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MT-CYB sequencing analysis in Frontotemporal lobar degeneration

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MT-CYB sequencing analysis in Frontotemporal lobar degeneration

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Abstract

Frontotemporal lobar degeneration (FTLD) is the second most common cause for dementia before 65 years of age. FTLD comprises variants with a very diverging spectrum, regarding the clinical presentation, genetic features, and neuropathology.

In the last decades remarkable progresses have been achieved in terms of genetic causes and their relation with neuropathology. Given the complexity of this disease, several aetiologies have been proposed. One of the theories includes variations in mitochondrial DNA (mtDNA) that affect the energy production in mitochondrial respiratory chain and consequently affect the tissue with higher energy demands, the central nervous system.

The aim of our study is to identify variations of *MT-CYB* gene in 70 DNA samples of patients with probable diagnosis of FTLD.

Total DNA of the samples was extracted from peripheral blood and *MT-CYB* gene was analysed and the sequence variations found were submitted to an *in silico* analysis. A total of 37 different sequence variations were identified in 44 patients (63%): 22 are synonymous, 2 are novel variations (m.15073C>T; m.15127C>T) that have not been reported to date in MITOMAP database and 15 are non-synonymous.

The results of the *in silico* analysis suggested that 3 variations may be pathogenic: m.14927A>C affects protein function according to SIFT and the amino acid is conserved in all mammals; m.15164T>C is predicted to be deleterious by *Provean*, it is an alteration in a critical position and is totally conserved in all species studied, and m.15465T>C affects protein function according to *Poly-Phen* and *SIFT* and is highly conserved in mammals.

Further investigation is important to clarify a possible relationship between mtDNA variations and pathophysiology of FTLD. However, our study represents a significant improvement in the complex field of dementia such like FTLD.

Keywords: Frontotemporal lobar degeneration, mitochondrial DNA, sequence variations, *MT-CYB* gene.

Abbreviations

AD	Alzheimer's Disease
aFTLD-U	Atypical frontotemporal lobar degeneration with ubiquitinated inclusions
AGD	Argyrophilic grain disease
APOE	Apolipoprotein E
avPPA	Agrammatical variant of PPA
BIBD	Basophilic inclusion body disease
bvFTD	Behavioural variant frontotemporal dementia
C9ORF72	Chromosome 9 open reading frame 21
CBS	Cortico basal syndrome
CDR	Clinical Dementia Rating
CHMP2B	Chromatin-modifying protein 2B
CHMP2B	Charged multivescicular body protein 2B
CST 3B	Cystatin C gene
DLDH	Dementia lacking distinctive histopathology
FTD	Frontotemporal dementia
FTD-3	Frontotemporal dementia linked to chromosome 3
FTD–MND	FTD with motor neuron disease
FTLD	Frontotemporal lobar degeneration
FTLD-U	FTLD-Ubiquitin
FTLD-UPS	FTLD ubiquitin proteasome system
FUS	Fused in sarcoma
LHON	Leber's hereditary optic neuropathy
LNVC	Left ventricular noncompaction
MAPT	Microtubule-associated protein tau
MDD	Major depressive disorder
MMSE	Mini-Mental State Examination
MRC	Mitochondrial respiratory chain
MSTD	Multiple system tauopathy with dementia
MT-CYB	Gene encoding cytochrome b
mtDNA	Mitochondrial DNA
NFT-dementia	Neurofibrillary tangle predominant dementia
Ni	No inclusions
NIFID	Neuronal intermediate filament inclusion disease
PGRN or GRN	Progranulin
PiD	Pick's disease
PPA	Primary progressive aphasia
PSP	Progressive supranuclear palsy
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
svPPA	Semantic variant of primary progressive aphasia
TAR	Transactive response
TARDBP	TAR DNA-binding protein 43
TARDBP	Transactive response DNA binding protein

TDP-43	TAR DNA-binding protein 43 kDa
VCP	Valosin-containing protein
WMT-GGI	White matter tauopathy with globular glial inclusions
9p	Genetic locus on chromosome 9p linked to familial amyotrophic lateral sclerosis and frontotemporal dementia

Introduction

In the last years, the interest in the study of diseases that are prevalent at older ages has increased, particularly because of ageing of the population worldwide. The prevalence of neurodegenerative dementias have also increased, including the frontotemporal lobar degeneration (FTLD), which is the most common cause of the non-Alzheimer type dementia worldwide (Borroni & Padovani, 2013). The FTLD accounts for about 9% of all cases of dementia (Salmon & Stuss, 2013) and is particularly prevalent in patients under than 65 years dementia (Neary *et al.*, 2005, Seelaar *et al.*, 2011, Sieben *et al.*, 2012), being the second most common early-onset dementia (Seelaar *et al.*, 2011).

The exact prevalence of FTLD remains uncertain, however the incidence appears to be equal in both genders (Neary *et al.*, 2005, Seelaar *et al.*, 2011). It is a clinically and pathologically heterogeneous spectrum of syndromes, characterized by a progressive decline in behavior or language associated difficulties with selective degeneration of the frontal and temporal cortex regions, with relative preservation of posterior regions (Seelaar *et al.*, 2011, Shinagawa, 2013).

Nomenclature of the FTLD spectrum phenotypes is complex as new pathological and genetic correlations have been recently discovered (Hales & Hu, 2013, Sieben *et al.*, 2012). Accordingly, current redefined clinical criteria recognize several different phenotypes, namely: behavioural variant frontotemporal dementia (bvFTD), the semantic variant of primary progressive aphasia (svPPA), the agrammatical variant of PPA (avPPA), FTD with motor neuron disease (FTD–MND), cortico basal syndrome (CBS) and progressive supranuclear palsy (PSP) (Borroni & Padovani, 2013, Shinagawa, 2013). The bvFTD subtype has the highest prevalence amongst the FTLD clinical syndromes, accounting for approximately 70% of all cases (Pan & Chen, 2013). The bvFTD, svPPA and avPPA phenotypes all share an insidious onset and inexorably progressive decline. Emotional

blunting, loss of empathy, apathy, selfishness and neglect of personal hygiene are typical of bvFTD, but that may be seen in all subtypes (Seelaar *et al.*, 2011).

In contrast to Alzheimer's Disease (AD) patients, subjects affected with FTLD usually perform relatively well in visuospatial ability and memory tasks (Grazina *et al.*, 2004, Schlachetzki, 2011, Wang *et al.*, 2013).

The clinical heterogeneity in familiar and sporadic forms of FTLD is remarkable, with patients demonstrating variable mixtures of disinhibition, dementia, PSP, CBD, and MND (Pan & Chen, 2013, Sleegers *et al.*, 2010).

The autosomal dominant transmission of the disease suggested a genetic cause (Galimberti & Scarpini, 2012) and approximately 40% of patients with FTLD have a positive family history (Pan & Chen, 2013). However, only 10%−30% of family pedigrees show an autosomal dominant inheritance pattern (Riedl *et al.*, 2014, Sieben *et al.*, 2012).

Mutations in the genes of *microtubule-associated protein tau* (*MAPT*), accounting for 50% of familial cases of FTLD (Riedl *et al.*, 2014), and mutations in *progranulin* (*PGRN* or *GRN*), and *Chromosome 9 open reading frame 21* (*C9ORF72*) have been shown as the major causes of FTLD (Sieben *et al.*, 2012, Wang *et al.*, 2013). Minority of cases are caused by mutations in 4 other genes (Table 1): *valosin-containing protein* (*VCP*), *TAR DNA-binding protein 43* (*TARDBP*), *chromatin-modifying protein 2B* (*CHMP2B*), and fused in sarcoma (*FUS*) (Mackenzie *et al.*, 2010, Pan & Chen, 2013, Wang *et al.*, 2013). However, given the incomplete penetrance of such mutations, a large number of cases are apparently sporadic, making more difficult to suspect the presence of a causal mutation (Galimberti & Scarpini, 2012).

Table 1: Genetic factors in inherited FTLD (Adapted from Sieben et al., 2012).

Gene symbol	Chromosomal location	Gene name	Mutation frequency
C9orf72	9p21.2	Chromosome 9 open reading frame 21	14 – 48 %
GRN	17q21.32	Progranulin	3 – 26 %
MAPT	17q21.1	Microtubule-associated protein tau	0 – 50 %
CHMP2B	3p11.2	Charged multivesicular body protein 2B	<1%
VCP	9p13.3	Valosin-containing protein	<1%

In fact, the majority of the FTLD cases are sporadic without a definite known cause (Kalkonde *et al.*, 2012). It is possible that environmental or inflammatory autoimmune factors contribute to the risk of develop this dementia (Kalkonde *et al.*, 2012, Mcmillan *et al.*, 2013). In addition, the first candidate-gene studied in FTLD was the well-known risk factor for late onset sporadic AD, *APOE* (Galimberti & Scarpini, 2012) and an increase in the risk for FTLD has been reported with *APOE* genotypes 2 and 4 contributing to sporadic variant and (*CST3B)* gene in association with PGRN (Kalkonde *et al.*, 2012).

Considering the variability in clinical features and molecular genetics, it is not surprising that the neuropathology associated with FTLD is also heterogeneous (Riedl *et al.*, 2014). Thus, immunohistochemistry analysis allows subcategorization of these spectrum disorders into specific proteinopathies based on the major component of the inclusions, such as FTLD-tau: the cells contain inclusions of hyperphosphorylated tau protein (Mackenzie *et al.*, 2010), FTLD-Ubiquitin (FTLD-U). Over 50% of the FTLD patients present tau-negative ubiquitin staining inclusions. This FTLD-U inclusions were found to be composed of transactive response (TAR) DNA-binding protein 43 kDa (TDP-43), referred as FTLD-TDP, or inclusions of fused in-sarcoma protein (FUS) and TDP-43-negative, referred to as FTLD-FUS (Pan & Chen, 2013). However, in a small number of FTLD-U patients, the inclusion protein remains unclear (Mackenzie *et al.*, 2010); this group is referred as FTLD ubiquitin

proteasome system (FTLD-UPS). There is other type of dementia lacking distinctive histopathology (DLDH) and there are also other rare types, such like dementia with basophilic inclusion body or neuronal intermediate filament inclusion disease (Pan & Chen, 2013).

Moreover, given that the most cases of FTLD have abnormal intracellular accumulation of some disease-specific protein, it has become popular to classify FTLD into broad categories, based on the molecular defect thought to be most abundant, which allows pathological diagnosis (Riedl *et al.*, 2014).

One of the most intriguing issues in the FTLD field is the poor correspondence between neuropathological features and clinical phenotypes. So far, neuropathological characteristics are only predictable in known genetic defects (Borroni & Padovani, 2013). In fact, these genetic causes of FTLD have 100% correspondence between mutations detectable during life and the FTLD pathological substrate (Table 2), only visible at autopsy (Hales & Hu, 2013). In addition, genetic screening for patients with known family history of FTLD or early onset behavior/language disorders can inform the exact FTLD pathological substrate for both diagnosis and enrolment into therapeutic trials (Borroni & Padovani, 2013, Hales & Hu, 2013).

Table 2: Nomenclature for Frontotemporal Lobar Degeneration based on genetics (Adapted from Mackenzie *et al.*, 2010).

Major molecular class	Recognized subtype	Associated gene
FTLD-tau	PiD CBD PSP AGD MSTD NFT-dementia WMT-GGI Unclassifiable	*MAPT*
FTLD-TDP	Types 1-4 Unclassifiable	*GRN* *VCP* *9p* *TARDBP*
FTLD-UPS	FTD-3	*CHMP2B*
FTLD-FUS	aFTLD-U NIFID BIBD	*FUS*
FTLD-ni	Not known	Not known

The pathophysiological mechanisms underlying the remaining 60% to 80% sporadic cases of FTLD are unclear (Wang *et al.*, 2013). One possible theory is that mutations located in mitochondrial DNA (mtDNA) have a key part in neurodegeneration (Grazina *et al.*, 2004, Swerdlow & Khan, 2004).

Primary respiratory chain diseases can be thought as the resulting of mutations in mtDNA or nuclear genes encoding mitochondrial respiratory chain (MRC) subunits. Since mtDNA encodes 13 proteins of the MRC, the mutations of mtDNA usually induce defects of MRC (Schapira, 2012). The mitochondrial theory of ageing proposes that mutations in mtDNA occur throughout life, due to either oxidative damage, with the increase of reactive oxygen species (ROS) production, or to errors of the mtDNA polymerase, and these mutations clonally expand to cause cellular dysfunction (Greaves *et al.*, 2012). Additionally,

the clinical features usually affect particularly tissues in which there is a high metabolic demand, such as the central nervous system, skeletal muscle or heart (Greaves *et al.*, 2012, Leonard & Schapira, 2000).

Mitochondrial dysfunction has been identified in several neurodegenerative disorders like AD (Dimauro & Schon, 2008, Grazina *et al.*, 2006, Grazina *et al.*, 2005), Huntington's disease (Dimauro & Schon, 2008, Lee *et al.*, 2009), Parkinson's disease (Dimauro & Schon, 2008, Federico *et al.*, 2012) and amyotrophic lateral sclerosis (Dimauro & Schon, 2008, Lee *et al.*, 2009), among others.

Evidences indicate that mitochondrial function is affected in AD, including reduction in brain energy metabolism, metabolic enzyme and MRC proteins deficiency (Dimauro & Schon, 2008, Grazina *et al.*, 2006).

As mitochondria play a role in the pathogenesis of several neurodegenerative diseases, it would be reasonable to invoke similar mechanisms for FTLD.

Furthermore, the neuropathological and clinical overlapping of AD and FTLD occur in some patients, such as focal TDP-43 lesions in AD and, conversely, mild AD pathology in FTLD-TDP add further complexity (Hales & Hu, 2013). Thus, mtDNA sequence variations may be an important factor for both diseases (Grazina *et al.*, 2004).

In mitochondrial cytopathies, isolated or combined deficiencies in Complex III of MRC are rare, but mutations in mtDNA gene encoding cytochrome b (*MT-CYB*) account for the most described to date (Schapira, 2012).

According to these references, the aim of this study is sequencing the *MT-CYB* gene in patients diagnosed with FTLD in the scope of a project funded by FCT (PI Professor Manuela Grazina).

Patients and Methods

The DNA samples included in the study belong to 70 patients (31 males and 39 females; mean age: 63 years; range: 38 to 82 years of age) with probable diagnosis of FTLD according to the Diagnostic and Statistical Manual of Mental Disorders – fourth edition (DSM-IV-TR) criteria (APA, 1994) and FTLD was classified following the Lund and Manchester clinical criteria (Groups, 1994) revised by the Work Group on Frontotemporal Dementia and Pick's Disease (Mcknann *et al.*, 2001), and more recently according to the International Behavioural Variant Frontotemporal Dementia Criteria Consortium for bvFTD (Rascovsky *et al.*, 2011). A control population was included, for setting reference values in our sample, composed by individuals without subjective cognitive complaints, whom performed on the normal range for education in the Mini-Mental State Examination (MMSE) and were independent in their instrumental daily life activities. This study had the approval of the Ethics Committee, following the Tenets of the Helsinki Declaration, and informed consent was obtained from all participants. Global cognitive impairment was quantified using the MMSE (Folstein *et al.*, 1975).

The patients were followed at Dementia Consultation of the Neurological Unit of the "Centro Hospitalar e Universitário de Coimbra". The mean age of onset was 58 years, ranging from 34 to 79 years. Taking into account the clinical variants, 60 patients (85.7%) presented bvFTD, while 4 (5.7%) had bvFTD with CBS, other 4 (5.7%) presented avPPA and 2 (2.8%) showed svPPA. A table with patients' characterization can be found in appendix 1.

The arbitrary cut off at 65 years was used to differentiate the early onset (below) from late onset (equal or above) cases.

Total cellular DNA (nuclear plus mitochondrial) was extracted from peripheral blood leukocytes, isolated after erythrocytes lysis using a standard phenol-chloroform method. After amplification of mtDNA fragments with PCR, the automated sequencing analysis was

performed using 3130 ABI Prism sequencing system with BigDye® Terminator Ready Reaction Mix 3.1 (Applied Biosystems) and specific primers (Landsverk *et al.*, 2012) that flanked the gene sequence in order to study *MT-CYB*. The sequences obtained were compared with the human mtDNA revised Cambridge reference sequence (Andrews *et al.*, 1999), obtained from GenBank. The Sequencing Analysis® v.5.4 and SeqScape® v.2.5 softwares (Applied Biosystems) allowed the identification of sequence variations. All variations were classified using the MITOMAP database (http://www.mitomap.org) and the frequency was obtained in the Human Mitochondrial Genome Database and from (Pereira *et al.*, 2009), in order to estimate its frequency in general population.

The next step performed was an *in silico* analysis of the non-synonymous variations using different bioinformatic tools: *Polyphen v. 2.2.2*, which is an automatic tool used to predict the possible impact of an amino acid substitution on the structure and function of the corresponding protein, using information of the sequence, phylogenetic and structural characteristics of the substituted sequence (Adzhubei *et al.*, 2010); *SIFT*, that gives the same type of prediction based on aligned sequences provided by the user, accounting on conservation of the alignment sequences in different species (Kumar *et al.*, 2009); *Mutation Assessor v. 2* that predicts the functional impact of amino acid substitution in protein, based on evolutionary conservation of the affected amino acid in protein homologous (Reva *et al.*, 2011); *Provean* is similar to the former but calculates the mutation score by the average of each sub-family average score (Choi *et al.*, 2012).

When alignment of sequences was necessary, the sequences of 14 different species, from *UniProt*® database, were selected (*Homo sapiens, Gorilla, Pan troglodytes, Pongo pygmaeus, Pan-paniscus, Rattus norvegicus, Mus Musculus, Bos Taurus, Gallus gallus, Xenopus laevis, Danio rerio, Strongylocentrotus purpuratus, Drosophilla melanogaster, Caenorhabditis elegans*). Evolutionary conservation was performed to all sequence variations

using *ClustalW2®*, which is a multiple sequence alignment program that uses seeded guide trees to generate alignments, enabling to visualize the alignment of the selected sequences (Larkin *et al.*, 2007).

The novel sequence variations found were confirmed by a second method, such as Restriction fragment length polymorphism (RFLP) analysis using specific endonucleases: BseMII for m.15073C>T and Alu I for m.15127C>T (Thermo Scientific) following the manufactures' instructions.

A statistical analysis was performed to correlate some variables with the genetic variations founded in *MT-CYB* in different patients. The t-test or Mann-Whitney test, in case of data that did not follow a Gaussian distribution, were used to compare age, age of onset, Clinical Dementia Rating (CDR) staging and the MMSE score of the different groups of patients. Contingency tables were used to compare genetic frequencies according to gender, age of onset (cut off patients with and without variations and <65 and the same with ≥65), MMSE class (defined from the score, with >22=0 and ≤22=1) and clinical outcome, following analysis with Fisher's exact test. The software used for statistical analysis was GraphPad and the result is considered significant when *p* value <0.05 (Motulsky, 1999).

Results

Sequence variations in *MT-CYB* gene were found in 44 patients (Figure 1), from a total of 70 patients. We have found only one variation in 14 patients, 2 variations in 11 patients, 3 variations in 10 patients, 4 variations in 7 patients and 5 variations in 2 patients.
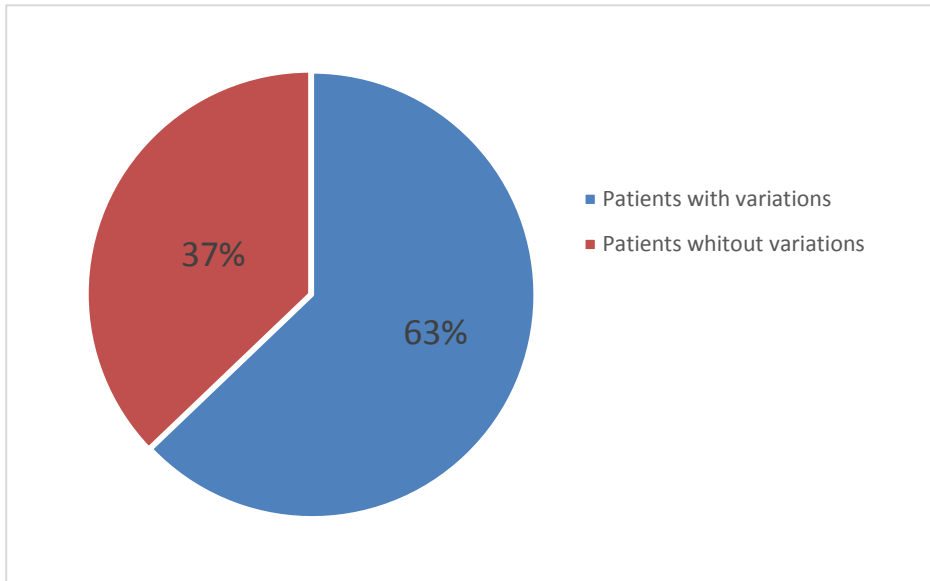
Figure 1: Graphical representation of the percentage of variations found in the patients' population under study.

A total of 37 different sequence variations were found (Figure 2), 15 of these are non-synonymous (41%) while 22 of these are synonymous (59%). Sequence variations are listed in Table 3. All variations were found in homoplasmy.

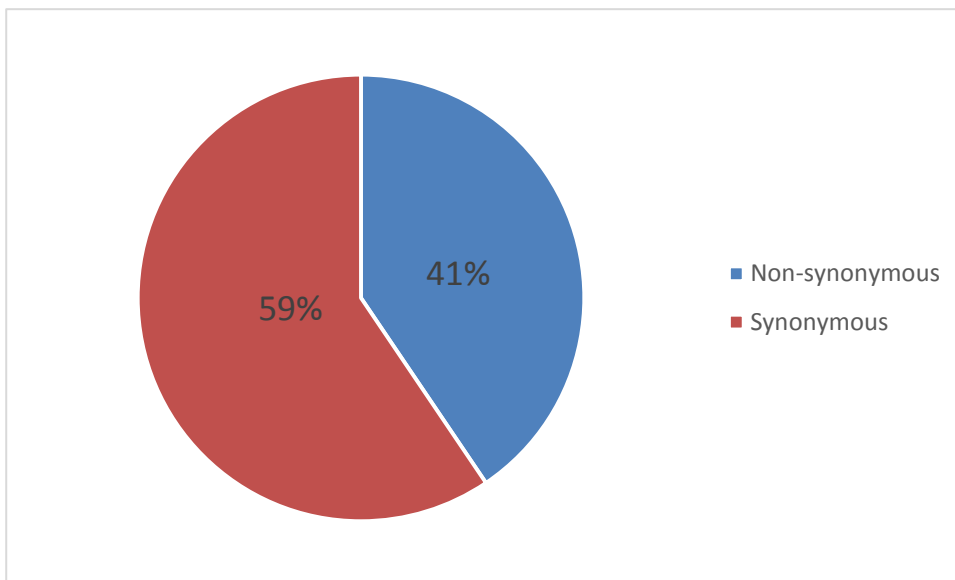**Figure 2:** Graphical representation of the percentage of the type of variations identified in the patients' population under study.

The majority of the variations identified were found in MITOMAP database: 25 have been described as "polymorphism", 10 as "polymorphism" and "somatic mutation". Two sequence variations were not reported in MITOMAP (m.15073C>T; m.15127C>T), being considered as "novel".

Table 3: mtDNA sequence variations of *MT-CYB* gene identified in FTLD patients.

Sequence Variation	Aminoacid Change	Reported in MITOMAP	Frequency in the sample (n=70)	Frequency in published sequences n=5140 (Pereira *et al.*, 2009)
m.14766C>T	Thr7Ile	Polymorphism	0.47	0.80
m.14798T>C	Phe18Leu	Polymorphism	0.16	0.07
m.14866C>T	Synonymous	Polymorphism; Pancreatic cancer cell line	0.03	0.001
m.14869G>A	Synonymous	Polymorphism; Breast tumor	0.01	0.004
m.14872C>T	Synonymous	Polymorphism	0.03	0.006
m.14883C>T	Thr46Ile	Polymorphism	0.01	0.0002
m.14902C>T	Synonymous	Polymorphism	0.01	0.001
m.14905G>A	Synonymous	Polymorphism	0.03	0.04
m.14927A>G	Thr61Ala	Polymorphism	0.01	0.007
m.14979T>C	Ile78Thr	Polymorphism	0.01	0.01
m.15013A>G	Synonymous	Polymorphism; Pituitary adenoma	0.01	NR
m.15043G>A	Synonymous	Polymorphism; MDD-associated	0.04	0.28
m.15073C>T	Synonymous	NOVEL	0.01	NR
m.15097T>C	Synonymous	Polymorphism	0.01	0.0004
m.15127C>T	Synonymous	NOVEL	0.01	NR
m.15164T>C	Phe140Leu	Polymorphism	0.01	0.0002
m.15217G>A	Synonymous	Polymorphism	0.04	0.01
m.15244A>G	Synonymous	Polymorphism	0.01	0.01
m.15257G>A	Asp171Asn	Polymorphism; LHON	0.06	0.007
m.15299T>C	Synonymous	Polymorphism	0.01	0.001
m.15301G>A	Synonymous	Polymorphism; Tumor	0.01	0.37
m.15314G>A	Ala190Thr	Polymorphism	0.03	0.007
m.15452C>A	Leu236Ile	Polymorphism	0.17	0.07
m.15465T>C	Met240Thr	Polymorphism	0.01	0.0002
m.15530T>C	Synonymous	Polymorphism	0.01	0.006
m.15607A>G	Synonymous	Polymorphism	0.03	0.04
m.15616C>T	Synonymous	Polymorphism	0.01	0.0002
m.15626C>T	Synonymous	Polymorphism	0.01	0.002
m.15629T>C	Synonymous	Polymorphism	0.01	0.009
m.15679A>G	Synonymous	Polymorphism	0.01	0.0008
m.15693T>C	Met316Thr	Polymorphism; Possibly LVNC cardiomyopathy-associated; Breast cancer	0.03	0.01
m.15784T>C	Synonymous	Polymorphism; Pancreatic cancer cell line	0.01	0.04
m.15812G>A	Val356Met	Polymorphism; LHON; Breast cancer	0.03	0.005

m.15833C>T	Synonymous	Polymorphism	0.01	0.005
m.15848A>G	Thr368Ala	Polymorphism	0.01	0.001
m.15884G>A	Ala380Thr	Polymorphism	0.01	0.01
m.15884G>C	Ala380Pro	Polymorphism; Pancreatic cancer cell line	0.01	0.01

NR: Not reported in published sequences (Pereira *et al.*, 2009).

The results of *in silico* analysis performed in the non-synonymous variations are presented on Table 4, suggesting that there are 5 alterations with possible impact in protein function. The tests scores that predict an impact in protein function are highlighted.

Table 4: The *in silico* analysis results for the non-synonymous variations found in FTLD patients.

Sequence Variation	Amino acid Change	Poly-Phen 2.2.2		SIFT		Provean		Mutation Assessor		Evolutionary conservation	
		Score	Prediction	Score	Prediction	Score	Prediction	Score	Prediction	Gene	Amino acid
m.14766C>T	Thr7Ile	0	BENIGN	0.24	TOLERATED	-2.35	Neutral	-2.49	Neutral	77%*	36%
m.14798T>C	Phe18Leu	0	BENIGN	1	TOLERATED	-3.09	Neutral	-0.49	Neutral	86%	64%
m.14883C>T	Thr46Ile	0	BENIGN	1	TOLERATED	1.94	Neutral	-1.425	Neutral	29%	29%
m.14927A>G	Thr61Ala	0	BENIGN	0.04	AFECT PROTEIN FUNCTION	-1.93	Neutral	2.95	Medium damage	79%	64%
m.14979T>C	Ile78Thr	0	BENIGN	0.07	TOLERATED	-2.50	Deleterious	2.11	Medium damage	93%	29%
m.15164T>C	Phe140Leu	0.012	BENIGN	0.18	TOLERATED	-4.85	Deleterious	3.42	Medium damage	100%	100%
m.15257G>A	Asp171Asn	0.001	BENIGN	0.45	TOLERATED	-3.56	Deleterious	2	Medium damage	86%	86%
m.15314G>A	Ala190Thr	0	BENIGN	0.35	TOLERATED	-1.67	Neutral	1.12	Low	43%	43%
m.15452C>A	Leu236Ile	0.029	BENIGN	0.55	TOLERATED	-0.79	Neutral	0.68	Neutral	57%	71%
m.15465T>C	Met240Thr	0.987	PROBABLY DAMAGING	0.02	AFECT PROTEIN FUNCTION	0.23	Neutral	0.42	Neutral	86%	57%
m.15693T>C	Met316Thr	0	BENIGN	0.61	TOLERATED	1.04	Neutral	-0.425	Neutral	57%	50%
m.15812G>A	Val356Met	0.004	BENIGN	0.15	TOLERATED	-0.73	Neutral	1.57	Low	57%	57%
m.15848A>G	Thr368Ala	0	BENIGN	0.31	TOLERATED	-0.46	Neutral	0.435	Neutral	86%	29%
m.15884G>A	Ala380Thr	0	BENIGN	1	TOLERATED	-0.01	Neutral	-0.345	Neutral	18%	29%#
m.15884G>C	Ala380Pro	0.167	BENIGN	0.22	TOLERATED	-0.30	Neutral	0.695	Neutral	18%	29%#

* Sequence is not available in all species *(Caenorhabditis elegans)*.

\# Sequence is not available in all species (*Mus Musculus, Bos Taurus, Xenopus laevis, Strongylocentrotus purpuratus, Drosophilla melanogaster, Caenorhabditis elegans*).

The results of the evolutionary conservation analysis for missense alterations, with location of the substituted amino acid position are described in Figure 3.
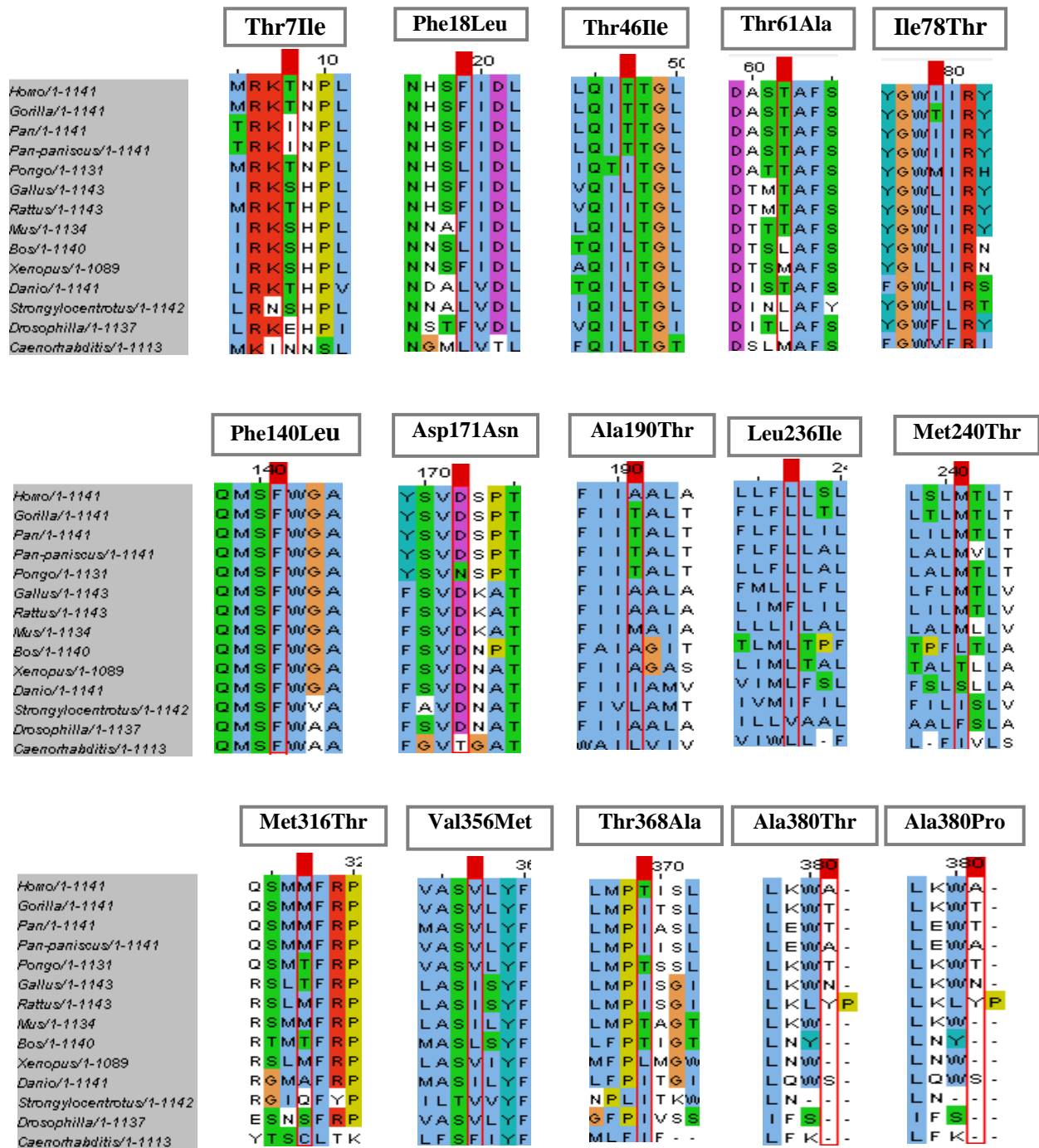

Figure 3: Aligned sequences for the non-synonymous variations in *MT-CYB* gene.

For the synonymous variations identified in FTLD patients, only evolutionary conservation analysis was performed and the results are listed in Table 5. The novel variations found are highlighted in red at the table.

Table 5: Evolutionary conservation performed in synonymous variations.

Sequence Variation	Evolutionary conservation	
	Gene	Amino acid
m.14866C>T	71%	93%
m.14869G>A	7%	100%
m.14872C>T	50%	71%
m.14902C>T	36%	100%
m.14905G>A	21%	93%
m.15013A>G	50%	57%
m.15043G>A	7%	86%
m.15073C>T	50%	43%
m.15097T>C	21%	43%
m.15127C>T	50%	100%
m.15217G>A	23%	100%
m.15244A>G	43%	93%
m.15299T>C	50%	93%
m.15301G>A	14%	93%
m.15530T>C	14%	93%
m.15607A>G	93%	100%
m.15616C>T	21%	100%
m.15626C>T	79%	100%
m.15629T>C	29%	43%
m.15679A>G	79%	100% *
m.15784T>C	29%	100%
m.15833C>T	57%	79%

* Sequence is not available in all species *(Caenorhabditis elegans).*

Statistical analysis was performed to test the correlation according to the presence/absence of *MT-CYB* gene variations and different demographic or clinical variables of the patients (age, age of onset, gender, CDR stage and MMSE class and clinical outcome). The *p* values are shown in Table 6.

Significant results are observed for age (p=0.0214), CDR stage (p=0.0183) and MMSE (p=0.0041).

The correlation between patients who has *MT-CYB* variations and the gender, age of onset (cut off 65 years), MMSE class and clinical outcome, compared to the correlation between patients without alterations and the clinical characteristics referred are represented in Table 7.

The results found were not significant for all variables analyzed (*p* value >0.05).

Table 6: Statistical analysis using t-test or Mann-Whitney test to evaluate if variations in *MT-CYB* affect age of the patients, age of onset of disease, CDR staging or the MMSE score.

	p value	
	T-test	Mann-Whitney
Age	0.0214	
Age of onset	0.0514	
CDR stage		0.0183
MMSE score		0.0041

Table 7: Contingency analysis using Fisher's exact test, sorting the patients by presence or absence of mtDNA variations in *MTCYB* gene and by gender, age of onset, MMSE class and clinical outcome (results not shown).

	p value
Gender	1.0000
Age of onset*	1.0000
MMSE class#	0.0585
Clinical outcome‡	0.4782

* Cut off patients with and without variations and <65 and the same with ≥65.
\# MMSE class is defined from the score, with >22=0 and ≤22=1.
‡ bvFTD versus language variants.

Discussion

In the present study, a higher number of synonymous variations were found, 22 (59%), compared to non-synonymous variations, 15 (41%), as presented in Figure 2. Among synonymous variations, 2 are "NOVEL" (m.15073C>T; m.15127C>T), meaning that they are not reported in MITOMAP database (http://mitomap.org). These variations are present in only 1 patient each one, with a frequency in the sample of 0.01 (Table 3). According to the evolutionary conservation analysis, m.15073C>T affects a nucleotide that is conserved in 50% of the species and an amino acid conserved only in 43% of the species under study. On the other hand, m.15127C>T leads to an alteration of a nucleotide also conserved in 50% of the species; however, the corresponding amino acid is conserved in 100% of the species analyzed, meaning that it could have an important and vital role to the protein structure/ function. According to the "codon usage" database, the triplets that codify the same amino acid are not used with the same frequency; one is always preferred instead of another. In fact,

the frequency of the preference is already achieved, however there is not a clearly explanation for the fact. Concerning m.15073C>T, the frequency of codon usage is affected, from UAC (22.2) to UAU (12.9) and for m.15127C>T the frequency of codon usage is GCC (29.6) to GCU (14.0). The variation results in the choice of a codon that has a lower frequency of use. It could have an impact in the modulation of pathophysiological processes (Nakamura *et al.*, 2000).

A total of 37 different variations were found, 15 of which lead to amino acid change in the mitochondrial CYT-B protein. In order to infer the possible pathogenicity of the variation, *in silico* analysis was performed and the results suggest that the variations that could have an impact in protein function are m.14927A>G, m.14979T>C, m.15164T>C, m.15257G>A, m.15465T>C, however some results of the different software tools are in disagreement (Table 4), which may be explained by the differences in the parameters considered for analysis in each case.

Regarding the variation m.14927A>G, it was found in 1 patient of our cohort (0.01) and it has a frequency of 0.007 (n=36) in published (n=5140) sequences (Pereira *et al.*, 2009). This alteration results in the change of an amino acid threonine, polar, to an isoleucine, nonpolar, in position 7 of the protein, which possible indicates an impact in the protein function; only *SIFT* predicts this possible impact (score 0.04), *Mutation Assessor* predicts medium damage (score 2.95), but the result in *Poly-Phen* and *Provean* is benign. According to evolutionary conservation, the amino acid threonine is not totally conserved (63%). Nevertheless, it is interesting to notice that all the species in which the amino acid is conserved are mammals. In MITOMAP this variation has been reported associated with Parkinson's disease, encephalomyopathy and hypertrophic cardiomyopathy.

The m.14979T>C variation is classified as deleterious by *Provean software* (score -2.50) and *Mutation assessor* predicts (score 2.11) a medium damage effect. However, the

change of a nonpolar isoleucine to a polar threonine, in position 78, is not enough to justify the results obtained in the software analyses. In addition, the amino acid is poorly conserved in other species and it is not likely to have the impact to alter the protein function.

The m.15164T>C is predicted to alter the protein function by *Provean*, classified as deleterious (score 4.85), and by *Mutation Assessor* as medium damage (score 3.42). Concerning the evolutionary conservation, it is highly conserved in nucleotide sequence (100%) and in amino acid (100%). This variation changes an aromatic phenylalanine to a polar leucine on a highly conserved site of the protein, leading to a total modification in its chemical structure. Moreover, taking into account the low frequency in our sample, 0.0003 (n=1) and in published sequences (n=5140), 0.0002 (n=1) (Grazina *et al.*, 2006), it is likely that the alteration has a pathological significance.

Nevertheless m.15257G>A has been reported (Howell *et al.*, 1993) in MITOMAP associated with Leber's hereditary optic neuropathy (LHON), the results suggest that it does not have an impact in protein function. This variation has a frequency of 0.06 (n=4) in our sample, much higher than in published sequences (37 out of 5140, 0.007) (Pereira *et al.*, 2009). According to the present analysis, it appears to have a benign character by *Poly-Phen* and *SIFT*. It is classified as deleterious (score -3.56) by *Provean*, as medium damage by *Mutation Assessor* (score 2) and the amino acid is 86% conserved in other species. However, the change of a polar aspartate to an asparagine (Asp171Asn), which is equally polar, suggests that it should not have major impact in functional outcome.

Another variation is m.15465T>C that changes a polar and sulphureted methionine to a nonpolar threonine in position 240 and it could be a deleterious variation. This hypothesis is supported by the result found in *Poly-Phen* (score 0.987) and *SIFT* (score 0.02), predicting an impact in protein function. According to the evolutionary conservation analysis, this amino acid is highly conserved in mammals. In addition, it reveals a low frequency in our sample,

0.01 (n=1), but much lower in published sequences (n=5140), 0.0002 (n=1) (Pereira *et al.*, 2009), decreasing the possibility of being a polymorphism and corroborates the major impact in protein function. In MITOMAP, it is not reported in association with pathologies.

All the non-synonymous variations were already reported in MITOMAP database; however, only a few are reported in association with pathologies.

On the other hand, it is important to complete the study with the results of statistical analysis. Interesting data emerged from the present study, namely the significant results concerning the analysis of CDR staging (p=0.0183) and MMSE score (p=0.0041), revealing a positive correlation with the presence of *MT-CYB* variations in FTLD patients, which reinforces the hypothesis of genetic variations contributing to the development of FTLD. Since the results are only referred to diagnostic scales, it is likely that those alterations are involved in the pathophysiology of particular disease forms.

A positive correlation between the presence of *MT-CYB* variations and particular patients' characteristics, such as age has also been found. However, it does not have a correspondence to age of onset, which does not allow taking any clearly conclusion. Nevertheless, this hypothesis points towards the existence of factors related to age, but apparently independent of disease onset for this gene.

In order to better understand the significance of the results, it would be interesting the analysis of a study group of healthy age matched subjects, as a control group for the genetic investigation performed.

Furthermore, a functional study of altered complex III and cytochrome b could help to prove the possible pathogenicity of these genetic variations.

On the other hand, it is relevant to mention that the sample was obtained from the peripheral blood leukocytes. Taking into account that the alterations were found in homoplasmy, the results could 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).

Most of the alterations identified have been previously described as polymorphisms. However, we cannot exclude the possibility of these variations being involved in the pathogenesis of FTLD. It should be noted the fact that a set of changes, which, when taken separately, are not pathogenic, but along with other changes, either in other mitochondrial genes, nuclear genes and environmental factors, they could cause MRC dysfunction (Grazina *et al.*, 2006). It is also important to understand the cellular processes that lead to neurodegeneration (Hales & Hu, 2013), which is highly complex.

Recently, remarkable progresses have been made regarding the understanding of the genetic causes, molecular basis, and neuropathological features of FTLD. Most common genetic alterations that cause FTLD have been discovered and the major pathological proteins have been identified (Riedl *et al.*, 2014).

Although significant progress has occurred in diagnosing FTLD, challenges remain in accurately identifying patients with the high sensitivity and specificity at the earliest possible stage. Establishing measures that elucidate the underlying cellular process responsible for specific FTLD pathology will be essential for developing successful therapeutic trials (Hales & Hu, 2013). And variations in mtDNA may play an important role.

In order to reinforce the data obtained in this study is important to carry out further studies that allow us to understand the possible existence of a relationship between mtDNA variations and the pathophysiology of FTLD.

Conclusion

The present study reports new data concerning mtDNA variations in FTLD patients. The results did not show any variation that is clearly pathogenic, but some of them have a

high probability of contributing to alter the normal MRC function, together with other factors, can cause mitochondrial dysfunction, which is a central event previously reported in neurodegeneration. These variations are m.14927A>C; m.15164T>C; m.15465T>C. *In silico* analysis suggest a major impact in protein function, as well as the highly conservation in species and low frequency in patients.

On the other hand, the statistical analysis for evaluating the correlation of patients' characteristics, like CDR staging and MMSE score, with the presence/absence of genetic variants, revealed significant results: a positive correlation between patients that have variations in *MT-CYB* and their lower CDR staging and MMSE scores.

In addition, two "novel" synonymous variations were found that have not been reported in MITOMAP database. It is not clear if they are 100% benign, or if the substitution of a nucleotide, apparently codifying an amino acid that have the same structural characteristics of the original, would functionally remains exactly the same protein?

According to the "Mitochondrial cascade hypothesis" (Swerdlow & Khan, 2004), polymorphic variations in MRC subunits encoding genes lead to MRC efficiency and increase in mitochondrial ROS production, that correlates with mtDNA damage. Furthermore, somatic mtDNA mutations decrease MRC and OXPHOS efficiency, enhancing ROS production.

Thus, changes in mtDNA may affect the age of onset of a particular disease, contributing to the neurodegenerative process, which may be related to the impairment of MRC (Grazina *et al.*, 2006). Additionally, mutations in mtDNA can have a cumulative effect, increasing the probability of developing an energy failure of the MRC (Grazina *et al.*, 2004).

Our study is original and it represents an important contribution to the understanding of this complex disease and to the achievement of successful diagnosis and treatment of these patients.

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Appendix 1: Patients' characterization and data from genetic analysis of *MT-CYB* gene.

Patient	Gender	Age	Diagnostic	Age of onset	CDR stage	MMSE score	mtDNA variations
1	M	73	bvFTD	73	1	28	m.14766C>T m.15257G>A m.15452C>A m.15530T>C m.15812G>A
2	F	77	bvFTD	73	3	10	None
3	F	69	bvFTD	69	1	19	None
4	F	68	bvFTD	66	2	15	None
5	M	64	bvFTD	62	0.5	28	m.14766C>T
6	M	45	bvFTD	40	3	7	None
7	M	75	bvFTD	64	1	27	m.14766C>T m.14798T>C m.15452C>A
8	F	49	avPPA	48	1	30	m.14766C>T m.14798T>C m.15452C>A m.15616C>T
9	F	78	bvFTD	78	1	28	m.14766C>T m.15073C>T
10	M	58	bvFTD	44	3	6	None
11	M	43	CBS	42	1	22	None
12	M	65	bvFTD	63	0.5	29	m.15833C>T
13	M	64	bvFTD	60	1	22	m.14766C>T m.14798T>C m.15452C>A
14	M	71	bvFTD	68	0.5	30	m.14766C>T m.15884G>C
15	M	70	bvFTD	67	1	25	m.14869G>A
16	F	81	bvFTD	75	2	13	None
17	F	77	bvFTD	64	1	21	m.14766C>T m.15043G>A
18	M	43	bvFTD	43	2	16	None
19	F	53	bvFTD	52	3	0	None
20	F	65	bvFTD	63	3	1	None
21	F	54	bvFTD	53	2	18	m.15465T>C
22	M	54	svPPA	52	1	21	m.14766C>T m.14866C>T m.15693T>C
23	F	71	bvFTD	67	3	14	m.14766C>T m.14798T>C m.15127C>T
24	M	51	bvFTD	51	1	25	m.14872C>T

25	M	60	avPPA	58	3	9	m.14766C>T m.14798T>C
26	M	69	bvFTD	54	0.5	28	m.14766C>T m.14798T>C m.15097T>C m.15452C>A
27	F	64	bvFTD	63	1	25	m.14766C>T m.14798T>C m.15626C>T
28	F	65	bvFTD	64	3	11	m.14766C>T m.15314G>A
29	M	46	svPPA	41	3	10	None
30	M	65	bvFTD	63	1	29	m.15164T>C
31	M	61	bvFTD	56	2	12	None
32	M	67	bvFTD	59	1	27	m.14766C>T m.15217G>A
33	M	72	bvFTD	69	2	13	None
34	M	66	bvFTD	63	1	29	m.14766C>T m.15257G>A m.15452C>A m.15679A>G
35	M	68	bvFTD	67	1	27	m.14766C>T m.15257G>A m.15452C>A m.15812G>A
36	M	70	bvFTD	69	1	20	m.14766C>T m.15043G>A
37	F	82	bvFTD	78	1	20	m.14766C>T m.15314G>A
38	F	69	avPPA	56	1	13	m.14766C>T m.14905G>A m.15452C>A m.15607A>G
39	F	62	bvFTD	59	2	17	None
40	F	74	bvFTD	72	1	15	m.14766C>T
41	F	48	bvFTD	47	3	0	m.14883C>T
42	M	64	bvFTD	61	1	24	None
43	M	74	bvFTD	73	2	16	None
44	F	59	bvFTD	53	3	0	m.14872C>T
45	F	59	bvFTD	55	3	0	None
46	F	59	bvFTD	57	1	18	m.15013A>G
47	F	60	bvFTD	56	1	15	None
48	F	65	avPPA	62	3	4	None
49	F	71	bvFTD	68	2	12	m.14766C>T m.14927A>G
50	F	58	CBS	58	1	18	None

51	F	59	bvFTD	58	2	16	m.14766C>T m.15244A>G m.15301G>A m.15629T>C m.15784T>C
52	F	54	bvFTD	54	1	26	None
53	F	55	bvFTD	54	1	22	m.14766C>T m.14798T>C m.15452C>A
54	F	54	bvFTD	50	2	19	m.14766C>T m.14798T>C m.15452C>A
55	F	74	bvFTD	73	1	17	m.14766C>T m.14798T>C m.15217G>A m.15452C>A
56	F	75	bvFTD	72	1	20	None
57	F	62	CBS	60	1	20	None
58	F	49	bvFTD	48	0.5	30	m.14979T>C
59	F	50	bvFTD	50	2	17	m.14766C>T
60	F	74	bvFTD	69	2	15	m.14766C>T m.14866C>T m.15693T>C
61	F	75	bvFTD	74	1	27	None
62	F	46	bvFTD	43	1	22	m.14766C>T m.15884G>A
63	F	81	bvFTD	79	1	21	m.14766C>T m.15043G>A m.15299T>C
64	F	66	bvFTD	60	3	16	m.14766C>T m.14798T>C m.15257G>A
65	M	48	bvFTD	45	2	13	m.15848A>G
66	M	38	bvFTD	34	1	25	None
67	M	54	CBS	53	3	0	m.14902C>T m.15217G>A
68	M	70	bvFTD	68	1	22	m.14766C>T m.14905G>A m.15452C>A m.15607A>G
69	M	76	bvFTD	70	1	28	m.14766C>T
70	M	59	bvFTD	56	1	21	None

Appendix 2: Guide for Authors from "Genes, Brain and Behavior"

Author Guidelines

Last update: July 2014

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