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Analysis of Mitochondrial Genome in Frontotemporal Lobar Degeneration: Contribution of *RNRs* and Correlation with the Biochemical Phenotype

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Manuela Grazina (Universidade de Coimbra, Faculdade de Medicina) e sob a orientação interna do Professor Doutor António Portugal (Universidade de Coimbra, Faculdade de Ciências e Tecnologia).

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Resumo

A degenerescência lobar frontotemporal (DLFT) é uma doença neurodegenerativa heterogénea, no que diz respeito aos aspetos clínicos, neuropatológicos, genéticos e sintomáticos. É considerada a segunda demência pré-senil mais comum, a seguir à doença de Alzheimer (DA), e a quarta mais comum do tipo senil. Devido ao envolvimento seletivo dos lobos frontal e temporal, a DLFT é caraterizada por mudanças progressivas no comportamento, disfunção executiva e/ou dificuldades na linguagem. Em alguns doentes verifica-se uma sobreposição clínica e neuropatológica com a doença de Alzheimer, o que coloca a hipótese de existirem semelhanças na fisiopatologia, como o envolvimento do DNA mitocondrial (mtDNA). O objetivo do nosso estudo é realizar a sequenciação dos genes no mtDNA que codificam os rRNAs, de modo a identificar alterações em doentes com DLFT, investigando o seu envolvimento na doença.

Foram analisadas 101 amostras de DNA provenientes de doentes com diagnóstico provável de DLFT, incluindo 52 mulheres e 49 homens (faixa etária: 38-84, média de 64±10), seguidos no Departamento de Neurologia do Centro Hospitalar e Universitário de Coimbra. O DNA total foi extraído a partir de sangue periférico, e foi efetuada a análise da sequência dos 2 genes de rRNAs mitocondriais, por sequenciação de Sanger. As variantes encontradas foram submetidas a análise *in silico*. Em 87 doentes foram identificadas 45 variações diferentes. Destas, 5 alterações foram consideradas como provavelmente patogénicas, de acordo com a análise *in silico*: m.1243T>C devido a alterações estruturais; apresenta elevada conservação nas espécies analisadas e encontra-se localizada numa região *stem*. A variação m.1971A>G ocorre em heteroplasmia e é 100% conservada. A alteração m.2098G>A apresenta um valor mínimo de energia livre elevado bem como elevado grau de conservação evolutiva e ainda devido ao facto da sua localização numa região *stem*. A variação m.2363A>G apresenta elevada conservação e localização numa região *stem*, causando alterações estruturais. A variação m.3036G>A, que ocorre em heteroplasmia, causa várias alterações, quer no mínimo de energia livre, quer estruturais; a conservação evolutiva é elevada nas 14 espécies analisadas e se encontrar localizada numa região *stem*.

Apesar dos resultados obtidos serem originais e interessantes e algumas novas perspetivas para o estudo do envolvimento da disfunção mitocondrial na DLFT, são necessários estudos adicionais para melhor clarificar a relação entre as variações do mtDNA

identificadas e a DLFT. No entanto, este é estudo original sendo o primeiro a investigar a sequência completa dos rRNAs mitocondriais na DLFT.

1. General Introduction

1.1 Frontotemporal Lobar Degeneration

Frontotemporal lobar degeneration (FTLD) is the second most common type of presenile dementia after Alzheimer's disease (AD) and the forth of senile dementia. FTLD is a heterogeneous neurodegenerative disease characterized by a progressive decline in behaviour or language difficulties, resulting from the atrophy of the frontal and anterior temporal lobes of the brain.

In 1892, Arnold Pick described the first clinical case of dementia, in which the patient presented cognitive impairment, progressive aphasia and changes in social behaviour, manifestations that were associated with temporal and frontal lobe atrophy (Harro Seelaar et al., 2010). Arnold Pick and Alois Alzheimer demonstrated neuronal inclusions that were later shown to be tau-positive at histopathology analysis (Alzheimer, 1911). Later on, research groups of Lund (Sweden) and Manchester (England) published the first clinical and neuropathological criteria for the diagnosis of frontotemporal dementia (FTD) (Statement, 1994). Clinical and neurological overlap between FTLD and other diseases, such as AD, has been described, leading to the development of many studies focused on possible pathophysiological similarities.

1.2 FTLD Epidemiology

Presently, FTLD is the umbrella term for distinguish a heterogeneous group of neurodegenerative diseases, including clinical, neuropathological and genetic features, characterized by progressive alterations in behaviour, executive dysfunction and/or language impairment, with frontal and temporal lobar atrophy (Josephs et al., 2011; Seltman & Matthews, 2012; Pan & Chen, 2013).

The FTLD onset of disease occurs before the age of 65 years in 75-80% of the patients, being mostly considered as a presenile dementia (Sieben et al., 2012), and it has an equal distribution among females and males (Galimberti & Scarpini, 2010). The disease has a familial component: 30-50% of the FTLD patients refer to at least one relative with similar symptomatology, and in 10-23% the disease segregates in the family with an autosomal dominant inheritance pattern (Goldman et al., 2005; Goldman et al., 2008).

Several studies have been made in order to clarify the prevalence of FTLD. Although the existence of population limitations, the estimated prevalence is 54 per 100,000 inhabitants over 75 years-old, 78 per 100,000 inhabitants aged 66 – 75 (Borroni et al., 2010), and 15 – 22 per 100,000 inhabitants aged 45 – 64 years, (Borroni et al., 2010; Ratnavalli et al., 2011), which is almost half the prevalence of AD in this age group (Seelaar et al., 2011).

1.3 Clinical variants of FTLD

Given its heterogeneity, FTLD is classified according to clinical characteristics.

The disease spectrum includes some subtypes with a higher prevalence. The term frontotemporal lobar degeneration describes a pathological syndrome, and has evolved from a captious onset of behavioural change or language impairment damage and cognitive decline to a severe and more generalized dementia, accompanied by progressive cerebral hypometabolism and atrophy of preferentially frontal and temporal lobes (Seelaar et al., 2010). The clinical classification groups of FTLD has been enlarged and distinct syndromes have been established, taking into account the several discovers made and giving a special attention to the clinical characteristics: the behavioural variant of FTD (bvFTD), and the language difficulties, primary progressive aphasia (PPA), which is still divided into two sub-groups, the agrammatic variant (avPPA) and the semantic variant of PPA (svPPA) (Borroni & Padovani, 2013).

The bvFTD is characterized by severe changes in behaviour and personality such as disinhibition, apathy, loss of empathy or stereotypic behaviour, leading to a loss of social competence. Executive functions are reduced, at least on the beginning of the disease, whereas memory and perceptuospatial skills are preserved. It accounts for more than 50% of the FTLD patients and onset is usually before 65 years-old, being the average before 58 years-old (Rabinovici & Miller, 2010; Seelaar et al., 2010; Sieben et al., 2012).

The avPPA is featured by grammatical errors and difficulties in preparing a speech, with a relatively preserved language comprehension. This sub-group is the second most prevalent presentation of FTLD, represented by 25%. The svPPA reveals a lack of comprehension and conceptual knowledge with problems in recalling words, while speech production is preserved. It represents 20-25% of the FTLD patients.

Recently redefining clinical criteria recognize several other phenotypes. Diseases like FTD with motor neuron disease (FTD-MND), coticobasal syndrome (CBS) and progressive supranuclear palsy (PSP) are now considered as part of the FTLD spectrum (Seelaar et al., 2011; Borroni & Padovani, 2013).

1.4 Neuropathological variants of FTLD

Since a clinical phenotype presentation may be associated with several different pathologies, additional information, such as histological analysis of brain tissue, is required for defining the specific disease (Grossman, 2011). The major pathological hallmark of FTLD is selective atrophy of the frontal and temporal cortex, with neuronal loss and gliosis (Seelaar et al., 2011). Neuropathology features of FTLD have been classified according to different inclusion proteins found in degenerating neurons (Borroni & Padovani, 2013).

Currently, five subtypes are accepted: FTLD-tau (tau pathology), with neuron and glial cells containing inclusions of hyperphosphorylated tau protein; FTLD-TDP, in which patients present tau-negative ubiquitin staining inclusions composed of transactive response (TAR) DNA-binding protein 43 (TDP-43) (Lipton et al., 2004; Neumann et al., 2006; Cairns et al., 2007; MacKenzie et al., 2010; Sieben et al., 2012); FTLD-UPS with ubiquitin-positive and TDP-43-negative histopathology (FTLD-UPS), FTLD presenting inclusions of the fused-in-sarcoma protein (MacKenzie et al., 2010; Sieben et al., 2012), and FTLD-ni, without any inclusions identified (MacKenzie et al., 2010).

One of the most intriguing issues in the FTLD field is the poor correspondence between neuropathological features and clinical phenotypes. Indeed, neuropathological characteristics are predictable only in cases with known genetic defects (Borroni & Padovani, 2013; Rohrer et al., 2011).

A correlation between neuropathological characteristics and genetic mutations has been found (MacKenzie et al., 2010). About 40% of FTLD cases are FTLD-tau, including all cases with *MAPT* gene mutations. In FTLD-TDP pathology cases, patients have mutations in *PGRN*, *TARDBP*, *VCP*, *C9ORF72.* Some cases of FTLD-UPS presented mutations in the *CHMP2B* gene*.* Additionally, although the majority of cases did not have mutations in *FUS* gene, there is a correlation between FTLD-FUS and mutations in *FUS* gene (Goldman et al., 2011) (Table I).

Table I: Neuropathological characterization of FTLD and associated genes (adapted from Mackenzie et al., 2010)

FTLD-tau	FTLD with tau-negative and ubiquitin-positive			FTLD-ni
	FTLD-TDP	**FTLD-FUS**	**FTLD-UPS**	
Mutations in genes				
MAPT 50%	*PGRN* *VCP* *TARDBP* *C9ORF72*	*FUS*	*CHMP2B*	No mutations identified

1.5 Genetic variants of FTLD

Positive family history was observed in 40 – 50% of the FTLD patients (Rohrer et al., 2011; Sieben et al., 2012), wherein bvFTD patients have a higher frequency (30 – 50%) than the patients with svPPA or avPPA. An autosomal dominant mode of inheritance is found in 10 – 27% of all FTLD patients (Stevens et al., 1998; Chow, et al., 1999; Rosso et al., 2003; Goldman et al., 2005; Seelaar et al., 2008; Rohrer et al., 2009; Seelaar et al., 2011).

Genetic heterogeneity of FTLD is observed by the identification of mutations in several nuclear genes. The most prevalent mutations occur in *MAPT* and *PGRN*, representing nearly 50% of the familial cases. While mutations in the nuclear genes, *VCP*, *CHMP2B*, *TARDP* and *FUS* are found in less than 5% of the patients (Seelaar et al., 2011; Sieben et al., 2012). More recently, mutations in *C9orf72* have been reported as responsible for FTLD, but the causative gene defect has yet to be discovered (Seelaar et al., 2011).

Gijselinck et al. 2012, evaluated patients with the repeat expansion in *C9orf72* gene, with the purpose of finding differences in clinical presentation when compared with patients carrying *MAPT* or *PGRN* mutations and also with patients without known pathogenic mutations. The results showed that patients with *C9orf72* repeat expansion had an early disease onset, and a familial history of FTLD and/or FTD-MND, presenting bvFTD, avPPA and concomitant CBS symptoms, while patients carrying *MAPT* mutations had mainly bvFTD presentation. It was then proposed that *C9orf72* genetic screening should

have priority in patients with FTD-MND, as in cases where a genetic svPPA is suspected, *PGRN* mutations should be taken into account in avPPA or CBS phenotypes, and *MAPT* mutations in bvFTD and also in PSP phenotypes. The algorithm proposed is defended not only to be applied for familial cases, but should be considered also for patients with apparently sporadic FLTD (Gijselinck et al., 2012; Borroni & Padovani, 2013). However, it should be kept always present that the distribution of genetic mutations can differ according to ethnic origins and it is therefore necessary better knowledge of FTLD genetic epidemiology (Borroni & Padovani, 2013).

More recently, mutations in the *CHCHD10* gene, responsible for encoding a mitochondrial protein that is located at the enriched junctions in the intermembrane space, have been identified in a large family with FTLD/ALS (Chaussenot et al., 2014; Johnson et al., 2014). It may play a role in cristae morphology maintenance or in oxidative phosphorylation (Martherus et al., 2010; Bannwarth et al., 2014).

The pattern of brain atrophy might also be helpful for the genetic diagnostics. Usually, patients with *MAPT* mutations show symmetrical brain atrophy, involving mainly the temporal lobes, while patients carrying *PGRN* mutations have more often an asymmetrical pattern of atrophy with frontoparietal involvement, and patients carrying *C9orf72* expansion showed a more generalized and symmetrical atrophy (Gijselinck et al., 2012; Whitwell et al., 2012 Borroni & Padovani, 2013;).

1.6 Etiological mechanisms of FTLD

Since FTLD is a heterogeneous disease, with different genes associated to several variants, there is a wide range of possible mechanisms involved in its development. Studies performed by several research groups throughout the world have been making big investigation efforts around this theme, in order to better understand the basic principles of the disease, so that in future it will be possible to effectively arrest its progression, or even prevent its disclosure.

It is known that one of the major proteins involved in FTLD is tau, the microtubule-associated protein, encoded by *MAPT* gene, located at chromosome 17q21.32, and it has a mutation estimated frequency up to 50% (Poorkaj et al., 1998; Rabinovici & Miller, 2010; Halliday et al., 2012; Pan & Chen, 2013). Human tau is alternatively cleaved, giving rise to

six isoforms, three isoforms containing three amino-acids repeats (3R), and three isoforms with four repeats (4R). This sequence is encoded on exon 10 of *MAPT* gene, in a way that the inclusion of this exon leads to 4R tau isoforms, and its exclusion leads to 3R isoforms (Halliday et al., 2012; Pan & Chen, 2013). In normal brain, identical amounts of 4R and 3R tau are present, while pathological tau may be predominantly composed of 4R either or 3R. Tau protein in neurons binds to axonal microtubules, promotes microtubule assembly and stabilization, and is also involved in signal transduction. Many *MAPT* mutations alter the normal ratio (1:1) between 3R and 4R in favour of 4R, which binds poorly to microtubule and shows an increased affinity for self-aggregation. Thus, there are some hypotheses indicating that mutations in the *MAPT* gene may be involved in the neurodegeneration process (Rabinovici & Miller, 2010). The first hypothesis proposes that mutant protein may have a decreased binding affinity to microtubules, leading to impaired microtubules assembly, stability and ultimately to disruption of axonal transport. The impairment of normal tau function, with bundling into tangle or Pick body-type structures, is considered key to FTLD-tau pathology. The second hypothesis suggests that mutant tau may have an increased affinity to self-aggregate into filaments, insoluble inclusions that are neurotoxic. The increase in 4R tau transcripts are involved in neurite outgrowth and cell death, and decrease in transcripts levels are involved in neuronal survival. Changes in tau isoforms expression may therefore precipitate a toxic gain of function over time. At last, the third hypothesis proposes that these mechanisms are not mutually exclusive, and that often act synergistically (Rabinovici & Miller, 2010; Halliday et al., 2012).

The *PGRN* gene encodes progranulin (PGRN), a ubiquitously expressed growth factor precursor, located at chromosome 17q21.32. It has been associated to FTLD, with a mutation estimated frequency of 3-26%, among a significant number of familial FTLD cases (Rabinovici & Miller, 2010; Ward & Miller, 2011; Halliday et al., 2012; Pan & Chen, 2013). In the majority of patients, these are null mutations leading to nonsense-mediated decay of the mutant mRNAs, leading to PGRN haploinsufficiency via decreased PGRN expression, and reduction in PGRN protein levels (~30-50% of normal). The PGRN is a secreted glycoprotein that regulates several cellular functions including cell proliferation, cell migration and inflammation (Ward & Miller, 2011). Presumptive mechanisms by which loss of PGRN function may lead to neurodegeneration include chronic depletion of neurotrophic support and inadequate response to injury (Rabinovici & Miller, 2010). The

first PGRN receptor discovered was sortilin, a transmembrane receptor involved in the trafficking of hydrolases to the lysosome and in binding of neurotrophin (in the brain, sortilin is mainly expressed in neurons). Given the high affinity of PGRN for sortilin, it is tempting to hypothesise that PGRN mediates neuronal survival, through influencing proNGF-/sortilin-induced apoptosis (Hu et al., 2010; Ward & Miller, 2011). Dysregulation of the immune system is a common observation in many neurodegenerative diseases, and microglia are central mediators of the immune response in the central nervous system (CNS). Despite the fact that microglia express very low levels of sortilin they have a strong signalling response to PGRN, which might have indirect effects on neuronal survival via tumor necrosis factor receptor (TNFR), expressed by myeloid cells (Ward & Miller, 2011). Some data suggests that PGRN can directly regulate intracellular signalling by binding to TNFR and inhibiting TNFRα-mediated pathways. In patients with haploinsufficiency, it is possible that a mild illness (like a mild infection) initiate a positive feedback loop that leads to an abnormal proinflammatory state. Over the lifetime of a PGRN-deficient organism, the accumulation of systemic insults could ultimately lead to neurotoxicity and neurodegeneration (Ward & Miller, 2011). One of the pathogenic hallmarks of PGRN-deficient FTLD is exclusion of TDP-43 from the nucleus and the accumulation of this protein within the cytoplasm of affected neurons. The TDP-43 is the major component of the tau-negative and ubiquitin-positive inclusions and it is known to be involved in multiple cellular processes, including gene transcription, alternative splicing and stabilization of mRNA, microRNA biogenesis, apoptosis and cell division (Ward & Miller, 2011; Arai, 2014). In newly formed motor neurons, TDP-43 is dramatically upregulated and translocated from the nucleus to cytoplasm, then returns to its normal localization in the nucleus once axon repair has been completed, in these same neurons, PGRN expression decreases as TDP-43 redistributes to the cytoplasm. This suggests that PGRN may be involved in the regulation of TDP-43 localization during normal neuronal repair processes, and suggests also a possible mechanism for pathogenic TDP-43 accumulation in patients with FTLD secondary to chronically reduced expression of PGRN. However, the loss of PGRN may have neurotoxic effects independent of TDP-43. Recent studies propose that TDP-43 accumulation can occur in a significant fraction of patients with end-stage AD, suggesting that TDP-43 may not be absolutely essential for early stages of PGRN-deficient FTLD, and

perhaps abnormalities in TDP-43 function may be a more widespread phenomenon in a variety of end-stage type of dementia (Ward & Miller, 2011).

The *VCP* gene encodes a ubiquitously expressed member of a family of ATPases, the vasolin containing protein and it is associated with a wide range of cellular functions (Weihl et al., 2009). It is located at chromosome 3p11.2, and it has been associated with FTLD. Its mutation frequency is estimated as <1% (Pan & Chen, 2013). The *VCP* has been implicated in multiple cellular processes, as mitosis, cell death, organelle biogenesis, membrane fusion and protein degradation, and may also be involved in protein inclusion formation. Histone deacetylase 6 (HDAC6) is an aggresome essential protein, responsible for the trafficking of misfolded proteins formed in the setting of UPS impairment to the aggresome where they are degraded via autophagy, and it binds to ubiquitinated proteins and VCP. Overexpression of HDAC6 decreases protein aggregate toxicity, enhances the autophagic degradation of protein aggregates, and rescue model organisms from neurodegeneration (Weihl et al., 2009). Additionally, neuronal and glial TDP-43 inclusions are found in familial cases associated to *VCP* gene mutations (Halliday et al., 2012).

The *TARDBP* is a gene located at chromosome 1p36.22, coding for the protein expression of TDP-43 is controlled by an auto-regulatory mechanism and both over- and under- expression result in impaired neuronal viability. Cellular stress causes redistribution of TDP-43 to the cytoplasm where its intrinsic self-aggregation property leads to inclusion body formation or trafficking stress granules. Aggregation is accompanied by several post-translational modifications including phosphorylation, ubiquitination and cleavage. These inclusion bodies may act as TDP-43 barriers and hinder translocation to the nucleus, where TDP normally regulates mRNA. Alternatively, these inclusion bodies may have the effect of dehydrate soluble TDP-43, resulting in an overall depletion of nuclear and usable TDP-43, causing an increase in cellular stress and resulting in neurodegeneration (Halliday et al., 2012).

The *C9orf72* encodes twelve exons and is located at chromosome 9p21.2 (Woollacott & Mead, 2014). It may undergo alternative splicing to three transcript variants, all expressed in the brain, but as two undergo identical translation, only two different protein isoforms are produced (Vatovec et al., 2014). A GGGGCC (G4C2) hexonucleotide repeat expansion is the most common pathogenic mutation of this locus in patients with familial FTLD, with an estimated frequency of 3-48% (Woollacott & Mead, 2014). The

function of the C9orf72 protein is unclear. However, recent studies suggest that it may be related to differential expression of normal and neoplasic (DENN)-like proteins, which are GDP/GTP exchanged factors that lead to activation of Rab-GTPases and maintenance of vesicular trafficking (Zhang et al., 2012; Levine et al., 2013; Woollacott & Mead, 2014). Despite the ATG start condon, the repeat expansion is translated in all reading frames into dipeptide repeat (DPR) proteins, which form insoluble ubiquitinated p62-positive aggregates that are more abundant in the cerebral cortex and cerebellum. Mutation carriers have a G4C2 hexonucleotide repeat expansion either in the first intron or in the promoter region, depending on the isoform of the C9orf72 transcript (May et al., 2014). The normal repeat region typically comprises a small number of G4C2 repeats, with less than 30 repeats. Nevertheless an intermediate range has been established, where the pathological number of repeats is not clearly established, ranging from 20-100 repeats (Woollacott & Mead, 2014). Nevertheless, unambiguously pathogenic C9orf72 expansions are typically found to have an expansion size from 500 to >5000 repeats in FTLD patients. Additionally, recent evidence suggests that the size of the expanded repeats varies between tissues of the same individual (Vatovec et al., 2014). Therefore, there are some major hypotheses of pathogenic mechanisms for the expanded repeat mutation. Recently, it has been demonstrated that *C9orf72* colocalizes with Rab proteins in neuronal cell lines, regulating autophagy and endosomal trafficking (Farg et al., 2014). The first hypothesis states that the disruption of such trafficking and defects in membrane-related were considered as one of the causes of FTLD, and were also linked to other neurodegenerative diseases (DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Farg et al., 2014; Vatovec et al., 2014). In addition, C9orf72 was also found to interact with hnRNP A1 and hnRNP A2/B1, proteins previously found to bind to the hexanucleotide repeat expansion in RNA. Overexpression of C9orf72 induced cellular stress, resulting in stress granule formation that were positive for hnRNP A1 and hnRNP A2/B1, possible indicating a role of C9orf72 in nuclear cytoplasmic-transport (Farg et al., 2014; Vatovec et al., 2014). The second hypothesis raised is related to repeat-associated RNA toxicity, a mechanism shown in other neurodegenerative repeat expansion disorders (Todd & Paulson, 2010; Nalavade et al., 2013; Vatovec et al., 2014). The RNA toxicity mechanism involves accumulation of RNA species that sequester and perturb the function of specific RNA binding proteins. The G4C2 repeat-induced nucleolar stress could be responsible, at least in part, for build-up of

absorptive transcripts in the cytoplasm and may represent a novel etiological mechanism in FTLD (Haeusler et al., 2014; Vatovec et al., 2014). Finally, the third hypothesis implies the repeat-associated non-ATG (RAN) translocation of pathogenic repeat from sense and antisense direction, resulting in dipeptide repeat proteins (DPRs), which accumulate as aggregates in the cytoplasm and it has been proposed as a contributing factor in FTLD (Zu et al., 2013; Gendron et al., 2014; Vatovec et al., 2014;). The RAN translocation is a non-ATG initiated form of translocation that takes place in repeat regions forming stable stem-loops or hairpins. It typically occurs across all three reading frames of a single strand and results in a long peptide consisting of single, or several, repeated amino-acid residues. When expanded G4C2 repeats occurs the translocation of the sense transcript in all three reading frames results in the dipeptide repeat protein, DPR (Haeusler et al., 2014; Vatovec et al., 2014). Besides to the common TDP-43 aggregates in FTLD, *C9orf72* repeat expantion mutation carriers have abundant-shaped TDP-43-negative neuronal cytoplasmic inclusions (NCI) than stain positive for markers of the ubiquitin proteasome system (UPS), such as p62 or ubiquitin proteins. However, the link between TDP-43 and DPR aggregates is unclear, questioning a major role of DPS in FTLD etiology (MacKenzie et al., 2013; Zu et al., 2013; Vatovec et al., 2014;).

The *FUS* gene provides instructions to produce the fused in sarcoma protein (FUS). It is located at chromosome 16p11.2, and, in about 5-10% of FTLD patients, abnormal accumulation of FUS in cytoplasmic inclusions are the defining hallmark cell lesion (Rabinovici & Miller, 2010; Halliday et al., 2012). The FUS is a ubiquitously expressed DNA/RNA binding protein that can bind to a large number of RNA targets. It belongs to the Fus – Ewing RNA-binding – TATA-binding protein-associated factor 2N (FET) protein family. It is mainly localized to the nucleus, but, under physiological it conditions continuously shuttles between the nuclear and cytoplasmic compartments. Example evidence suggested that it might be involved in several cellular processes, including cell proliferation, DNA repair, transcription regulation, and multiple levels of RNA and microRNA processing (Halliday et al., 2012; Deng et al., 2014). The protein FUS has already been associated with neurodegenerative diseases, and more relevant for this report, FUS aggregation has been observed in FTLD (Woulfe et al., 2010; Deng et al., 2014), and identified as the characteristic marker in tau-negative and/or TDP-43 negative FTLD, defined as FTLD-FUS (Neumann et al., 2009; MacKenzie et al., 2010; Deng et al., 2014). The FTLD-FUS includes three distinct

clinic-pathologies: atypical FTLD-U, with neuronal intranuclear inclusions, neuronal intermediate filament inclusion disease (NIFID), and basophilic inclusion body disease, BIBD (MacKenzie et al., 2010; Deng et al., 2014).

The FUS and TDP-43 proteins share many common features, most their involved in cellular processes and the pathology (Mackenzie et al., 2010; Deng et al., 2014). Further evidence supporting a role of FUS in neurodegeneration comes from other studies with two proteins of FET family, Ewing RNA-binding protein (EWS) and TATA-binding protein-associated factor 2N (TAF-15), also related to FTLD (Neumann et al., 2011; MacKenzie & Neumann, 2012; Deng et al., 2014). The proteins EWS and TAF-15 co-accumulate in FUS-positive cytoplasmic inclusions, and cells with inclusions showed a reduction in nuclear staining for all three FET proteins (Neumann et al., 2011; Deng et al., 2014). Although mutant forms of FUS protein are claimed to disrupt RNA and protein homeostasis, to have aggregation tendency and altered subcellular localization, the pathogenic mechanism by which mutant FUS causes neurodegeneration is still unclear. However, knockdown of *FUS* or its homologous animal model, has been shown to result in abnormal development, reduced viability, motor deficits and abnormal neuronal morphology, with some of these deficits being rescued by expression of *FUS* transgenes (Halliday et al., 2012; Deng et al., 2014). On the other hand, there are some evidence against a toxic-gain-of function mechanism, such as the absence of an abnormal molecular species in FTLD-FUS (Neumann et al., 2009: Halliday et al., 2012), the lack of neurodegeneration in some anatomical regions with abundant cytoplasmic FUS inclusions (MacKenzie et al., 2011; Halliday et al., 2012), and the absence of FUS aggregates in some models of toxicity (Halliday et al., 2012; Lanson & Pandey, 2012). It should be noted that all FET proteins, along with transportin-1, co-accumulate in the cellular inclusions of FTLD-FUS, indicating that dysregulations of several other RNA-binding proteins are also associated with FTLD (Neumann et al., 2011; Gao & Taylor, 2012; Halliday et al., 2012; Davidson et al., 2013). The majority of FUS mutations have been shown to disrupt a region characterized as non-classical nuclear localization sequence, leading to impaired transportin-mediated nuclear import of FUS, with redistribution of the protein to the cytoplasm (Ito et al., 2011; Halliday et al., 2012). However, the mechanisms bellow FUS are much unclear. In human pathology, inclusion bearing cells often retain their physiological nuclear FUS staining, arguing against a loss of nuclear function (Neumann et al., 2009; Neumann et al., 2009; Halliday et al., 2012).

Besides, the severity of phenotype correlates with the level of cytoplasmic FUS, supporting the idea of neurotoxic effect of cytoplasmic FUS. This might be interceded by abnormal interaction with cytoplasmic RNA targets or protein binding partners, resulting in disturbance of RNA metabolism, or by a gain of novel function of disease-associated FUS isoforms unrelated to its native function (Halliday et al., 2012). A combined effect, in which a loss of normal FUS function and a gain of toxic properties act together to develop neurodegeneration, should also be taken into account (Deng et al., 2014). Overall, contribution of *FUS* gene might be humble, considering that mutations in a critical domain of a gene potentially cause a monogenic form of disease, whereas nucleotide variants in a non-critical region might only increase susceptibility to the disorder (Deng et al., 2014).

The *CHMP2B* gene encodes a component of the heterometric endosomal sorting complexes required for transport III (ESCRT-III) complexe with functions in the endosomal-lysosomal and the autophagic protein degradation pathway (Tsang et al., 2006; Pan & Chen, 2013). It is located at chromosome 3p11.2, and it has been associated with familial FTLD with a mutation estimated frequency of <1% (Pan & Chen, 2013). The *CHMP2B* mutations were associated with enlarged vacuoles in cortical neurons in the frontal, temporal, parietal and occipital cortices, due to impaired endosome-lysosome fusion, and impaired autophagy. Ubiquitin-immunoreactive NCI do not stain for tau, TDP-43 or FUS antibodies, consistent with a pathological classification of FTLD-UPS (Holm et al., 2009; MacKenzie et al., 2011; Sieben et al., 2012). The gene is expressed in neurons of all major brain regions, and mutations affect the C-terminal end of protein, due the aberrant splicing (Sieben et al., 2012). Mutations have been described in patients with FTLD; however, their pathogenic nature remains unclear (Cruts et al., 2012; Sieben et al., 2012).

The figure I summarizes the molecular and genetic classification of FTLD.

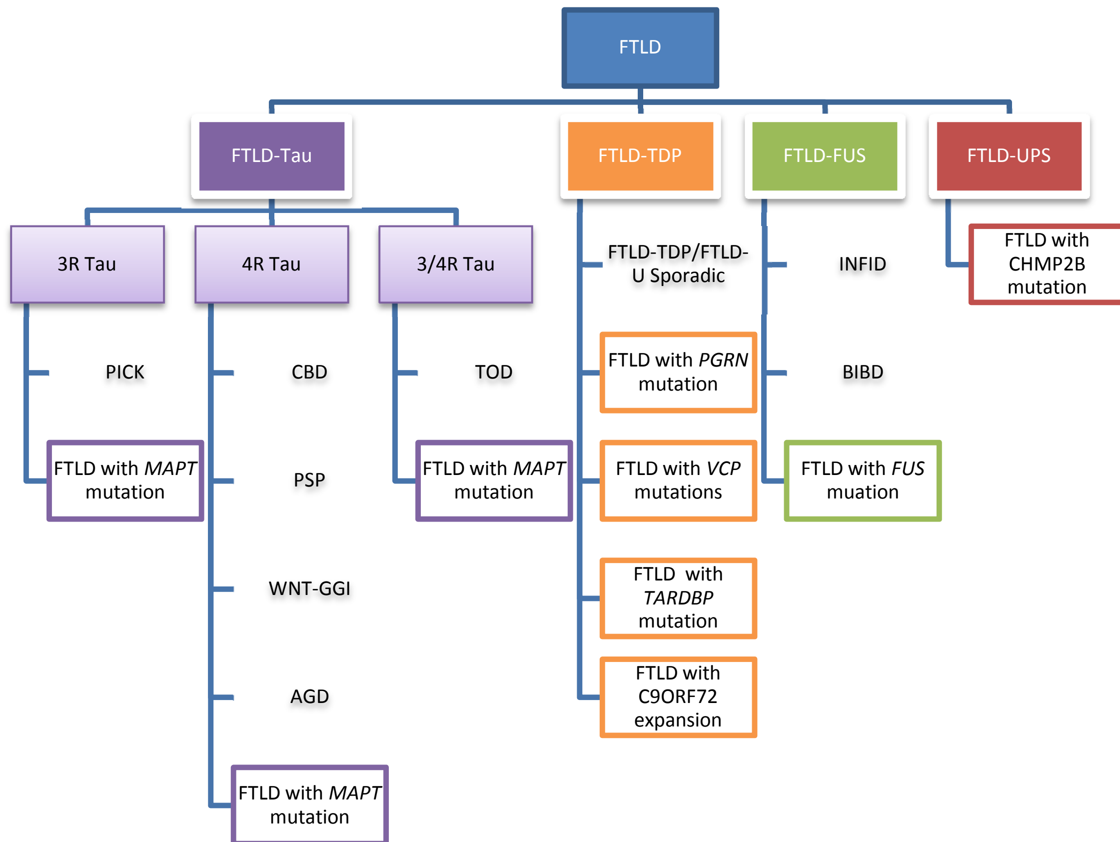

Figure I: Molecular and genetic classification of FTLD (adapted from Halliday et al., 2012)

1.7 Mitochondrial DNA and Neurodegeneration

Human mitochondrial genome, mtDNA (Figure II), contain multiple copies of a 16,568 pb, double-strained, circular DNA molecule – mtDNA which is maternally inherited (Anderson et al., 1981; Andrews et al., 1999), although in one case of paternal inheritance and recombination was reported (Taylor et al., 2003). The mtDNA codes for 13 polypeptides, all of which are components of the mitochondrial respiratory chain (MRC), 7 subunits (*ND1*, *2*, *3*, *4*, *4L*, *5* and *6*) of the 46 subunits composing complex I, one of the 11 subunits of complex III, 3 of the 13 subunits of complex IV, and 2 of the 17 subunits of complex V. It mtDNA also encodes the *12S* and *16S rRNA* genes and the 22 tRNA genes (Falkenberg et al., 2007), which are required for mitochondrial protein synthesis(Dhillon & Fenech, 2014).

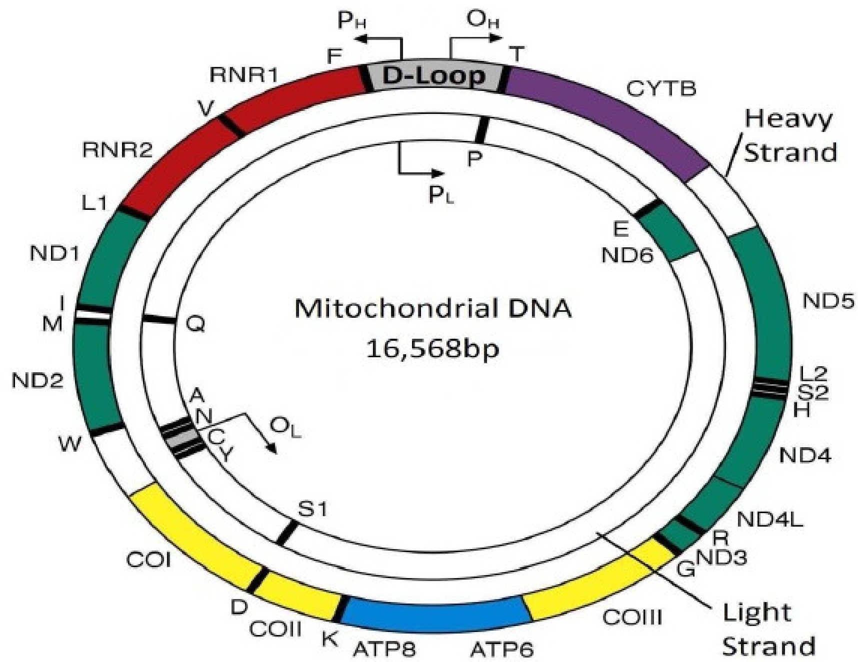

Figure II: Human mitochondrial DNA (adapted from Greaves et al., 2012). ND1 – NADH dehydrogenase, subunit 1; ND2 – NADH dehydrogenase, subunit 2; ND3 – NADH dehydrogenase, subunit 3; ND4 – NADH dehydrogenase, subunit 4; ND4L – NADH dehydrogenase, subunit 4L; ND5 – NADH dehydrogenase, subunit 5; ND6 – NADH dehydrogenase, subunit 6; CYTB – Cythocrome B; COI – cytochrome c oxidase I; COII – cytochrome c oxidase II; COIII – cytochrome c oxidase III; ATP6 – ATP synthase F0 subunit 6; ATP8 – ATP synthase F0 subunit 8; RNR1 – Mitochondrially encoded 12S RNA; RNR2 – Mitochondrially encoded 16S RNA; T – Mitochondrially encoded tRNA threonine; P - Mitochondrially encoded tRNA proline; E - Mitochondrially encoded tRNA glutamic acid; L2 - Mitochondrially encoded tRNA leucine 2; S2 - Mitochondrially encoded tRNA serine 2; H - Mitochondrially encoded tRNA histidine; R - Mitochondrially encoded tRNA arginine; G - Mitochondrially encoded tRNA glycine; K - Mitochondrially encoded tRNA lysine; D - Mitochondrially encoded tRNA aspartic acid; S1 - Mitochondrially encoded tRNA serine 1; Y - Mitochondrially encoded tRNA tyrosine; C - Mitochondrially encoded tRNA cysteine; N - Mitochondrially encoded tRNA asparagines; A - Mitochondrially encoded tRNA alanine; W - Mitochondrially encoded tRNA tryptophan; M - Mitochondrially encoded tRNA methionine: I - Mitochondrially encoded tRNA isoleucine; Q - Mitochondrially encoded tRNA glutamine; L1 - Mitochondrially encoded tRNA leucine 1; V - Mitochondrially encoded tRNA valine; F - Mitochondrially encoded tRNA phenylalanine.

The mtDNA has its own genetic code, it is semiautonomous, depending also on the replication and transcription factors of nuclear origin, being the essential regulatory sequences located at the D-loop (non-coding region of the mtDNA). Replication is bidirectional, stands from the origin of replication of heavy strand (HS), located on the D-loop going clockwise, to allow the synthesis of the HS, followed by the light strand (LS) replication. The mtDNA transcription is asymmetrical, as the two chain promoters, heavy strand promoter (PH) and light strand promoter (PL) are both located on the D-loop and the two components work in opposite directions, and HS is transcribed to clockwise direction (Grazina, 2004).

As stated above, mtDNA is maternally inherited, and do not undergo recombination. Thereby the only way that mtDNA sequence may change is through the accumulation of sequence variations along the maternal lineage. The high mutation rate of

mtDNA results from the lack of protective histones, and limited mtDNA repair systems (Wallace, 1994; Grazina, 2004). However, studies have revealed that mitochondria has several mechanisms for genetic maintenance, including a robust base excision repair (BER) mechanism that uses both mitochondrial proteins and nuclear proteins that translocate into mitochondria. Also mitochondrial fusion and fission events and mtDNA degradation may allow the organelles to decrease toxic effects of damage (Cline, 2012).

The major mitochondrial event is the production of adenosine-5'-triphosphate (ATP), the primary source of high-energy compounds in the cell (Morán et al., 2012). Mitochondrial ATP is generated via oxidative phosphorylation (OXPHOS), that occurs in MRC, located at the inner mitochondrial membrane, and comprises the five multiprotein complexes plus two transporters (Grazina, 2004). Besides ATP synthesis, there is also the reactive oxygen species (ROS) production and mtDNA is located close to the main source of ROS formation, becoming very susceptible to damage (Reddy & Reddy, 2011). The correct biosynthesis of the OXPHOS complexes is a highly intricate regulated process, that requires the combined action of the two genomes (Morán et al., 2012).

Most of human cells contain hundreds of mitochondria and thousands of mtDNA copies. When there is a mtDNA mutation, a mixture of wild type and mutant molecules may coexist, and this situation is called heteroplasmy; in turn, the presence of pure mutant or wild type molecules is called homoplasmy.

In case of heteroplasmy, as the percentage of mutant molecules increase, OXPHOS enzyme activity decrease. When the energy threshold is reached, the probability of disease manifestation increases (Wallace, 1994; Grazina, 2004). The percentage of mutated DNA may vary in different patients, organs and even between cells of the same tissue (Samuels et al., 2006).

Although most mitochondrial proteins are encoded by nuclear DNA, mtDNA defects can cause numerous diseases, many of which are associated with neuronal degeneration (Beal, 2005). The nuclear genes coding for maintenance and expression of mtDNA; mitochondrial protein synthesis import, and proteolysis; mitochondrial fusion, fission and stability; iron-sulfur cluster synthesis; key metabolic pathways like kress cycle or fatty acid oxidation, and cell survival control (apoptosis), are essencial and may influence diseases involving mitochondrial dysfunction. The nuclear DNA has a direct influence in mtDNA, as mutations in nuclear genes responsible for mtDNA maintenance may be responsible for

mtDNA deletions or depletion (Finsterer, 2009). The majority of large-scale deletions seem to occur spontaneously (Schon et al., 1989), whereas multiple mtDNA deletions may also be inherited either as an autosomal dominant or autosomal recessive traits (Zeviani et al., 1990; Nishino et al., 2000), influenced by mutations affecting nuclear genes, such as *POLG*..

Neural tissue accounts for a large share of the body's total oxygen metabolism at rest, rendering neurons particularly susceptible to ROS production. With an increasing amount of mutant mtDNA in these tissues, the mitochondrial energy output could drop below a critical threshold required for the normal cell function and resulting in the appearance of various specific pathological clinical symptoms (Wallace, 1992). Dysfunction of mitochondrial energy metabolism leads to reduced ATP production, impaired calcium buffering and generation of ROS, beingits role increasingly recognized in both aging (Beal, 2005; Wallace, 2005; Reddy, 2007) and neurodegenerative diseases (Wallace, 1999; Hirai et al., 2001; Swerdlow & Khan, 2004; Wallace, 2005; Reddy & Beal, 2005; Beal, 2005; Martin & Martin, 2006; Abeliovich & Beal, 2006; Swerdlow, 2007; Kim et al., 2007; Trushina & McMurray, 2007; Fukui & Moraes, 2007).

Many novel mtDNA variations have been associated to age-related diseases, such as cancer and neurodegenerative diseases (Taylor & Turnbull, 2005), although the exact link between aging and neurodegenerative diseases remains unclear (Reddy, 2008; Lapointe & Hekimi, 2010). Electrons pass along the MRC complexes and create an electrochemical gradient by executing the ejection of protons from the matrix across the inner mitochondrial membrane complexes. ATP is produced by the dissipation of this proton gradient through ATP synthase (complex V). Therefore, age-related neurodegenerative diseases may be the result of abnormalities in mitochondrial function and/or structure (Reddy, 2007; Dhillon & Fenech, 2014). A long-standing hypothesis postulates that mutations in the mitochondrial genome limit mammalian lifespan and accelerate ageing (Linnane et al., 1989; Reeve et al., 2008). Therefore, several studies using genetically engineered animals, reinforced a major theory of aging, which postulates that mutations in mtDNA and oxidative damage may contribute to the aging process and neurodegenerative diseases (Beal, 2005; Vinogradov & Grivennikova, 2005; Reddy, 2006; Federico et al., 2012).

It is clear that the transport of mitochondria is accomplished through axons and dendrites, to satisfy cell energy demands (Reddy & Beal, 2008). If the mitochondria in the

cell are damaged or degraded, those will be transported to the synaptic terminal, being responsible for reduction in the production of ATP. Since this is a region that requires high levels of energy, complications may arise from this situation (Reddy, 2008; Dhillon & Fenech, 2014). Patients harbouring mtDNA mutations are often severely neurological impaired due to neurodegeneration in the central nervous system (Dhillon & Fenech, 2014).

The half-time of neuronal mitochondria is around one month (Reddy, 2007), occurring a constant replacement in all cell types, including neuronal cells. This process is called mitochondrial recycling and ensures mitochondrial function in neurons (D. T. W. Chang & Reynolds, 2006). Recent evidence suggests that the mitochondrial recycling occurs by two processes, fusion and fission (Dupuis, 2014). The fusion process is the integration of two mitochondria, whereas the fission is the fragmentation of one mitochondrion (Reddy, 2008). Given these processes, the formation of mitochondria can take place through pre-existing mitochondria by the fission process (Chang & Reynolds, 2006; Reddy, 2007). Fusion provides a defense mechanism against harmful mutations in mtDNA, by functional complementation of mtDNA gene products. Cells that showed a deficiency on fusion presented a decline on mitochondrial function (Chan, 2006). Since the number of normal functional mitochondria at the synaptic terminal is lower, it is possible that in this case the fusion process exhibits a lower rate. The absence of this process may lead to an increase of ROS and decline of ATP levels, and may be the causative reason for decreased neurotransmission, particularly in a disease state (Reddy, 2008). On the other hand, mitochondrial fission consists in the fragmentation of mitochondria and it plays an important role in apoptosis (Bossy-Wetzel et al., 2003). In short, mitochondrial fission is activated by Drp1 and Fis1 molecules, which in turn, are activated by increased of ROS levels. Decreased levels of Drp1 and Fis1 reduce apoptosis. After induction of apoptosis, mitochondrial fusion is reduced, and the over-expression of mitofusins can reduce apoptosis (Reddy, 2008). Evidence suggests that increased mitochondrial ROS is responsible for changes in mitochondrial morphology (Barsoum et al., 2006; Yoon et al., 2006; Kim et al., 2007). Accordingly, it is possible that in late-onset neurodegenerative diseases, the production of ROS cause mitochondrial fragmentation, which may lead to mitochondrial dysfunction and, consequently, to neuronal cell death (Reddy, 2008).

Due to the fact that mtDNA is highly polymorphic, mitochondria within cells may vary from each other with respect to their mtDNA content and heteroplasmy status. This may be more prevalent in non-dividing tissues, because selection process against cells with defective mitochondrial genomes is likely to be less effective than in rapidly proliferating tissues. However, significant differences in biochemical phenotype and pathological consequence is not observed until the number of mutated mtDNA molecules reaches a critical threshold level of bioenergetics impairment which varies depending on the type of accumulated mutations and ATP requirement of the tissue (Taylor & Turnbull, 2005). Researchers found that several tissues from aged individuals have lower mitochondrial function than those from younger individuals (Cooper et al., 1992).

The mechanism by which mitochondrial dysfunction results in cognitive decline or dementia remains unclear. A possible mechanism involves the depletion of energy that causes dysfunction of cortical neurons. This event leads to increased oxidative stress and impairs functions of cortical neurons and glia cells. The mitochondrial defects result in apoptosis of cortical neurons, or focal paroxysmal discharges inhibiting physiological membrane functions (Finsterer, 2009).

Studies on AD suggested an association of the disease with mitochondrial dysfunction and mtDNA alterations, although the exact mechanisms are still being debated (Grazina et al., 2006; Swerdlow et al., 2014). In 2001, Hirai et al. demonstrated that mitochondria degeneration occurs early in the course of the disease (Hirai et al., 2001). Moreover, mitochondrial energy metabolism key enzymes, specially cytochrome c oxidase (Castellani et al., 2002), have been shown to be severely affected in AD (Blass, 2001; Eckert et al., 2003). It has also been reported that genes coding for subunits of the OXPHOS system are differentially expressed in patients with AD, suggesting that mtDNA defects may be responsible for the heterogeneity of the phenotype observed in AD patients (Manczak et al., 2004). Apolipoprotein E polymorphism ε4 (APOE4) is the strongest known genetic risk factor for AD (Mahley et al., 2006). More recently, the accumulation of APOE fragments in mitochondria were reported affecting its function (S. Chang et al., 2005; Mahley et al., 2006; Nakamura et al., 2009). However, in 2010, Lakatos et al. developed a study in which the results indicated that the mitochondrial haplogroup UK may confer genetic susceptibility to AD independently of the APOE4 allele (Lakatos et al., 2010).

Also, the mitochondrial dysfunction has been suggested to be involved in PD, arising from mtDNA variations or acquired mutations in PD pathogenesis. One possible hypothesis of the relationship of mtDNA mutations with observed features of PD, addresses the accumulation of somatic mtDNA mutations throughout the body that gives rise to a progressive decline of mitochondrial function (Andalib et al., 2014).

However, although the role of mtDNA damage has been conclusively connected to neuronal impairment in mitochondrial diseases, its role in age-related neurodegenerative diseases remains speculative (Pinto & Moraes, 2014).

1.8 Mitochondrial DNA and FTLD

Mitochondrial dysfunction due to mtDNA alterations has been implicated in the neuronal death in neurodegenerative disorders (Cortopassi & Arnheim, 1990; Corral-Debrinski et al., 1992). In 2004, Mawrin et al. using real-time PCR, quantified the 5 kB common mtDNA deletion (CD) in several brain regions from neurodegenerative disorders patients and controls, in frozen autopsy tissue (Mawrin et al., 2004). They confirmed that CD levels increase with age, reaching highest levels in the basal ganglia. High CD levels were also found in affected regions in patients with FTLD, Parkinson's disease (PD) and dementia with Lewy bodies, but not in AD, suggesting that in neurodegenerative disorders mtDNA damage may occur in a region-specific distribution.

In 2004, Grazina et al. identified a FTLD patient (54-year-old woman) with two mtDNA transitions, one already known (m.3316G>A) and another unreported (m.3337G>A) (Grazina et al., 2004). It was verified that MRC complex I activity was reduced in leukocytes of this patient (36% of the control mean activity). The first alteration (m.3316G>A) promotes an alteration of a nonpolar alanine to a polar threonine in an essentially hydrophobic peptide, a substitution described in diabetes mellitus patients (Odawara et al., 1996; Matsuura et al., 1999; Fukuda et al., 2000), however, the FTLD patient in study did not present any clinical evidence of diabetes mellitus. This may be consistent with the other variation described. The second alteration (m.3337G>A) promotes the change of a valine to a methionine in the NADH Dehydrogenase subunit 1 (ND1) peptide. This two alterations in the same region of the *MTND1* gene, suggested a possible involvement of mitochondrial ND1 in FTLD etiopathogenesis. Both alterations combined may have a

functional meaning causing the reduction of MRC complex activity and contributing to the pathogenesis. The same group reported three mtDNA variants in two AD patients, one with a change at position m.3199T>C of the *16S rRNA* mtDNA gene and other with two changes at positions m.3197T>C of the *16S rRNA* gene and m.3338T>C of the *MTND1* gene. Combining all the data, mutations in the 3197 – 3338 may decrease the age of onset neurodegenerative disorders (Grazina et al., 2005). The results suggested that mutation in mtDNA *ND1* nucleotides 3337 – 3340 do not account for a primary risk for developing FTLD. However, it is not excluded that other sequence regions of *ND1* or other mtDNA genes could be involved in the pathogenesis or onset of FTLD.

A study aiming to test the hypothesis that the major European mtDNA haplogroups play a role in the onset of FTLD(Rose et al., 2008) did not find significant differences between patients with sporadic FTLD and controls or between patients with familial FTLD and controls either in the frequency distribution of haplogroups or sub-haplogroups. However, these results may be explained by two hypotheses. Firstly, major European mtDNA haplogroups do not affect susceptibility to FTLD, at least in the studied population. Secondly, the sample size had no sufficient statistical power to reveal associations, either because the influence of mtDNA haplogroups on FTLD is quite low or because only some rare mtDNA haplotypes contribute to FTLD (Samuels et al., 2006). In any case, the results presented may provide a reliable starting point for further investigations on the relationships between FTLD and mitochondrial genome. Mitochondrial dysfunction leads to reduced ATP production, increased generation of ROS and also impaired calcium buffering (Beal, 2005). The brain is especially vulnerable to oxidative damage and mutations in mtDNA or in genes involved in mtDNA maintenance are associated with variable spectrum of mitochondrial disorders, affecting the brain (Spinazzola & Zeviani, 2005; Copeland, 2008). Genes like *POLG1*, encoding the catalytic subunit of DNA polymerase ɣ, *TWIKLE*, coding for the mitochondrial helicase Twinkle, and *ANT1*, encoding the adenine nucleotide translocator, are important for mtDNA replication. Taking into account this information, and the report on FTD and mtDNA haplogroup by Rose et al. in 2008, in 2010, Krüger et al., reported the role of mtDNA and its maintenance enzymes in AD and FTLD Finnish patients. Patients and controls were collected from a defined region of Finland, the Northern Ostrobothnia, for the determination of mtDNA haplogroups and

the analysis of two common mtDNA mutations (m.3243A>G, m.8344A>G) in the patients. Since encephalopathy and cognitive decline are common features in mitochondrial diseases, it was performed the screening of five common *POLG1* mutations (T251I, A467T, P587L, W748S and Y955C) and all the coding exons of the *PEO1* and *ANT1* genes were screened for mutations. This study showed a significant association between haplogroups IWX and FTLD. Although the frequency of haplogroup W was not significantly higher in FTLD patients compared to controls, it is quite interesting that its frequency was higher in FTLD patients compared to the controls and AD patients. Interestingly, the non-synonymous/synonymous rate in the mtDNA encoded complex I genes (*MTND*) is higher within haplogroup cluster IWX than in the remaining European haplogoups (Autere et al., 2004) thus suggesting that the relative excess of non-synonymous mutations in cluster IWX may have a role in the risk of developing FTLD. Another possible explanation for the association observed between cluster IWX and FTLD may be a recent founder effect. In this investigation, the two common mtDNA mutations (m.3243A>G, m.8344A>G), and five *POLG1* mutations, were not found, neither any pathogenic mutations in the *PEO1* or *ANT1* genes. However, the presence of other mitochondrial mutations cannot be excluded as the whole mitochondrial genome was not screened (Krüger et al., 2010).

Gaspar and colleagues developed a study in which the main objective was to sequence the 7 mitochondrial encoded complex I genes (*MT-ND*), investigate MRC complexe I activity and correlating these results with FTLD features, in a sample of 70 Caucasian patients. In a total of 161 different variations, 5 were found to be possible pathogenic (m.12634A>G and m.13630A>G) or probably pathogenic (m.4172T>A; m.11087T>C and m.14000T>A). Although no disease has been associated to these variations, they may contribute to the pathogenic events occurring in FTLD. The complex I activity was significantly decreased, and severely decreased in 7% of patients, suggesting that complex I deficiency may be involved in FTLD pathogenesis of the disease, although it was not possible to establish a straightly forward genotype-phenotype correlation (Gaspar et al., 2015).

1.9 Mitochondrial DNA – *MT-RNR1* and *MT-RNR2* genes

Mitochondrial ribosomes, compared to cytoplasmic ribosomes of nuclear origin, present some differences, in particular related to RNA and protein composition, size, and antibiotic sensitivity (Scheffler, 1999). However, all the machinery of protein synthesis is similar.

The small subunit of the mitochondrial ribosome has a capacity of sedimentation of 12S and is responsible for mediating the interaction between the mRNA codon and anticodon of the tRNA. It is encoded by *MT-RNR1* gene, with 954pb, from nucleotide 648 to nucleotide 1601 of mtDNA. The large subunit presents a capacity of sedimentation 16S, responsible for the catalysis of peptide bond formation. This is encoded by the *MT-RNR2* gene having 1557pb, from nucleotide 1,671 to 3,228 of mtDNA (Petsko and Ringe, 2008).

The *MT-RNR2* does not only encode for the ribosomal large subunit, but also a functional peptide, Humanin (HN). This protein is a 24-amino acid peptide encoded by the mitochondrial 16S rRNA gene, was discovered by screening a cDNA library from the occipital cortex of a patient with AD (Maximov et al., 2013). It is a suppressor of neuronal cell death induced by genetic variations, causing early-onset AD (Maximov et al., 2002). The HN peptide and its derivatives improve memory and learning in transgenic mouse models of AD, and restore it in rodents treated with amyloid-β-peptides, scopolamine and 3'-quinuclidinyl benzilate (Maximov et al., 2013).

The mitochondrial oxidative phosphorylation system is composed by multimeric enzymes. It has been established that key subunits of these enzymes, translated to mitochondrial ribosomes, are the players of assembly-dependent translational regulation. Translational control plays a primordial role in the regulation of gene expression, allowing the coordinated accumulation of proteins that have to be coregulated, such as those that are subunits of a multimeric enzyme. Moreover, protein synthesis consumes a large proportion of the cellular energetic resources and therefore needs to be precisely controlled. Among the regulatory strategies, translational control represents a solution to the problem (Fontanesi, 2013). During the course of evolution, most of the mitochondrial protein-coding genes have been transferred to the nuclear genome However the mitochondrial genome encodes only 13 proteins in humans. The presence of a protein-coding genome, although small, requires the preservation of a functional translation

apparatus in mitochondria. The products of mitochondrial translation must be produced in the correct stoichiometry in order to ensure the correct assembly of the MRC components (Kuzmenko et al., 2014). Therefore, the study of the genes encoding for the mitochondrial ribosomes subunits becomes extremely important and relevant, as it may be the cause of abnormalities in their assembly and posterior translational errors.

2. Fundamentals of methods

2.1 Polymerase chain reaction

The amplification of the 2 mitochondrial rRNA encoding genes was performed by *Polymerase Chain Reaction* (PCR).

PCR technique was initially described by Kary Mullis in 1985, contributing to advances in cellular and molecular Biology, allowing the analysis of nucleic acids (DNA and RNA) from any source. As a reaction highly specific and sensitive, it allows *in vitro* production of multiple copies, very rapid and selective, of a particular DNA fragment (Markham, 1993).

2.2 Agarose gel electrophoresis

PCR procedure was followed by agarose gel electrophoresis for the separation of DNA fragments, in order to verify the success of amplification.

This technique is advantageous and effective to separate and identify DNA fragments. The agarose gel is an easy and economical way of separation, which allows separating DNA molecules based on their size, under an electric field, since the DNA molecules are negatively charged at neutral pH, they will migrate to the positive pole (Videira, 2001).

2.3 ExoSAP-IT® purification

After the amplification of the samples, PCR products mixture may further contain dNTP's and *primers* that were not incorporated.

Samples were purified with ExoSAP-IT®, consisting of exonuclease I (exo I), responsible for the degradation of residual single stranded *primers* and DNA fragments, and shrimp alkaline phosphatase (SAP) that promotes hydrolysis of dNTP's, which are the main factors interfering with PCR sequencing (Werle et al., 1994).

2.4 Sequencing PCR

The most common procedure for DNA sequencing is the controlled interruption of the enzymatic replication, method developed by Fred Sanger (Viljoen and Nel, 2005).

The sequencing PCR involves the synthesis of single stranded DNA using the DNA previously amplified in PCR template. Synthesized chains are terminated prematurely with various possible sizes. The synthesis begins at the *primer* binding site and ending with the incorporation of a terminator nucleotide that lack the hydroxyl group at the 3' position of the deoxyribose, preventing the establishment of the phospho-diester bonds and avoiding the incorporation of new nucleotides to the DNA strand. When a terminator nucleotide is incorporated, the synthesis of new chain ends (Buitrago and Jimenez, 2001).

2.5 Sephadex® purification

Purification using Sephadex® is a technique extremely advantageous since it is simple and fast, allowing to obtain high levels of purity, as it completely removes the ddNTP's, dNTP's and *primers*.

2.6 Automatic sequencing

Automated sequencing is based on a capillary electrophoresis at which the samples are submitted, after purification of the sequencing reaction products. Applied Biosystems DNA sequencers detect fluorescence from four different dyes that are used to identify the A, C, G and T terminators. Fluorescence is identified when ddNTP's pass through a laser beam that excites the fluorophores and this information is sent to a computer and converted to an electropherogram. Each dye has a fluorescence wavelength of emission,

when excited by argon ion laser, allowing detection and distinction of all four bases (Healthcare, 2009).

This method has a high resolution, reproducibility, being a very reliable and widely used method.

Analysis of Mitochondrial Genome in Frontotemporal Lobar Degeneration: Contribution of RNRs and Correlation with the Biochemical Phenotype

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Abbreviations

AD	Alzheimer's disease
avPPA	Agramatic variant of PPA
bvFTD	Behavioural variant of FTD
CBS	Corticosal syndrome
DEAF	Maternally inherited DEAF-ness or aminoglycoside-induced DEAFness
FTD	Frontotemporal dementia
FTLD	Frontotemporal lobar degeneration
FTD-MND	FTD with motor neuron disease
FTLD-FUS	FTLD with inclusions of the fused in sarcoma protein
FTLD-ni	FTLD without inclusions
FTLD-tau	FTLD with tau pathology
FTLD-TDP	FTLD with TAR-DNA binding protein (TDP-43)
FTLD-UPS	FTLD with ubiquitin-positive and TDP-43-negative histopathology
MCR	Mitochondrial Chain Respiratory
OXPHOS	Oxidative Phosphorilation
LHON	Leber Hereditary Optic Neuropathy
mtDNA	Mitochondrial DNA
MELAS	Lactic Acidosis and Stroke-like episodes
PLA	Lopogenic progressive aphasia
PNFA	Progressive nonfluent aphasia
PPA	Primary progressive aphasia
PPS	Progressive supranuclear palsy
ROS	Reactive Oxygen Species
SD	Semantic dementia
svPPA	Semantic variation of PPA

Abstract

The Frontotemporal lobar degeneration (FTLD) is a heterogeneous neurodegenerative dementia; It is the second most common type of presenile dementia and the among senile dementias. Due to the selective involvement of the frontal and temporal lobes, it is characterized by progressive changes in behaviour, executive dysfunction and/ or language difficulties. Clinical and neurological overlaps between FTLD and other diseases, such as AD, have been described. This led to the development of many studies on possible pathophysiological similarities, as the involvement of mitochondrial DNA (mtDNA).

DNA samples of 100 patients with probable diagnosis of FTLD according to the standard criteria of DSM-IV, followed at Neurology Unit of the Centro Hospitalar e Universitário de Coimbra, 52 females and 49 males (age range of patients is between 38-84 years; mean: 64 ± 10), were investigated. Total DNA was extracted from peripheral blood and the 2 *MT-RNR* mitochondrial genes were sequenced. A total of 45 variations were identified in 87 patients. From these, 5 variations were considered to be probably pathogenic, according to *in silico* analysis: m.1243T>C, due to the change of minimum free energy and its structure, high percentage of evolutionary conservation, and the location in a stem region; m.1971A>G due to the facts of being heteroplasmic and 100% conserved; the m.2098G>A variation due to its high change of minimum free energy, highly conservation and location in a stem region; m.2363A>G, for inducing binding minimum free energy and structure changes, high evolutionary conservation and location in a stem region; and m.3036G>A, due to heteroplasmy, change of both minimum free energy and structure, highly conservation and location in a stem region.

Additional studies are needed to better understand the relationship between mtDNA variations found and FTLD. However, this is an original study, being the first to investigate the sequence of *MT-RNR* genes in FTLD patients.

Keywords: mitochondrial DNA; FTLD; *MT-RNR* genes; sequence variations.

Introduction

The term FTLD is the umbrella for referring a heterogeneous group of neurodegenerative diseases, including clinical, neuropathological and genetic features. It is the second most common type of presenile dementia and the forth of senile dementia. Due to the selective involvement of the frontal and temporal lobes, it is characterized by progressive alterations in behaviour, executive dysfunction and/or language impairment (Josephs et al., 2011; Pan & Chen, 2013; Seltman & Matthews, 2012). The FTLD onset occurs before the age of 65 years, in 75-80% of the patients, being considered a presenile dementia (Sieben et al., 2012), and it has an equal distribution among female and male (Galimberti & Scarpini, 2010). The disease has a familial component: 30-50% of the FTLD patients report at least one relative with similar symptomatology, and in 10-23% of cases the disease segregates in the family with an autosomal dominant inheritance pattern (Goldman et al., 2005; Goldman et al., 2008). The estimated prevalence is 54 per 100,000 inhabitants over 75 years-old, 78 per 100,000 inhabitants aged 66 – 75 (Borroni et al., 2010), and 15 – 22 per 100,000 inhabitants aged 45 – 64 years (Borroni et al., 2010; Ratnavalli et al., 2002; Seelaar et al., 2011), which is almost half of the prevalence of AD in this age group (Seelaar et al., 2011).

The clinical spectrum of FTLD has been recently enlarged and distinct syndromes have been established, taking into account the several discoveries made and giving a special attention to the clinical characteristics: the behavioural variant of FTD (bvFTD), and the language difficulties, primary progressive aphasia (PPA), which stills divided into two sub-groups, the agrammatic variant (avPPA) and the semantic variant of PPA (svPPA) (Borroni & Padovani, 2013). Recently redefining clinical criteria recognize several other phenotypes. Diseases like FTD with motor neuron disease (FTD-MND), corticobasal syndrome (CBS) and progressive supranuclear palsy (PSP) are now considered as part of the FTLD spectrum (Borroni & Padovani, 2013; Harro Seelaar et al., 2011).

Neuropathology features of FTLD have been classified according to different inclusion proteins found in degenerating neurons (Borroni & Padovani, 2013). Currently, five subtypes are accepted: FTLD-tau (tau pathology), with neuron and glial cells containing

inclusions of hyperphosphorylated tau protein; FTLD-TDP, in which patients present tau-negative ubiquitin staining inclusions composed of transactive response (TAR) DNA-binding protein 43 (TDP-43) (Lipton et al., 2004; Neumann et al., 2006; Cairns et al., 2007; MacKenzie et al., 2010; Sieben et al., 2012); FTLD-UPS, with ubiquitin-positive and TDP-43-negative histopathology, FTLD-FUS, presenting inclusions of the fused-in-sarcoma protein (MacKenzie et al., 2010; Sieben et al., 2012); and FTLD-ni, without any inclusions identified (MacKenzie et al., 2010). One of the most intriguing issues in the FTLD field is the poor correspondence between neuropathological features and clinical phenotypes. Indeed, neuropathological characteristics are predictable only in cases with known genetic defects (Rohrer et al., 2011;Borroni & Padovani, 2013). A correlation between neuropathological characteristics and genetic mutations has been found (MacKenzie et al., 2010). About 40% of FTLD cases are FTLD-tau, including all cases with *MAPT* gene mutation. In FTLD-TDP cases, patients have mutations in genes *PGRN*, *TARDBP*, *VCP* and *C9ORF72.* Some cases of FTLD-UPS presented mutations in the charged *CHMP2B* gene*.* Additionally, although the majority of cases did not have mutations in *FUS* gene, there is a correlation between FTLD-FUS and mutations in *FUS* gene (Goldman et al., 2011).

Genetic heterogeneity of FTLD is observed by the identification of mutations in several nuclear genes. The most prevalent mutations occur in the microtubule associated tau gene (*MAPT*) and progranulin gene (*PGRN*), representing nearly 50% of the familial cases. While mutations in the nuclear genes, vasolin containing protein (*VCP*), charged multivesicular body protein 2B (*CHMP2B*), TAR-DNA binding protein (*TARDP*) and fused-in-sarcoma (*FUS*) are found in less than 5% (Seelaar et al., 2011; Sieben et al., 2012). More recently, mutations in chromosome 9 open reading frame 72 (*C9orf72*) have been reported as responsible for FTLD, but the causative gene defect has yet to be discovered (Seelaar et al., 2011). More recently, mutations in the *CHCHD10* gene, responsible for encoding a mitochondrial protein that is located at the enriched junctions in the intermembrane space, have been identified in a large family with FTLD/ALS (Chaussenot et al., 2014; Johnson et al., 2014). It may play a role in cristae morphology maintenance or in oxidative phosphorylation (Martherus et al., 2010; Bannwarth et al., 2014).

Clinical and neurological overlaps between FTLD and other diseases, such as AD, have been described. This led to the development of many studies on possible pathophysiological similarities, as the involvement of mitochondrial DNA (mtDNA) (Grazina

et al., 2004; Grazina et al., 2006). For this reason, it is important to study the role of mtDNA in FTLD. Polymorphisms and specific haplogroups in mtDNA have also been associated to neurodegenerative diseases (Grazina et al., 2006; Gaspar et al., 2015;). Mitochondrial changes, including mitochondrial respiratory chain (MRC) dysfunction, are related to increased reactive oxygen species (ROS) production and mtDNA somatic mutations that accumulate throughout the years, leading to energy insufficiency, signalling defects, apoptosis and replicative senescence, culminates in loss of cell function (Taylor & Turnbull, 2005; Gaspar et al., 2015;). Human mitochondrial genome contain multiple copies of a 16,568 pb, double-strained, circular DNA molecule, codes for 13 polypeptides, essential components of the MRC (Reddy, 2008). The mtDNA also encodes the *12S* and *16S rRNA* genes and the 22 tRNA genes (Falkenberg et al., 2007).

The prsent work includes the study of the 2 *MT-RNRs* genes coded in mtDNA. The small subunit of the mitochondrial ribosome has a capacity of sedimentation of 12S and is responsible for mediating the interaction between the mRNA codon and anticodon of the tRNA, encoded by *MT-RNR1* gene, with 954pb, from nucleotide 648 to nucleotide 1,601 of mtDNA. The large subunit presents a capacity of sedimentation 16S, and is responsible for the catalysis of peptide bond formation. This is encoded by the *MT-RNR2* gene having 1,557pb, from nucleotide 1,671 to 3,228 of mtDNA (Gregory and Dagmar, 2008). Due to the central role in translation, the malfunction of mutant rRNAs could be important in the pathologies occurring in mitochondria. Disorders related to mt-rRNAs have been associated with several clinical phenotypes, including maternally inherited DEAFness or aminoglycoside-induced DEAFness (DEAF), Leber Hereditary Optic Neuropathy (LHON), Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS), and others (http://www.mitomap.org/MITOMAP). Changes in mitochondrial genes encoding rRNAs have also been described in neurodegenerative diseases such as AD and PD (Shoffner et al., 1993; Tanaka et al., 2010).

To our knowledge, there is no previous investigation the of mt-rRNAs enconding genes involvement in FTLD. So it is relevant to study their contribution to this pathology.

Objectives

The aim of this study is to sequence the mitochondrial genes coding for *12S* and *16S MT-RNR*, in order to identify alterations possibly linked to FTLD.

Patients and Methods

Samples

DNA samples of 101 patients (age range: 38-84; mean: 64 ± 10; 52 females and 49 males) with probable diagnosis of FTLD, according to the standard criteria of DSM-IV (Scheffler, 1999; Statement, 1994), obtained at Neurology Unit of the "Centro Hospitalar e Universitário de Coimbra", were investigated. Total DNA was extracted from peripheral blood by standard phenol chloroform method (Treco DA., 1999).

The scaling of dementia was obtained by scoring the CDR (Clinical Dementia Rating) and MMSE (Mini-mental state examination). In CDR, the scale is between 0 and 3, the higher values correspond to higher degree of dementia, while in MMSE, the scale is between 0 and 30, and the lower values correspond to higher degree of dementia (Folstein, Folstein, & McHugh, 1975).

Polymerase chain reaction (PCR)

The reaction was performed in a total volume of 25 µL, containing 100 ng/µL of DNA, 0.2 µM of each primer (Life Technologies, Carlsband, United States of America), forward and reverse (sequences may be provided upon request), 0.12 mM dNTPs (GE Healthcare Life Sciences, Upssala, Sweden), 0.09 mM $MgCl_2$, 0.04 U of Taq DNA polymerase and 1x complete buffer of Taq DNA polymerase (Thermo Scientific, Waltham, Uited States of America). The amplification conditions included initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 45 seconds, 50 - 60°C for 45 seconds, 72°C for 60 seconds, and a final extension step at 72°C for 5 minutes.

Agarose gel electrophoresis

PCR products were mixed with loading dye (1:1) and then loaded to 1% agarose gel for 1 hour, at 100 Volts, using a molecular weight marker (NZY DNA ladder VI). After

migration, ethidium bromide labelled DNA molecules were visualized under ultraviolet irradiation.

ExoSAP-IT® purification

ExoSAP-IT® was added to the PCR product as recommended, since the enzymes are active with the buffer used in PCR, it is not necessary to use a distinct buffer ("ExoSAP-IT For PCR Product Cleanup | Affymetrix,"). A volume of 3.5µl was used per sample (Gaspar et al., 2015).

Sequencing PCR

The amplification conditions were an initial denaturation at 96°C for 2 minutes followed by 45 cycles at 96°C for 10 seconds, 55°C for 5 seconds, 60°C for 4 minutes. The reaction mix was composed by 1µl BigDye® Terminator Ready Reaction Mix v.3.1, 5X sequencing buffer (Life Technologies, Carlsband, United States of America), 2,5µM of *primer* forward or reverse and H_2O mili Q.

Sephadex® purification

After sequencing PCR, samples were submitted to standard Sephadex® purification, gel filtration. Molecules of smaller size are retained in the pores of the matrix, whereas fragments of DNA, larger than 20pb, are filtered (Healthcare, 2009).

Automatic sequencing

After the automated sequencing, the results were analysed using two different software tools, *Sequencing Analysis v5.4®* and *SeqScape v2.5®*, which allow the comparison of obtained data with the reference sequence. It enables the detection of any alteration in the sequences under study.

***In silico* analysis**

After the analysis of all sequences and the identification of the alterations, an *in silico* analysis was performed. The database MITOMAP® was used in order to see if the variations have been reported (http://www.mitomap.org/MITOMAP). The sequences were then analysed with the computer software RNAfold (Hofacker, 2009) in order to make a prediction of changes in the secondary structure of rRNA, based on minimum energy requirements (Ballana et al., 2006). Evolutionary conservation was performed for all alterations in 14 species: *Homo sapiens, Pan paniscus, Pan troglodytes, Pan panicus, Pongo pygmaeus, Gorilla gorilla, Mus musculus, Rattus norvegicus, Bos Taurus, Gallus gallus, Xenopus laevis, Danio rerio, Strongylocentrotus purpuratos, Drosophilla melanogaster,*

Caenorthabditis elegans. The sequence alignment was performed using ClustalW® (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Results

A total of 100 patients (51 females and 49 males) with FTLD were included in this study. From these, 87 (44 females and 43 males) present 45 different genetic variations in *MT-RNR* genes, 19 in the *MT-RNR1* and 33 in the *MT-RNR2* (Figure 1). We have found 30 patients with only one variation, 33 patients with 2 variations, 13 patients with 3 variations, 8 patients with 4 variations, 2 patients with 5 variations and 1 patient with 6 variations (Table 1).

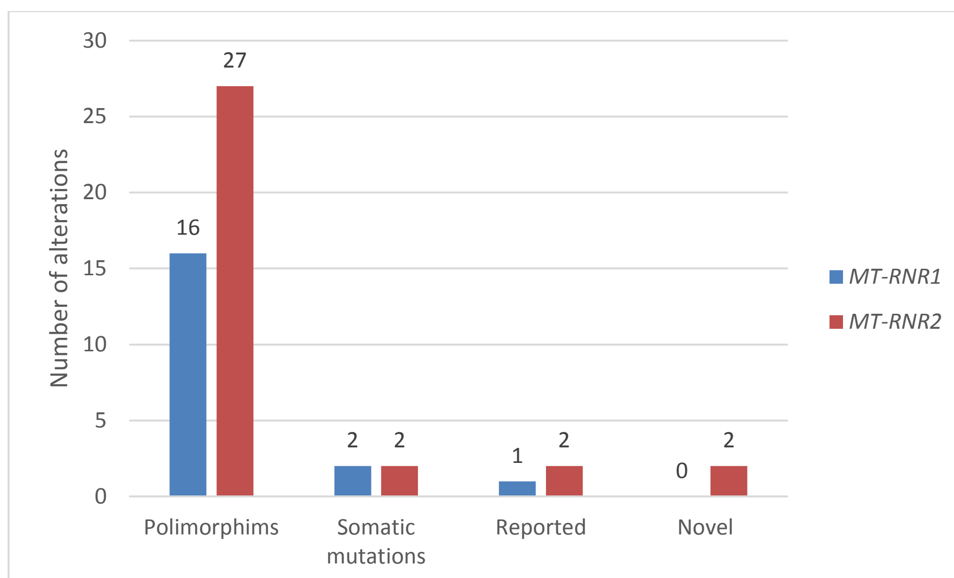

Figure 1: Sequence variations found per gene according to status in MITOMAP.

Concerning the CDR scores, 18 patients presented the maximum score for dementia, and 14 of these have mtDNA variations. In relation to MMSE, 37 patients exhibited maximum score dementia, 32 presenting variations (Table 1).

Table 1: Patients characterization and results of the mtDNA sequence variations found in *MT-RNR* genes.

Patient	Clinical Variant	Gender	Age	Age of onset	CDR	MMSE	mtDNA sequence variation	Gene
1	bvFTD	F	53	52	3	0	m.2706A>G	*MT-RNR2*
2	bvFTD	F	65	64	3	11	m.3010G>A	*MT-RNR2*
3	bvFTD	F	74	73	1	17	m.2772C>T	*MT-RNR2*
4	CBS	M	54	53	3	0	none	none

5	CBS	F	58	54	1	18	none	none
6	Familiar bvFTD	M	58	44	3	6	m.2706A>G m.3010G>A	*MT-RNR2* *MT-RNR2*
7	Esporadic bvFTD + psychiatric alt.	F	54	53	2	18	m.2706A>G m.3010G>A	*MT-RNR2* *MT-RNR2*
8	Esporadic bvFTD	F	71	68	2	12	m.1719G>A m.2706A>G	*MT-RNR2* *MT-RNR2*
9	Semantic dementia	M	46	41	3	10	m.2706A>G	*MT-RNR2*
10	bvFTD	F	64	63	1	25	m.2706A>G	*MT-RNR2*
11	bvFTD	F	59	55	3	0	none	none
12	Familiar bvFTD	F	75	74	1	27	m.2706A>G m.3010G>A	*MT-RNR2* *MT-RNR2*
13	bvFTD	F	77	73	3	10	m.709G>A m.1243T>C m.2706A>G	*MT-RNR1* *MT-RNR1* *MT-RNR2*
14	bvFTD	F	46	43	1	22	m.3010G>A	*MT-RNR2*
15	bvFTD	F	81	79	1	21	m.2706A>G m.2707A>G	*MT-RNR2* *MT-RNR2*
16	Familiar bvFTD	M	66	63	1	29	m.3010G>A	*MT-RNR2*
17	Familiar bvFTD	M	75	64	1	27	none	none
18	Esporadic bvFTD	F	62	59	2	17	m.750A>A m.1438A>A	*MT-RNR1* *MT-RNR1*
19	bvFTD	F	74	69	2	15	none	none
20	PPA	F	49	48	1	30	m.1811A>G m.2217C>T m.2706A>G	*MT-RNR2* *MT-RNR2* *MT-RNR2*
21	MDP (manic depressive depression = bipolar)+ bvFTD	M	64	62	0.5	28	m.2259C>T	*MT-RNR2*
22	bvFTD	F	50	50	2	17	m.1189T>C m.1811A>G m.2706A>G	*MT-RNR1* *MT-RNR2* *MT-RNR2*
23	Esporadic bvFTD	F	60	57	1	18	m.2706A>G m.2833A>G m.3010G>A	*MT-RNR2* *MT-RNR2* *MT-RNR2*
24	Familiar bvFTD	M	64	60	1	22	m.1189T>C m.1811A>G m.2706A>G	*MT-RNR1* *MT-RNR2* *MT-RNR2*

25	bvFTD (Korea without being associated with Huntington)	F	71	67	3	14	m.1719G>A m.2706A>G	*MT-RNR2* *MT-RNR2*
26	Semantic dementia	M	54	52	1	21	m.3010G>A	*MT-RNR2*
27	bvFTD	F	65	63	3	1	m.2706A>G	*MT-RNR2*
28	Esporadic bvFTD	F	49	48	0.5	30	m.3010G>A	*MT-RNR2*
29	Esporadic bvFTD	F	59	57	1	18	m.1719G>A m.2706A>G	*MT-RNR1* *MT-RNR2*
30	Esporadic bvFTD	F	54	50	2	19	m.3010G>A	*MT-RNR2*
31	bvFTD	M	51	51	1	25	m.2706A>G	*MT-RNR2*
32	Familiar bvFTD	M	68	67	1	27	m.2706A>G	*MT-RNR2*
33	bvFTD	F	68	66	2	15	m.1719G>A m.2706A>G	*MT-RNR2* *MT-RNR2*
34	Familiar bvFTD	M	64	61	1	24	m.709G>A m.930G>A m.1888G>A m.2706A>G m.3010G>A	*MT-RNR1* *MT-RNR1* *MT-RNR2* *MT-RNR2* *MT-RNR2*
35	bvFTD	M	70	69	1	20	m.3010G>A	*MT-RNR2*
36	Esporadic bvFTD	F	66	60	3	16	m.709G>A m.1243T>C m.2706A>G	*MT-RNR1* *MT-RNR1* *MT-RNR2*
37	bvFTD	M	74	73	2	16	m.2706A>G	*MT-RNR2*
38	bvFTD	F	54	54	1	26	m.2626T>C	*MT-RNR2*
39	Esporadic bvFTD	F	59	58	2	16	m.2259C>T	*MT-RNR2*
40	bvFTD	F	78	78	1	28	m.2706A>G	*MT-RNR2*
41	bvFTD	F	82	78	1	20	m.2248T>C m.3010G>A	*MT-RNR2* *MT-RNR2*
42	bvFTD	M	73	73	1	28	m.1375C>T m.3010G>A	*MT-RNR2* *MT-RNR2*
43	CBS	M	43	42	1	22	m.2706A>G	*MT-RNR2*
44	Esporadic bvFTD	M	38	34	1	25	m.2363A>G m.3010G>A	*MT-RNR2* *MT-RNR2*

45	bvFTD	M	72	69	2	13	m.709G>A m.769G>A m.1018G>A m.2416T>C m.2706A>G m.2789C>T	*MT-RNR1* *MT-RNR1* *MT-RNR1* *MT-RNR2* *MT-RNR2* *MT-RNR2*
46	bvFTD	F	81	75	2	13	m.3010G>A	*MT-RNR2*
47	bvFTD	M	45	40	3	7	m.2706A>G m.3010G>A	*MT-RNR2* *MT-RNR2*
48	bvFTD	M	43	43	2	16	m.2706A>G m.3010G>A	*MT-RNR2* *MT-RNR2*
49	Familiar bvFTD	F	75	72	1	20	none	none
50	PPA	M	60	58	3	9	none	none
51	MDP+bvFTD	F	55	54	1	22	m.1189T>C m.1811A>G m.2706A>G m.3197T>C	*MT-RNR1* *MT-RNR2* *MT-RNR2* *MT-RNR2*
52	PPA+ parkinsonism	F	54	62	3	4	m.1811A>G m.2706A>G	*MT-RNR2* *MT-RNR2*
53	Esporadic bvFTD	M	65	63	0.5	29	none	none
54	bvFTD	M	71	68	0.5	30	m.1393G>A m.1721C>T m.2706A>G m.2755A>G m.3197T>C	*MT-RNR1* *MT-RNR2* *MT-RNR2* *MT-RNR2* *MT-RNR2*
55	bvFTD	M	65	63	1	29	m.669T>C m.1719G>A m.2702G>A m.2706A>G	*MT-RNR1* *MT-RNR2* *MT-RNR2* *MT-RNR2*
56	PPA	F	69	56	1	13	m.1189T>C m.1811A>G m.2706A>G	*MT-RNR1* *MT-RNR2* *MT-RNR2*
57	bvFTD	F	77	64	1	21	none	none
58	bvFTD	M	48	45	2	13	m.709G>A m.1888G>A m.2706A>G	*MT-RNR1* *MT-RNR2* *MT-RNR2*
59	CBS	F	62	60	1	20	m.1721C>T m.2706A>G m.2755A>G m.3197T>C	*MT-RNR2* *MT-RNR2* *MT-RNR2* *MT-RNR2*
60	bvFTD	M	64	62	2	16	m.1719G>A m.1971A>G m.2706A>G	*MT-RNR2* *MT-RNR2* *MT-RNR2*

61	bvFTD	M	77	74	1	26	m.3036G>A	*MT-RNR2*
62	bvFTD	M	67	61	2	10	m.1211G>A m.2706A>G	*MT-RNR1* *MT-RNR2*
63	bvFTD	F	74	72	0.5	26	m.2706A>G m.3197T>C	*MT-RNR2* *MT-RNR2*
64	bvFTD	M	55	52	0.5	30	none	none
65	bvFTD	F	76	75	1	26	none	none
66	bvFTD	M	84	80	1	25	m.709G>A m.2706A>G	*MT-RNR1* *MT-RNR2*
67	bvFTD	F	76	76	3	0	m.709G>A m.930G>A m.1888G>A m.1992C>T m.2706A>G	*MT-RNR1* *MT-RNR1* *MT-RNR2* *MT-RNR2* *MT-RNR2*
68	bvFTD	M	67	63	1	23	m.2706A>G m.3203A>G	*MT-RNR2* *MT-RNR2*
69	bvFTD	M	57	55	1	27	m.1406T>C m.1811A>G	*MT-RNR1* *MT-RNR2*
70	bvFTD	M	70	68	1	22	m.3010A>G	*MT-RNR2*
71	bvFTD	F	68	67	1	25	m.2706A>G m.3010A>G	*MT-RNR2* *MT-RNR2*
72	bvFTD	M	75	72	0.5	30	m.709G>A m.930G>A m.1888G>A m.2706A>G	*MT-RNR1* *MT-RNR1* *MT-RNR2* *MT-RNR2*
73	bvFTD	F	76		3		m.709G>A m.930G>A m.1888G>A m.2706A>G	*MT-RNR1* *MT-RNR1* *MT-RNR2* *MT-RNR2*
74	bvFTD	F	64	62	1	26	m.2706A>G m.3197T>C	*MT-RNR2* *MT-RNR2*
75	FTD	F	76	70	3	0	m.1189T>C m.1811A>G m.1849C>T m.2706A>G	*MT-RNR1* *MT-RNR2* *MT-RNR2* *MT-RNR2*
76	bvFTD	F	70	61	1	20	none	none
77	bvFTD	M	49	47	1	22	m.2706A>G	*MT-RNR2*
78	bvFTD	F	77	74	0.5	20	m.2098G>A m.3010G>A	*MT-RNR2* *MT-RNR2*
79	Semantic Dementia	M	63	58	1	24	m.2706A>G m.3010G>A	*MT-RNR2* *MT-RNR2*
80	Semantic Dementia	M	63	61	2	12	m.2706A>G	*MT-RNR2*

81	bvFTD	M	55	52	1	24	m.961T>C m.965_966insCC m.2164C>T m.2706A>G	*MT-RNR1* *MT-RNR1* *MT-RNR2* *MT-RNR2*
82	bvFTD	M	60	55	1	29	m.1721C>T m.2706A>G	*MT-RNR2* *MT-RNR2*
83	bvFTD	F	79	77	1	25	m.1719G>A m.2706A>G	*MT-RNR2* *MT-RNR2*
84	bvFTD	M	62		1	21	m.1719G>A m.2706A>G	*MT-RNR2* *MT-RNR2*
85	bvFTD	M	71	66	1	21	m.2706A>G	*MT-RNR2*
86	PPA	M	72	70	2	13	m.2706A>G	*MT-RNR2*
87	bvFTD	F	74	73	1	26	m.3010G>A	*MT-RNR2*
88	bvFTD	F	65	64	1	27	m.2706A>G	*MT-RNR2*
89	bvFTD	F	74	72	1	15	m.1811A>G m.2706A>G	*MT-RNR2* *MT-RNR2*
90	bvFTD	F	69	69	1	19	m.2706A>G m.3010G>A	*MT-RNR2* *MT-RNR2*
91	bvFTD	F	59	53	3	0	m.3010G>A	*MT-RNR2*
92	bvFTD	M	70	67	1	25	none	none
93	bvFTD	M	67	59	1	27	m.709A>G m.2442T>C m.2706A>G	*MT-RNR2* *MT-RNR2* *MT-RNR2*
94	bvFTD	M	61	56	2	12	m.1719G>A m.2706A>G	*MT-RNR2* *MT-RNR2*
95	bvFTD	M	76	70	1	28	m.1719G>A m.2706A>G	*MT-RNR2* *MT-RNR2*
96	Familiar BvFTD	M	59	56	1	21	m.961T>C m.965_966insCC m.965_966insCCC	*MT-RNR1* *MT-RNR1* *MT-RNR1*
97	bvFTD	M	69	54	0,5	28	m.961T>C m.965_966insCC m.965_966insCCC	*MT-RNR1* *MT-RNR1* *MT-RNR1*
98	bvFTD	M	74		1	27	m.1811A>G m.2294A>G m.2706A>G m.3010G>A	*MT-RNR2* *MT-RNR2* *MT-RNR2* *MT-RNR2*
99	PPA	F	70	68	1	13	m.1811A>G m.2706A>G	*MT-RNR2* *MT-RNR2*
100	Semantic dementia	M	72		1	25	m.1811A>G m.2706A>G	*MT-RNR2* *MT-RNR2*

Abbreviations: CBS=Corticobasal Syndrome; bvFTD=behavioural variant of Frontotemporal Dementia; MDP=Manic Depressive Depression; PPA=Primary Progressive Aphasia.

The table 2 summarizes the description and classification of the mtDNA sequence variations identified in *MT-RNR* genes.

The variations identified were found in MITOMAP database: 43 have been described as “polymorphism”; 3 as “polymorphism” and “mutation”; 4 variations as “polymorphism” and “somatic mutation”; and 2 are novel, m.1849C>T (Figure: 3) and m.3036G>A (Figure 7).

In silico analysis showed that the minimum free energy was changed in 27 of the 45 found sequence variations found, and 12 of these are predicted to alter the rRNA structure.

There are 5 variations probably pathogenic, according to the *in silico* analysis, 4 of them causing structure and binding minimum free energy changes: the m.1243T>C (*MT-RNR1*) variation has been already associated with hearing loss, LHON disease and, in homoplasmy, to pancreatic cancer cell line (Figure 2); the m.2363A>G (*MT-RNR2*) was not related to any disease so far (Figure 6); and the variation m.3036G>A, is heteroplasmic and so far not reported on MITOMAP (Figure 7). Although it does not seem to be causing structure changes, the m.2098G>A (*MT-RNR2*) variation, has been associated with LHON disease, has a diminish binding minimum free energy and also a high percentage of conservation (Figure 5). The m.1971A>G variation, although does not seem to be causing structure or minimum free energy changes, it is present in heteroplasmy and is 100% conserved (Figure 4). The most frequent variation found is m.2706A>G (*MT-RNR2*) with a high rate of conservation.

Table 2: *In silico* analysis results of the sequence variations found in *MT-RNR* genes.

Sequence variation	*MT-RNR*	Results in MITOMAP	Frequency in the sample (n)	Frequency in database (n) %	Minimum free energy kcal/mol		Structure	Evolutionary conservation %
					Normal	Changed		
m.669T>C	*MT-RNR1*	Polymorphisms Mutation (DEAF)	1	0.99	-226.50	-227.80	**Changed**	62%
m.709G>A	*MT-RNR1*	Polymorphisms	10	9.90	-226.50	-226.6	**Changed**	54%
m.750G>A	*MT-RNR1*	Polymorphisms	1	0.99	-226.50	-223.6	**Changed**	8%
m.769G>A	*MT-RNR1*	Polymorphisms	1	0.99	-226.50	-225.6	Normal	56%
m.930G>A	*MT-RNR1*	Polymorphisms	4	3.96	-226.50	-226.6	Normal	25%
m.961T>C	*MT-RNR1*	Polymorphisms	3	2.97	-226.50	-226.50	Normal	42%
m.965_966insCC	*MT-RNR1*	Polymorphisms	3	2.97	-226.50	-226.40	Normal	
m.965_966insCCC	*MT-RNR1*	Polymorphisms	2	1.98	-226.50	-226.40	Normal	
m.1018G>A	*MT-RNR1*	Polymorphisms	1	0.99	-226.50	-226.5	Normal	7%
m.1189T>C	*MT-RNR1*	Polymorphisms	5	4.95	-226.50	-227.7	Normal	57%
m.1211G>A	*MT-RNR1*	Polymorphisms	1	0.99	-226.50	-226.5	Normal	43%
m.1243T>C	*MT-RNR1*	Polymorphism Somatic Mutation	2	1.98	-226.50	-225.5	**Changed**	86%
m.1375C>T	*MT-RNR1*	Polymorphisms	1	0.99	-226.50	-228.2	Normal	50%
m.1393G>A	*MT-RNR1*	Polymorphisms	1	0.99	-226.50	-226.2	Normal	36%
m.1406T>C	*MT-RNR1*	Polymorphism Somatic Mutation	1	0.99	-226.50	-225.6	Normal	40%
m.1438G>A	*MT-RNR1*	Polymorphisms	1	0.99	-226.50	-226.4	Normal	43%
m.1719G>A	*MT-RNR2*	Polymorphisms	10	9.90	-337.37	-337.37	Normal	8%

m.1721C>T	*MT-RNR2*	Polymorphism Somatic Mutation	3	2.97	-337.37	-339.97	**Changed**	67%
m.1811A>G	*MT-RNR2*	Polymorphism Somatic Mutation	12	11.88	-337.37	-338.87	Normal	79%
m.1849C>T	*MT-RNR2*	Novel	1	0.99	-337.37	-338.17	**Changed**	15%
m.1888G>A	*MT-RNR2*	Polymorphisms	5	4.95	-337.37	-337.37	Normal	25%
m.1971A>G	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.87	Normal	100%
m.1992C>T	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.37	Normal	73%
m.2098G>A	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-333.77	Normal	90%
m.2164C>T	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.37	Normal	67%
m.2217C>T	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.37	Normal	36%
m.2248T>C	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.37	Normal	82%
m.2259C>T	*MT-RNR2*	Polymorphisms	2	1.98	-337.37	-337.87	**Changed**	43%
m.2294A>G	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.37	Normal	75%
m.2363A>G	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-338.3	**Changed**	93%
m.2416T>C	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.37	Normal	18%
m.2442T>C	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.67	**Changed**	27%
m.2589A>G	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-336.77	Normal	79%
m.2626T>C	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-332.77	**Changed**	57%
m.2702G>A	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-331.57	Normal	62%
m.2706A>G	*MT-RNR2*	Polymorphisms	62	61.39	-337.37	-337.87	**Changed**	86%
m.2707A>G	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.37	Normal	86%
m.2755A>G	*MT-RNR2*	Polymorphism Mutation (Possibly LVNC-associated)	2	1.98	-337.37	-337.37	Normal	89%
m.2772C>T	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.67	Normal	50%

m.2789C>T	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.37	Normal	42%
m.2833A>G	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.37	Normal	85%
m.3010G>A	*MT-RNR2*	Polymorphism Mutation (Cyclic Vomiting Syndrome with Migraine)	26	25.74	-337.37	-337.07	Normal	36%
m.3036G>A	*MT-RNR2*	Novel	1	0.99	-337.37	-338.27	**Changed**	93%
m.3197T>C	*MT-RNR2*	Polymorphisms	3	2.97	-337.37	-337.37	Normal	50%
m.3203A>G	*MT-RNR2*	Polymorphisms	1	0.99	-337.37	-337.37	Normal	70%

Abbreviations: DEAF = Deafness; LVNC = Left Ventricular Non-Compaction

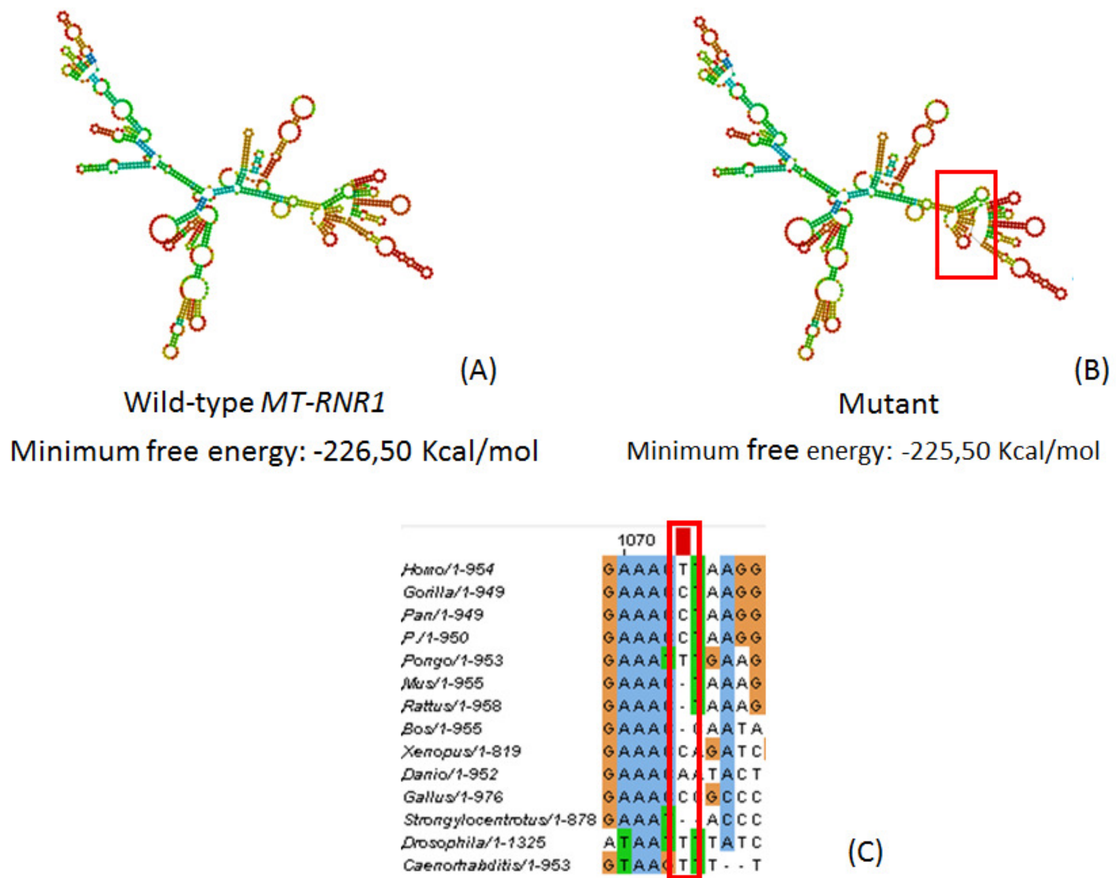

Figure 2: Results from *in silico* analysis using RNAfold software and Clustal W for rRNA12S structure with m.1243T>C variation. A – wild type and B – “mutated”; C – Evolutionary conservation for the nucleotide position.

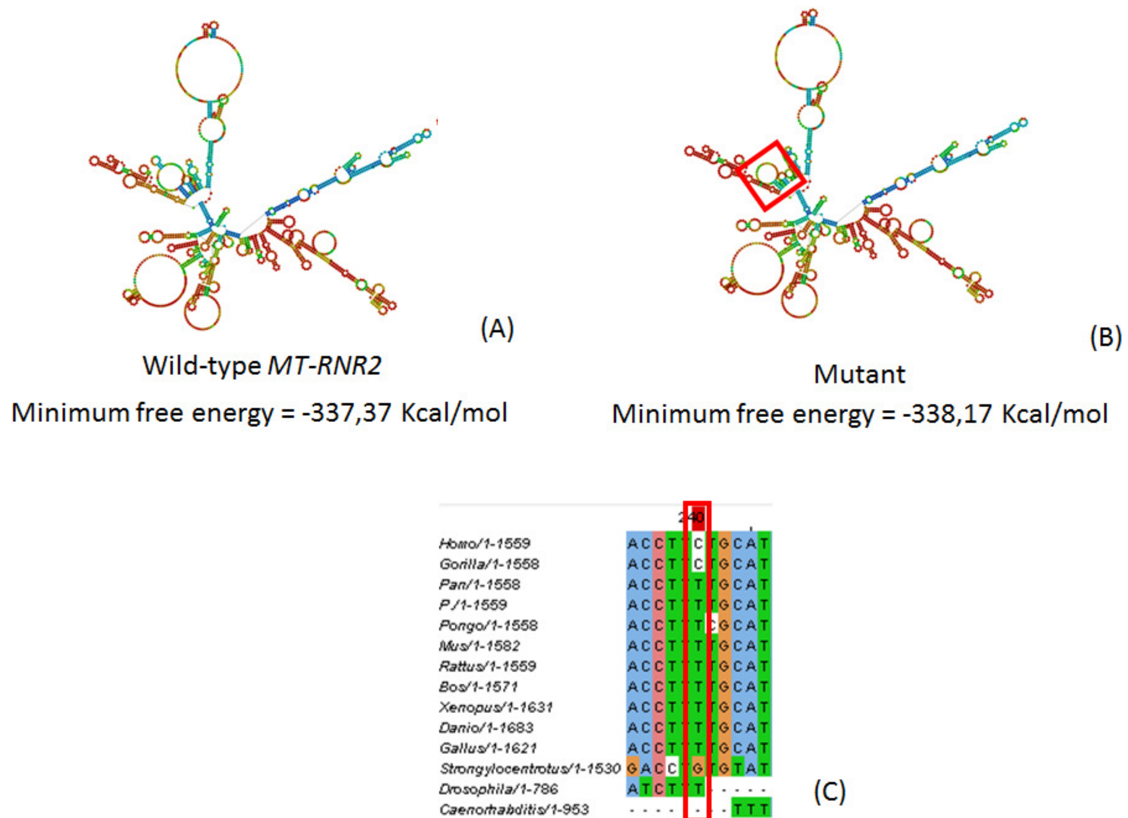

Figure 3: Results from *in silico* analysis using RNAfold software and Clustal W for rRNA16S structure with m.1849 A>C variation. A – wild type and B – “mutated”; C – Evolutionary conservation for the nucleotide position.

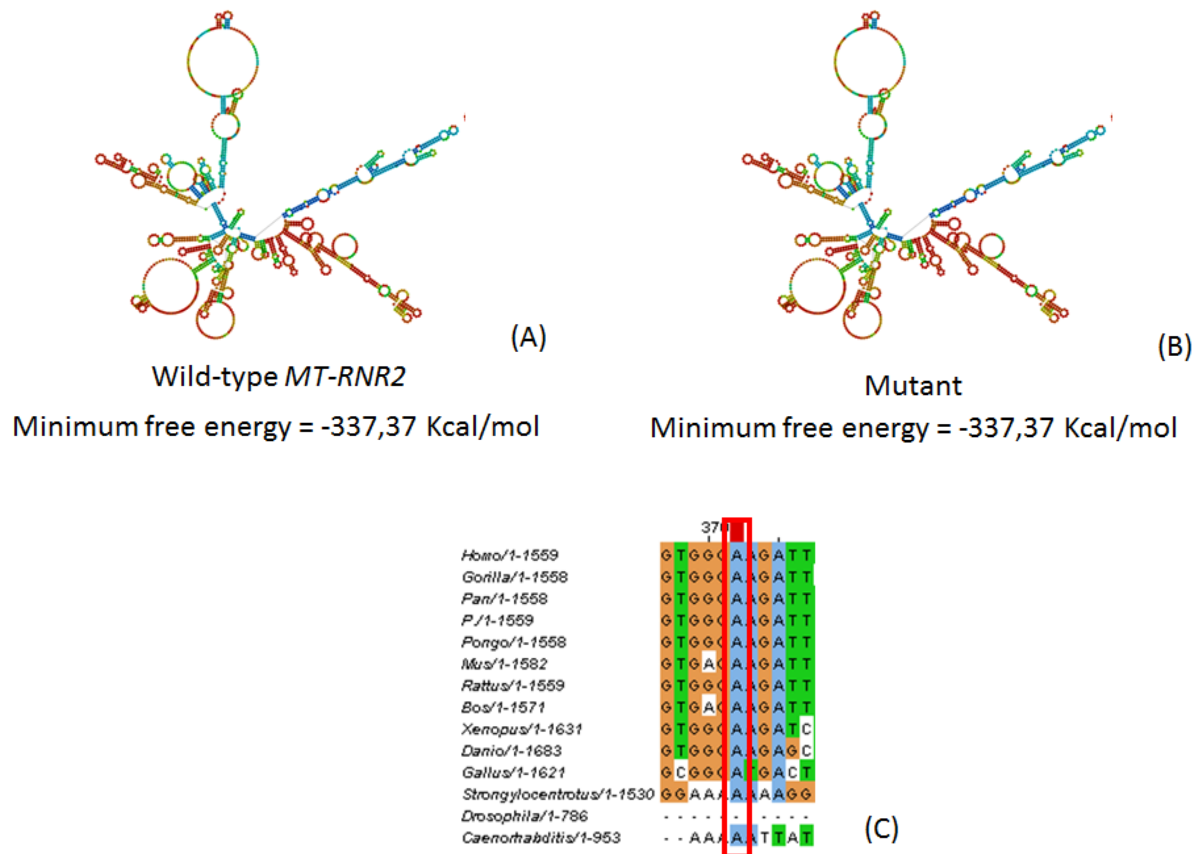

Figure 4: Results from *in silico* analysis using RNAfold software and Clustal W for rRNA16S structure with m.1971A>G variation. A – wild type and B – “mutated”; C – Evolutionary conservation for the nucleotide position.

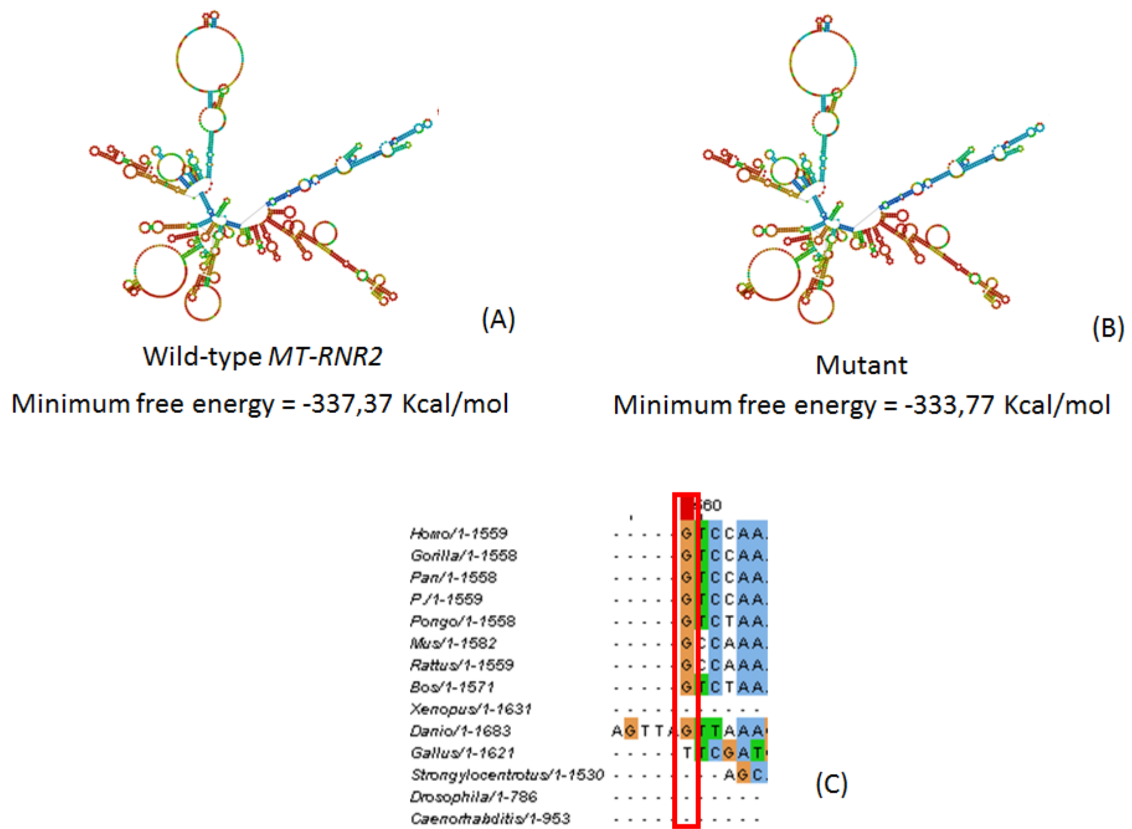

Figure 5: Results from *in silico* analysis using RNAfold software and Clustal W for rRNA16S structure with m.2098G>A variation. A – wild type and B – “mutated”; C – Evolutionary conservation for the nucleotide position.

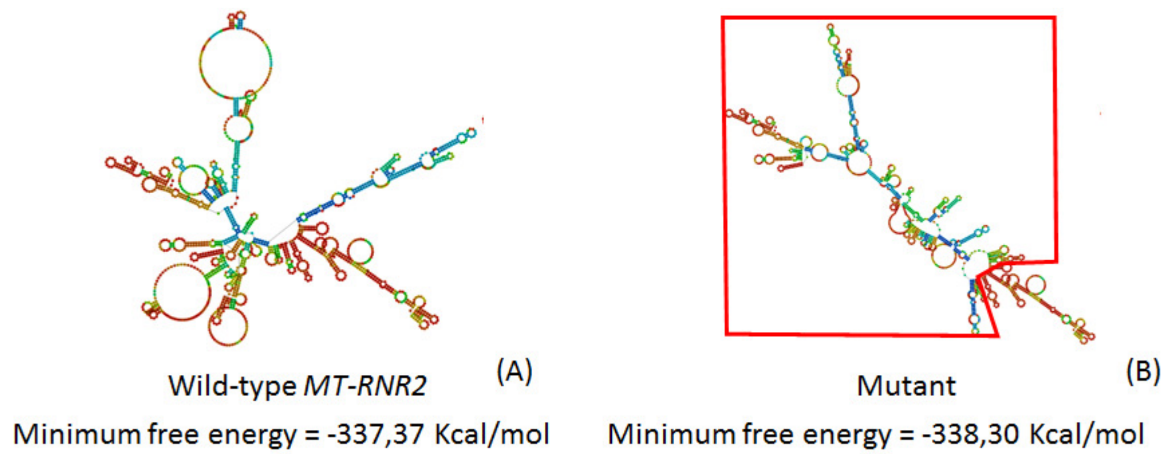

Figure 6: Results from *in silico* analysis using RNAfold software and Clustal W for rRNA16S structure with m.2363A>G variation. A – wild type and B – "mutated"; C – Evolutionary conservation for the nucleotide position.

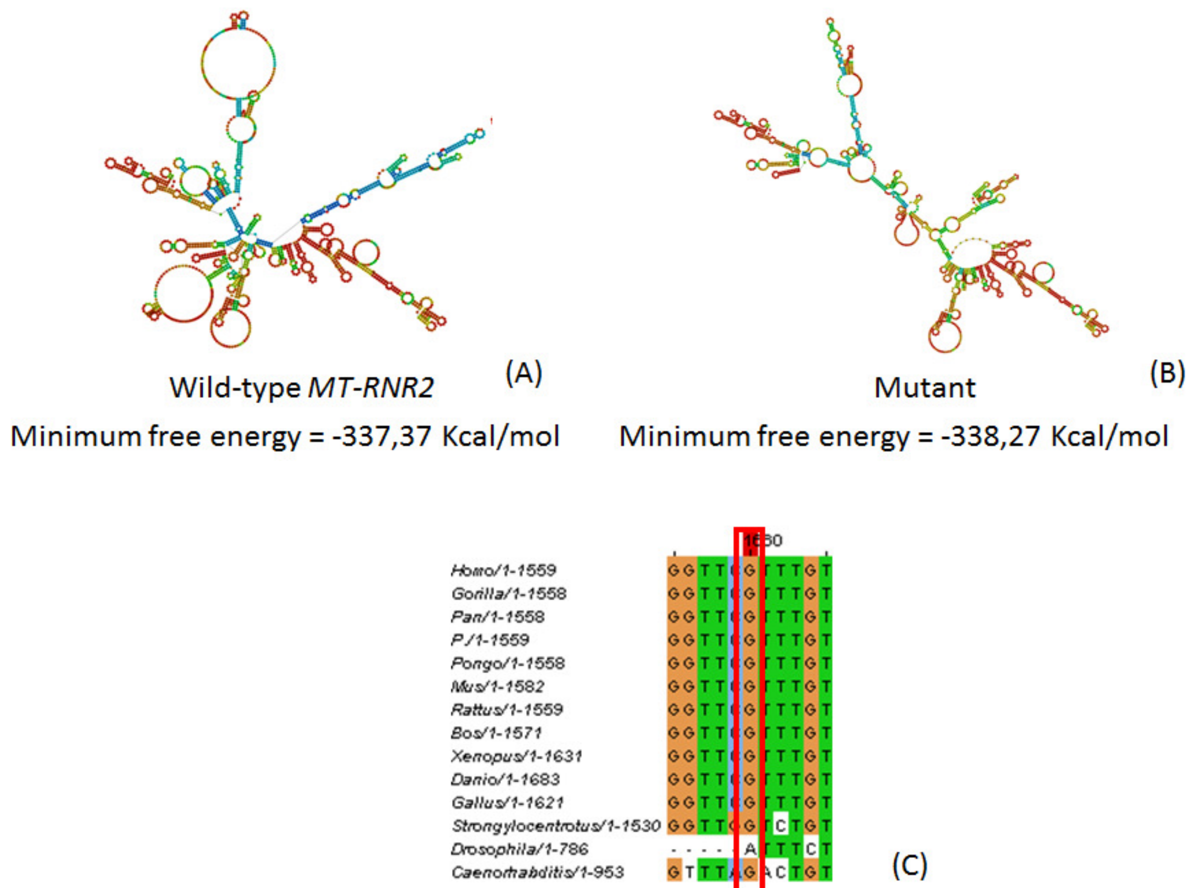

Figure 7: Results from *in silico* analysis using RNAfold software and Clustal W for rRNA12S structure with m.3036G>A variation. A – wild type and B – "mutated"; C – Evolutionary conservation for the nucleotide position.

Discussion

Mitochondrial ribosomes are fundamental structures for the protein biosynthesis. They are the key for translation and thereby for the correct protein formation. Human mitochondrial ribosomes arouse interest as there are some point mutations reported in their genes and neurodegenerative diseases.

Most of the identified variations of the present study have been previously described as polymorphisms (http://www.mitomap.org/MITOMAP). However, it is not possible to exclude the possibility of these variations to be involved in the pathogenesis of FTLD. It should be noted that a set of changes, that are not pathogenic per se, but, along with other changes, either in mitochondrial or nuclear genes and environmental factors, may create susceptibility, for certain diseases (Autere et al., 2004), as a result of

DNA expression modulation, or might cause "dysfunction/damage" (Grazina et al., 2006). It is also important to understand the cellular processes that lead to neurodegeneration (Hales & Hu, 2013), which are highly complex. In addition we have identified two novel variations that are new, m.1849C>T and m.3036G>A, that have not been described, to our knowledge and according to the databases MITOMAP, 2015, mtDB and the Pereira et al. study, (http://www.mitomap.org/MITOMAP; Ingman & Gyllensten, 2006; Pereira et al., 2009).

The minimum free energy is a criterion to predict the stability of rRNA structure, since a mutation in *MT-RNR* can alter the secondary structure of the corresponding rRNA. Lower minimum free energy is related to a more stable structure, but it is not the only variable to taken into account, because of biological complexity (Ballana et al., 2006). Evolutionary conservation is also an important feature of pathogenicity, as high percentage of evolutionary conservation suggests that a sequence variation may potentially be pathogenic or its alteration may cause further damage (Yarham et al., 2011).

In 45 variations identified in the present study, 27 are predicted to have structure changes and/or minimum free energy variations, but only 5 present a high evolutionary conservation score (Table 2). In 4 variations, despite of having high percentage of conservation, the corresponding structures and minimum free energy do not change (m.1971A>G; m.2707A>G; m.2755A>G; m.2833A>G). There are 6 variations (m.1971A>G; m.2098G>A; m.2363A>G; m.2706A>G; m.2707A>G; m.2755A>G; m.2833A>G) that are reported in MITOMAP as polymorphisms but the percentage of conservation is high (85-100%). Our data shows that variations described as pathogenic mutations, affect mostly high conserved nucleotides, whereas most polymorphic variations affect rather non-conserved nucleotides, as expected, some exceptions are reported suggesting that other criteria, such as structural changes, must be considered for more accurate conclusions. It is clear that mutations related with secondary structure conformation are a main cause for an uneven distribution of mtDNA diversity, mainly in *MT-RNR* genes, in which the secondary structure is relevant for functionality (Pereira et al., 2009).

From the 45 variations found in the present study, there are 7 variations that are located in the stems and 38 in non-stem regions (figures 2-7). The *MT-RNR* genes

present higher proportion of polymorphism on loop regions than in stem regions (Pereira et al., 2009).

Nineteen variations are located in *MT-RNR1* and, from these, 13 are in a non-stem region. On the other hand, 33 variations are found in *MT-RNR2* and 25 are located in non-stem regions, which indicates higher variability in *MT-RNR2*, especially in non-stem regions. This is in agreement with the study by Pereira and colleagues (2009).

The m.1971A>G and m.3036G>A variations are present in heteroplasmy, but it was not possible to determine the exact percentage. Furthermore, because mtDNA is present in multiple copies, a pathogenic mtDNA mutation often results in heteroplasmy. The clinical expression of mtDNA mutations is extremely heterogeneous, depending on the percentage of mutant loads in affected tissues, the energy demands of the tissue, and the type of mutation (Wong, 2007).

The variation m.2626T>C, predicted to cause changes of structure and minimum free energy, was mentioned by Takasaki in a study with the aim of examining the differences between Japanese centenarians, Japanese AD patients and Japanese PD patients, and their mitochondrial single nucleotide polymorphism (mtSNP) frequencies at individual mitochondrial mtDNA positions of the entire mitochondrial-genome (Takasaki, 2008). Also, the variation m.3197T>C, arouse interest due to its localization. It is positioned in the nucleotide following a reported variation in two neurodegenerative diseases, AD and PD, m.3196G>A (http://www.mitomap.org/MITOMAP), both featuring overlaps with FTLD.

After *in silico* analysis, 5 sequence variations have high probability of being pathogenic: m.1243T>C due to the change of minimum free energy and in its structure, the high percentage of evolutionary conservation, and the location in a stem region; m.1971A>G due to heteroplasmy and since it is 100% conserved; the m.2098G>A variation due to its high change of minimum free energy, highly conservation and location in a stem region; m.2363A>G induces binding minimum free energy and structure changes, has high evolutionary conservation and it is located in a stem region; and m.3036G>A due to the heteroplasmy presented, change of both minimum free energy and structure, highly conservation and location in a stem region.

It is relevant to mention that the sample was obtained from the peripheral blood leukocytes. Taking into account that the variations were mostly found in homoplasmy,

the results can be extrapolated to the brain tissue. However, there might be somatic mutations in heteroplasmy in the brain that are not present in the blood cells (Grazina et al., 2003). Testing other tissues, such as muscle or skin fibroblasts, post-mortem brain tissue, would add more evidence to this association.

It is unclear yet how the mutant *MT-RNRs* can cause mitochondrial dysfunction. There is a current approach stating that a combination of different mutations affecting mitochondrial genes is responsible for a variety of clinical diseases (Robert et al., 2004). Additionally, given the role of *MT-RNRs* in mitochondrial ribosomes and translocation, mutations in these genes may affect the ability to produce mitochondrial proteins.

Although significant progresses have occurred in diagnosing FTLD, challenges remain in identifying accurately patients with the high sensitivity and specificity at the earliest possible stage. Establishing measures that elucidate the underlying cellular process responsible for FTLD pathology will be essential for developing successful therapeutic trials (Hales and Hu, 2013), where variations in mtDNA may play an important role. In this context the study of maternal lineage would also be an important trait to assess, but unfortunately, in the majority of the cases, it is impossible due to the age of patients.

In general, more research needs to be accomplished in order to achieve more accurate conclusions on the relationship between mtDNA variations and the pathophysiology of FTLD.

It would also important to complement this study with functional analyses to prove the possible pathogenicity of the reported variations.

Conclusion

The analysis of the *MT-RNRs* variations pointed out that there is no sufficient evidence to classify the variations as pathogenic causes of FTLD. However, our results report new data regarding the presence of mtDNA variations in patients with FTLD. Most of the detected variations altered the structure and/or minimum free energy of *MT-RNRs*, although not always in concordance with each other. The number of variations in the stem region is higher, which is in agreement with the literature, concerning the involvement of *MT-RNR* folding genes in diseases. The evolutionary conservation is not always in agreement with the results obtained for the structure and/or minimum free

energy, for predicting of pathogenicity. Therefore, due to the heterogeneity of results, it is difficult to classify as variations pathogenic. Nevertheless, according to all the pathogenicity criteria studied, from the 45 variations identified, the more likely to be pathogenic or potentially cause damage are: m.1243C>T in *MT-RNR1*, and m.1971A>G, m.2098G>A, m.2363A>G and m.3036G>A, all in *MT-RNR2*.

In addition, two "novel" variation was found, however it is not clear if it represents a risk factor.

Moreover, according to the "Mitochondrial cascade hypothesis" (Swerdlow & Khan, 2004), polymorphic variations in MCR subunits encoding genes establish MCR efficiency and basal mitochondrial ROS production, that correlates with mtDNA damage. Subsequently, somatic mtDNA mutation decreases MCR efficiency, leading to reduced OXPHOS and/or increased ROS production. For this reason, mtDNA mutations might modify age onset, contributing to neurodegeneration process, probably due to an impairment of MCR and/or translation mechanisms (Grazina et al., 2006). Moreover, mtDNA mutations may have a cumulative effect, increasing the probability to develop an energy failure (Grazina et al., 2004).

Alterations in *MT-RNRs*, although nonspecific, may induce protein synthesis anomalies that could result in OXPHOS deficiency, compromising the enzymatic activity of most MRC complexes.

Furthermore, it is necessary a deeper biochemical research in order to clarify the relationship between mtDNA and FTLD considering also the involvement of nuclear genes. Genotype/phenotype correlation can involve nuclear and mitochondrial interactions, but the exact mechanism is still unknown.

The report and identification of mtDNA variations is not only a valuable contribution towards the better understanding of the FTLD ethiology, but also a way to improve diagnosis protocols of the pathology in a near future, and a way to develop new long term treatment strategies (Grazina et al., 2006).

In conclusion, to our knowledge, the present work is the first report of a complete sequence of the 2 *MT-RNR* genes in FTLD patients and it represents an important contribution to the field. Although more research is needed to determine whether the *MT-RNR* variations play a direct pathogenic role in FTLD.

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