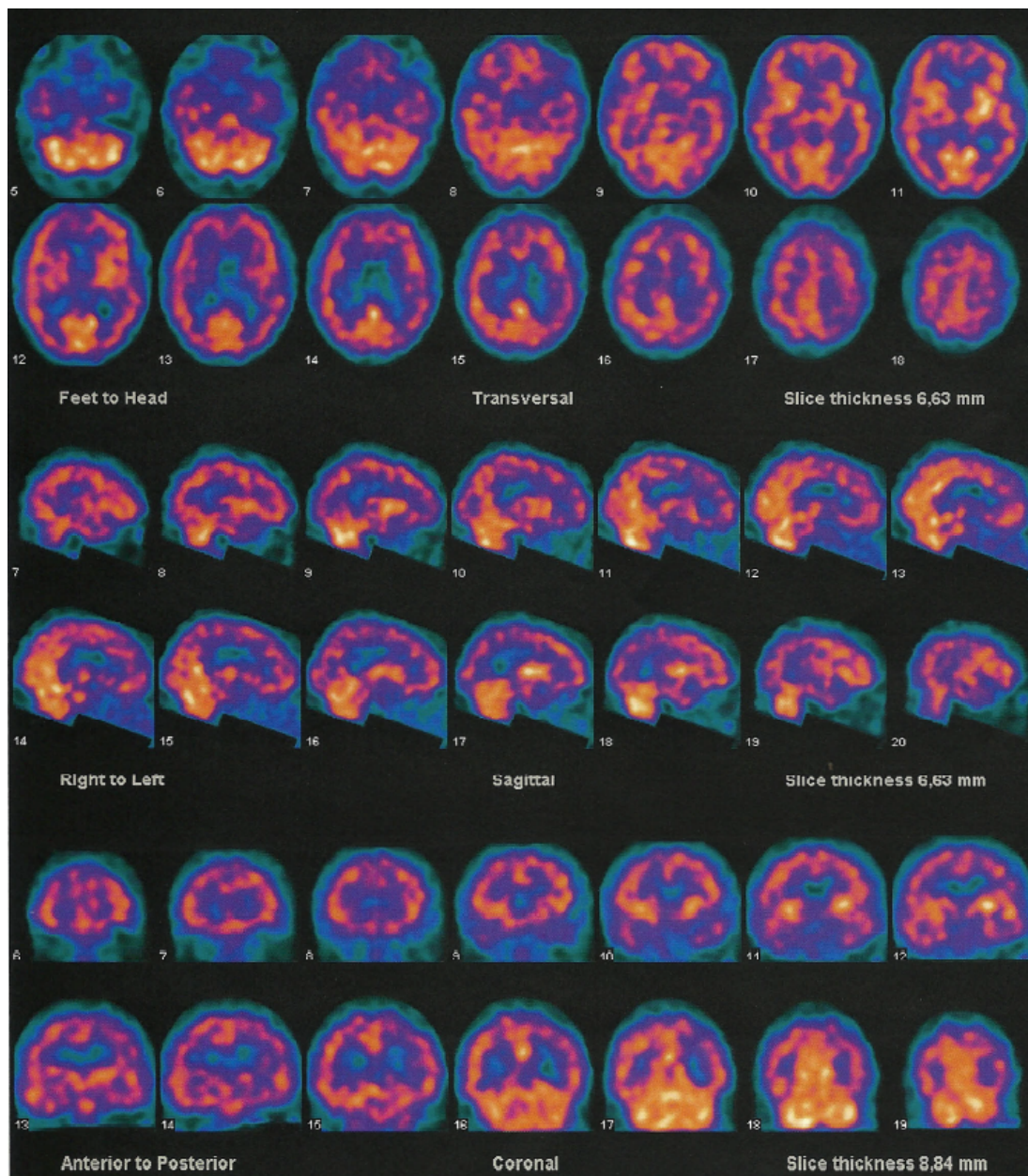


Table 1 – Summary of the genetic, phenotypic and imaging information from all evaluated family members

Case	Mutation status		Investigation (Age/years)	Clinical phenotype			Imaging data	
	<i>C9orf72</i> repeat expansion	<i>SQSTM1</i> , p.P392L		Cognitive impairment	Other neurological signs	Paget	Brain imaging	Bone scintigraphy
II1	n/a	n/a	-	Dementia (60 years)	Parkinsonism	Possible	n/a	n/a
II2	n/a	n/a	-	No (90 years)	Parkinsonism	No	n/a	n/a
II4	+	-	80	Dementia (81 years)	No	No	MRI = Asymmetric atrophy (hipocampus) PiB-PET = negative	negative
III1	+	-	69	Frontal Cognitive Impairment	No	No	Not done	negative
III2	+	-	65	Frontal Cognitive Impairment	No	No	Not done	negative
III3	+	+	58	Dementia (58 years)	Tetraparesis, grasp reflexes	yes	MRI = Cortical-subcortical left hemisphere atrophy SPECT = frontal hypoperfusion	positive
III4	+	+	60	No	No	yes	Not done	positive
III5	-	+	57	Mental Developmental Disorder	No	yes	Not done	positive
III6	-	-	52	No	No	No	Not done	negative

n/a=not available; + = present; - = absent; SPECT= Single Photon Emission Computed Tomography; MRI= **Magnetic resonance imaging**; Pittsburgh Compound-B (PiB) positron emission tomography (PET);

Table2 – Individual neuropsychological and psychological data available for the evaluated cases

	#II4	#III1	#III2	&III3	&III4	□III5	III6
Behavior*							
Impulsivity	-	√	√	√	-	-	-
Rigidity/perseveration	-	-	√	√	-	-	-
Apathy	√	-	-	√	-	√	-
Loss of attention	√	√	√	√		√	-
MMSE (≤22)**	22/30	29/30	30/30	23/30	30/30	24/30	28/30
MoCA (<17)**	18/30	23/30	24/30	n/a	30/30	13/30	28/30
Comprehensive Neuropsychological Assessment*							
Executive							
Psychomotor slowing	√	-	-	√	-	√	-
Sequencing impairment	√	√	-	√	-	-	-
Verbal/ motor perseveration	-	-	-	√	-	-	-
Generation impairment	√	-	-	√	-	-	-
Planning /set shifting impairment	√	√	√	√	-	√	-
Impairment abstraction	-	-	-	√	-	√	-
Language							
Reduced fluency	-	-	-	√	-	-	-
Word finding difficulty	-	-	-	-	-	-	-
Comprehension deficit	-	-	-	-	-	-	-
Echolalia	-	-	-	√	-	-	-
Memory							
Short term impairment	-	-	-	-	-	√	-
Retrograde information	-	-	-	-	-	√	-
Verbal learning impairment	√	-	-	√	-	√	-
Verbal consolidation impairment	√	-	-	-	-	-	-
Visual learning impairment	√	-	√	√	-	-	-
Visual consolidation impairment	√	√	√	-	-	-	-
Visuospatial construction impairment	-	-	-	-	-	-	-
Apraxia	-			-	-	-	-

MoCA = The Montreal Cognitive Assessment; **MMSE= The Mini-Mental State Examination**; √= present; - = absent;

= *C9orf72* expansion carrier; &=double mutation carrier; □= SQSTM1 p. P392L carrier

* Qualitative evaluation of specific domains

**Cut off for Dementia considering the normative data for the Portuguese population