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Nuclear and Mitochondrial Genetic Risk Factors in Frontotemporal Dementia

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SOMMARIO

La Demenza Frontotemporale (FTD) rappresenta, dopo la Malattia di Alzheimer, la forma più comune di demenza degenerativa primaria e costituisce pertanto un importante problema a livello sociale ed economico. Le caratteristiche cliniche salienti della FTD sono le alterazioni del comportamento e/o i disturbi del linguaggio determinati da una degenerazione focale del SNC a livello dei lobi frontale e/o temporale. La patologia può presentarsi in forma sporadica oppure può mostrare aggregazione familiare, o, raramente, si può osservare un modello di eredità autosomico dominante.

La FTD, è una sindrome complessa che presenta quadri clinici e neuropatologici estremamente eterogenei che spesso si sovrappongono ad altri disordini neurodegenerativi. Tale eterogeneità fenotipica, dovuta in parte ad una eterogeneità genetica, costituisce uno dei maggiori problemi nella identificazione dei fattori genetici coinvolti nella sua patogenesi.

Obiettivo del presente studio è stato quello di analizzare la possibile associazione tra la FTD e la variabilità di due geni nucleari, *TAU* e *APOE* e del genoma mitocondriale (mtDNA).

Per superare le difficoltà relative alla eterogeneità della FTD, il nostro studio si è incentrato su una attenta selezione dei casi (pazienti FTD) e dei controlli , reclutati in una popolazione geneticamente omogenea (Calabria) e su una rigorosa applicazione di criteri diagnostici internazionali.

Sono stati dunque analizzati 114 pazienti con FTD, di cui 68 sporadici and 46 familiari, e un gruppo di 180 controlli.

I dati ottenuti dalla nostra analisi hanno dimostrato l'assenza di associazione significativa fra aplotipi H1/H2 del gene *TAU* e la FTD. Al contrario, lo studio della variabilità del gene *APOE* nella suscettibilità alla FTD ha stabilito che l'allele ε4 costituisce fattore genetico di rischio per la FTD (O.R.= 2.68 with 95% CI=1.51–4.76; p=0.001), mentre l'allele ε2 costituisce fattore di protezione (O.R.= 0.28 with 95% CI =0.12–0.66; p=0.003). Inoltre, è risultato che l'aplotipo H1 del gene *TAU* può aumentare l'effetto protettivo conferito dall'allele ε2 (O.R.= 0.31 with 95% CI = 0.13–0.73; p=0.007). I risultati di questo studio sono già stati oggetto di pubblicazione (Bernardi et al., 2006). Un breve

commento al lavoro pubblicato è stato inserito nella sezione "Results and Discussion" a pag. 35. Copia del lavoro è stata inserita nella "End Section" del presente manoscritto.

Nella seconda parte del nostro studio, abbiamo voluto investigare se la variabilità ereditaria del DNA mitocondriale (aplogruppi/sotto-aplogruppi) fosse associata alla FTD.

Dalla suddetta analisi non è emersa nessuna differenza statisticamente significativa nella distribuzione delle frequenze degli aplogruppi/sotto-aplogruppi del mtDNA tra pazienti FTD sporadici o familiari e controlli.

Tuttavia, questi risultati non escludono che un aplotipo del mtDNA possa influenzare la suscettibilità alla FTD. Infatti, sebbene il nostro campione sia, a nostra conoscenza, il più numeroso finora analizzato, non possiamo comunque escludere che la dimensione del campione sia insufficiente nel rivelare un aplotipo raro responsabile delle ipotetiche differenze.

Nel complesso, i risultati conseguiti nel presente studio possono essere riassunti come segue:

i) La variabilità del gene *APOE* è associata alla FTD, confermando un ruolo generale di tale variabilità nelle patologie neurodegenerative;

ii) La FTD, sia nella forma sporadica che in quella familiare, si presenta senza un coinvolgimento della variabilità del gene *TAU*.

iii) La variabilità ereditaria del mtDNA non risulta associata alla FTD, per lo meno nella popolazione Calabrese.

SUMMARY

Frontotemporal dementia (FTD) is the second most common form of degenerative dementia after Alzheimer's Disease (AD) thus having an important impact on socio-economic level. The clinical hallmarks of FTD are behaviour and/or language dysfunction caused by a focal degeneration mainly affecting the frontal and temporal brain regions. The disease can be sporadic or showing a familial aggregation, or, more rarely, presenting with an autosomal dominant pattern of inheritance. Characteristic of the disease is a phenotypic complexity which often shows overlapping features with other neurodegenerative diseases. The phenotypic heterogeneity, which is at least in part due to genetic heterogeneity, is the major cause of the difficulties in disentangling genetic factors affecting susceptibility to FTD.

Aim of the present study was to explore the association between FTD and variability of two nuclear genes, *TAU* and *APOE*, and of the mitochondrial genome. To overcome the problems of FTD heterogeneity, we paid a special attention to the recruitment of the cases (as well as of the controls) who were collected in a genetically homogeneous population (Calabria, southern Italy) and with a strict application of international diagnostic criteria. A total of 114 FTD cases (68 sporadic and 46 familial) *plus* 180 controls were analysed

The study failed to reveal association between *TAU* haplotypes (H1 and H2) and FTD. On the contrary, we found that *APOE* ε4 allele increased FTD risk (O.R.= 2.68 with 95% CI=1.51–4.76; p=0.001), while a protective effect was associated with the *APOE* ε2 allele (O.R.= 0.28 with 95% CI =0.12–0.66; p=0.003). Moreover, the protective effect of ε2 was increased by the H1 haplotype (O.R.= 0.31 with 95% CI = 0.13–0.73; p=0.007). Results of this part of the study have been already published (Bernardi et al., 2006). A brief comment to the paper is shown in the "Results and Discussion" section at page 35 and the reprint is annexed in the "End Section" of the present manuscript.

In the second part of the study, we evaluated whether mtDNA inherited variability (haplogroups/sub-haplogroups) was associated with FTD. No statistically significant difference in the frequency distributions of mtDNA haplogroups/sub-haplogroups was found between sporadic or familial FTD patients and controls. However, the results do not exclude that a rare haplotype could affect susceptibility to FTD. Really, although the

sample we analyzed is, at our knowledge, one of the greatest screened till now, we cannot exclude that the sample size could be unable to reveal a rare mtDNA type responsible for hypothetical differences.

On the whole, results we pursued in the present study can be summarized as follows.

i) *APOE* variability is associated with FTD, and this confirms a general role of *APOE* variability in neurodegenerative diseases;

ii) Both sporadic and familial FTD can occur without involvement of *TAU* variability;

iii) MtDNA inherited variability is not associated with FTD, at least in the Calabrian population.

LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
APOE	Apolipoprotein-E gene
ApoE	Apolipoprotein-E gene product
ATP	Adenosine Triphosphate
bp	base pair
CR	Control Region
D-loop	Displacement loop
FTD	Frontotemporal Dementia
FTD-MND	Frontotemporal lobar degeneration with motor neuron disease
FTDP-17	Frontotemporal dementia with parkinsonism linked to chromosome 17
Hg	haplogroup
HVS	hypervariable segment
MAPT	Microtubule-Associated Protein tau gene
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
NFT_S	Neurofibrillary Tangles
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association Work Group
nt	nucleotide
OXPHOS	Oxidative Phosphorylation
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
rCRS	revised Cambridge Reference Sequence
RFLP	Restriction fragment lenght polymorphism
ROS	Reactive Oxygen Species
SE	Standard Error
subHg	Subhaplogroup
TBE	Tris Borato EDTA

1.INTRODUCTION

Advances in medicine and improvements in nutrition have contributed to increase lifespan of human populations across the world, but a consistent prevalence of age-related cognitive disorders and dementing illnesses paralleled such an increase. Indeed, common neurodegenerative disorders affect million of people and represent a considerable drain of health-care resources (Mayeux , 2003).

The most common cause of dementia is Alzheimer's Disease (AD) which involves deterioration of cognitive functioning with prominent impairment in memory. Frontotemporal Dementia (FTD) is another frequent cause of dementia, which is characterized by behavioural disorders as well as cognition.

1.1 Frontotemporal Dementia (FTD)

Frontotemporal dementia (FTD) is a primary neurodegenerative dementia, meaning that a gradual loss of neurons is responsible for the progressive brain dysfunction. FTD is the second most common form of presenile dementia after AD (Dermaut et al., 2005), accounting for approximatively 20% of cases (Knopman et al., 1990; Talbot, 1997).

The hallmark of FTD is a gradual, progressive decline in behavior and/or language caused by a focal degeneration mainly affecting the frontal and temporal brain regions, (Lund & Manchester Groups, 1994; Neary et al., 1998; McKhann et al., 2001; Lee et al., 2001), (Fig. 1).

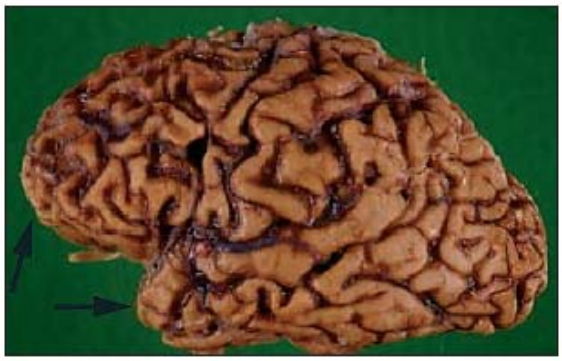

Fig.1. Brain of a patient with frontotemporal dementia showing atrophy of the frontal and anterior temporal lobes *(Neary et al., 2005).*

From a clinical point of view there is an early change in personal and social behaviour with relatively preservation of memory often associated with disinhibition. FTD patients also display a language deterioration that progresses to mutism.

The clinical features can be complicated by neurological signs, such as motor neuron signs and/or parkinsonism (Sjogren & Wallin, 2001).

The onset of FTD symptoms typically occurs in the middle years of life (45–65 years); nevertheless it has been seen before 30 and later than 80 years of age (Snowden et al., 1996; Ratnavalli et al., 2002; Rosso et al., 2003). FTD occurs equally in men and women and the average duration of the disease has been estimated in about 8 years (Snowden et al., 2002).

The majority of FTD cases are sporadic, however, a positive family history is present in approximately 10%-50% of patients (Knopman et al., 1990; Stevens et al., 1998; Houlden et al., 1999**;** Rizzu et al., 1999; Morris et al., 2001; Poorkaj et al., 2001). Only a subset of familial cases exhibits an autosomal dominant pattern of inheritance (Chow et al., 1999; Poorkaj et al., 2001).

Diagnosis is generally made according to international criteria that allow to differentiate among different types of dementia and is based on clinicopathological features, namely, peculiar clinical profiles in combination with characteristic brain lesions (Graeber et al., 1997; Forstl, 1999; Duckett & Stern, 1999; McKhann et al., 2001).

Due to a great variability in the clinical phenotype, FTD often mimics other dementing disorders; as a result, FTD has been frequently misdiagnosed primarily with Alzheimer's Disease (AD) (Neary et al., 1988; Brun & Gustafson, 1999).

The distinction between AD and FTD can be difficult, especially in presenile cases, which often have an atypical presentation; moreover, the NINCDS-ADRDA criteria (McKhann et al., 1984), widely utilized for Alzheimer Disease (AD), do not accurately differentiate FTD. Clinical diagnostic criteria, introduced by the Lund-Manchester group (Brun et al., 1994), and following revisions (Foster et al., 1997; McKhann et al., 2001) have been established for FTD; consequently, starting from the past decade, FTD has been increasingly recognized.

The combination of clinical observations, neurological examination, neuropsychological tests, information gained from laboratory investigations and morphological and functional brain imaging (e.g.CT, MRI, SPECT) are necessary to achieve accuracy of clinical

diagnosis. Finally, the diagnosis of FTD should be based on neuropathological examination, that only definitively differentiates from other types of dementia.

Several classification systems for FTD have been proposed and an increasing number of disorders and phenotypes has been included into FTD. Based on recent criteria (McKhann et al., 2001), FTD corresponds to a broad dementing syndrome comprising a group of related conditions sharing many clinical features and resulting from progressive degeneration of the temporal and frontal lobes of the brain.

These related phenotypes include Pick's disease (PiD), frontotemporal lobar degeneration (FTLD), frontotemporal lobar degeneration with motor neuron disease (FTD-MND), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), and neurofibrillary tangle dementia (Brun et al., 1994; McKhann et al., 2001).

Therefore, substantial heterogeneity exists among FTD phenotypes.

1.2 Pathological hallmarks

FTD presents with different neuropathological abnormalities (Lund & Manchester Group, 1994; McKhann et al., 2001). Although some findings are specific to one or two of the FTD subtypes, there is a general profile of FTD brain pathology that includes macroscopically atrophy or shrinkage of the frontal and temporal lobes. At microscopic level neuronal loss, gliosis, spongiosis and cellular inclusions are present. For instance, affected neurons frequently display intracellular inclusions, named neurofibrillary tangles (NFTs), primarily composed of hyperphosphorylated tau protein (Spillantini et al., 2000) (Fig. 2).

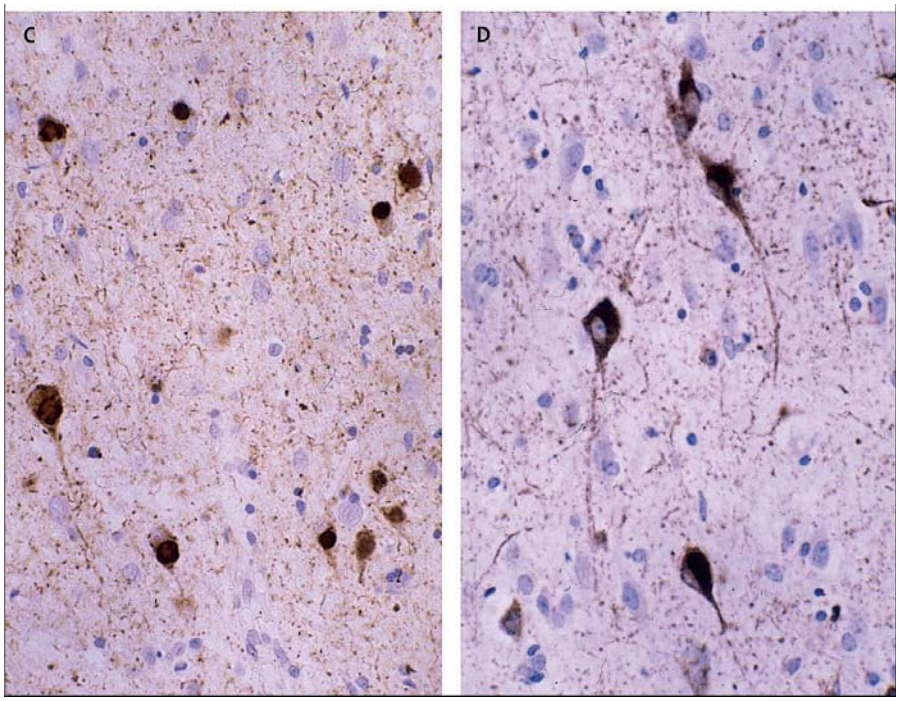

Fig 2. Neurofibrillary tangles in patient with frontotemporal dementia and parkinsonism associated with chromosome 17 (FTDP-17) (Neary et al., 2005)

However, based on immunohistochemical staining and the pattern of intracellular inclusions, three predominant patterns of histopathological changes have been recognized (Brun, 1987; Mann et al., 1993; Neary et al., 1993; Hodges et al., 2004) and can be summarized as follows:

- Tau-immunopositive neuronal inclusions sometimes associated with balloned neurons and/or argentophilic Pick bodies.
- Tau-negative, ubiquitin-positive inclusions and evidence of motor neuron disease (MND-type)
- No tau- or ubiquitin-positive intraneuronal inclusions or pathology (Dementia lacking distinctive histology, DLDH)

Furthermore, a very recent study showed that, in absence of tau pathology, neuronal inclusions can be composed of hyperphosphorylated, ubiquitinated and cleaved form of TAR DNA binding protein (TDP43) (Neumann et al., 2006).

Nevertheless, there is no correlation between a particular clinical phenotype and the underlying histological changes (Hodges et al., 2004; Taniguchi et al., 2004). As an example, Grimes et al (1999) described a patient with the typical clinical features of Cortico Basal Degeneration (CBD) and the histological features of FTD-MND.

In addition, a significant heterogeneity among clinical and neuropathological patterns exists and a coexistence of Pick's disease and CBD has also been reported within the same family (Bugiani et al., 1999).

The great clinical and neuropathological heterogeneity suggests the existence of several genetic factors underlying or modifying the pathogenesis of this prevalent and untreatable disorder.

1.3 Genetic factors

The occurrence of a family history of FTD in about 10%-50% of cases suggests a genetic background underlying the aetiology of this disease.

However, the genetic contribution to FTD has been identified only in a small fraction of the cases. A subset of autosomal dominant FTD cases presenting with parkinsonism has been linked to chromosome 17 (FTDP-17) (Wilhelmsen et al., 1994) and pathogenic mutations in the gene coding for microtubule-associated-protein tau (*TAU* gene) have been identified segregating with FTDP-17 (Clark et al 1998; Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998)

1.3.1 TAU gene

The human *TAU* gene or Microtubule-Associated-Protein tau gene *(MAPT)* (OMIM, NM 016841) is located at chromosome 17q21-22 (Hutton et al., 1998) where it spans over 100 kb and contains 16 exons (Andreadis et al., 1992) (Fig.3).

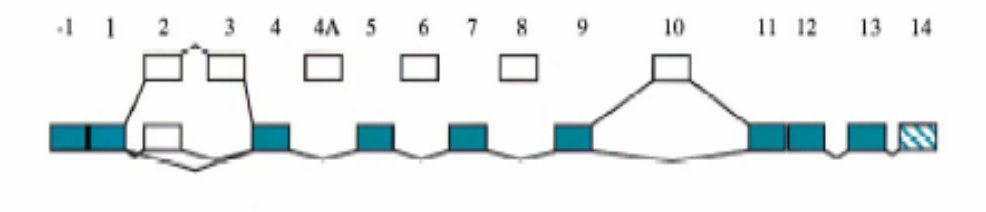

Fig. 3. Schematic representation of the *TAU* gene. Alternatively spliced exons 2,3 and 10 are shown above the constitutive exons. Exons 4A, 6 and 8 are generally not spliced into human tau mRNA and most transcripts retain the intron between exons 13 and 14 (van Slegtenhorst et al., 2000).

Tau protein

Tau is one of the most abundant axonal microtubule (MT)-associated proteins having a major role in both assembly of tubulin into microtubules and stability of microtubules (Garcia & Cleveland, 2001).

Exons 2, 3 and 10 of the *TAU* gene are alternatively spliced yielding to six different tau mRNAs corresponding to six different protein isoforms ranging from 48 to 67 kDa (Goedert et al., 1989; van Slegtenhorst et al., 2000). In particular, the alternative splicing of exon 10 generates tau isoforms containing either three binding repeat domains (3R; exon

10-) or four binding repeat domains (4R; exon 10+) of 31 or 32 amino acids at the C-terminus (Goedert & Jakes, 1990; van Slegtenhorst et al., 2000). These repeats constitute the microtubule binding domains of the protein (Goedert et al., 1989) (Fig.4). *TAU* exon 10 splicing is also developmentally regulated, so that only 3R tau is present in human fetal brain, while the 3R/4R ratio is approximately 1/1 in adult brain (Goedert et al., 1989).
Moreover, alternative splicing of exons 2 and 3 leads to the absence (0N) or presence(1N and 2N) of inserts of 29 residues or 58 residues close to the N-terminus of tau proteins (van Slegtenhorst et al., 2000). Thus the longest tau isoform of 441 residues has four repeats and two inserts, while the shortest isoform of 352 residues has three repeats and no insert (Fig. 4). Finally, *TAU* exon 4A is alternatively spliced yielding to other tau isoforms in peripheral neurons, as well as in other non-brain tissues (Goedert et al., 1992; Ingelsson et al., 1996).

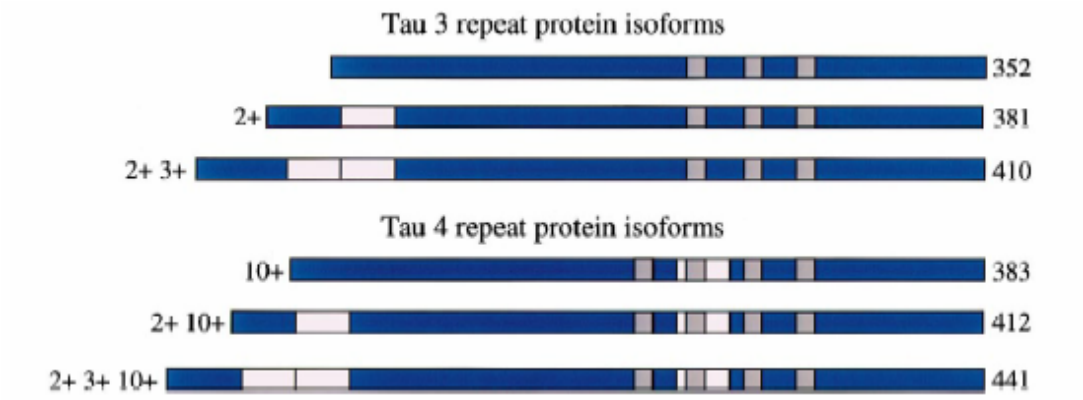

Fig. 4. The six tau protein isoforms in human brain. Alternatively spliced exons 2, 3 and 10 (pink boxes). Exon 9–12 encode microtubule binding repeats (grey boxes). Alternative splicing of exon 10 gives rise to Tau isoforms with 4 binding repeats (4R; exon 10 +) or 3 binding repeats (3R; exon 10-) (van Slegtenhorst et. al., 2000).

All six tau protein isoforms are required for normal axonal function, with microtubule binding being regulated by a number of binding repeats and phosphorylation state of the protein (Buée et al., 2000; Forman et al., 2000; Lee et al., 2001).

TAU gene mutations

To date, two categories of *TAU* gene mutations have been described (Lee et al., 2001; Ingram & Spillantini, 2002).

- Missense mutations (exons 1, 9, 10, 11, 12, and 13) affecting microtubule affinity;

- Splice site mutations (intron 10 close to the 5' splice site) altering the alternative splicing.

Missense mutations, located within or around the microtubule binding domains, alter binding properties of the tau protein leading to microtubule destabilization and increase of cytosolic tau. Splice site mutations alter the splicing of exon 10 thus producing an increase in the levels of 4R tau isoforms and affecting the 3R:4R ratio (Fig. 5).

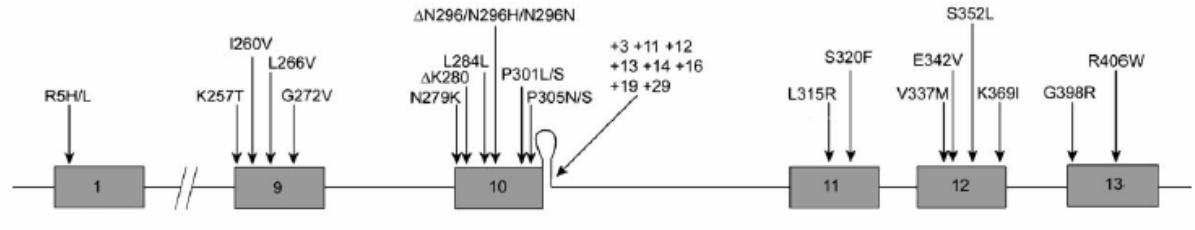

Fig. 5 Mutations in the TAU gene associated with FTDP-17. Exons 1 and 9–13 of the *TAU* gene are shown with their mutations. A predicted stem-loop structure in the pre-mRNA, found at the boundary between exon 10 and the following intron, is also shown. Twenty missense mutations, two deletion mutations, two silent mutations, and eight intronic mutations are shown (Brandt et al., 2005).

Potential pathogenic mechanisms of TAU mutations

Analysis of *TAU* gene mutations showed a variety of effects on the biology and function of the protein, and several molecular mechanisms might explain tau-mediated neuronal cell death (Hutton et al., 1998; Spillantini et al., 1998b; Lee et al., 2001).

Interestingly, almost all mutations are clustered in the known functional region or the C-terminus of the protein (exon 9-13) (Goedert et al. 1998) and can affect both the affinity of tau for microtubules and the binding capacity (Hong et al., 1998).

One potential pathogenic mechanism is a "loss-of-function" effect: tau regulates microtubule dynamics improperly, leading to under- or overstabilized microtubules that cannot perform their normal essential cellular functions thus leading to cell death. A second potential pathogenic effect of these mutations is a "gain-of-toxic function": tau dissociates from microtubles and aggregates into abnormal, cytotoxic neurofibrillary tangles.

Furthermore, some *TAU* gene mutations may have a double pathogenic mechanism: tau dissociates from microtubules and aggregates into abnormal neurofibrillary tangles. The

sequestration of tau into these aggregates results in tau decrease, leading to over-active microtubles that cannot perform their essential functions, thus leading to cell death (Fig. 6).

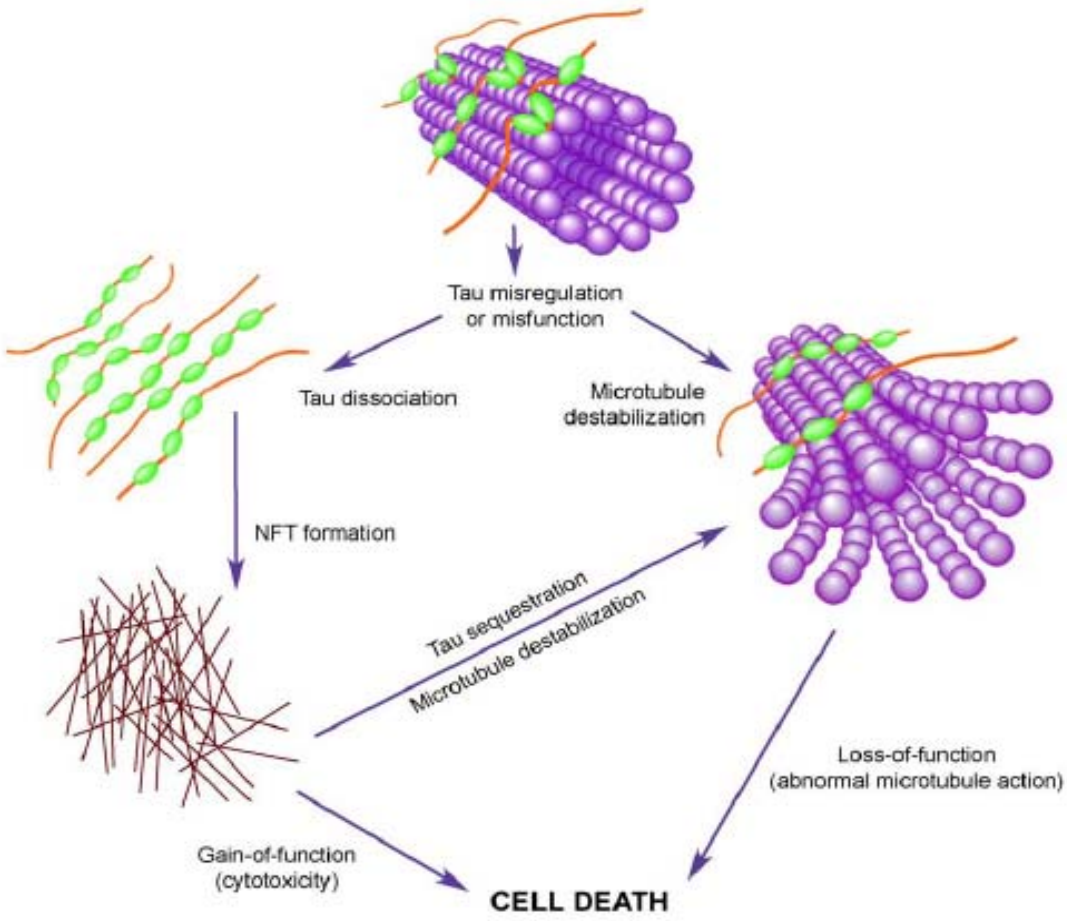

Fig. 6. Three possible pathways of tau-mediated neuronal cell death.
Pathway 1— Loss-of-function model: tau misfunction, yielding to microtubules misregulation and destabilization, severely impinges the intraneuronal transport system leading to cell death.
Pathway 2— Gain-of-function model: tau dissociates from microtubules and tends to polymerise into insoluble, cytotoxic neurofibrillary tangles (NFT).
Pathway 3—This model incorporates elements of Pathways 1 and 2: This double pathogenic mechanism involves initially a reduction of tau to bind microtubules and subsequently an increasing tendency of the unbound tau to form insoluble filaments (Feinstein et al., 2005).

The presence of various mutations and different pathogenic mechanisms might explain the existence of the wide range of clinical and neuropathological features observed in FTD. Nevertheless, although some *TAU* mutations show similar phenotypes, in many cases clinical heterogeneity is observed also when the same *TAU* mutation occurs, suggesting that additional genetic or epigenetic factors influence the phenotypic manifestations of FTD (van Swieten et al., 1999; Reed et al., 2001; Bird et al., 1999; Bugiani et al., 1999).

Thus, although alteration in *TAU* are sufficient to cause neurodegeneration, the causal link between tau molecule and clinical symptoms is not yet clear.

On the whole, the discovery of mutations in *TAU* gene has been helpful to shed light on FTD and other neurodegenerative diseases named "tauopathies" (e.g. AD; PSP; CBD).

However, although *TAU* gene likely represents the first and most obvious candidate in the puzzle of FTD genetics, the frequency of *TAU*-gene mutations is low in the whole FTD case records (Houlden et al., 1999a; Froelich Fabre et al., 2001). Furthermore, in FTD cases with autosomal dominant inheritance patterns, *TAU* mutations account for 7%-50% of the cases (Rosso et al., 2002; Hutton et al., 1998; Morris et al. 2001). The absence of *TAU* mutations or abnormality in the tau protein in many familial FTD and in the majority of sporadic FTD cases clearly indicates that other causes exist for the development of FTD (Tolnay et al., 2002).

Currently, efforts are focused on identifying other genes and risk factors implicated in FTD pathological phenotypes.

TAU Haplotypes

In addition to mutations, a series of polymorphisms, which are in complete linkage disequilibrium, have been described in the *TAU* gene, mostly inherited as two distinct haplotypes designated H1 and H2 (Baker et al., 1999).

Genetic association studies showed that the H1H1 *TAU* genotype has a greater frequency in patients showing corticobasal degeneration (CBD) or progressive supranuclear palsy (PSP) than in control subjects, thus suggesting that this genotype may increase susceptibility to these neurodegenerative disorders (Baker et al., 1999; Verpillat et al., 2002). The mechanism for this association is not clear because *TAU* risk haplotype (H1) is also common in the general population. In fact, association studies in different populations showed conflicting results (Hughes et al., 2003; Morris et al., 2002; Verpillat et al., 2002a; Sobrido et al., 2003).

Furthermore, a recent meta-analysis showed that H1 haplotype is also frequently associated with Parkinson's Disease (PD) (Healy et al., 2004) thus suggesting common and uncharacterized tau-related pathogenic mechanisms shared by PD and FTD.

Other genetic factors involved in FTD.

FTD is a genetically heterogeneous disease and genetic linkage to other chromosomes have been described in addition to linkage to chromosome 17.

In particular, a syndrome of FTD coupled with familial amyotrophic lateral sclerosis (ALS) has been linked to chromosomes 9, even if no gene mutation in this candidate region has been identified yet (Hosler et al., 2000; Wilhelmsen et al., 2004).

Moreover, linkage to chromosome 3p11–12 has been reported in a Danish family showing FTD with frontotemporal atrophy, neuronal loss, and gliosis (Brown et al., 1995). Unexpectedly, the recent finding that mutations in the gene coding for Charged Multivesicular Body Protein 2B (*CHMBP2B*) on chromosome 3 cause FTD (Skibinski et al., 2005) provides a link between dysfunction in endosomal trafficking or lysosomal activity and neuronal degeneration.

Interestingly, recent reports (Baker et al., 2006; Cruts et al., 2006) showed that some cases of FTD are caused by mutations in progranulin (*PGRN*) gene on chromosome 17, which encodes for a widely expressed secreted glycoprotein involved in development, wound repair, inflammation and neuronal survival. FTD patients lacking tau-pathology and with *PGRN* gene mutations showed neuronal inclusions of ubiquitinated TDP43 (Neumann et al., 2006).

In a large Italian kindred segregating FTD, in which ambiguous linkage to the 17q21 locus and absence of *TAU* gene mutations were reported (Curcio et al., 2002), a *PGRN* gene mutation has been recently identified as cause of FTD (personal communication).

1.3.2 APOE gene

Following the identification of *TAU* gene mutations in familial forms of FTD, there was an increasing interest on genetic factors that may predispose to the disease.

The human Apolipoprotein E gene (*APOE*) emerged as the major genetic susceptibility factor for AD in many populations (Kamboh, 2004) accounting for approximately half of late onset familial and sporadic AD cases (Corder et al., 1993; Kehoe et al., 1999). Furthermore, it has been suggested that the *APOE* polymorphism may contribute, with different alleles, to increase the risk of other neurodegenerative disorders such as PD and FTD (Smith J.D., 2000; Verpillat et al., 2002a).

The *APOE* gene (OMIM, NM 107741) is located on the long arm of chromosome 19, in the 19q13.2 region, where it spans over 3.7 kb. The gene contains 4 exons and encodes for a 34 kDa glycoprotein (apoE) of 299 amino acid. The major function of the protein is to redistribute lipids and to participate to cholesterol homeostasis.

The three common alleles of *APOE* locus, termed ε2, ε3 and ε4, have frequencies of 7-8%, 77-78% and 14-16%, in the Caucasian population, respectively. These alleles encode the apoE protein isoforms E2, E3, E4.

ApoE protein

ApoE plays a fundamental role in lipid binding and transport function for normal lipid homeostasis, in the maintenance and repair of injured neurons, maintaining synapto-dendritic connections, and scavenging toxins (Rocchi et al., 2003). It is synthesized mainly by liver, neurons and astrocytes in the brain, and also by macrophages and monocytes (Siest et al., 1995). ApoE synthesis in the Central Nervous System (CNS) increases dramatically after injury (Weisgraber & Mahley, 1996).

The three apoE protein isoforms differ for aminoacids in position 112 or 158, where E3 contains cysteine and arginine (Cys-112 and Arg-158), E2 contains cysteine (Cys-112 and Cys-158) and E4 arginine in both positions (Arg-112 and Arg-158). These small differences cause significant differences in the three dimensional structure and functional properties of the protein (Mahley, 2000).

The three apoE isoforms have specific properties and differ in their ability to carry out critical functions of the apoE: E3 and E2 are effective in maintaining and repairing neuronal cells; conversely, E4 is less efficient in this process (Michikawa et al., 2000). Additionally, the amino acid substitutions in E4, result in a structure that causes this isoform to bind preferentially very low density lipoproteins (VLDL), while E3 and E2 bind preferentially high-density lipoproteins (HDL) (Beffert et al., 1998).

On the whole, the differences among the three apoE isoforms have special relevance in several diseases, such as cardiovascular disease, and in a variety of neurodegenerative disorders including AD, Lewy Body Disease and frontal lobe dementia (for a review: Kamboh et al., 2004). Insights on the role of apoE isoforms in neuropathology came from *in vitro* studies demonstrating that the E2 and E3 isoforms bind more readily tau, as compared to E4, and thereby prevent abnormal tau hyperphosphorylation and destabilization of the neuronal cytoskeleton (Strittmatter et al., 1994). In addition, E4

decreases neuronal extension and branching, and inhibits the ability of tau to stabilize microtubules thus inducing their collapse *in vitro* (DeMattos, 1998). However, the molecular interaction between apoE and tau molecules, and consequent damage to the neuronal cytoskeleton, remains to be clarified.

The *APOE* gene has been proposed as possible risk factor also for FTD. However, contrastant results have been presented for association between *APOE* and FTD. Some studies showed an increase of ε4 allele frequency in FTD and dose effect on age at onset (Farrer et al., 1995; Stevens et al., 1997), whereas other studies failed to replicate the results (Minthon et al., 1997; Geschwind et al., 1998; Houlden et al., 1999b).

A meta-analysis on all published data detected a significant increase of ε2-allele frequency in FTD patients (Verpillat et al. 2002b).

Moreover, few studies reported an interaction between TAU haplotypes and APOE gene in FTD. Ingelson and colleagues demonstrated, in a group of FTD sporadic patients, that H1 haplotype in combination with APOE ε4 allele increased the risk of FTD (Ingelson et al., 2001). Also the interactive effect between H1 haplotype and APOE ε2 allele has been reported to increase the risk of FTD (Verpillat et al., 2002b).

1.3.3 MtDNA and neurodegenerative disorders.

Several lines of evidence suggest that mitochondrial dysfunction may play an important role in the pathogenesis of common neurodegenerative diseases with multifactorial aetiology. It is indeed recognized that mitochondrial defects contribute to the pathogenesis of both AD and PD (Bonilla et al., 1999; Leonard et al., 2000). Mitochondria, which are cytoplasmic and semi-autonomously functioning organelles, have a fundamental role in cellular energy metabolism. This includes, among others, the fatty acid β-oxidation, the citric acid cycle and the oxidative phosphorylation (OXPHOS), the essential respiratory pathway for adenosine triphosphate (ATP) production.

As mitochondria are the major site of energy production in the cell, generating approximately 90% of cellular ATP (Wallace, 1997), it is not surprising that an energy dependant tissue such as brain may be affected by mitochondrial dysfunction.

OXPHOS

The mitochondrial respiratory chain involves a group of five enzyme complexes (I–V) situated on the inner mitochondrial membrane. Each complex is composed by multiple subunits, the largest being complex I with 46 polypeptide components. The function of complexes I–IV is the electron (e-) transfer that generates the proton gradient across the inner mitochondrial membrane; this proton flow is harnessed by complex V to synthesise ATP from adenosine diphosphate (ADP) and inorganic phosphate (Fig.7).

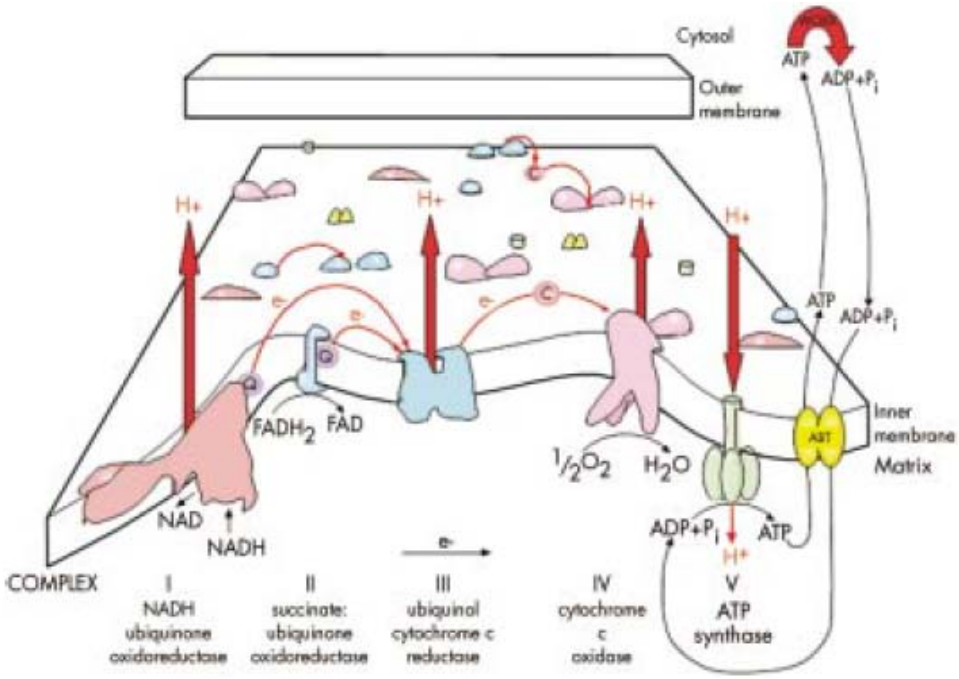

Fig. 7: ***Schematic diagram of the respiratory chain.***
Electrons are passed down the chain and protons are pumped from the matrix to the space between the inner and outer mitochondrial membranes. This creates an electrochemical gradient (membrane potential) which drives ATP production via ATPase (complex V) (Chinnery & Schon, 2003).

mtDNA

Mitochondria have their own DNA and the respiratory chain subunits of the OXPHOS system are encoded both by nuclear and mitochondrial genome (mtDNA) which is present in hundreds to thousands of copies in every cell.

Human mtDNA is a small circular double-stranded molecule, 16,569 base pairs (bp) in length, that codes for 13 subunits of the oxidative phosphorylation system and includes 7 of the 46 polypeptides of complex I (NADH dehydrogenase), 1 of the 11 polypeptides of

complex III, 3 of the 13 polypeptides of complex IV (cytochrome c oxidase), and 2 of the 16 proteins of complex V (ATP-synthetase).

Furthermore, mtDNA also codes for 2 ribosomal RNAs (16S and 23S rRNA), and 22 transfer RNAs (tRNAs) that are needed for intramitochondrial protein synthesis (Anderson S., et al., 1981), (Fig. 8).

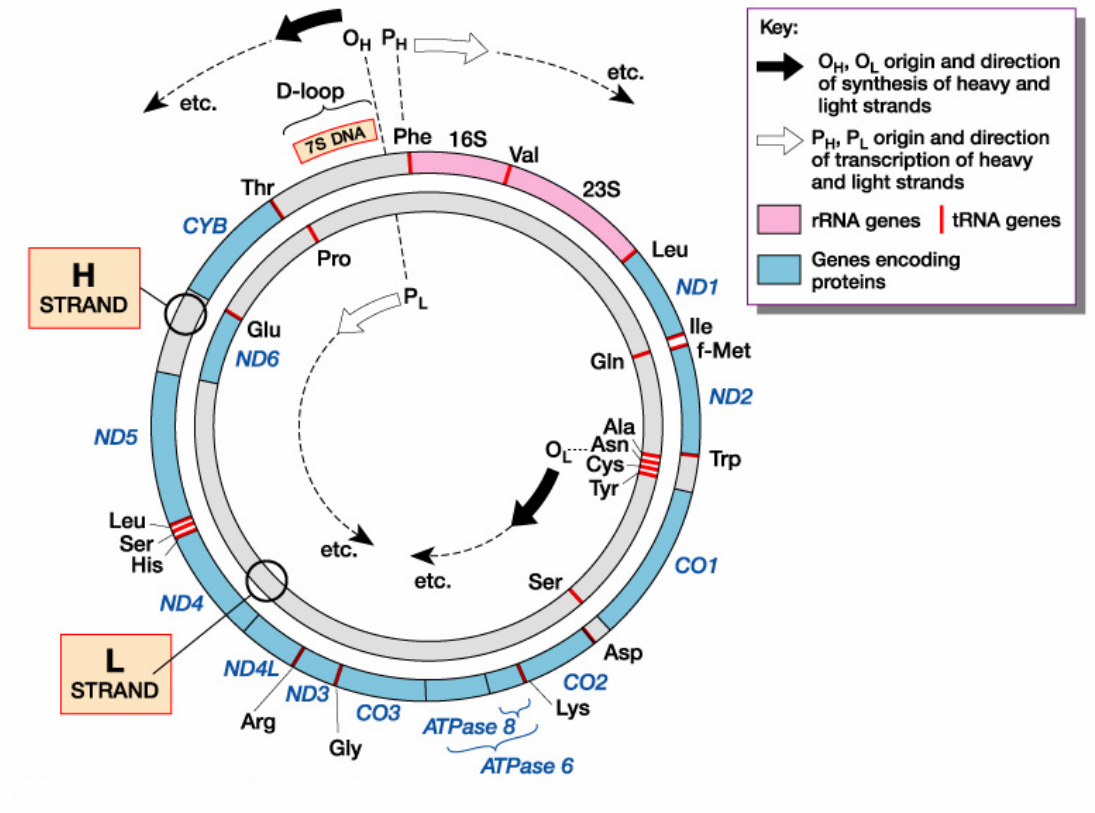

Fig 8: Map of the human mitochondrial genome (mtDNA)
ND1, **2**, **3**, **4L**, **4**, **5**, **6** are NADH dehydrogenase subunits of the of complex I; **CYB** is Cytochrome B of complex III. **CO1, 2, 3** are cytochrome c oxidase polypeptides of complex IVand ATP-synthetase, **ATPase 6** and **8**, are proteins of complex V.

MtDNA consists of coding DNA, with the exception of a 1121 bp long fragment, named displacement loop (D-Loop), that has mainly regulatory functions and is therefore also termed control region (CR). The D-loop is the most variable region of mtDNA and contains the replication origin of the heavy H-strand (O_H) and the promoter positions for the transcription of both H-strand (P_H) and light L-strand (P_L). Transcription is initiated at P_H or P_L and progresses around the mtDNA, generating a polycistronic message.

ROS production

Althought mtDNA codes for only a small portion of all proteins forming the subunits of the mitochondrial respiratory chain, all of them are essential for OXPHOS. What is more, the same mitochondrial machinery that carries out respiration is also a major source of endogenous toxic free radicals, namely reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and superoxide radical (O_2^{-}•) (Wallace, 1999). ROS are physiologically significant byproducts of normal cellular respiration and are potentially toxic (Melov, 2004). In fact, when ROS levels exceed the cellular antioxidant defenses, a deleterious condition known as oxidative stress occurs that results in substantial damage to various cellular costituents, including mtDNA (Beckman & Ames, 1998).

Mitochondria are essential for neuronal function because neurons are highly dependent on OXPHOS for their energetic needs, and oxidative damage has been found in all classes of organic molecules (proteins, lipids, nucleic acids and sugars) that are critical for neuronal structural and functional integrity (Perry et al., 2002). High lipid content, relatively high oxidative metabolism and low level of antioxidant defenses make the brain very susceptible to oxidative stress (Emerit et al., 2004). Additionally, neurons are terminally differentiated cells and damaged cells do not have the possibility to be replaced.

The free radical injury and disruption of mitochondrial function have been implicated in the initiation and/or progression of neurodegenerative disease such as AD and PD. Mitochondrial dysfunction has been described in AD patients, especially in carriers of *APOE*-ε4 genotype (Gibson et al., 2000) as the ε4 allele is associated with increased oxidative stress (Ramassamy et al., 2001). Excessive lipid peroxidation, protein oxidation, DNA and RNA oxidation and glyco-oxidation have all been documented in AD brains (Bonilla et al., 1999). Furthermore, reduced complex IV activity has been found in the brain and platelets of AD patients, but the pathologic relevance of this mitochondrial defect is not known (Kish et al., 1992; Parker et al., 1994; Mutisya et al., 1994). However, no direct cause-effect relationship has yet been established between oxidative damage, mtDNA defects and AD, so that more work is required in this area.

Besides the key role of mitochondria in cell energy production, these organelles are also involved in cell death pathways accomplished via the mitochondrial permeability transition pore (mtPTP) (Kokoszka et al., 2004). ROS generated during respiration may induce mutations in mitochondrial DNA (Beckman & Ames, 1998) and any damage to mtDNA,

by inhibiting mitochondrial respiratory function, may increase the propensity for mtPTP activation that finally drives cell death by apoptosis and, consequently, loss of tissue function (Emerit et al., 2004).

In AD and PD the neural cell death occurs predominantly by apoptosis (Emerit et al., 2004) and the brains of AD patients have increased caspase activity, a classical apoptotic marker (Coskun et al., 2003). Because of these reasons, mitochondrial dysfunction is an attractive candidate for an executioner's role in neuronal degeneration.

Overall, a clear involvement of oxidative stress, mitochondria dysfunction and neuronal damage/death in the pathogenesis of neurodegenerative disease is indicated by several studies. The hypothesis that mitochondrial abnormalities have a role in neuronal diseases suggests also the existence of selective defects for both AD and PD (Howell et al., 2005; Shapira, 2002). The relevant genetic factors still remain elusive and somatic mutations as well as inherited mtDNA variations, might be involved in neurodegenerative diseases.

MtDNA variability

While most of human cells contain two copies of nuclear DNA (nDNA), they contain many more copies of mtDNA (from 1000 to 100 000, depending on the cell type). Unlike nuclear DNA which replicates only once during each cell cycle, mtDNA replication is independent of the cell cycle and may occur even in non-dividing cells (Birky, 2001).

In addition to high copy number, mtDNA has a mutation rate higher than that of nDNA due to lack of histones and inefficient repair systems. Moreover, mtDNA is more exposed to mutagenic events than nDNA because of its localization close to the mitochondrial respiratory chain, a source of DNA damaging free radicals. The D-loop region, especially, is more susceptible to mutagenesis (Chinnery et al., 1999) resulting the most variable region in the mitochondrial genome with the most polymorphic nucleotide sites concentrated in the hypervariable segments HVS-I and HVS-II (Wilkinson-Herbots et al., 1996).

Due to these particular features, mtDNA has a high level of variability. Specifically, a somatic or intra-individual variability and a hereditary or inter-individual variability of mtDNA exist.

When a new mtDNA mutation arises in a cell, a mixed intracellular population of mutant and normal mtDNA molecules is generated, which can coexist in the same cell, tissue or

organ, a state known as heteroplasmy. Cell containing only wild-type DNA or only mutated DNA are called homoplasmic (Wallace et al., 1999b). A very low level of heteroplasmy does not impair the respiratory chain function of cell or tissue, but if mutant mtDNA exceeds a certain level, OXPHOS dysfunction occurs and symptoms arise. This is known as threshold effect (DiMauro & Schon, 2003). Heteroplasmy can be observed in post-mitotic tissues such as central nervous system characterized by high energy demand (Chomyn & Attardi G., 2003). MtDNA mutations may accumulate in the brain; moreover, the rate of accumulation seems to be faster in brain of patients with dementia than in normal ones (Chinnery et al., 1999).

A dissertation on the literature on mtDNA heteroplasmy in neurodegenerative diseases would be out of the aims of this PhD thesis, which is instead focussed on mtDNA inherited variability.

MtDNA inherited variability: haplogroups and sub-haplogroups

Several unique properties of human mtDNA, including high copy number, maternal inheritance, lack of recombination, and high mutation rate have made mtDNA very appropriate in studies of human evolution, migration, and population history (Ingman et al., 2000). In fact, selectively neutral or almost neutral variants accumulate sequentially along radiating maternal lineages, becoming prevalent through genetic drift. Consequently, mutations which occurred ten thousands of years ago are nowadays present in high frequency, and are population- and continent-specific, creating groups of related mtDNA haplotypes, or haplogroups, sharing a specific set of stable polymorphic restriction sites (Torroni & Wallace, 1994; Wallace, 1994; Wallace, 1995).

The classification of mtDNA haplogroups is based on information obtained from RFLP analysis of the coding region and from the nucleotide sequence of the control-region (Torroni et al., 1996). Haplogroups are coded with capital letters and subclusters with a running number (Ballinger et al., 1992; Torroni et al., 1996). The three major ethnic groups (Africans, Asians and Native Americans, and Europeans) show different patterns of haplogroups. Three African (L_1, L_2, L_3), seven Asian (C, D, G, E, A, B, F) and nine European (H, T, U, V, W, X, J, I) main mtDNA haplogroups have been identified (Torroni et al., 1994; Torroni et al., 1996) (Fig. 9).

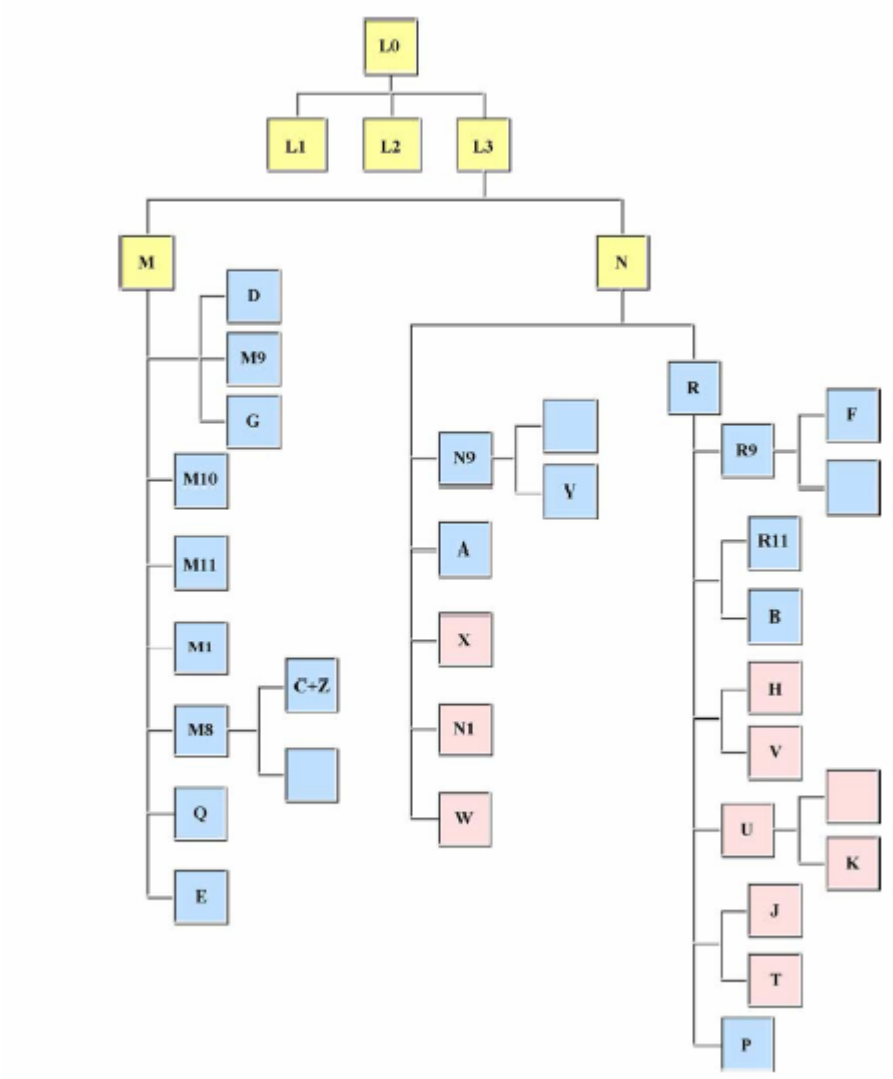

Fig. 9: Simplified network of major mtDNA haplogroups. The African haplogroups and superclades M and N that descend from subSaharan L3 are shown in yellow. Asian/Native Americans haplogroups are shown in light blue while European haplogroups are shown in pink-colored boxes. Empty boxes indicate additional subgroups that are not displayed (Herrnstadt & Howell, 2004).

The phylogenetics of haplogroups is complex and multiple subclades exist. For example, haplogroup U comprises phylogeographically different subhaplogroups such as U1, U2, U3, U4, U5, the oldest subclade, U6, U7, U8, U9 and K (Achilli et al., 2005).

The most prevalent European haplogroup H comprises numerous sub-haplogroups identifiable by characteristic mutations (Achilli et al., 2004) (Fig.10).

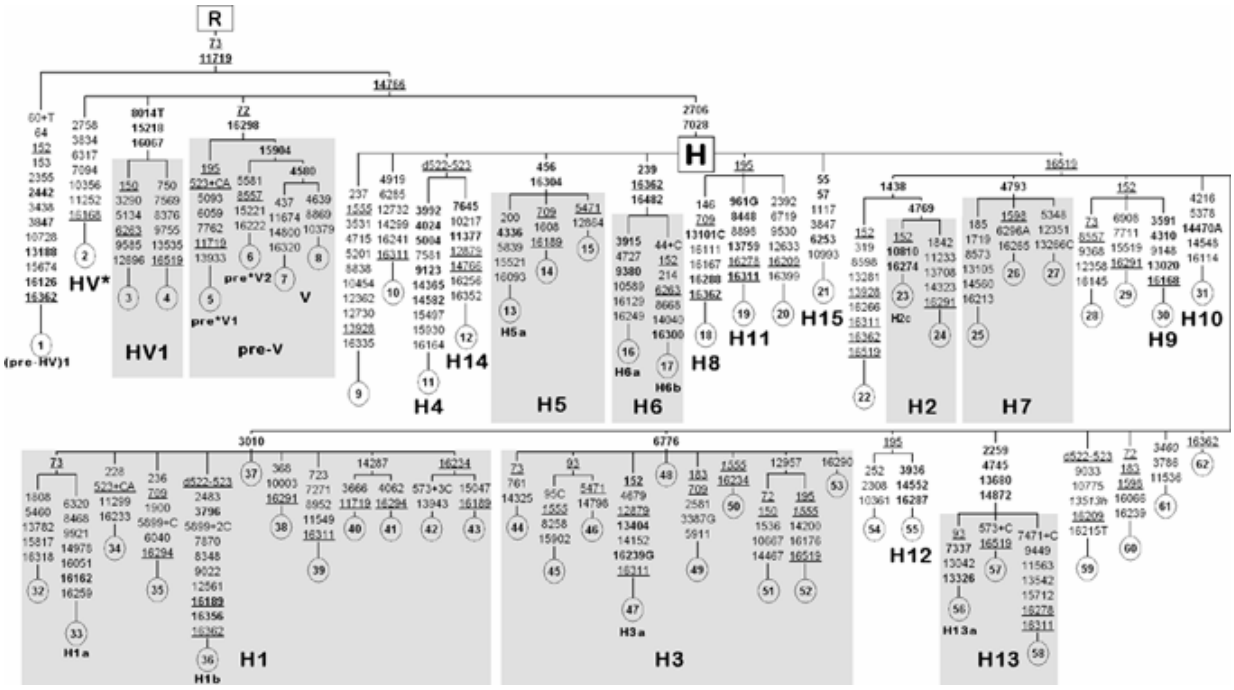

Fig. 10: ***Phylogenetic tree of mtDNA haplogroup H***. It encompasses 62 entire mtDNA sequences, all mutations relative to the root of R, and the 15 sub-haplogroups (H1-H15) identified by Achilli et al.,2004.

Most of these H sub-haplogroups likely arose/expanded about 10,000-12,000 years ago. In contrast to haplogroup H as a whole, which harbors a rather uniform and elevated frequency within Europe (30%–50%), their spatial frequency patterns are very different in European regions (Achilli et al., 2004). For example, among the H-sub-haplogroups, H1 and H2 have been defined in a sample of Finns (Finnilä et al., 2001). Based on mtDNA complete sequences, two further sub-groups were described by Herrnstadt and coworkers (Herrnstadt et al., 2002) : H3, the next most common sub-haplogroup after H1, and the rare H4. Moreover, Quintáns and colleagues (Quintáns et al., 2004) further identified H5, H6, and H7. Additional sub-haplogroups (H16-H21) have been recently proposed by Roostalu et al (2006).

Haplogroup and sub-haplogroups in association studies

Population genetic studies are based on the assumption that mtDNA polymorphisms are neutral from an evolutionary point of view. However, it has been suggested that some mtDNA types have been selected on the basis of their effect on mitochondrial function (Moilanen & Majamaa, 2003).

Therefore, the geographical variation seen in mtDNA haplogroups may reflect the action of natural selection on specific mtDNA lineages in different climatic conditions (Mishmar et al., 2003;Ruiz-Pesini et al., 2004).

The presumed "neutral" variation that defines haplogroups and subhaplogroups has also been associated to a wide range of common complex diseases and phenotypes (Wallace D.C., 2005). In particular, the role of mtDNA variation in the pathogenesis of neurodegenerative diseases has been the focus of several studies (Howell et al., 2005).

The first study reporting haplogroup effects in modulating the penetrance of mutations showed that specific pathogenic mutations of the Leber Hereditary Optic Neuropathy (LHON) were preferentially associated with the haplogroup J (Torroni et al., 1997). Following these results, association studies became a current approach to disentangle the role of mtDNA variations in a variety of disorders and phenotypes.

Examples of associations studies are showed in the Table 1.

Trait	***Haplogroups significantly associated with the trait***	***References***
Longevity	***Haplogroup J***	***DeBenedictis et al. 1999; Ross et al. 2001; Niemi et al. 2003.***
Lewy body dementia	***Haplogroup H***	***Chinnery et al. 2000***
Occipital stroke in migraine	***Haplogroup U***	***Majamaa et al. 1998***
Asthenozoospermia	***Haplogroup T***	***Ruiz-Pesini et al. 2000***
Alzheimer's Disease	***Haplogroups U and K***	***Carrieri et al., 2001***
Sprint athletic performances	***Haplogroups J and K***	***Niemi & Majamaa 2005***
Migraine without aura and cyclic vomiting syndrome	***Haplogroup H***	***Wang et al. 2004***
Bipolar disorder	***Haplogroup D***	***Munakata et al. 2004***

Table 1 : Examples of haplogroup-associations studies.

However, in some cases associations studies across different populations showed contrasting results. For instance, an increased risk of PD was associated both with the

superhaplogroup JT (Ross et al., 2003) and the supercluster JTIWX (Autere et al., 2004); on the contrary, in other studies, a reduced risk of PD was associated with haplogroups J and K (van der Walt et al., 2003), the supercluster UKJT (Pyle et al., 2005), or haplogroup K alone (Ghezzi et al., 2005). To explain the lack of reproducibility Samuels et al. (2006), examining the problem of the statistical power in mtDNA association analyses, established that very large sample sizes are required to detect differences in haplogroup frequencies between cases an controls (Samuels et al., 2006).

Moreover, inconsistent results may also be explained by the low resolution of the mtDNA haplogroup structure currently used in such association studies (Carelli et al., 2006; Saxena et al., 2006).

Haplogroups have been defined on the basis of evolutionarily ancient, stable polymorphisms deeply rooted in the world mtDNA phylogeny, and therefore almost all the association studies have ignored the majority of more recent sequence variations.

The molecular dissection of major mtDNA haplogroups into the "younger" sub-haplogroups with more restricted geographic and ethnical distributions can be extremely informative in disease studies. For example, Achilli and coworkers showed that the previously reported excess of H among Spanish families affected by nonsyndromic sensorineural deafness was caused entirely by H3 sub-haplogroup (Achilli et al., 2004).

2. AIMS OF THE STUDY

Data emerging from clinical and genetic studies on FTD provide evidence of a complex and multifactorial neurodegenerative disease. We collected a sample of unrelated subjects recruited in Calabria (southern Italy) and including 114 FTD patients and 180 controls matched for age, gender, ethnic origin and geographical area with the cases.

In my PhD, the focus was on following topics:

1. ***TAU gene variability in FTD*** . The *TAU* gene encodes for tau protein, the axonal microtubule (MT)-associated proteins that have a major role in the assembly and stability of microtubules. Since conflicting results have been obtained as it regard *TAU* gene variability (H1 haplotype) in FTD risk in different populations, aim of the study was to investigated the role of H1/H2 haplotypes of *TAU* gene in a large sample carefully selected as for clinical and neuropsychological features.

2. ***APOE gene variability in FTD.*** ApoE plays a fundamental role in lipid homeostasis, maintaining synapto-dendritic connections and scavenging toxins. *APOE*-ε4 allele has been consistently associated with AD and other types of dementia, but a possible role in FTD is still controversial. Aim of the study was to investigate the role of *APOE* gene variability in FTD. Furthermore, since studies suggested that the interaction between ε2/ε4 *APOE* alleles and H1 haplotype is a risk factor for FTD, we also explored this aspect of the problem.

The results relevant to points 1 and 2 have been already published (Bernardi et al., 2006). A brief comment to the paper is shown in the "Results and Discussion" section at page 35 and the reprint is annexed in the "End Section" of the present manuscript.

3. ***MtDNA inherited variability in FTD***. The analysis of mtDNA has been the focus of numerous studies investigating its role as a contributing factor to the pathogenesis of neurodegenerative diseases, but the possibility that mtDNA variability is a significant factor in FTD has not been explored yet. Therefore, aim of the work was to investigate the patterns of mtDNA haplogroups and sub-haplogroups in our samples.

3. MtDNA inherited variability in FTD

3.1 Subjects and Methods

3.1.1 Sample

We analyzed a sample of 294 subjects that comprised **114** unrelated ***FTD patients*** and **180** ethnically, age and sex matched healthy ***Controls***. All individuals were recruited in a genetically homogeneous population (Calabria, southern Italy) and their Calabrian ancestry had been ascertained up to the grandparents generation.

Patients and Controls

All the FTD patients were collected and diagnosed at the Regional Neurogenetics Centre of Calabria (Director: A.C. Bruni). Here, thanks to a team of neurologists, psychologists, biologists, etc.; each patient was tested, visited and then subjected to blood withdrawal by venipuncture. Thereafter, blood samples were processed for both haematological and DNA analyses.

The differential diagnosis of FTD was made following stringent international criteria, among others Lund-Manchester group criteria and NINCDS-ADRDA (Brun A., et al., 1994; McKhann G, et al., 1984), in combination with clinical observations, neuropsychological evaluation (e.g. by MMSE), information gained from laboratory investigations, morphological and functional brain imaging (e.g.CT, MRI, SPECT). A detailed description of diagnosis and neuropsychological procedures is reported in the "Materials and Methods" section of the annexed reprint (Bernardi et al., 2006).

The FTD sample was further subdivided into sporadic and familial cases: if FTD was diagnosed in one patient without further members of the family affected, the case was defined as "sporadic". On the contrary, if FTD was diagnosed in a subject who had a positive family history for FTD, the case was defined as "familial". In such a case, a sole affected subject per family was randomly selected for the genetic study. Patients whose family history was compatible with the hypothesis of monogenic FTD were not included in our study. The distribution of our FTD sample across the Calabrian region is shown in Fig.11.

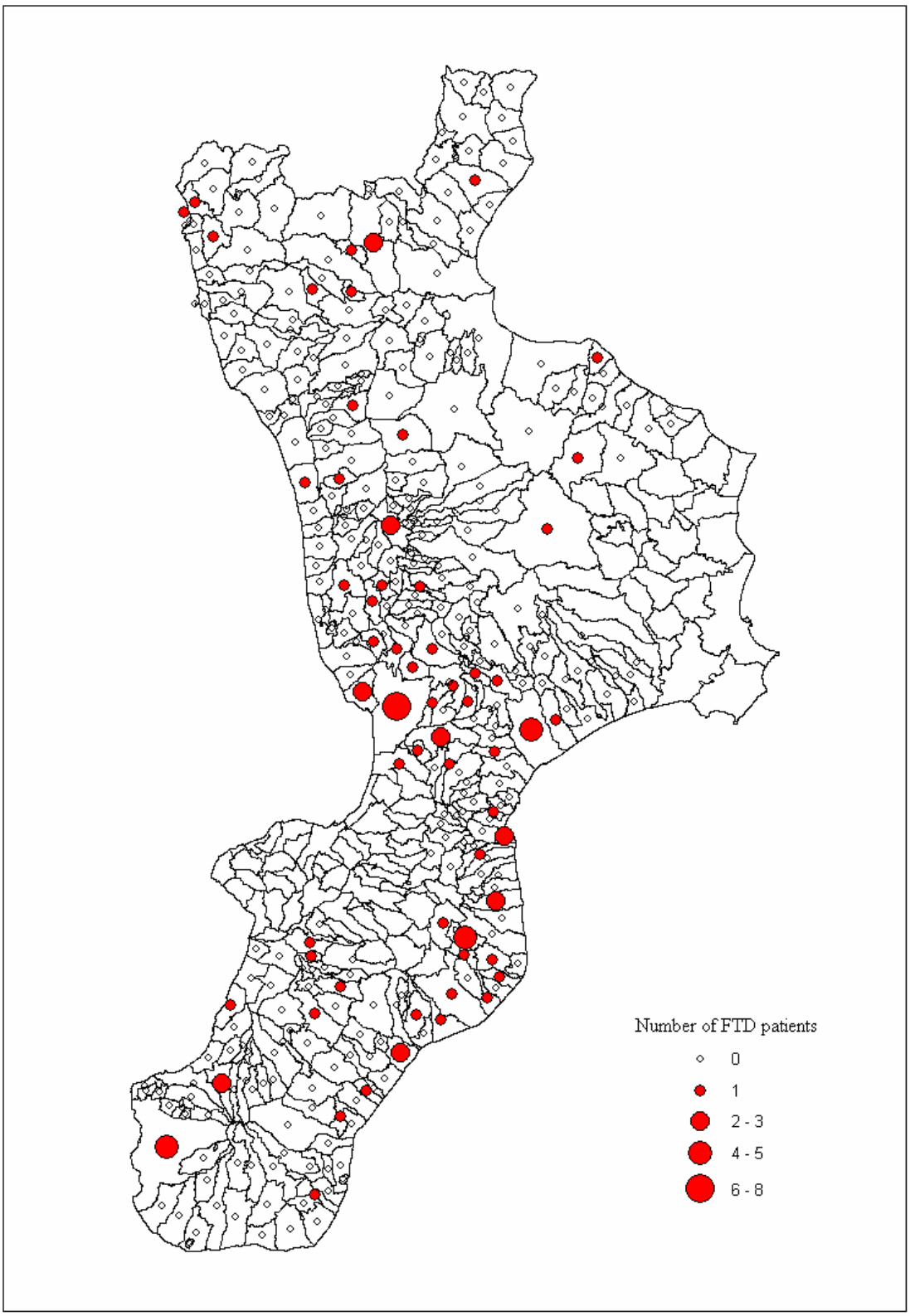

Fig.11: Distribution of FTD patients across the Calabrian region. Red circles denote the place of origin of FTD patients.

Table 2 reports the number of examined sporadic and familial FTD patients (a) and controls (b) stratified by sex.

a)	*Sporadic FTD*	*Familial FTD*	*Total*
Men	**30** ***(44.1%)***	**23** ***(50.0%)***	**53** ***(46.5%)***
Women	**38** ***(55.9%)***	**23** ***(50.0%)***	**61** ***(53.5%)***
Total	**68** ***(100%)***	**46** ***(100%)***	**114** ***(100%)***
Median age	***68.0 years***	***64.5 years***	

b)	*CONTROLS*
Men	**84** ***(46.7%)***
Women	**96** ***(53.3%)***
Total	**180** ***(100%)***
Median age	***66.5 years***

Table 2: Number of subjects in FTD (a) and controls (b) stratified by sex.

Age frequency distributions of sporadic FTD patients (a), familial FTD patients (b) and and control sample (c) are shown in Figures 12.

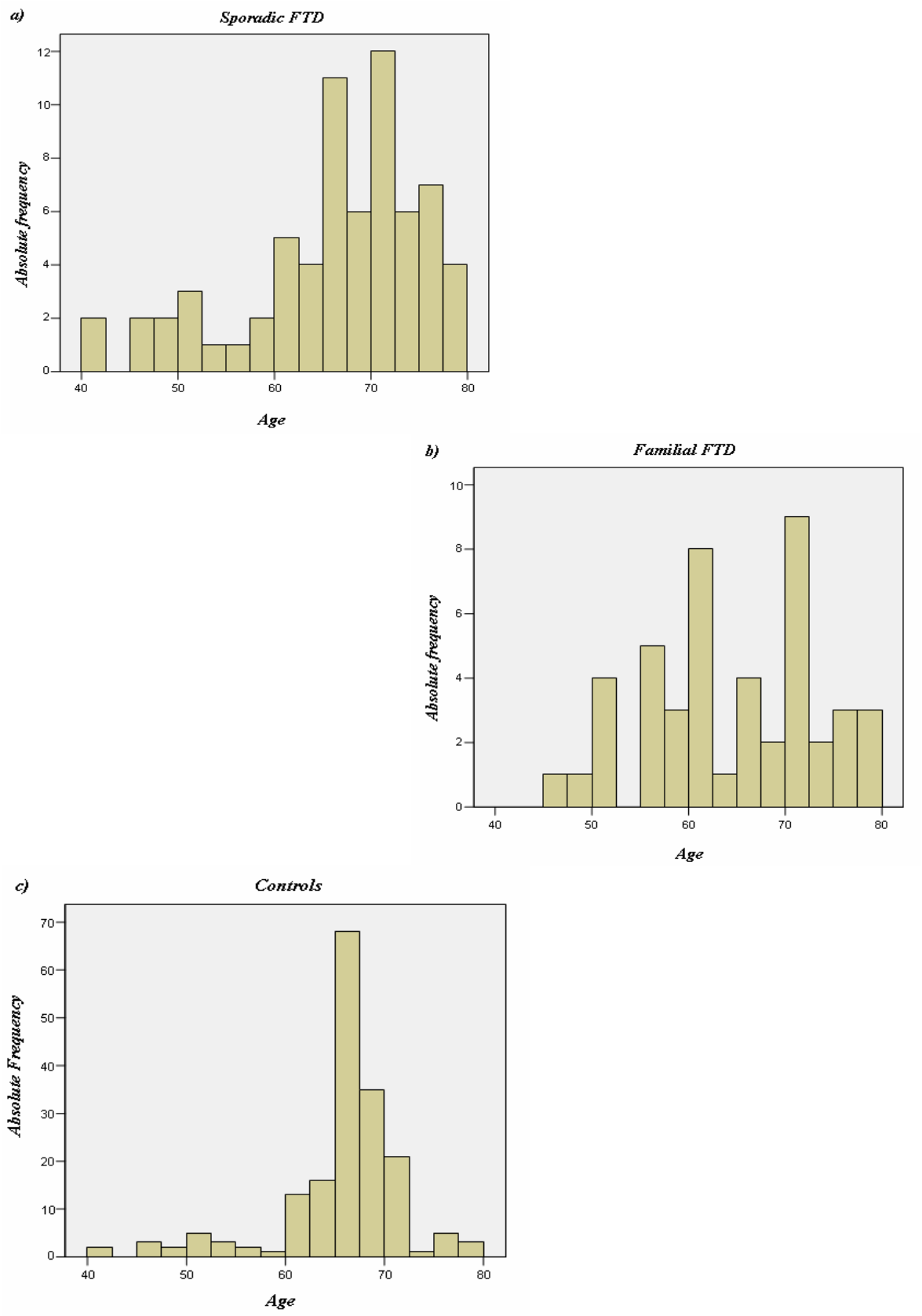

Fig. 12: Age frequency distribution in sporadic FTD patients (a) familial FTD patients (b) and in the control group (c). Homogeneity between samples was verified by Mann-Whitney test.

The same complete set of clinical-laboratory procedures and neurological assessment of cognitive status we used for patients, was performed also in the control group.
Written informed consent was obtained from all the persons participating to the study; for disabled FTD patients the agreement was given by their legal tutors.

Blood samples

DNA from blood buffy coats was isolated in the recruitment center above mentioned by using standard procedures.

3.1.2 Analysis of mtDNA variability

Haplogroups

Haplogroup typing was performed by PCR combined with restriction analysis (RFLP) (Torroni et al., 1996). MtDNA fragments, containing the polymorphic restriction sites that characterize each European haplogroup (Table 3), were amplified from each individual and submitted to restriction analyses.

Hg	***Polymorphic restriction sites***								
	G1719Ac	**G4580A**q	**C7028T**a	**G8251A**b	**G9055A**n	**A10398G**c	**A12308G**g	**G13368A**m	**G13708A**p
H	1	1	0#	0	1	0#	0	0	1
I	0#	1	1#	1#	1	1#	0	0	1
J	1	1	1#	0	1	1#	0	0	0#
K	1	1	1#	0	0#	1#	1#	0	1
T	1	1	1#	0	1	0#	0	1#	1
U	1	1	1#	0	1	0#	1#	0	1
V	1	0#	1#	0	1	0#	0	0	1
W	1	1	1#	1#	1	0#	0	0	1
X	0#	1	1#	0	1	0#	0	0	1

Table 3: Identification of mtDNA haplogroups by restriction analysis (a: AluI, b: AvaII, c: DdeI, g: HinfI, m: BamHI, n: HaeII, q: NlaIII, p: BstoI; 1/0 presence/absence of restriction site, # associations of sites that characterize each haplogroup) of the specific target sequence (Torroni et al., 1996).

Total DNA was amplified in 25 µl reaction mixtures containing 200 ng of genomic DNA, 1U of Taq DNA polymerase (Eppendorf AG, Germany), 0.3 µM of each primer, 0.2 mM dNTPs, 1.5 mM $Mg(OAc)_2$ and 1X reaction buffer. Amplifications were performed in an

Eppendorf thermal cycler. Cycling conditions were the same for all the fragments, but with different specific annealing temperature. Specifically, the initial denaturation at 93°C for 30s was followed by 35 cycles at 93°C for 15s, specific primers annealing temperature for 20s, 72°C for 1' and a final extension of 72°C for 12'. Details on mtDNA amplification, specific primers and corresponding annealing temperatures are shown in Table 4.

Hg	PRIMERS SEQUENCE	Annealing T. °C	Fragment length bp	Fragment positions nt
H	FOR:5'AAGCAATATGAAATGATCTG-3' REV: 5'-CGTAGGTTTGGTCTAGG-3'	47	242	6890-7131
M	FOR:5'-TCCTTTTACCCCTACCATGAG-3' REV:5'-ATTATTCCTTCTAGGCATAGTAG-3'	57	309	10270-10579
T	FOR:5'-GCTTAGGCGCTATCACCAC-3' REV:5'-ATATCTTGTTCATTGTAAG-3'	50	232	13172-13403
U	FOR:5'-CTCAACCCCGACATCATTACC-3' REV:5'-ATTACTTTTATTTGGAGTTGCACCAAGAT-3'	59	234	12104-12338
V	FOR:5'-GGAGCTTAAACCCCCTTA-3' REV:5'-GGTAGTATTGGTTATGGTTC-3'	50	432	4308-4739
X	FOR:5'-AACACAAAGCACCCAACTTACACTTAGGA-3' REV:5'-CTTTGGCTCTCCTTGCAAAGT-3'	62	273	1615-1894
I, W	FOR:5'-AGCAAACCACAGTTTCATGC-3' REV:5'-TTTCACTGTAAAGAGGTGTTGG-3'	53	179	8188-8366
J	FOR:5'-CCTCCCTGACAAGCGCCTATAGC-3' REV:5'-CTAGGGCTGTTAGAAGTCCT-3'	60	261	13583-13843
K	FOR:5'-CCTAGCCATGGCCATCC-3' REV:5'-GGCTTACTAGAAGTGTGAAAAC-3'	53	356	8829-9184

Table 4: Sequence of each couple of primers and annealing temperature used in PCR for mtDNA haplogroups (Hg) detection.

The amplified fragments, after digestion by appropriate restriction enzymes (Table 4), were separeted by 2% agarose gel electrophoresis. By this procedure, each mtDNA was ascribed to one of the nine haplogroups (H, I, J, K, T, U, V, W, X) specific to Europeans. MtDNA that were non-classifiable within a haplogroup were grouped as "Others" (Torroni et al., 1996).

HVS-I analysis

PCR

The HVS-I target region was a fragment of 405 bp spanning from nt 15996 to nt 16401. This fragment was amplified by using by 5'-CACCATTAGCACCCAAAGCT-3' forward primer and 5'-TGATTTCACGGAGGATGGTG -3' reverse primer (0.3 µM each) in a final volume reaction mixture of 25 µl, containing 200 ng DNA, 1.5 mM $Mg(OAc)_2$, 0.2 mM dNTPs, and 1 U Taq DNA polymerase and 1X reaction buffer (Eppendorf AG, Germany). Amplification was performed in an Eppendorf thermal cycler at the following conditions: pre-denaturation at 93°C for 30s, followed by 35 cycles at 93°C for 15s, 60°C for 20s, 72°C for 1' and a final extension of 72°C for 12'.

PCR fragments were then checked by an 2% agarose gel electrophoresis in TBE buffer and stained with ethidium bromide.

Sequencing

The amplified fragments were purified by QIAquick PCR purification Kit (Qiagen) and sequenced by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) on an ABI 310 automated sequencer (Applied Biosystems). Sequencing reaction mixtures contained 4 µl of Terminator Ready Reaction Mix, 200 ng of template, 3.2 pmol of each primer, 4 µl of 5X reaction buffer in a total volume of 20 µl. Cycle sequencing was performed for 25 cycles at 96°C for 10s, 50°C for 5s, 60°C for 4' in an Eppendorf thermal cycler. The extension products were purified by using Amersham spin columns (Amersham Biosciences).

Sequences were aligned by using Genalys 2.0 beta software and compared to revised Cambridge Reference Sequence (rCRS).

HVS-II analysis

PCR

For HVS-II analysis, a fragment of 732 bp spanning from nt 16411 to nt 580 was amplified by 5'-CGTGAAATCAATATCCCGCAC-3' forward primer and 5'-TTGAGGAGGTAAGCTACATA -3' reverse primer (0.4 µM each) in a final volume reaction mixture of 25 µl, containing 200 ng DNA, 1.5 mM $Mg(OAc)_2$, 0.2 mM dNTPs, and 1.5 U Taq DNA polymerase and 1X reaction buffer (Eppendorf AG, Germany). PCR conditions were the following: pre-denaturation at 94°C for 30s, followed by 35 cycles at 94°C for 15s, 52°C for 20s, 72°C for 1' and a final extension of 72°C for 12'. Amplification was performed in an Eppendorf thermal cycler and products were visualized by 2% agarose gel electrophoresis in TBE buffer with ethidium bromide staining.

Sequencing

HVS-II sequence analysis was performed as previously described for HVS-I, in a reaction mixture containing 2 µl of Terminator Ready Reaction Mix, 200 ng of template, 3.2 pmol of each primer, 2 µl of 5X reaction buffer in a total volume of 10 µl. Cycle sequencing was performed as follows: pre-denaturation at 96°C for 2', and 25 cycles at 96°C for 30s, 50°C for 5s, 60°C for 4' in an Eppendorf thermal cycler. The extension products were purified and sequenced as above described.

H Sub-haplogroups

Coding-region diagnostic markers used to define some H sub-haplogroups are reported in Table 5.

SubHg	Coding-regions diagnostic markers	PRIMERS SEQUENCE	Annealing T. °C	PCR fragment length bp	Fragment positions nt
H1	G3010A	FOR: 5' TTCGAGCAGTACATGCTAAGA-3' REV: 5'- AACCCTGTTCTTGGGTGGGTG-3'	51	387	2841-3228
H2 **H7** **H13**	4769A A4793G A4745G	FOR: 5'- TAGGCCTAGAAATAAACATGC-3' REV: 5'- CGTTTAGTGAGGGAGAGATTT-3'	51	359	4561-4920
H3	T6776C	FOR: 5'- AATAATCTCCCATATTGTAAC-3' REV: 5'-GGCCACCTACGGTGAAAAGAA-3'	51	301	6659-6960

Table 5: Coding-region diagnostic markers relevant to H sub-haplogroups (subHg) together with sequence and annealing temperature of each couple of primers.

PCR

The specific mtDNA target regions were amplified by using the primers (0.3 μM each) shown in Table 5 at the same conditions above reported for HVS-I, in a final volume reaction mixture of 25 μl, containing 200 ng DNA, 1.5 mM $Mg(OAc)_2$, 0.2 mM dNTPs, and 1 U Taq DNA polymerase and 1X reaction buffer (Eppendorf AG, Germany). All amplifications were performed in an Eppendorf thermal cycler at the conditions already described for HVS-I amplification, except for the annealing temperature as reported in Table 6. PCR products were checked by 2% agarose gel electrophoresis in TBE buffer with ethidium bromide. Then, amplified fragments were sequenced by using the same procedure and conditions above described for HVS-I sequencing.

3.1.3 Statistical analyses.

Non-parametric Mann-Whitney-U test was used to verify if age frequency distribution was different between samples.

The null hypothesis of homogeneity between haplogroup (Hg)/subhaplogroup (subHg) frequency distributions in pair of samples was tested by permutation tests (Weir 1996).

Standard Errors (SE) for Hg/subHg were computed according to the hypothesis of a binomial distribution of random resampling. The formula for the binomial SE is given by:

$$SE = \sqrt{\frac{p_i \times (1 - p_i)}{N}};$$

Where p_i is the frequency of the i-th mitochondrial Hg/subHg in the sample and N is the total number of the haplogroups/subhaplogroups in the same sample.

4. RESULTS and DISCUSSION

4.1 Comment to the published paper: Bernardi L., et al., 2006.

We collected a sample of 100 unrelated FTD patients (cases) and 180 ethnically, age and sex matched healthy controls. Both cases and controls was carefully recruited and selected in a genetically homogeneous population (Calabria, southern Italy).

Diagnosis of FTD in affected subjects was made using multiple diagnostic operational criteria and was based on specific clinico-neuropsychological features and neuroradiological profiles.

We investigated *TAU* and *APOE* gene variability in order to verify possible associations of FTD with *TAU* haplotypes (H1/H2) and/or *APOE* alleles (ε2/ε3/ε4).

By applying a logistic regression analysis we found that both H1 and H2 haplotypes do not affect the risk of FTD in our population. This finding, in agreement with previous studies performed in other populations, revealed that *TAU* gene variability is not involved in FTD.

The analysis of *APOE* gene variability showed a rather important effect of this gene in the susceptibility to FTD. In particular, the *APOE*-ε4 allele was found to increase the risk to develop FTD (O.R.= 2.68 with 95% CI=1.51–4.76; p=0.001) while, *APOE*-ε2 allele was found to decrease it (O.R.= 0.28 with 95% CI =0.12–0.66; p=0.003). The association of the ε4/ε2 alleles with FTD is in accord with the well established allele-specific anti-oxidant effects exerted by the three ApoE isoforms and confirms the significant role played by *APOE* variability in common dementing disorders.

The analysis of the interactions between *APOE* and *TAU* variability showed a small but significant effect exerted by the H1 haplotype in increasing the protective effect of the ε2 allele (O.R.= 0.31 with 95% CI = 0.13–0.73; p=0.007), thus demostrating an interaction between nuclear genetic factors on the risk to develop FTD.

4.2 Haplogroups analysis.

Sporadic FTD (n=68)and familial FTD (n=46) patients, and healthy controls (n=180) were screened for mtDNA haplogroups.

First, haplogroup analysis was carried out according to sex in each sample. Since significant differences were not found between men and women, men and women samples were pooled to increased the power of the analysis.

Data are reported in Table 6.

MtDNA HAPLOGROUPS	***Sporadic FTD***		***Familial FTD***		***CONTROLS***	
	N	***(% ± SE)***	***N***	***(% ± SE)***	***N***	***(% ± SE)***
H	**30**	**(44.1 ± 6.0)**	**16**	**(34.8 ± 7.0)**	**49**	**(27.2 ± 3.3)**
I	**1**	**(1.5 ± 1.5)**	**2**	**(4.3 ± 3.0)**	**8**	**(4.4 ± 1.5)**
J	**4**	**(5.9 ± 2.9)**	**3**	**(6.5 ± 3.6)**	**24**	**(13.3 ± 2.5)**
K	**10**	**(14.7 ± 4.3)**	**4**	**(8.7 ± 4.2)**	**22**	**(12.2 ± 2.4)**
T	**5**	**(7.4 ± 3.2)**	**7**	**(15.2 ± 5.3)**	**21**	**(11.7 ± 2.4)**
U	**7**	**(10.3 ± 3.7)**	**8**	**(17.4 ± 5.6)**	**18**	**(10.0 ± 2.2)**
V	**3**	**(4.4 ± 2.5)**	**1**	**(2.2 ± 2.2)**	**2**	**(1.1 ± 0.8)**
W	**1**	**(1.5 ± 1.5)**	**1**	**(2.2 ± 2.2)**	**4**	**(2.2 ± 1.1)**
X	**4**	**(5.9 ± 2.9)**	**0**	**(0.0 ± 0.0)**	**9**	**(5.0 ± 1.6)**
OTHERS	**3**	**(4.4 ± 2.5)**	**4**	**(8.7 ± 4.2)**	**23**	**(12.8 ± 2.5)**
Total	**68**	**100**	**46**	**100**	**180**	**100**

Table 6: MtDNA haplogroups frequencies distributions in sporadic FTD/familial FTD patients and controls: Absolute (N), relative frequencies (%) and their standard errors (SE) are shown. MtDNAs that were non-classifiable within any specific haplogroup were grouped as "others".

All nine haplogroups characteristic of Europeans were found in the three sample groups. They included 95,6%, 91,3% and 87,2% of the mtDNAs in sporadic FTD, familial FTD and controls, respectively.

By applying permutation tests, no difference was found in the distribution of haplogroups between sporadic FTD patients and controls (p=0.078) or familial FTD patients and controls (p=0.508).

Therefore, the apparent difference relevant to single haplogroups (for example H) is probably only caused by random phenomena due to the sample size.

4.3. HVS-I analysis.

In the next step of our study we analyzed the mtDNA HVS-I region (nt16024-nt16383) of the D-Loop. The target region was PCR amplified and sequenced in the whole sample. Sequences were aligned and compared to revised the Cambridge Reference Sequence (rCRS) by using Genalys 2.0 beta software. Sequences without ambiguities were obtained between positions 16014 and 16399.

On the whole, we observed 649 positions which differed from the rCRS for a total of 186 sequence variant sites. In particular, we found 96 variants sites in FTD patients and 90 in controls.

By using the variant sites, we constructed a total of 174 different haplotypes, 24 of which were exclusive of familial FTD, 27 of sporadic FTD and 99 were exclusive of controls; the remaining 8 were shared between FTD patients and controls.

Table 7 (see annexed sheet) reports all mtDNA sequence variants found in the HVS-I, with haplotype absolute frequencies in each sample.

By applying permutation tests to data in Table 8 no difference was found between sporadic FTD patients and controls (p=0.99) or familial FTD patients and controls (p=1).

Therefore, by HVS-I sequencing and haplotype analysis we did not evidence statistically significant association between FTD and HVS-I variability.

4.4. Sub-haplogroups analysis

The study of mtDNA inherited variability was further extended by dissecting major haplogroups, into sub-haplogroups.

The data on HVS-I sequences enabled us not only to verify diagnostic haplogroup markers, but also provided some information for sub-haplogroup assignment. Then, to accomplish sub-haplogroups analysis, the samples were analyzed by HVS-II (nt57-nt576) sequencing and screened for a few additional coding-region diagnostic markers relevant to H sub-haplogroups (G3010A ; 4769A ; A4745G ; A4793G ; T6776C).

Table 8 summarizes the specific diagnostic sites used for our analysis.

Sub-Hg	HVS1	HVS2	CODING REGION	References
H1	-------	-------	G3010A	(Finnila et al., 2001)
H2	-------	-------	4769A	(Finnila et al., 2001)
H3	-------	-------	T6776C	(Herrnstadt et al., 2002)
H5	T16304C	C456T	-------	(Quintans et al ., 2004)
H6	T16362C; A16482G	T239C	-------	(Quintans et al., 2004)
H7	-------	-------	A4793G	(Quintans et al., 2004)
H8	T16288C; T16362C	-------	-------	(Loogvali et al., 2004)
H9	C16168T	-------	-------	(Achilli et al., 2004)
H11	T16311C	-------	-------	(Loogvali et al., 2004)
H13	-------	-------	A4745G	(Achilli et al., 2004)
H15	-------	T55C; T57C	-------	(Achilli et al., 2004)
I1	T16311C	--------	------	(Palanicamy et al., 2004)
I2	--------	T199C;T204C;T250C	------	(Palanicamy et al., 2004)
I3	--------	T152C;G207A;T239C	------	(Bandelt et al., 2005)
J1	--------	C462T	------	(Finnila et al., 2001)
J2	--------	C150T ; T152C	------	(Finnila et al., 2001)
K1	---------	C497T	------	(Palanicamy et al.,2004)
T1	A16163G T16189C	------	------	(Finnila et al., 2001)
T2	C16296T ; T16304C	------	------	(Finnila et al., 2000; Palanicamy et al., 2004)
U1	T16249C	C285T	------	(Macaulay et al., 1999)
U2	A16051G	------	------	(Finnila et al., 2001)
U3	A16343G	C150T	------	(Macaulay et al., 1999)
U4	T16356C	T195C	------	(Macaulay et al., 1999)
U5	C16270T	------	------	(Macaulay et al., 1999)
U6	T16172C	------	------	(Achilli et al., 2005)
U7	A16318T	T152C	------	(Palanicamy et al., 2004)
V	-------	-------	------	(Macaulay et al., 1999; Torroni et al., 1996)
X2	-------	A153G; T195C	-------	(Reidla et al., 2003)
N1b1	G16145A C16176G G16390A	T152C	-------	(Palanicamy et al., 2004)
W	-------	-------	-------	Torroni et al., 1996
L0	G16129A C16187T T16189C A16230G C16278T T16311C	-------	-------	(Kivisild et al., 2005)
L1	G16129A C16187T T16189C C16278T ; T16311C	-------	-------	(Kivisild et al., 2005)
L3	G16129A T16189C T16249C	C150T T195C	-------	(Salas et al., 2002-2004)
M1	T16311C	T195C	-------	(Kivisild et al., 2005)
M5	G16129A	-------	-------	(Thangaraj et al., 2005)
M8	T16298C	-------	-------	(Kong et al., 2005)
preHV1	T16126C T16362C	C64T	-------	(Richards et al., 2000)
preV	T16298C	T72C	-------	(Torroni et al., 2001)
R1	C16278T T16311C	C295A	-------	(Quintana-Murci et al., 2004)
T2	T16126C C16294T T16304C	------	------	(Palanicamy et al., 2004)
OTHER(?)	------	------	----- -	-----

Table 8: Polymorphisms in HVS-I, HVS-II and in the coding region of mtDNA that enabled us to define sub-haplogroups.

Table 9 shows mtDNA sub-haplogroup frequency distributions in FTD patients and controls.

SUB-Hg	FTD sporadic		FTD familial		CONTROLS	
	N	*(% ± SE)*	*N*	*(% ± SE)*	*N*	*(% ± SE)*
H1	**10**	**(14.7 ± 4.3)**	**3**	**(6.5 ± 3.6)**	**13**	**(7.2 ± 1.9)**
H2	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**	**2**	**(1.1 ± 0.8)**
H3	**1**	**(1.5 ± 1.5)**	**1**	**(2.2 ± 2.2)**	**1**	**(0.6 ± 0.6)**
H5	**3**	**(4.4 ± 2.5)**	**3**	**(6.5 ± 3.6)**	**4**	**(2.2 ± 1.1)**
H6	**1**	**(1.5 ± 1.5)**	**1**	**(2.2 ± 2.2)**	**2**	**(1.1 ± 0.8)**
H7	**2**	**(2.9 ± 2.0)**	**1**	**(2.2 ± 2.2)**	**1**	**(0.6 ± 0.6)**
H8	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**	**3**	**(1.7 ± 1.0)**
H9	**1**	**(1.5 ± 1.5)**	**0**	**(0.0 ± 0.0)**	**1**	**(0.6 ± 0.6)**
H11	**1**	**(1.5 ± 1.5)**	**1**	**(2.2 ± 2.2)**	**2**	**(1.1 ± 0.8)**
H13	**1**	**(1.5 ± 1.5)**	**0**	**(0.0 ± 0.0)**	**5**	**(2.8 ± 1.2)**
H15	**1**	**(1.5 ± 1.5)**	**1**	**(2.2 ± 2.2)**	**1**	**(0.6 ± 0.6)**
H*	**9**	**(13.2 ± 4.1)**	**5**	**(10.9 ± 4.6)**	**14**	**(7.8 ± 2.0)**
I1	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**	**4**	**(2.2 ± 1.1)**
I2	**1**	**(1.5 ± 1.5)**	**2**	**(4.3 ± 3.0)**	**1**	**(0.6 ± 0.6)**
I3	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**	**3**	**(1.7 ± 1.0)**
J1	**3**	**(4.4 ± 2.5)**	**2**	**(4.3 ± 3.0)**	**19**	**(10.6 ± 2.3)**
J2	**1**	**(1.5 ± 1.5)**	**1**	**(2.2 ± 2.2)**	**5**	**(2.8 ± 1.2)**
K1	**10**	**(14.7 ± 4.3)**	**4**	**(8.7 ± 4.2)**	**22**	**(12.2 ± 2.4)**
T1	**3**	**(4.4 ± 2.5)**	**4**	**(8.7 ± 4.2)**	**8**	**(4.4 ± 1.5)**
T2	**2**	**(2.9 ± 2.0)**	**3**	**(6.5 ± 3.6)**	**13**	**(7.2 ± 1.9)**
U1	**1**	**(1.5 ± 1.5)**	**1**	**(2.2 ± 2.2)**	**1**	**(0.6 ± 0.6)**
U2	**1**	**(1.5 ± 1.5)**	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**
U3	**2**	**(2.9 ± 2.0)**	**1**	**(2.2 ± 2.2)**	**3**	**(1.7 ± 1.0)**
U5	**2**	**(2.9 ± 2.0)**	**4**	**(8.7 ± 4.2)**	**13**	**(7.2 ± 1.9)**
U6	**0**	**(0.0 ± 0.0)**	**1**	**(2.2 ± 2.2)**	**0**	**(0.0 ± 0.0)**
U7	**1**	**(1.5 ± 1.5)**	**1**	**(2.2 ± 2.2)**	**0**	**(0.0 ± 0.0)**
U4	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**	**1**	**(0.6 ± 0.6)**
V	**3**	**(4.4 ± 2.5)**	**1**	**(2.2 ± 2.2)**	**2**	**(1.1 ± 0.8)**
W	**1**	**(1.5 ± 1.5)**	**1**	**(2.2 ± 2.2)**	**4**	**(2.2 ± 1.1)**
X2	**3**	**(4.4 ± 2.5)**	**0**	**(0.0 ± 0.0)**	**7**	**(3.9 ± 1.4)**
N1b1	**1**	**(1.5 ± 1.5)**	**0**	**(0.0 ± 0.0)**	**2**	**(1.1 ± 0.8)**
L0	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**	**2**	**(1.1 ± 0.8)**
L1	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**	**2**	**(1.1 ± 0.8)**
L3	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**	**1**	**(0.6 ± 0.6)**
M1	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**	**4**	**(2.2 ± 1.1)**
M5	**0**	**(0.0 ± 0.0)**	**1**	**(2.2 ± 2.2)**	**0**	**(0.0 ± 0.0)**
M8	**1**	**(1.5 ± 1.5)**	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**
preHV1	**0**	**(0.0 ± 0.0)**	**1**	**(2.2 ± 2.2)**	**0**	**(0.0 ± 0.0)**
preV	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**	**8**	**(4.4 ± 1.5)**
R1	**0**	**(0.0 ± 0.0)**	**0**	**(0.0 ± 0.0)**	**2**	**(1.1 ± 0.8)**
T2	**1**	**(1.5 ± 1.5)**	**1**	**(2.2 ± 2.2)**	**1**	**(0.6 ± 0.6)**
OTHER (?)	**1**	**(1.5 ± 1.5)**	**1**	**(2.2 ± 2.2)**	**3**	**(1.7 ± 1.0)**
Total	**68**	**100**	**46**	**100**	**180**	**100**

Table 9: MtDNA sub-haplogroup frequency distributions in FTD patients and controls: Absolute (N) and relative frequencies (%) are shown. H-mtDNAs that were non-classifiable within the specific sub-haplogroup were grouped as " **H*** "

As previously observed in haplogroup analysis, also in this case a diversity between sample groups seemed to emerge and to differentiate sporadic FTD patients and controls. However, also in this case, permutation tests applied to the data set in Table 10 did not show differences between sporadic FTD patients and controls (p=1) or familial FTD patients and controls (p=0.99).

In summary, the comparative analysis between each group of FTD patients and the group of controls did not show statistically significant difference in the frequency distributions of mtDNA haplogroups/sub-haplogroups. Therefore, apparent differences between sample groups in the frequency of single haplogroups/ sub-haplogroups are only due to chance and are not significant if we take into account multiple comparisons.

5. CONCLUDING REMARKS

As remarked in the Introduction, FTD is a complex degenerative dementing syndrome which is characterised by a broad spectrum of phenotypes overlapping with those of other neurodegenerative disorders. It is likely that a genetic heterogeneity corresponds to such a phenotypic complexity, and this could account for the contrasting data reported in literature on FTD genetic risk factors. Generally, when we deal with the genetics of complex traits, a crucial point for overcoming heterogeneity problems is to assemble a sample collection whose size comes out from a balance between appropriate numerousness and phenotypic homogeneity. The collection of FTD patients screened in the present study not only is one of the largest till now examined, but has some important homogeneity features. First, it was collected in a population (Calabrians) characterised by a high level of genetic homogeneity, due to the geographical isolation of the region until recent years. Second, an adequate team of neurologists, psychologists and physicians carried out a huge effort to exactly define homogeneous FTD phenotypes (see, for example, the distinction between sporadic and familial FTD in data analysis). Third, the group of controls was matched with cases for ethnicity, genetic origin, sex, and age; most importantly, the same neuropsychological tests used for cases were applied to the whole control sample in order to exclude the presence of latent forms of dementia. Therefore, the quality of the sample has been the starting point for exploring possible effects of *TAU*, *APOE* and mtDNA genetic variability on susceptibility to FTD.

Taking into account their biological role (see Introduction), *TAU* and *APOE* genes are obvious candidate in such an analysis. In fact, several studies searched for a possible association between *TAU* or *APOE* variability and FTD, but contrasting results are reported in literature. The results of the study we carried out in our large and carefully selected sample (Bernardi et al., 2006) can be summarised as follows. First, H1 and H2 *TAU* gene haplotypes are not related to FTD. Second, allele ε4 increases ($p=0.001$) while allele ε2 decreases ($p=0.003$) the probability of disease. Interestingly, a small but significant effect was observed by the H1 haplotype in increasing the protective effect of the ε2 allele ($p=0.007$). A significant interaction between H1 and ε2 had been already reported by Verpillat et al. (2002), but in that case the interaction resulted in *an increase of the risk* conferred by allele ε2. Thus, both the Verpillat's study (2002) and our study agree

in that H1 enhances the effect of ε2, but with opposite results on the risk of the disease. These contrasting results indicate that interactive effects between gene variants are much more complicated than simple one-by-one interactions, and that the whole genetic background (which is population specific), together with population specific environments, plays a substantial role in modulating the risk of a complex neurodegenerative disease, such as FTD.

In any case, the association we observed between *APOE* gene variability and FTD is in agreement with the well established allele specific anti-oxidant effects exerted by the three of apoE isoforms on neuronal tissues, and extended the association of the ε4/ε2 alleles with a type of dementia other than AD. Thus, according to our results, the ε4 allele may represent a general nuclear genetic risk factor for neurodegenerative diseases. However, the specific role of apoE in neurodegeneration remains to be elucidated. Additional studies are required to establish its relevance in health and in the disease pathogenesis. Intriguingly, studies in animal model have strongly suggested that brain apoE is a multifunctional molecule, with potential roles in amyloid deposition and clearance, microtubule stability, intracellular signalling, immune modulation, glucose metabolism, oxidative stress, and other cellular processes. New data on the role of apoE may give important clues and provide potential opportunities to design rational therapeutic and preventive strategies in some neurodegenerative diseases, including FTD.

As *APOE* and *TAU* genes are, also mtDNA is a good candidate in susceptibility to neurodegenerative diseases, because of the essential role played by mitochondria in energy metabolism and apoptosis. Our study failed to reveal significant associations between mtDNA inherited variability and FTD, both at haplogroup and sub-haplogroup level. This negative finding can be explained by two alternative hypotheses: a) mtDNA inherited variability does not affect susceptibility to FTD; b) some rare mtDNA haplotypes may contribute to FTD, but the sample size has not sufficient statistical power to reveal significant associations. In other words, we cannot exclude that some significant effect may emerge by sequencing the entire mtDNA molecule in a very large sample. A recent paper by Samuels et al. (2006) analyzed the problem of the statistical power in association studies regarding mtDNA inherited variability. If the disease is quite uncommon (and therefore the sample size non sufficiently great) the detection of subtle associations is not reliable. In fact, although today mtDNA sequencing is not a problem from a technical point

of view, it is still a problem to chose the level of variability (haplogroup, sub-haplogroup, sequencing of the entire molecule) at which the analysis should be stopped in order to have a sample of sufficient power with respect to the number of variables. This is an open question that could be solved by further studies that consider *a-priori* biological hypotheses and then examine mtDNA variability accordingly.

On the whole, the studies carried out in the course of my PhD appointment to explore the association between the inherited variability of candidate loci and FTD confirm the complexity of the gene network modulating FTD risk.

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The effects of APOE and tau gene variability on risk of frontotemporal dementia

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Abstract

Frontotemporal dementia (FTD) is a complex dementing syndrome whose genetic/non genetic risk factors are mostly unknown. Aim of the present work was to investigate whether APOE and/or tau gene variability does affect the risk of FTD. A sample of FTD cases (sporadic: $n = 54$; familial: $n = 46$, one subject per family) was collected in a genetically homogeneous population (Calabria, southern Italy) and analyzed in comparison with an age- and sex-matched control group ($n = 180$) extracted from the same population. Logistic regression analysis showed that APOE gene variability affects the probability of disease, with allele ε4 increasing ($\exp^{(\beta 1)} = 2.68$ with [1.51–4.76] 95% confidence interval; $p = 0.001$) and allele ε2 decreasing ($\exp^{(\beta 1)} = 0.28$ with [0.12–0.66] 95% confidence interval; $p = 0.003$) the risk of FTD. On the contrary, tau gene variability was ineffectual ($\exp^{(\beta 1)}$ non significantly different from 1 for either H1 or H2 haplotypes), although a small effect was observed by the H1 haplotype in increasing the protective effect of the ε2 allele ($p = 0.007$).

Keywords: Frontotemporal dementia; Risk factors; Tau haplotypes; APOE; Alzheimer's disease; Lewy body dementia; Subcortical vascular dementia; Vascular dementia; Genetically homogeneous population

1. Introduction

Frontotemporal dementia (FTD) is a complex degenerative dementing syndrome whose broad phenotype often overlaps with that of other neurodegenerative disorders. Progresses in neuropathology, immunohistochemistry, biochemistry and genetics have shown that FTD is heterogeneous, encompassing many different diseases with similar clinical presentation. Its prevalence has been underestimated owing to the difficulty to ascertain early behavioral signs and to the fact that the NINCDS–ADRDA criteria [34], well established for Alzheimer disease (AD), failed to accurately differentiate FTD [53]. The recent introduction of specific operational criteria for FTD [9,20] led to an improvement of the diagnosis and to a better ascertainment of FTD cases. Neuropathological hallmarks of FTD consist, at macroscopic level, of bilateral or focal atrophy localised in frontal and temporal lobes. Neuronal loss, gliosis, Pick bodies and filamentous inclusions containing hyperphosphorylated tau protein [50] are variably combined in the different subgroups of FTD. Since hyperphospohorylated tau protein participates to the formation of neurofibrillary tangles, as in several neurodegenerative diseases [12,13,18], the tau gene has been assumed as an FTD candidate gene. However, tau mutations have been demonstrated as causal only in subgroups of FTD patients showing autosomal dominant pattern of inheritance [30,40,50]. On the contrary, the role of tau mutations in familial [6,44] and spo-

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radic [28,41] FTD is controversial, and evidence of genetic heterogeneity is growing with further genes probably modulating the risk of disease [8,27,57].

Tau gene sequencing [1] has revealed several polymorphisms which are in complete linkage disequilibrium, mostly inherited as two distinct haplotypes, H1 and H2, with relatively few recombination events [3]. Haplotype-based association studies have shown a significant association between the H1/H1 genotype and progressive supranuclear palsy (PSP) [3,14,17]. A similar association has also been found in corticobasal degeneration (CBD) [17,28], while the results are conflicting in both FTD [29,49,55,58] and Pick's disease (PiD) [38].

Apolipoprotein E (APOE) is established as the major genetic risk factor in AD, as well as in other neurodegenerative diseases, but its possible role in FTD is still debated [54]. The interaction of $\varepsilon 2/\varepsilon 4$ APOE alleles with H1 haplotype has also been suggested as a risk factor of developing FTD [31,55].

Aim of the present work was to investigate possible associations of FTD with tau haplotypes and APOE alleles, by applying a logistic regression analysis to a large sample of FTD patients recruited in a well defined and genetically homogeneous population.

2. Material and methods

2.1. Recruitment and selection of participants

One hundred consecutive unrelated persons diagnosed as affected by FTD (53 females and 47 males) were selected from a large group of demented outpatients recruited at the Regional Neurogenetics Centre (Calabria, southern Italy). According to familiarity (at least one first degree relative with dementia), the cases were classified as affected by familial FTD ($n = 46$ subjects, by including in the study only one case per family) or sporadic FTD ($n = 54$ subjects). Subjects whose pedigree analysis was compatible with the hypothesis of monogenic FTD were not present in the sample. According to the age of onset, the cases were defined as early onset cases ($n = 43$; onset ≤ 65 years) and late onset cases ($n = 57$; onset >65 years); the average age of onset was 65.1 years with 9.4 years standard deviation (range 40–83 years). The average duration of the disease was 8.3 years with 5.4 years standard deviation (range 2–40). The maximum follow-up was 8 years (mean 2.1 years with 1.3 years standard deviation).

A control group of 180 unrelated subjects (84 males and 96 females) was recruited in the same population, paying attention to match cases and controls for age, sex, ethnicity and origin in the area, as ascertained by genealogical analyses carried out on three generations. The controls were apparently healthy. In particular, all kinds of dementing diseases were excluded by using the same clinical-laboratory procedures used for patients, including MMSE for the assessment of cognitive status (Table 1).

Table 1
FTD patients and controls

	FTD patients, 100	Controls, 180
Mean age	65.1 ± 9.4	65.2 ± 6.7
Sex ratio (F:M)	1.1	1.1
MMSE*	***14.1 ± 7.2***	26 ± 3.7

Bold and italic represent values under the cut off.
* Calculated on the 86/100 affected testable subjects.

Informed written consent was obtained from all the individuals participating to the study (or from their legal guardians).

2.2. Diagnosis

Diagnosis of FTD resulted from clinical and neurological examination (including elicitation of primitive reflexes such as grasping, sucking, palmo-mental and snout), neuropsychology, morphologic and/or functional neuroradiology, blood count, complete chemistry profile including lipidic assessment, serum folate, Vitamin B_{12}, thyroid function and syphilis serology. The NINCDS–ADRDA [34], McKeith [33] Lund-Manchester Group criteria [9] and NINDS–AIREN criteria [46] also modified for subcortical ischemic vascular dementia [39,45] were used to establish a differential diagnosis between Alzheimer's disease, Lewy body disease (LBD), frontotemporal dementia, vascular dementia (VD) and subcortical ischemic vascular dementia (SIVD), respectively. All affected subjects were inquired for vascular risk factors such as hypertension, hypertrigliceridemia, hypercholesterolemia, cardiopathy, diabetes and alcoholic ingestion; cranial trauma, psychiatric diseases and disorders of the thyroid function were also systematically ascertained. Hachinski score was measured [25] in each case together with activities of daily living and instrumental activities of daily living.

Detailed clinical histories, for profoundly untestable demented patients, were reconstructed through relatives using a checklist of symptoms referring to L–M criteria [9] already used for FTD patients [15].

2.3. Neuropsychological procedures

Neuropsychological battery comprised an extensive assessment of all of cognitive functions: MMSE [19], 15 Rey's words test and 15 Rey's words recall test [43], Verbal Span [2], Corsi Span [35], Babcock story [5], Token test [16], Phonological verbal fluency [7], Phrase construction [22], Drawing copy and Drawing copy with hallmarks [23], Attentive matrices [51], GO/NO GO test [21], Verbal judgements test and Arithmetic judgements test [4], Raven progressive matrices [42].

2.4. Molecular analyses

Genomic DNA was extracted from blood buffy-coats by using standard phenol–chloroform procedures.

2.5. Screening for tau pathogenic mutations

Screening for tau (genomic contig NT 010783) pathogenic mutations was performed by DNA sequence analyses on affected subjects. Exons 9–13 were amplified with primers designed to flanking intronic sequences for exons 9–12 [40] and 13 [3]. A total of 200 ng of genomic DNA were used in a 50 μl reaction mixture containing 20 pmol of each primer, 0.2 mM dNTPs, 1 U Taq (Eppendorf AG, Germany), 1.5 mM $Mg(OAc)_2$ and 1 × buffer. Amplifications were performed in Eppendorf thermal cycler. Conditions for exons 10–12 were 35 cycles of 95 °C for 2 min, 65 °C for 30 s and 72 °C for 40 s with a final extension of 72 °C for 7 min. Conditions for exon 9 and 13 were the same, but with annealing temperature of 60 °C. For each exon, sequence analysis was performed in both directions by using the Big Dye kit (Perkin-Elmer) and relevant PCR primers, on an ABI310 automated sequencer.

2.6. Screening for tau haplotypes

Three polymorphisms (+18 C/T in intron 2; +34 T/C in intron 13; insertion/deletion Δ238 in intron 9; www.alzforum.org/res/com/mut/tau/taupolytable.asp) that are in complete linkage disequilibrium [3] were chosen to construct the haplotypes H1 and H2. The SNPs were spotted in both controls and patients by restriction analyses. The SNP +34 T/C which creates a Tsp5091 restriction enzyme recognition site was analyzed in controls by electrophoresis because of its simplicity. Given the perfect concordance between RFLP and sequence analysis results were compared to sequence data obtained in patients during the screening for tau pathogenic mutations.

The DNA target regions were amplified by using primers previously designed [3]. A total of 200 ng of genomic DNA were used in a 50 μl reaction mixture containing 20 pmol of each primer, 0.2 mM dNTPs, 1 U Taq (Eppendorf AG, Germany), 1.5 mM $Mg(OAc)_2$ and 1 × buffer. Amplifications were performed in Eppendorf thermal cycler. Conditions for the target sequences containing the +18 C/T variant of intron 2 or the +34 T/C variant of intron 13 were 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s with a final extension of 72 °C for 10 m. PCR products were digested with 5 U of the diagnostic restriction enzyme BsaH1 for intron 2 (37 °C overnight) and Tsp5091 for intron 13 (65 °C for 3 h) in a final volume of 20 μl. The digestion products were run on an 1% Methaphor gel with 1 × TBE and visualized by ethidium bromide staining.

PCR conditions for the target sequence containing the Δ238 polymorphism were the same as above described, but with annealing temperature of 62 °C. The presence of the Δ238 deletion was determined directly by visualizing PCR product on an 1% agarose gel.

2.7. Screening for APOE genotypes

APOE genotypes were determined by PCR–RFLP with the Kit Multigen APOE INRCA (Diatech, Ancona, Italy).

2.8. Statistical analyses

The effects of carrying given alleles on the probability to be part of the group of patients was carried out by logistic analysis, under the assumption that risk alleles are more frequent among patients than among controls. In our case, given a dichotomous variable ($D = 1$, 0), such as being part of patients ($D = 1$) or of healthy subjects ($D = 0$), and given a dichotomous genetic variable ($G = 1$, 0) indicating to be carriers ($G = 1$) or not ($G = 0$) of a certain allele at locus G, we calculate the ratio between the probability to be $D = 1$ having $G = 1$, and the probability to be $D = 1$ having $G = 0$. This indicates how much the probability of being $D = 1$ is influenced by being $G = 1$.When such a ratio is significantly higher than 1, the probability of being $D = 1$ is significantly influenced by being $G = 1$ (statistical significance checked by Wald's test). In such a way, the model can discriminate between $D = 1$ and $D = 0$.

The potential effect on the age of onset either by APOE or tau variability was investigated by non parametric Mann–Whitney test.

All statistical analysis were performed on SPSS 11.5.

3. Results

3.1. Diagnosis of FTD

All patients showed a clinico-neuropsychological and neuroradiological profile compatible with the diagnosis of FTD. Fig. 1 shows behavioral and cognitive symptoms displayed by testable and untestable FTD patients in the average period of follow-up. MMSE was performed in 86 patients; 14 patients were untestable mostly for the complete impairment of the language or for uncooperativeness. Forty-six patients underwent the complete battery of neuropsychological testing, while the remaining all underwent only part of the battery. Table 2 shows the results of neuropsycological assessment in the 46 patients who have been grouped according to MMSE value.

Hachinski score resulted ≤4 in all cases indicating a degenerative dementia.

Neuropathology was available in one case only and diagnosis was a frontotemporal ubiquitin positive, tau and alpha synuclein negative dementia [11]. Neuroradiological examination, such as magnetic resonance imaging (27 patients) and computed tomography (54 patients) showed atrophy of the frontotemporal lobes; in any case, no infarcts or white matter early confluent or confluent lesions according to the Wahlund scale [56] were evidenced; SPECT examination (49 persons) showed hypoperfusion in frontal, parietal and temporal areas.

Concerning the analysis of risk factors, hypertension was detected in 28% of patients, hypertrigliceridemia in 3%, hypercholesterolemia in 13%, cardiopathy in 8%, diabetes

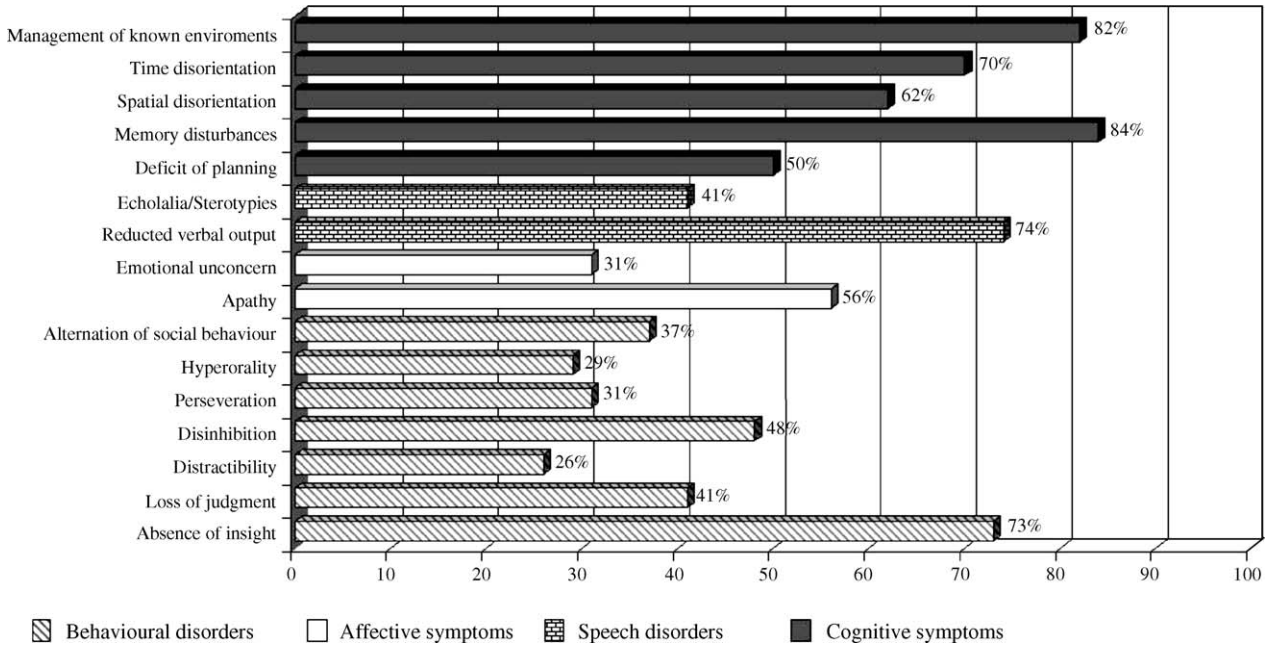

Fig. 1. Behavioral and cognitive symptoms displayed by testable and untestable FTD patients in the mean period of follow-up.

Table 2
Results of neuropsychological assessment in 46 patients who have been grouped according to MMSE value

	Mild cognitive impairment (MMSE 25-21), $N=12$		Mild dementia (MMSE 20-16), $N=12$		Moderate dementia (MMSE 15-11), $N=13$		Severe dementia (MMSE <11), $N=9$	
	T	Mean ± S.D.	T	Mean ± S.D.	T	Mean ± S.D.	T	Mean ± S.D.
Rey's words test	7	32.3 ± 4.1	9	***23.1 ± 8.7***	8	***17.3 ± 9.2***	2	***20.7 ± 0.7***
Rey's words recall test	7	7.2 ± 2.1	9	***3.2 ± 2.7***	9	***2.4 ± 2.8***	2	***0.0 ± 0.0***
Verbal span	5	4.1 ± 0.5	3	3.8 ± 0.1	5	***3.1 ± 0.4***	5	3.5 ± 1.1
Corsi span	9	4.0 ± 1.3	7	***3.5 ± 0.6***	6	3.9 ± 1.2	6	***2.5 ± 1.5***
Babcock story	6	***7.1 ± 5.6***	4	***3.7 ± 4.3***	5	***0.7 ± 1.5***	3	***0.8 ± 1.4***
Phonological verbal fluency	10	***15.2 ± 2.0***	9	*11.8 ± 6.0*	8	***10.8 ± 7.4***	6	***6.7 ± 9.7***
Phrase construction	2	16.2 ± 2.2	5	15.1 ± 7.3	3	15.2 ± 8.7	0	–
Token test	11	***4.6 ± 0.7***	10	***4.4 ± 0.8***	9	***4.2 ± 1.1***	7	***3.6 ± 1.4***
Immediate visive memory	5	16.4 ± 3.8	6	13.9 ± 3.5	4	***10.7 ± 4.3***	4	***12.2 ± 4.6***
Drawing copy	10	***5 ± 2.8***	10	***4.8 ± 2.7***	9	***3.9 ± 2.6***	6	***5.1 ± 3.4***
Drawing copy with hallmarks	7	***40.7 ± 23.8***	9	***32.8 ± 17.1***	8	***23.0 ± 17.4***	3	***28.4 ± 11.4***
Attentive matrices	12	44.5 ± 1.9	9	***36.1 ± 15.7***	10	***29.0 ± 20.4***	6	46.8 ± 17.7
GO/NO GO test	7	***3.9 ± 1.9***	6	***3.3 ± 1.5***	8	***2.5 ± 1.8***	5	***1.8 ± 2.1***
Raven progressive matrices	7	***18.3 ± 2.3***	8	***14.0 ± 4.0***	7	***12.0 ± 4.9***	3	***13.1 ± 4.5***
Verbal judgments	3	42.3 ± 8.2	2	***23 ± 7.1***	3	***20.9 ± 18.2***	2	***19.8 ± 27.9***
Arithmetic judgments	1	10 ± 0	1	***7***	2	***3.9 ± 5.6***	0	–

N: number of the patients; *T*: patients able to undergo the specific test; bold and italic represent values under the cut off; S.D.: standard deviation.

in 4% and alcoholic ingestion in 11%. Cranial trauma, psychiatric diseases and disorders of the thyroid function were reported in 2, 11 and 7% patients, respectively. No patient showed more than two of the above risk factors at the same time and no statistical difference was found by comparing early and late onset FTD groups. In control subjects, only main risk factors were available: hypertension was detected in 47%, hypertrigliceridemia in 48%, hypercholesterolemia in 26% and diabetes in 16%. The prevalence of the above risk factors in FTD patients was below that of the control group (Table 3) according to previous epidemiological studies (www.cuore.iss.it/fat_rischio/regioni/calabria/calabria.html).

3.2. Absence of tau pathogenic mutations

All the patients were screened for tau mutations in exons 9–13 to investigate the frequency of tau mutations in our

Table 3
Main risk factors in FTD patients and controls

Main risk factors	FTD patients, 100 (%)	Controls, 180 (%)
Hypertension	28	47
Hypercholesterolemia	13	48
Hypertrigliceridemia	3	26
Diabetes	4	16

Table 4
APOE genotypes and alleles frequencies in patients and controls

APOE genotypes	FTD		Controls	
	Observed	Expected	Observed	Expected
2/2	1	0.2	4	2.5
2/3	4	6.2	31	33.5
2/4	2	1.5	3	3.6
3/3	61	59.3	116	114.4
3/4	28	29.3	24	24.7
4/4	4	3.6	2	1.3
Total	100	100	180	180

Allele	Frequency (% ± S.E.)	Frequency (% ± S.E.)
2	8 (4.0 ± 1.4)	42 (11.7 ± 1.7)
3	154 (77.0 ± 3.0)	287 (79.7 ± 2.1)
4	38 (19.0 ± 2.8)	31 (8.6 ± 1.5)
Total	200	360

APOE genotypes and alleles in FTD patients and controls. Observed and expected (under Hardy–Weinberg equilibrium assumption) absolute frequencies are shown for genotypes. Absolute and relative (% ± S.E.) frequencies are shown for alleles.

Table 5
H1–H2 genotypes and haplotypes frequencies in patients and controls

Tau H1–H2 genotypes	FTD		Controls	
	Observed	Expected	Observed	Expected
H1H1	54	53.2	95	95.8
H1H2	38	38.6	70	69.4
H2H2	8	8.2	15	14.8
Total	100	100	180	180

Haplotypes	Frequency (% ± S.E.)	Frequency (% ± S.E.)
H1	146 (73 ± 3.1)	260 (72.2 ± 2.4)
H2	54 (27 ± 3.1)	100 (27.8 ± 2.4)
Total	200	360

H1–H2 genotypes and haplotypes in FTD patients and controls. Observed and expected (under Hardy–Weinberg equilibrium assumption) absolute frequencies are shown for genotypes. Absolute and relative (% ± S.E.) frequencies are shown for haplotypes.

familial and sporadic FTD cases. None of the previously reported, nor novel tau mutations were detected.

3.3. Logistic regression analysis

Both cases and controls were screened for APOE and haplotype tau variability. Since no significant difference was found between familial and sporadic cases, the two FTD samples were pooled to increase the power of the analyses (data relevant to familial and sporadic cases are shown at the website www.arn.it). Data on APOE and tau variability are reported in Tables 4 and 5, respectively. Logistic regression analysis of the main effects of APOE and tau variability on the FTD risk showed that while APOE alleles significantly modulate the risk, tau haplotypes are ineffectual. In fact, $\varepsilon 4$ allele increased the risk ($\exp^{(\beta 1)} = 2.68$ with 95% CI = 1.51–4.76; $p = 0.001$; $R^2 = 5.5\%$) while $\varepsilon 2$ decreased it ($\exp^{(\beta 1)} = 0.28$ with 95% CI = 0.12–0.66; $p = 0.003$; $R^2 = 5.1\%$), no significant effect being observed as for $\varepsilon 3$ ($\exp^{(\beta 1)} = 0.699$ with 95% CI = 0.252–1.938; $p = 0.492$). Dominant and/or additive effects of $\varepsilon 2$–$\varepsilon 4$ alleles were not calculated because of

Table 6
Cross tables reporting the absolute frequencies of H1 (H2) carriers/non-carriers with respect to carriers/non-carriers of $\varepsilon 2$ ($\varepsilon 4$) APOE alleles in patients and controls

	Patients		Controls	
	$\varepsilon 4$ carriers	$\varepsilon 4$ non-carriers	$\varepsilon 4$ carriers	$\varepsilon 4$ non-carriers
a				
H1 carriers	30	62	29	136
H1 non-carriers	4	4	0	15
Total	34	66	29	151
b				
H2 carriers	14	32	9	76
H2 non-carriers	20	34	20	75
Total	34	66	29	151
	Patients		Controls	
	$\varepsilon 2$ carriers	$\varepsilon 2$ non-carriers	$\varepsilon 2$ carriers	$\varepsilon 2$ non-carriers
c				
H1 carriers	7	85	35	130
H1 non-carriers	0	8	3	12
Total	7	93	38	142
d				
H2 carriers	5	41	13	72
H2 non-carriers	2	52	25	70
Total	7	93	38	142

the small number of homozygous carriers. For what concerns haplotype analysis, $\exp^{(\beta 1)}$ was not significantly different from 1 for either the haplotypes H1 ($\exp^{(\beta 1)} = 1.04$ with 95% CI = 0.42–2.55; $p = 0.92$) or H2 ($\exp^{(\beta 1)} = 0.95$ with 95% CI = 0.58–1.55; $p = 0.84$). Table 6 reports the absolute frequencies of H1 (H2) carriers/non-carriers with respect to carriers/non-carriers of ε2 (ε4) APOE alleles in patients and in controls. The analysis of the interactions between APOE and tau polymorphisms showed no effect of either H1 or H2 on modulating the risk estimated for ε4 allele (Table 6, sections a and b). In fact, the risk estimate due to the presence of ε4 allele was not changed by the simultaneous presence of either H1 ($\exp^{(\beta 1)} = 2{\cdot}23$ with 95% CI = 1.25–4.00; $p = 0.007$) or H2 ($\exp^{(\beta 1)} = 3.09$ with 95% CI = 1.29–7.43; $p = 0.012$) with ε4. On the contrary, the protective effect of ε2 was increased when H1 was simultaneously present in the genotype ($\exp^{(\beta 1)} = 0.31$ with 95% CI = 0.13–0.73; $p = 0.007$; $R^2 = 5.6\%$) with ε2, and decreased by the presence of the combination H2/ε2 ($\exp^{(\beta 1)} = 0.68$ with 95% CI = 0.23–1.96; $p = 0.470$) (see Table 6, sections c and d). It is worth noticing that H2 allele was present in 34.2% of ε2 carriers among controls but in 71.4% of cases with ε2 allele.

Multivariate logistic regression analysis including the effects of all the alleles was also performed; results were not significant because of the most important effect of ε2 and ε4 alleles with respect to H1 (H2) (data not shown). Results coming from the logistic separate analysis of sporadic and familial cases were in trend with the findings we obtained on a whole group although statistical significance was not reached because of the small sample size (data not shown).

No significant effect was observed on the age of onset either by APOE or tau variability.

4. Discussion

We analyzed a large clinical-based series of familial and sporadic unrelated FTD patients with the aim to verify if APOE and tau variability did affect FTD risk. All patients and controls came from a region of southern Italy (Calabria) that could be considered as a genetic isolate due to the geographic and historical isolation that occurred in the last three centuries. In this region, phenomena of founder effect had been already described for the genetic PS1 mutated Alzheimer's disease [10] and Manic depressive illness [26] strongly reinforcing the hypothesis of genetic homogeneity of this population.

Owing to the lack of neuropathological examinations (except one case) and MAPτ mutations, to minimize errors in diagnosis, we used multiple diagnostic criteria, based on extensive collection of signs and symptoms, gathering of vascular risk factors, neuropsychological testing and morphological and functional neuroradiology. Independently from the age at onset, all patients showed a homogeneous behavioral pattern of dementia characterized by an insidious onset, loss of social awareness, frequently disinhibition and absence of insight. Emotional unconcern, apathy, lack of empathy, loss of judgment and distractibility were reported in a high percentage of patients. These signs had been described also in SIVD [45], nevertheless, the absence of ischemic confluent lesions at MRI and CT in our series helped to distinguish the two illnesses. Reduction of the verbal output up to complete lost of language, persistence of management of known environment, stereotypes and hyperorality were the symptoms characterizing FTD in these patients. The course of the disease was progressive and without fluctuations in cognitive function as seen in Lewy body patients.

We started from searching for tau mutations in the whole group of FTD patients. None of the previously reported, nor novel tau mutations were detected. This finding is in line with data showing that the prevalence of tau mutations in FTD is generally very low [6,28,41]. What is more, the absence of tau mutations had been already found in a large Calabria kindred segregating FTD [15]. On the whole, these data suggest that, in the Calabrian population, tau mutations are not involved in FTD and reinforce the idea that genetic risk factors are population-specific. The lack of effect by tau variability on FTD risk is confirmed by the results of the H1/H2 haplotype analyses (Table 5), and is in line with data obtained in both FTD [49,58] and Pick's disease [38].

On the contrary, the effect of APOE variability on FTD risk was quite significant, with allele ε4 increasing and allele ε2 decreasing the risk (see Tables 4 and 6). Given the frequency of ε4 and ε2 carriers (16 and 21%, respectively), it is evident that the impact of this result on the population is important, thus suggesting that quite common variants may modulate the susceptibility to FTD in this population. As for allele ε4, our results are in agreement with studies showing this allele as an FTD risk factor [18,24,47,52]. On the other hand, the protective effect of ε2 allele has never been reported, at our knowledge, in FTD and is in contrast with studies reporting ε2 as an allele that increases the risk [32,54,55]. However, the finding that ε4 increases and ε2 decreases FTD risk, at least in our population, is in line with the well established allele-specific anti-oxidant effects exerted by the three ApoE isoforms on neuronal tissues [36].

The analysis of the interactions between APOE and tau variability in modulating FTD risk has been scarcely analyzed, and the reported data result in puzzling findings [29,31,48,58]. In our population sample, logistic regression analysis evidenced that H1/H2 haplotypes did not increase the ε4 allele risk and did not influence the onset of the disease, while the protective effect of ε2 was apparently increased by the H1 haplotype. A significant interaction between ε2 and H1 had been already observed in the Verpillat study [55], although in that case the interaction resulted in an increase of the risk conferred by allele ε2. In any case, both the cited and the present studies are in line with the observation that the H1 haplotype enhances the effect of the ε2 allele, increasing or decreasing FTD risk, probably according to the population-specific genetic background.

From the present study, two main conclusions can be drawn. First, the role played by APOE in predisposing to or protecting from common neurodegenerative disorders [37] is confirmed. Second, the importance of the population history in modeling the pattern of risk factors in complex neurodegenerative diseases as a consequence of population-specific linkage disequilibrium patterns [59].

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Table 7: Variant sites found in mtDNA HVS-I. For each haplotype the absolute frequency in sporadic FTD (S) patients, familial FTD (F) and controls (C) is reported. rCRS refers to revised Cambridge Reference Sequence (MITOMAP: A Human Mitochondrial Genome Database.http://www.mitomap.org 2006). 16000 nt have to be added to align the sequence with rCRS.

controls (C)	controls + FTD (F or S)
sporadic FTD (S)	sporadic FTD + familial FTD
familial FTD (F)	