# GENETIC AND BIOCHEMICAL ANALYSES OF FRONTOTEMPORAL DEMENTIA

A THESIS PRESENTED BY

**ASWATHY P M**

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SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

THIRUVANANTHAPURAM

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF

**DOCTOR OF PHILOSOPHY**

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# DECLARATION

I, Mrs. Aswathy P M, hereby certify that I had personally carried out the work depicted in the thesis entitled, **"Genetic and Biochemical Analyses of Frontotemporal Dementia"**. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date: 24th June 2016 Aswathy P M

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The thesis entitled **"Genetic and Biochemical Analyses of Frontotemporal Dementia"** was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date. Clearance was obtained from the Institutional Ethics Committee for carrying out the study.

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The thesis entitled

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Submitted by

**ASWATHY P M**

For the Degree of

**Doctor of Philosophy**

of

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

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# ABBREVIATIONS

| | |
|---|---|
| AD | Alzheimer's disease |
| ALS | Amyotrophic lateral sclerosis |
| ANOVA | Analysis of variance |
| APOE | Apolipoprotein E |
| bp | base pair |
| bvFTD | Behavioral variant FTD |
| C9ORF72 | Chromosome 9 open reading frame 72 |
| CBD | Corticobasal degeneration |
| CBS | Corticobasal syndrome |
| cDNA | Complementary DNA |
| CHMP2B | Charged multi-vesicular protein 2B |
| CNS | Central nervous system |
| CSF | Cerebrospinal fluid |
| ddNTPS | Di-deoxy nucleotide triphosphates |
| DLBD | Dementia with Lewy bodies |
| DLDH | Dementia lacking distinctive histopathology |
| DNA | Deoxy-ribonucleic acid |
| DNs | Dystrophic neurites |
| dNTPs | Deoxy-nucleotide triphosphates |
| ELISA | Enzyme-linked immunosorbent assay |
| ESCRT | Endosomal sorting complex required for transport |
| FTD | Frontotemporal dementia |
| FTDP-17 | Frontotemporal dementia and Parkinsonism linked to chromosome 17 |
| FTD-U | FTD with ubiquitinated inclusions |
| FTLD | Frontotemporal lobar degeneration |
| FUS | Fused in sarcoma |
| GWAS | Genome wide association analysis |

| | |
|---|---|
| IBMPFD | Inclusion body myopathy with Paget's disease of the bone and frontotemporal dementia |
| IVS | Intervening sequence |
| Kb | Kilobase |
| kD | Kilodalton |
| LD | Linkage disequilibrium |
| *MAPT* | Microtubule-associated protein tau |
| MMSE | Mini mental state examination |
| MND | Motor neuron disease |
| MRI | Magnetic resonance imaging |
| mRNA | messenger RNA |
| NCIs | Neuronal cytoplasmic inclusions |
| NDDs | Neurodegenerative disorders |
| NFT | Neurofibrillary tangles |
| NIIs | Neuronal intra-nuclear inclusions |
| nM | Nanomol |
| NMD | Nonsense-mediated decay |
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| PGRN | Progranulin |
| PHF | Paired helical filaments |
| PiD | Pick's disease |
| PNFA | Progressive non-fluent aphasia |
| PNS | Peripheral nervous system |
| PSEN | Presenilin |
| PSP | Progressive supranuclear palsy |
| RFLP | Restriction fragment length polymorphism |
| RNA | Ribonucleic acid |
| SD | Semantic dementia |
| SNP | Single nucleotide polymorphism |

| | |
|---|---|
| SSP | Sequence-specific primer |
| SSRIs | Selective serotonin re-uptake inhibitors |
| Taq | Thermus aquaticus |
| TBARS | Thiobarbituric acid reactive substances |
| TDP-43 | Trans-activation response DNA binding protein with molecular weight of 43 kD |
| TMEM106B | Trans-membrane protein 106B |
| UTR | Untranslated region |
| VCP | Valosin containing protein |
| VD | Vascular dementia |

# SYNOPSIS

## Background of the study

Frontotemporal dementia (FTD) is a devastating clinical syndrome associated with the progressive degeneration of frontal and anterior temporal lobes of the brain which is specifically involved in the behavior control, decision-making, emotions and language. It affects the people under the age of 65 and is the second most common cause of presenile dementia after Alzheimer's disease (AD). Based on the specific brain region involved, three different clinical manifestations are known: behavioral variant FTD, progressive non-fluent aphasia (PNFA) and semantic dementia (SD). FTD is a genetically complex disorder and there is no known treatment to stop or reverse the neurodegeneration in the affected individuals. Up to 50% of FTD cases possess a family history of similar dementia and in remaining cases the disease is sporadic in nature without any known causes. Familial cases shows an autosomal dominant pattern of inheritance and currently, a number of genetic mutations have been identified in familial FTD patients. Mutations in the microtubule-associated protein tau (*MAPT*), progranulin (*PGRN*) and chromosome 9 open reading frame 72 (*C9ORF72*) are the most common genetic causes for FTD. FTD is a proteinopathy with abnormal protein inclusions in the cytoplasm or nuclei of neuronal and glial cells in affected brain regions. Neuropathologically, FTD is characterized by the deposition of tau or trans-active response DNA-binding protein with molecular weight of 43 kDa (TDP-43) positive inclusions that are pathologically modified and aggregated in the degenerating brain regions.

In some patients, the genes do not directly cause disease in the classical Mendelian pattern but alter the risk of development of disease. Some genes have been described

as genetic risk factors that increase an individual's susceptibility to develop FTD rather than directly causing the disease. *MAPT* genomic region encompasses two distinct haplotypes termed as H1 and H2. Even though H1 has been established as a genetic risk factor for sporadic tauopathies like progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD), the association of H1 haplotype with FTD lacks consensus. Case-control studies have not been consistent in showing association between *MAPT* haplotypes and FTD, producing contradictory findings. For *PGRN*, a single nucleotide polymorphism (SNP) located in the 3'-untranslated region of *PGRN* (rs5848) has recently been associated with increased risk of developing FTD. The ε4 allele of apolipoprotein gene (*APOE*) is a well-known risk factor for late onset and familial AD. Case-control studies have not been consistent in showing association between *APOE* polymorphisms and FTD producing contradictory findings.

Biomarker analysis in CSF is becoming increasingly useful at identifying a specific histopathologic abnormality in patients with familial as well as sporadic FTD. Increased cerebrospinal fluid (CSF) tau protein has been detected in FTD. A significant reduction in CSF or plasma progranulin protein levels can also potentially serve as a biomarker of FTD which help to distinguish the carriers of *PGRN* mutation from normal controls. Since one of the risk factors for developing FTD is age, the processes involved in aging process are also suggested to be involved in the pathogenesis of FTD.

## Objectives

1. To determine the prevalence of known genetic mutations in *MAPT* and *PGRN* associated with a south Indian cohort of FTD patients
2. To evaluate *MAPT* haplotypes, *PGRN* rs5848 polymorphism and *APOE* polymorphisms as genetic susceptibility factors in FTD
3. To quantify the plasma levels of tau, progranulin, TDP-43 and lipid peroxides in order to serve as biomarker profile in FTD

## Hypothesis

The commonest genetic variations on *MAPT*, *PGRN* and *APOE* may be responsible for the genetic predisposition to FTD in south Indian cohorts and plasma proteins such as tau, progranulin and TDP-43 and the oxidative stress marker such as lipid peroxides in serum may serve as biomarkers that are able to distinguish FTD patients invariably from the age-matched controls.

## Methods

Study participants were recruited from patients attending the Memory and Neurobehavioral Clinic (MNC) at SCTIMST, after obtaining approval from the Institutional Ethical Committee and written informed consent from all participants or their caregivers. The study cohort consisted of 529 participants comprising FTD (n=116), AD (n=132), vascular dementia (VD) (n=36), other dementia groups (comprising progressive supranuclear palsy (PSP), corticobasal syndrome (CBS), Dementia with Lewy bodies (DLBD) and mixed dementia cases) (n=37), mild cognitive impairment (MCI) (n=78), and cognitively unimpaired controls (n=130)

who were matched with the patients for age, sex and ethnicity. Clinical assessment was done by a neurologist according to the standard criteria (Consensus criteria for FTD). Venous blood samples were collected from all participants and separated into plasma, serum and DNA. The candidate genes selected for mutation analysis included *MAPT* and *PGRN* and the mutation screening was performed through direct DNA sequencing. Briefly, all the exons harboring pathogenic mutations were subjected to polymerase chain reaction (PCR) using the primers flanking the adjacent intronic regions. Amplified fragments were directly sequenced using the BigDye Terminator Cycle sequencing kit and products were analyzed in an automated sequencer. Mutation screening was done using Sequencher software. In order to determine the association of known genetic risk factors, genetic association analyses of known variants have been conducted with age-matched cognitively normal controls. This included *MAPT* haplotypes, *PGRN* 3'-UTR rs5848 polymorphism and *APOE* polymorphisms. Genotyping was performed by PCR, direct DNA sequencing, RFLP-PCR and SSP-PCR. Genotype as well as allele frequencies were determined and checked for deviation from Hardy-Weinberg equilibrium. Statistical analyses were performed using the GraphPad Prism 5.0 software. Biochemical assays were carried out through enzyme-linked immunosorbent assay (ELISA), dot blot assay and thiobarbituric acid reacting substances (TBARS) assay. Total tau protein in plasma was assessed through a sandwich ELISA kit from Invitrogen. Plasma concentration of progranulin was carried out through Human PGRN ELISA kit from Adipogen. Plasma TDP-43 ELISA was carried out through a sandwich ELISA kit from USCN life sciences. Lipid peroxides in serum samples were quantified through TBARS assay.

## Major findings

A positive family history was noticed in 17/116 FTD cases (15%), but the *MAPT* mutation analysis revealed no pathogenic mutations in either familial or sporadic FTD patients. Several non-pathogenic single nucleotide polymorphisms (SNPs) were detected and IVS9-48 was a novel intronic variation detected through this study. *MAPT* haplotype frequencies were determined in 399 patients comprising FTD (n=116), AD (n=132), VD (n=36), other dementia subgroups (n=37) and MCI (n=78) and compared with 130 controls. *MAPT* genotype frequencies in the entire study group did not vary significantly from Hardy-Weinberg equilibrium. The frequency of H1H1 genotype in whole FTD group was 85% compared to 91.5% in controls, 94.7% in AD, 94% in VD, 96% in MCI, and 80% in other dementia groups. However, there were no statistically significant differences in the distribution of *MAPT* genotype frequencies between FTD (except SD, p value=0.01) or other dementia groups and controls. In SD, a significant association in the genotype frequency was observed with an overrepresentation of H2H2 genotype when compared to controls (p=0.01). When *MAPT* haplotype frequencies were compared, there were no statistically significant differences in the distribution in FTD or other dementia groups with controls. The H2 allele did not show any significant association with age at onset or familial occurrence of the disease.

Evaluation of plasma tau in FTD (n=23) and controls (n=10) showed that there is a trend towards increase in the tau concentration in FTD but did not show a statistically significant difference when compared with controls (p=0.82). This

implies that plasma tau protein is of limited value in discriminating the FTD from controls.

When plasma concentrations of progranulin were measured in FTD (n=60) and controls (n=36), 20% of the FTD patients carried a reduction in the protein level. *PGRN* mutation analysis in the whole FTD patients (n=116) revealed one novel pathogenic mutation in one familial FTD patient showing significantly reduced level of plasma progranulin (28 ng/mL). This mutation was located on exon 12 and was found to introduce a premature termination codon producing a polypeptide chain terminated at 503$^{rd}$ amino acid residue. This prematurely terminated mRNA may probably undergo nonsense-mediated decay resulting in the haploinsufficiency of progranulin. A series of reported as well as novel SNPs were detected and were found to be non-pathogenic. Two novel intronic variations were detected in intron 2 (IVS1-41 and IVS1-29).

The association analysis of *PGRN* rs5848 C>T polymorphism showed that variation at rs5848 does not contribute susceptibility to FTD (n=116) when compared with controls (n=130). However, the correlation of plasma progranulin levels and rs5848 polymorphisms in FTD (n=60) revealed a statistically significant reduction on the *PGRN* expression in homozygous T allele carriers when compared with homozygous C allele carriers, which may explain the reduced progranulin levels in patients without *PGRN* mutations (p=0.8).

The association analysis of *APOE* locus failed to find a significant association of ε2 or ε4 allele with disease risk in FTD when compared with controls. However, ε3ε4 genotype was significantly associated with increased plasma progranulin levels in

FTD (p=0.01). The analysis of plasma TDP-43 levels failed to find a significant difference between FTD patients (n=29) and controls (n=13) (p=0.14). TDP-43 was barely detectable in some patients and controls. Serum malondialdehyde (MDA) levels determined through TBARS assay showed a significant increase in FTD (n=21) compared with controls (n=20) (p=0.0001).

## Significance/Implications of the findings

To the best of our knowledge, this is the first report to analyze the genetic mutations in *MAPT* and *PGRN* in a south Indian cohort of FTD patients. The study has evaluated the contribution of known genetic causative or risk factors in pathogenesis of FTD. *MAPT* and *PGRN* genes were screened for mutations and found that known genetic mutations in *MAPT* and *PGRN* are rare cause of FTD in south Indian population. However, the identification of one novel mutation in *PGRN* with the proposed uniform disease mechanism of haploinsufficiency does not rule out the occurrence of novel mutations in the south Indian cohorts.

Since the study cohort comprised both familial and sporadic FTD cases, association analyses were combined to verify the existence of the risk haplotypes or genotypes in the study cohort. However, none of the risk alleles associated with *MAPT, PGRN* or *APOE* showed a strong or statistically significant correlation compared with controls. The contradictory results obtained through this study may be due to the ethnic difference of the study population from other studies; however this needs to be validated in large number of samples.

The biochemical analyses evaluating plasma levels of tau, progranulin, TDP-43 and serum levels of lipid peroxides were performed through this study. The plasma

concentrations of tau and TDP-43 were not found to be the predictability markers for neurodegeneration in FTD. Although there was no statistically significant difference in plasma progranulin levels between FTD and controls, a proportion of FTD cases were shown reduced level of progranulin up to 50% of the normal protein levels. Since only one patient harbored the *PGRN* mutation, we speculated that some other pathogenic mechanism at *PGRN* loci might be involved in the reduced expression. To address this question, we have correlated the plasma progranulin levels with rs5848 polymorphism. This revealed that the presence of T allele was associated with a significant reduction of progranulin levels in FTD patients. This may clearly implicate the role of common genetic variants other than mutations as genetic susceptibility factors for FTD. The correlation of plasma progranulin with *APOE* genotype in FTD revealed that the average plasma progranulin level is significantly increased in ε3ε4 carriers compared to ε3ε3 carriers. The increase in progranulin levels observed may be related to the inflammatory response associated with the pathogenesis. Moreover, a subset of sporadic FTD patients have shown a significantly higher level of lipid peroxides in serum compared with controls which suggests that oxidative stress might be associated with the pathogenesis.

This study provided evidence for the existence of genetic heterogeneity associated with FTD. More comprehensive screening studies are essential to establish the genetic linkage of other putative loci, which can definitely find out underlying genetic abnormality leading to the pathogenesis of FTD in this study cohort.

# I. INTRODUCTION

## I.1. Dementia

Dementia is defined as a group of clinical syndromes affecting the cognitive decline due to neurodegeneration i.e., a decline in memory or other thinking skills severe enough to reduce a person's ability to perform everyday activities. It is a major health problem in aging society. Whereas normal aging is associated with a slow and steady loss of brain cells (Bartzokis et al., 2003; Fotenos et al., 2005; Jernigan et al., 2001) dementia is characterized by gradual onset and progressive decline of cognitive functions that interfere with the everyday life. Globally, the prevalence of dementia is estimated to double every twenty years during the first half of this century, increasing from approximately 35 million in 2010 to over 115 million in 2050 due to increased life expectancy (Alzheimer's Disease International, 2009).

A number of neurodegenerative diseases (NDDs) cause dementia, including Alzheimer's disease (AD), dementia with Lewy bodies (DLBD), vascular dementia (VD) and frontotemporal dementia (FTD). The most common cause of dementia is AD accounting for 60-80% of cases after the age of 65 years. Numerous other NDDs have an associated dementia; including corticobasal degeneration (CBD), progressive supranuclear palsy (PSP) and amyotrophic lateral sclerosis (ALS). The pattern of neurodegeneration in dementia shows regional selectivity of brain cell loss. Majority of dementia cases are associated with aberrant deposition of proteins in the affected brain regions. Dementia can present either as familial disorder or as apparently sporadic, implying the existence of both genetic as well as environmental causes.

While dementia mainly affects people in the older age groups, the incidence of early-onset dementia (presenile dementia) is increasing worldwide affecting people under the age of 65. Due to its multiple etiological factors and in many cases, the unusual presentation, presenile dementia poses a significant diagnostic challenge for clinicians, often leading to delay in diagnosis.

## I.2. Frontotemporal dementia (FTD)

The frontal lobe, located on the front part of cerebral hemispheres, is considered as the most vital part of the brain and it is mainly concerned with the execution of behavior, i.e., it is involved in the emotional control, personality, motor functions, thinking, problem solving, memory, attention, language initiation, judgment, impulse control and social behavior. There is an asymmetric difference in the functional organization of right and left frontal lobes. The left frontal lobes are more specialized for language related functions and the right frontal lobe plays a role in social recognition and emotions. Temporal lobe is located along the sides of the brain is associated with perception and recognition of auditory stimuli, memory, and speech.

There are a few conditions, in which the frontal cortex is the primary site of diseases. FTD is the typical example for the disorder that primarily involves frontal lobes. It (OMIM #600274) is the second most common cause of presenile dementia next to AD. FTD comes under the pathological diagnosis termed as frontotemporal lobar degeneration (FTLD) accounting for 5-15% of all cases of dementia (Bird et al., 2003; Ratnavalli et al., 2002). It is a devastating disorder for patient and caregiver and the economic and emotional consequences are more detrimental because it

strikes otherwise healthy individuals in their middle age, who are often at the peak of their careers and parenting responsibilities. Incidence estimates of FTD range from 3.3 per 100,000 for 50-59 year olds to 8.9 per 100,000 for 60-69 year olds (Knopman et al., 2004).

Based on several aspects, FTD is considered as a heterogeneous syndrome. Clinically, FTD comprises a heterogeneous group of dementing disorders associated with the degeneration of frontal and anterior temporal lobes. Based on the predominance of pathologic involvement of the prefrontal cortex and anterior temporal lobes, the clinical features are characterized by varying degree of progressive deterioration in behavior, personality and/or language with relative preservation of memory (The Lund, Manchester Criteria, 1994; McKhann et al., 2001; Neary et al., 1998). The lobar atrophy may be symmetrical or asymmetrical. Based on the pathology and clinical presentations, FTD is broadly divided into behavioral variant (bvFTD) and language variant syndromes based on the presence of fluent or non-fluent speech output as progressive non-fluent aphasia (PNFA) and semantic dementia (SD) (Neary et al., 1998). These three syndromes; bvFTD, PNFA and SD often tend to overlap and as the disease progresses, the clinical symptoms tend to converge, eventually resulting in mutism and behavioral disturbances. FTD is sometimes associated with parkinsonism or motor neuron disease (MND) (Lomen-Hoerth et al., 2002). Moreover, FTD has been related to two other sporadic dementing disorders such as PSP and CBD. PSP is characterized by early falls, vertical (especially down) gaze, supranuclear palsy and axial greater than appendicular rigidity (Litvan et al., 1996). CBD is a complex disorder with multiple

clinical phenotypes and the features can evolve and develop into behavioral features, personality changes, and executive dysfunction similar to those observed in bvFTD.

The neuropathology of FTD shows the degeneration of frontal and temporal lobes with neuronal loss and gliosis assisted with varying levels of cytoplasmic and/or nuclear inclusions in neurons and glia. The microscopic pathology also shows heterogeneity in different forms of FTD. Majority of FTD cases are characterized by the deposition and/or abnormal processing of the tau protein (Spillantini et al., 1998). The remaining cases display ubiquitin-positive inclusions in the affected regions. Up to 10% of these ubiquitin-positive cases contain small, ubiquitinated nuclear protein termed as TAR DNA binding protein with molecular weight of 43Kda (TDP-43) (Cairns et al., 2007; Neumann et al., 2006).

## I.3. Genetics of FTD

The genome of any given individual will contain millions of sequence variants of which the vast majority will have no effect or will represent normal differences in phenotype. However, some may harbor pathogenic mutations that cause or predispose to disease. The genetic basis of FTD is not fully understood and currently it is a topic of active research. FTD shows an extensive degree of family history; i.e., up to 50% cases show a positive family history of similar illness in the first degree relative. A number of genetic mutations in different genes have been identified on chromosomes 1, 3, 9 and 17. These genetic mutations tend to run in families with a clear autosomal dominant pattern of inheritance. Among them, mutations in three genes were identified as the most common causative factor for FTD in different

populations (Cruts et al., 2012). Genetic mutations in gene encoding the microtubule-associated protein tau (*MAPT*) on chromosome 17 cause FTD with associated clinical features of Parkinson's disease and tau-positive inclusions in the affected brain regions. The second gene also located on chromosome 17, codes for progranulin (*PGRN*) and mutations in *PGRN* cause FTD associated with ubiquitin-positive inclusions in affected neurons and glia. Finally, a hexanucleotide repeat expansion within the promoter region of chromosome 9 open reading frame 72 (*C9ORF72*) has been identified as a major cause of FTD associated with ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011).

In addition to these genetic mutations, common variants in several genes have been shown to contribute the susceptibility to FTD. A large number of susceptibility loci have been identified through genome wide association analysis (GWAS) and these include *MAPT* haplotypes, *PGRN* rs5848 polymorphism, apolipoprotein E (*APOE*) polymorphisms etc. The exact mechanisms by which these variants contribute to developing FTD is yet to be elucidated and it is most likely that susceptibility acts through subtle changes in the gene expression of the target genes.

## I.4. Biochemical markers of FTD

The proteomic searches of cerebrospinal fluid (CSF) and peripheral blood for the identification of biochemical markers are limited in FTD. CSF closely reflects the composition of brain extracellular space but it is limited by the use of invasive procedure, lumbar puncture. So the identification of biomarkers in peripheral blood is necessary to reduce the need for invasive, expensive and time consuming tests.

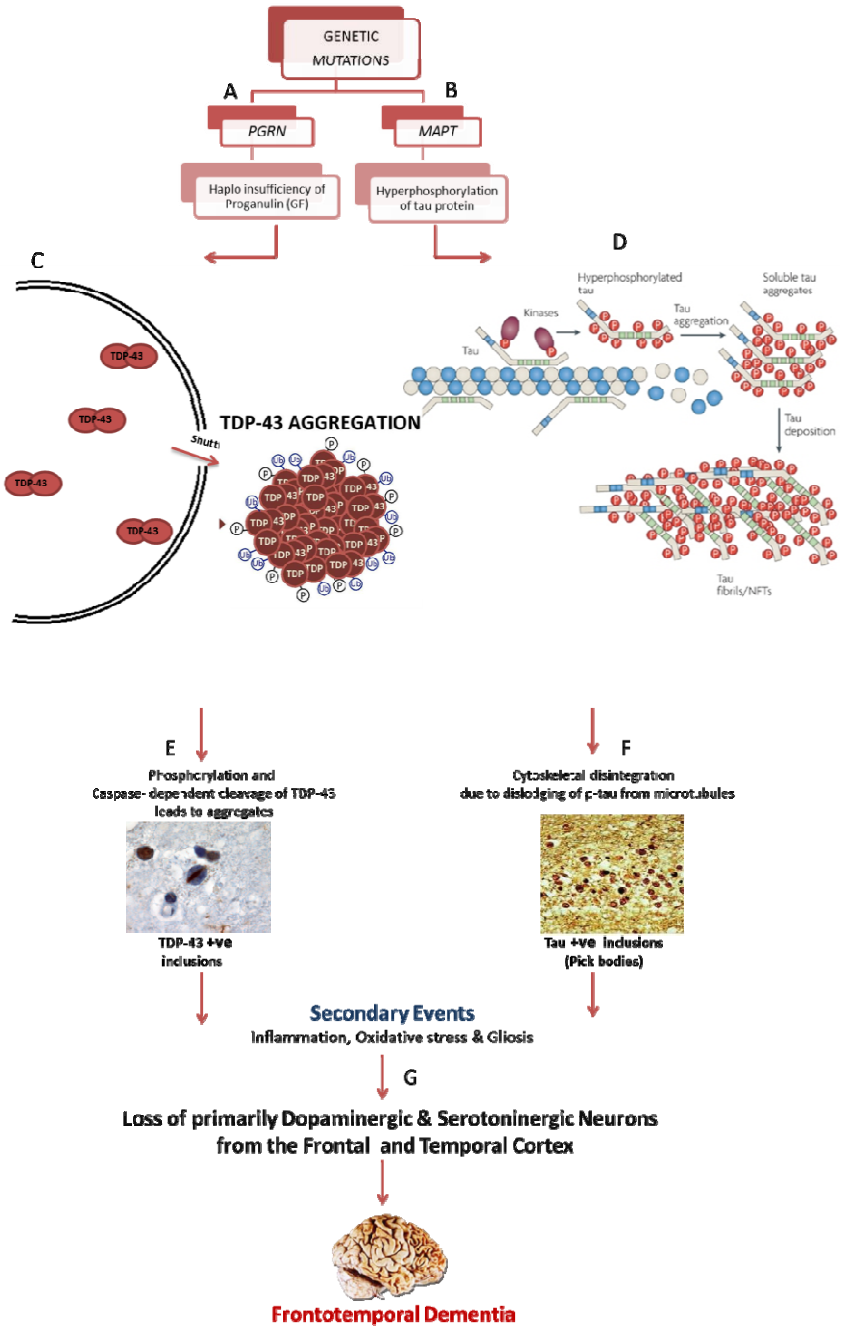
The evaluation of protein species that underlie neuropathology of FTD in the peripheral biological fluids may lead to earlier and better understanding of the disease status. *MAPT* mutations are associated with tau positive neuropathology (Hong et al., 1998; Lee et al., 2001; Zhukareva et al., 2001) and both *PGRN* and *C9ORF72* mutations are associated with TDP-43 positive neuropathology (Davidson et al., 2007; Hsiung et al., 2012). Combined with the genetic background, the diagnostic accuracy of biomarker analysis will definitely lead to improve the clinical diagnosis.

## 1.5. Pathogenesis of FTD

The exact pathogenic mechanism behind the selective vulnerability of neurodegeneration in FTD remains unknown. However, with the identification of genetic mutations in familial cases, several hypothetical pathogenic mechanisms have been proposed (Figure 1). Mutations in *MAPT* were found to be associated with tau-positive neuropathology (Spillantini et al., 1998). This mutations cause the production of abnormal tau proteins or over-expression of the 4 repeat (4R) tau isoforms which are more aggregation prone than 3 repeat (3R) tau isoforms. This may lead to dysfunction of tau proteins resulting in the inefficient binding of tau proteins to the microtubules leading to the aberrant assembly of microtubules and deposition of tau aggregates. This forms the neurofibrillary tangles or Pick bodies which disrupt the cellular integrity thereby leading to the neuronal loss and degeneration of associated brain regions. Progranulin is a secreted growth factor which has the neurotrophic as well as neuroprotective functions (Eriksen &

Mackenzie, 2008). Mutations in *PGRN* were demonstrated to result in the partial loss of progranulin which is the basis of pathogenesis in FTD with *PGRN* mutations (Gass et al., 2006). *PGRN* mutation carriers invariably show the neuropathological feature characterized by the pathologic accumulation of TDP-43 as nuclear and cytoplasmic inclusions (Mackenzie et al., 2010). TDP-43 is a nuclear protein and in *PGRN* mutation carriers, it is redistributed to the cytoplasm (Neumann et al., 2006). The normal function of TDP-43 in the brain is unclear, but it actively regulates the expression of numerous genes involved in neuronal development and functioning, and regulates alternative splicing of several pre-mRNA transcripts. Since progranulin is involved in a wide variety of cellular processes such as cell proliferation, tissue repair, wound healing and inflammation, progranulin deficiency could cause neuronal dysfunction and/or neurodegeneration via impaired lysosomal function, dysregulated apoptosis, mislocalization of TDP-43 and/or neuroinflammation. It remains unknown whether neuronal loss result from the loss of the normal functions of nuclear TDP-43 or gain of function of cytoplasmic TDP-43. The evidences from cellular models suggests that mislocalization of TDP-43 is a mechanism of neuronal death.

**Figure 1. Pathogenesis of FTD**




*Downstream pathology triggered by pathogenic mutations in PGRN and MAPT culminating in neuronal loss from selected brain regions causing FTD A) PGRN mutations result in haploinsufficiency of functional progranulin, which is a neuronal growth factor (GF) C) Loss of function of PGRN results in abnormal cytoplasmic shuttling of TDP-43 from nucleus E) In the cytoplasm, TDP-43 is phosphorylated and subjected to cleavage by caspases which lead to pathogenic aggregates termed*

*TDP-43 positive inclusions B) MAPT mutations result in abnormal hyperphosphorylation of tau protein and dislodging of phosphorylated tau (p-tau) from microtubules leading to the cytoskeletal disruption in neurons (D) P-tau aggregate as NFT/pick bodies (F) These pathogenic inclusions lead to secondary events including inflammation and oxidative stress which finally results in loss of dopaminergic and serotonergic neurons from frontal and temporal lobes of brain characteristic of FTD neuropathology.*

## I.6. Treatment for FTD

Treatment of dementias depends on its cause. The clinical, neuropathologic and genetic heterogeneity makes the diagnosis and treatment of FTD challenging and difficult. Currently, there is no specific pharmacological treatment to prevent, to slow down or to reverse the progression of FTD. However, certain medications were shown to relieve the symptoms associated with FTD. Since there is a deficiency of serotonergic neurons in FTD, selective serotonin re-uptake inhibitors (SSRIs), a type of anti-depressant may help control the loss of inhibitions, overeating and compulsive behavior seen in some people with FTD. Another medication prescribed is anti-psychotics used for treating severely challenging behavior that can alleviate extremely unrealistic or disorganized thinking such as hallucinations, delusions and aggression.

Pharmacological research is limited in FTD. The identification of disease-specific abnormal protein inclusions has lead to the elucidation of pathogenic mechanisms and molecular characterization of FTD. Although FTD is a heterogeneous disorder, majority of cases shows either tau (tauopathies) or TDP-43 (TDP-43 proteinopathies) positive neuropathology, thereby providing potential molecular targets for FTD drug discovery research. Currently, tau, progranulin and TDP-43-based therapeutic

approaches are underway. Since the molecular mechanisms of tau-mediated neurodegeneration involves the hyperphosphorylation and aggregation of tau, therapeutic approaches aim to inhibit the phosphorylation and aggregation of tau species, enhancement of tau clearance, and finally to stabilize microtubules. For *PGRN* mutations, there is a uniform disease mechanism of loss of function in all mutation carriers and drugs such as chloroquine, nimodipine and vorinostat have been shown to increase progranulin concentration (Capell et al., 2011; Cenik et al., 2011) suggesting their use in future clinical trials.

## I.7. Research perspectives

Risk factors for dementia such as age and genetics cannot be changed. But researchers continue to explore the impact of other risk factors on brain health and prevention of dementia. During the last two decades, the genetic etiological factors and the underlying neuropathology associated with FTD have been more or less delineated. Several genetic mutations were identified associated with the inheritable susceptibility to FTD. This leads to the frequent need for physicians to consider the merits of genetic counseling and genetic testing to identify the genetic abnormalities which will allow a more definitive diagnosis of the disease. However, the molecular mechanisms behind neurodegeneration in FTD still remain unknown. Moreover, biomarkers of cognitive decline in FTD should be identified in order to detect the biochemical and pathological alterations of pathophysiology in biological fluids. As FTD shows clinical and pathological heterogeneity, there exists a significant challenge for the accurate diagnosis and to conduct appropriate drug trials. In order

to adopt better treatment modalities and drug designing the exact pathogenic mechanisms leading to FTD should be delineated. Hence, revealing the genetic abnormality has implications for understanding the disease pathogenesis as well as for genetic counseling. So far, there are limited studies regarding the genetic or clinical characterization of FTD patients in the study population. Hence, we hypothesize that understanding the role of mutations in *MAPT* and *PGRN* as well as biochemical changes associated may help to adopt better interventional strategies in our study population.

The present study is aimed to delineate the genetic etiology of FTD patients in a south Indian clinical cohort. The role of genetic mutations in *MAPT* and *PGRN* were determined in the study cohort through direct sequencing and mutation analysis. To identify the predisposing genetic susceptibility factors associated with disease risk, association analyses were conducted in the same cohort with age and ethnicity-matched controls. Finally, the study combined biomarker analyses in the peripheral blood to detect the diagnostic value of the biochemical changes associated with pathogenesis in FTD. Before going to the details of investigations, an overview of the currently available information about FTD and associated molecular events are discussed through literature review.

# II. LITERATURE REVIEW

## II.1. FTD: A brief history

In 1892, Arnold Pick first described patients characterized by the circumscribed atrophy of frontal and temporal lobes, clinically presenting with dementia and aphasia (Pick, 1892). Subsequently in 1911, Alois Alzheimer characterized argyrophilicintraneuronal inclusions (now termed as Pick bodies) and swollen neurons (Pick's cells) in such cases (Alzheimer, 1911) and in 1922, Gans coined the term Pick's disease for such disorders. In the 1960s, the so-called Pick bodies were shown to contain abnormal filaments (Rewcastle & Ball, 1968). In 1974, different pathological subtypes of Pick's disease were described and in 1987, Arne Brun defined the cases based on two underlying pathologic entities, less common Pick's disease and the more frequent non-Pick lobar atrophy, those lacking Pick's bodies. During the same period, cases of progressive aphasia, non-fluent type termed as progressive non-fluent aphasia (PNFA) and fluent type termed as semantic dementia (SD), with different topographical distribution pattern of frontotemporal atrophy were described (Hodges et al., 1992; Mesulam, 1982; Warrington, 1975). Moreover, some reports had shown occasional clinical and pathological overlap of frontotemporal atrophy with another neurodegenerative condition termed as amyotrophic lateral sclerosis (ALS) (Neary et al., 1990). In 1990, Knopman described some cases without specific histopathology and termed them as dementia lacking distinctive histology (DLDH) which is similar to that observed in non-Pick lobar atrophy (Knopman et al., 1990). In the same period ubiquitin immunoreactive inclusions were characterized in a subset of these cases (Jackson et al., 1996).

In 1994, the first diagnostic criteria based on clinical and neuropathological findings were proposed by investigators in Lund, Sweden and Manchester (UK), and introduced the term frontotemporal dementia (FTD) to describe cases of behavioral and language impairment associated with frontal and temporal lobe atrophy and introduced a consensus criteria for clinical and neuropathological diagnoses of FTD (The Lund, Manchester Criteria, 1994). In 1998, Neary and colleagues further refined these criteria and proposed another term frontotemporal lobar degeneration (FTLD) that encompasses three clinical variants, behavioral variant FTD (bvFTD), and the two language variants; PNFA and SD (Neary et al., 1998). Recently, it has been recognized that FTD overlaps with two other dementing conditions termed as progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) (Forman et al., 2006; Hodges et al., 2004; Josephs et al., 2006; Kertesz et al., 2005).

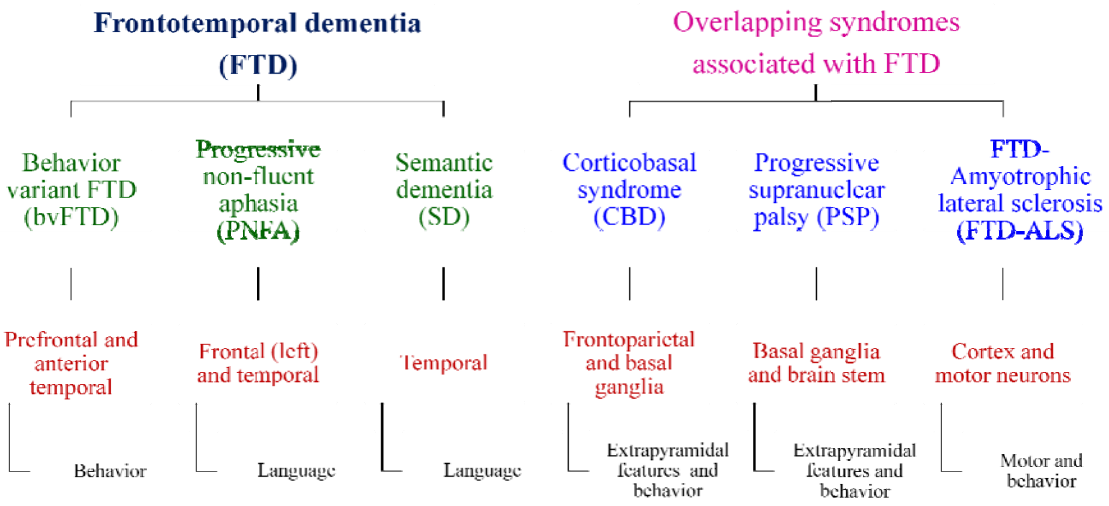
## II.2. FTD: Clinical syndromes and diagnosis

FTD accounts for 5-15% of all cases of dementia and is the second most common cause of dementia in the presenile age group (Bird et al., 2003). It is clinically characterized by progressive deterioration in behavior, personality and/or language, with relative preservation of memory (The Lund Manchester Criteria, 1994; McKhann et al., 2001; Neary et al., 1998). The associated neuropathology is characterized by selective degeneration of the frontal and temporal lobes (FTLD), usually with the presence of abnormal intracellular protein accumulations. So the current classification of FTD neuropathology is based on the identity of the predominant protein abnormality. Major subgroups include those characterized by

the pathological tau, TDP-43 and a group with unidentified ubiquitinated protein cellular inclusions. Currently, there are two terminologies used for the nomenclature of this clinico-pathological syndrome such as FTD and FTLD. Both terminologies are in wide use and there is no consensus as to which is more appropriate. Here, FTD is used as the general term for the clinical syndrome (including all behavioral and language variants) and the term FTLD is reserved for the associated pathology.

Depending on the difference in the topographical distribution of atrophy, different clinical manifestations are presented (Figure 2). Based on the consensus clinical criteria (Appendix I), there are mainly three clinical variants of FTD, bvFTD and two language variants; PNFA and SD (Neary et al., 1998), each associated with distinct regional pattern of atrophy. Overlap between the three syndromes can occur as the disease progresses to involve the frontal and temporal lobes more diffusely (Kertesz et al., 2005).

**Figure 2: FTD clinical subtypes, brain regions affected by associated pathology and the major clinical features shown by the syndromes**




### II.2.1. Behavioral variant FTD (bvFTD)

The most common clinical presentation of FTD is the bvFTD characterized by significant changes in personality, behavior and language. The insidious onset and highly progressive nature is a demarcating feature of bvFTD that distinguish it from other neurodegenerative disorders (Neary et al., 1998; Rossoret al., 2010). It is associated with the symmetric or asymmetric atrophy of the frontal, insular and/or anterior temporal lobes (Whitwell et al., 2009).

### II.2.2. Progressive non-fluent aphasia (PNFA)

PNFA affects the expressive language, presenting with insidious onset and progressive loss of language skills (Mesulam, 2001). It is associated with the asymmetric atrophy of left hemisphere involving frontal, temporal, insular and parietal components of the language network (Neary et al., 2005). The most common

presenting symptom is the word finding difficulty. The other clinical manifestations include changes in fluency and pronunciation, difficulty in language comprehension and motor speech.

### II.2.3. Semantic dementia (SD)

SD is also termed as temporal variant FTD which is a fluent aphasia. It is associated with bilateral atrophy of middle and inferior temporal neocortex (Neary et al., 2005). The most common clinical presentation is the abnormality of language including loss of memory for words or word meaning (Boxer & Miller, 2005).

### II.2.4. Overlapping syndromes associated with FTD (ALS, PSP and CBD)

FTD is often associated with parkinsonism or motor neuron disease (MND) (Lomen-Hoerth et al., 2002) (Figure 2). ALS is a MND where the neurodegeneration affects primarily the motor neurons of the motor cortex, brain stem and spinal cord. Approximately 15% of the FTD cases display symptoms of ALS and is referred to as FTD-ALS. There is an increasing recognition of a clinical (Lomen-Hoerth et al., 2002), neuropathological (Mackenzie & Feldman, 2005) and genetic overlap between FTD and ALS (Murphy et al., 2007). The co-morbidity of ALS predicts poorer survival in FTD patients and vice versa (Olney et al., 2005).

Some cases overlap with PSP/Steele-Richardson-Olszewski syndrome or CBD (Kertesz et al., 2005). CBD and PSP are rare, sporadic and slowly progressive neurodegenerative diseases that typically present with atypical parkinsonism and cognitive dysfunction (Rebeiz et al., 1968).Almost all cases of PSP and CBD are

sporadic in nature. As PSP and CBD progress, cognitive and/or behavioral dysfunction as well as language impairment similar to that in FTD may occur, and vice versa; patients presenting with either bvFTD or PNFA may develop movement disorders characteristic of CBD or PSP over the disease course (Kertesz et al., 2005).

## II.3. Epidemiology

### II.3.1. Incidence and prevalence

FTD is widely recognized as a young onset dementia affecting people in the presenile group with age of onset under 65 years. It accounts for 5-15% of all cases of dementia and 10-20% of early onset dementias (Harvey et al., 2003; Ratnavalli et al., 2002; Stevens et al., 2002; Yokota et al., 2005). The annual incidence rates for FTD have varied from 2.5 to 3.5 cases per 100,000 person-years for age 45-65 years (Garre-Olmo et al., 2010; Mercy et al., 2008). Population based studies from Japan, Netherlands and United Kingdom have found a prevalence of 2-15 cases per 100,000 people between 45-65 years (Harvey et al., 2003; Ikejima et al., 2009; Ratnavalli et al., 2002; Rosso et al., 2003), whereas a prevalence of 29 per 100,000 has been reported from Italy (Borroni et al., 2011). In India, the epidemiological studies on FTD are limited (Das et al., 2012). One study from East India on early onset dementias (under the age of 65 years) reported FTD (27%) as the second most common cause for dementia after possible AD (30%), and have reported a positive family history in 20% of FTD cases (Nandi et al., 2008). A clinic-based study from South India found that FTD accounts for 18.7% of total dementia cases (Alladi et al., 2011).

### II.3.2. Age and sex distribution

There is an equal incidence in male and female (Borroni et al., 2011; Chow et al., 2005; Ioannidis et al., 2012; Rosso et al., 2003), although male predominance has also been suggested (Ratnavalli et al., 2002). Age at onset is most commonly in the sixth decade, with a mean age at onset most commonly reported at around 59 years (Hodges et al., 2003; Johnson et al., 2005; Roberson et al., 2005; Rosso et al., 2003). Among the clinical subtypes, patients with bvFTD and SD have been suggested to have earlier age at onset than those with PNFA (Johnson et al., 2005). In general, age at onset in familial verses sporadic cases does not differ significantly (Godbolt et al., 2005; Piguet et al., 2004).

### II.3.3. Prognosis

The disease progresses steadily and often rapidly, ranging from less than 2 years in some individuals to more than 10 years in others. The average survival time from the onset of symptoms is estimated to be 6-11 years (Borroni et al., 2011; Hodges et al., 2003; Rascovsky et al., 2005; Roberson et al., 2005).

### II.3.4. Risk factors

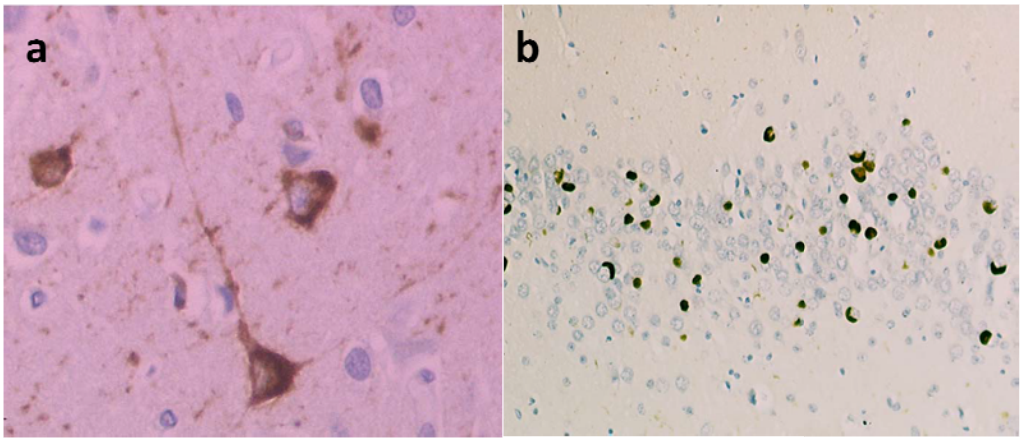
Studies on the modifiable risk factors of FTD are limited. Some studies have identified the traumatic brain injury as a risk factor for developing FTD (Rosso et al., 2003) since the frontal and temporal lobes are more susceptible to trauma. Hypothyroidism was also associated with increased risk for developing FTD (Rosso

et al., 2003). The other non-modifiable risk factors may include age, presence of family history, and possession of risky genotypes.

## II.4. Neuropathology of FTD

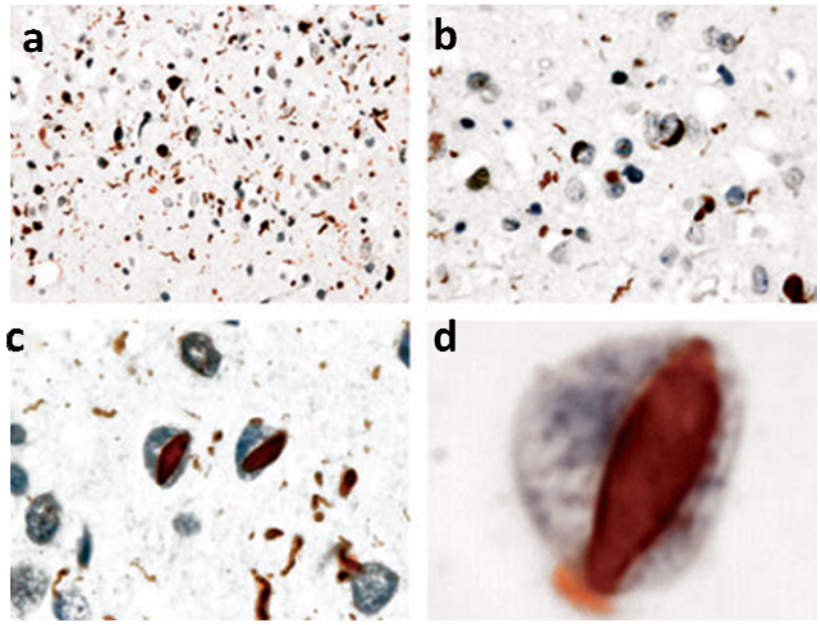
In the absence of a known pathogenic mutation, the specific diagnosis of definite FTD requires histopathologic confirmation or immunocytochemistry (Cairns et al., 2007; Gorno-Tempini et al., 2011; Rascovsky et al., 2011). At autopsy, the neuropathology assisted often includes selective changes on gross anatomy of frontal lobe, temporal lobe or both with asymmetric atrophy of the hemispheres and microscopic changes. Similar to the clinical heterogeneity, the neuropathology assisted is also heterogeneous (Cairns et al., 2007). Almost all cases of FTD are microscopically characterized by abnormal intracellular accumulation of disease-specific proteins in the cytoplasm and/or nuclei of both neurons and glial cells. Based on the immunoreactivity of protein inclusions to specific antibodies, FTD has been divided into different proteinopathies (Mackenzie et al., 2010). These include FTD with tau-positive inclusions (FTD-tau) (Figure 3), FTD with ubiquitinated TDP-43-positive inclusions (FTD-TDP) (Figure 4), FTD with ubiquitinated FUS-positive inclusions (FTD-FUS), FTD with ubiquitin positive TDP-43 and FUS-negative inclusions (FTD-U) and finally FTD with no demonstrable inclusions (previously known as DLDH).

**Figure 3: Neuropathology of FTD with *MAPT* mutations**




*Filamentous cytoplasmic inclusions made of hyperphosphorylated tau protein characterize a proportion of cases with FTD, including Pick's disease (a) Neurofibrillary tangles containing phosphorylated tau in FTD with MAPT mutations, (b) Pick bodies. Figure adapted from (Hutton et al., 1998.*

**Figure 4: Neuropathology of FTD with *PGRN* mutations**




*Numerous ubiquitin-immunoreactive dystrophic neurites and neuronal cytoplasmic inclusions (NCIs) in superficial layers of the neocortex which stains for TDP-43 (a,b). Neuronal intranuclear inclusions (NIIs) majority in characteristic lentiform shape (c, d). Scale bar = (a), 80 µm; (b), 40 µm; (c), 20 µm; (d), 5 µm. Figure adapted from Neumann et al., 2006.*

## II.5. Molecular genetics in FTD

### II.5.1. Genetic mutations in FTD

FTD is commonly presented as a sporadic disorder. However, it shows a strong genetic component with up to 50% of cases having a positive family history of similar illness and around 10-27% of patients showing a distinct pattern of autosomal dominant inheritance. Genetic linkage studies in these families have identified different genetic mutations on different chromosomes. So far, mutations in eight genes have been demonstrated to cause the pathogenesis of FTD (Table 1).

**Table 1: Mendelian genetics in FTD**

| Gene | Locus | Clinical presentation | Total FTD (%) | Familial FTD (%) | Neuropathology | Mean Age at onset |
|---|---|---|---|---|---|---|
| *MAPT* | 17q21.1 | bvFTD±parkinsonism | 5-10 | 10-25 | Tau | 52 |
| *PGRN* | 17q21.3 | bvFTD, PNFA | 5-10 | 10-25 | TDP-43 | 59 |
| *C9ORF72* | 9p21.2 | bvFTD±ALS | 12 | <25 | TDP-43 | 58 |
| *TARDBP* | 1p36.2 | FTD±ALS, ALS | <1 | <1 | TDP-43 | 55 |
| *CHMP2B* | 3p11.2 | bvFTD | <1 | <1 | UPS | 58 |
| *VCP* | 9p13.3 | IBMPFD | ~1 | <1 | TDP-43 | 55 |
| *FUS* | 16p11.2 | ALS±FTD, FTD | <1 | <1 | FUS | 49 |
| *UBQLN2* | Xp11.2 | ALS±FTD, FTD | <1 | NA | P62, UBQLN2 (TDP-43, FUS) | 40 |

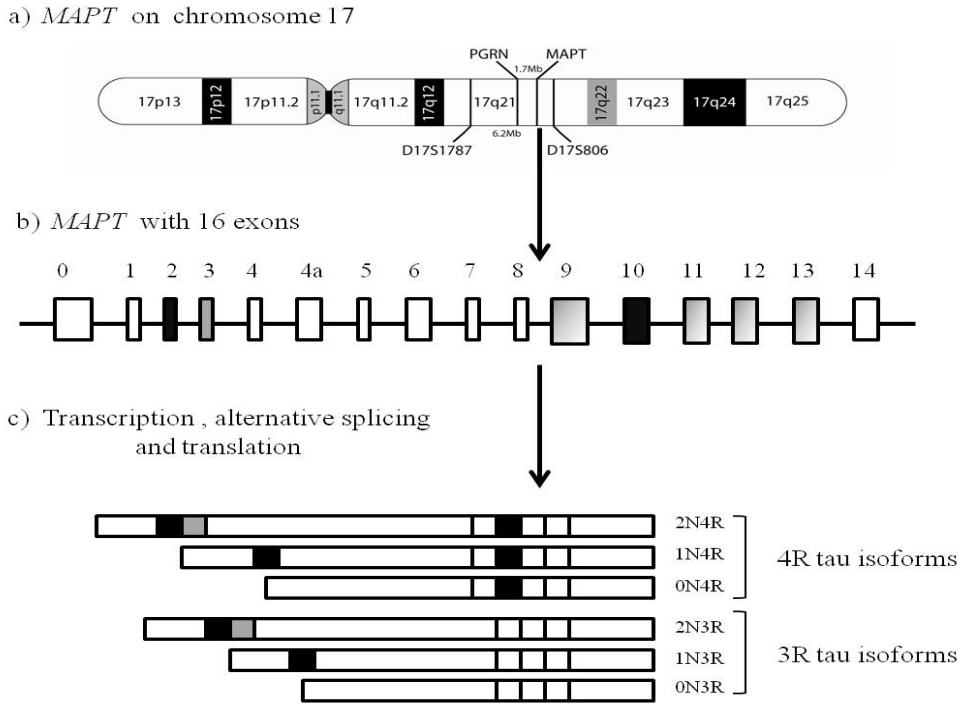
*MAPT: Microtubule-associated protein tau, PGRN: Progranulin, C9ORF72: Chromosome 9 open reading frame 72, TARDBP: Trans-active response DNA binding protein with molecular weight of 43 kDa, CHMP2B: Charged multi-vesicular protein 2B, VCP: Valosin-containing protein, FUS: Fused in sarcoma, UBQLN2: Ubiquilin 2, FTD: Frontotemporal dementia, bvFTD: Behavioral variant FTD, PNFA: Progressive non-fluent aphasia, ALS: Amyotrophic lateral sclerosis, IBMPFD: Inclusion body Myopathy and Paget's disease of the bone and/or FTD, UPS: Ubiquitin-proteasome system, NA: Not available*

#### *II.5.1.1 Microtubule-associated protein tau*

The human gene encoding microtubule-associated protein tau (*MAPT*) is located on chromosome 17q21 and consists of 16 exons (Figure 5) (Andreadis et al., 1992).

Exons 2, 3 and 10 are alternatively spliced and are specific to the adult central nervous system (CNS) (Andreadis et al., 1992). The alternative splicing of exons 2, 3 and 10 allows the production of six different tau isoforms in an adult human CNS; three of which containing 3 repeats (3R) of the 31 amino acid microtubule-binding sequence domain (2-3-10-, 2+3-10-, 2+3+10-) and three of which containing 4 repeats (4R) of this domain (2-3-10+, 2+3-10+, 2+3+10+) (Figure 5) (Goedert et al., 1989a; Goedert et al., 1989b; Kosik et al., 1989). These tau isoforms stabilize the microtubule cytoskeleton and promote microtubule assembly and dynamics, thereby playing an important role in neuronal integrity and axonal transport.

**Figure 5: Schematic representation of *MAPT* genomic structure**




*a) MAPT is located on the long arm of chromosome 17. b) MAPT with 16 exons shown as boxes. Exons 2, 3 and 10 are alternatively spliced exons. Exons 4a and 6 are not expressed in the major brain isoforms and exon 8 is not expressed in human MAPT transcripts. All the remaining exons are constitutively spliced. c) MAPT transcription, alternative splicing and translation produce 6 major tau isoforms that are named according to the number of N-terminal inserts and C-terminal microtubule-binding domains.*

Tau is a constitutively expressed, soluble protein in both the CNS and the peripheral nervous system (PNS) (Binder et al., 1985). In the CNS, tau is enriched in the axons of mature and growing neurons. Tau proteins can bind microtubules through repetitive sequences in the C-terminal region encoded by exons 9 to 12 (Lee et al., 1989). Reports show that adult 4R tau isoforms are more efficient at promoting microtubule assembly than 3R tau isoforms (Goedert & Jakes, 1990). In normal cerebral cortex, the ratio of 3R and 4R tau transcripts is approximately one : one. This delicate balance of 3R versus 4R tau isoforms appears to be critical for neuronal function and any mutations that disrupt this balance are suggested to lead to the development of tauopathy.

*II.5.1.1.1. MAPT mutations*

In 1994, the autosomal dominantly inherited form of FTD with parkinsonism was shown linkage with chromosome 17q21.2 (FTDP-17) (Wilhelmsen et al., 1994). These patients consistently showed severe frontotemporal atrophy with filamentous neuropathology made of hyperphosphorylated tau protein in neurons or in both neurons and glia (Spillantini et al., 1998). In 1998, the first mutations associated with FTDP-17 were identified in gene encoding the tau protein (*MAPT*) (Hutton et al., 1998) which provided the first evidence that mutations in *MAPT* alone are sufficient to cause neurodegeneration. Extensive mutation analyses revealed many other *MAPT* mutations in several additional FTDP-17 families. So far about 44 different mutations in a total of 123 families have been reported worldwide (www.molgen.ua.ac.be/FTDMutations). The spectra of mutations include several missense mutations, silent mutations and in-frame single codon deletions occurring

either in the coding region or in the non-coding region. Most of the coding region mutations are located on exons 1, 9, 10, 11, 12 and 13 and intronic mutations are located close to the splice donor site of the intron following exon 10. Together, *MAPT* mutations account for about 5-10% of total FTD cases and 10-25% of familial cases (Goldman et al., 2011; Seelaar et al., 2011).

*MAPT* mutations are associated with an autosomal dominant mode of inheritance and are completely penetrant in nature. The frequency of *MAPT* mutations varies significantly between populations and also depending on the clinical diagnosis and family history of the patients.

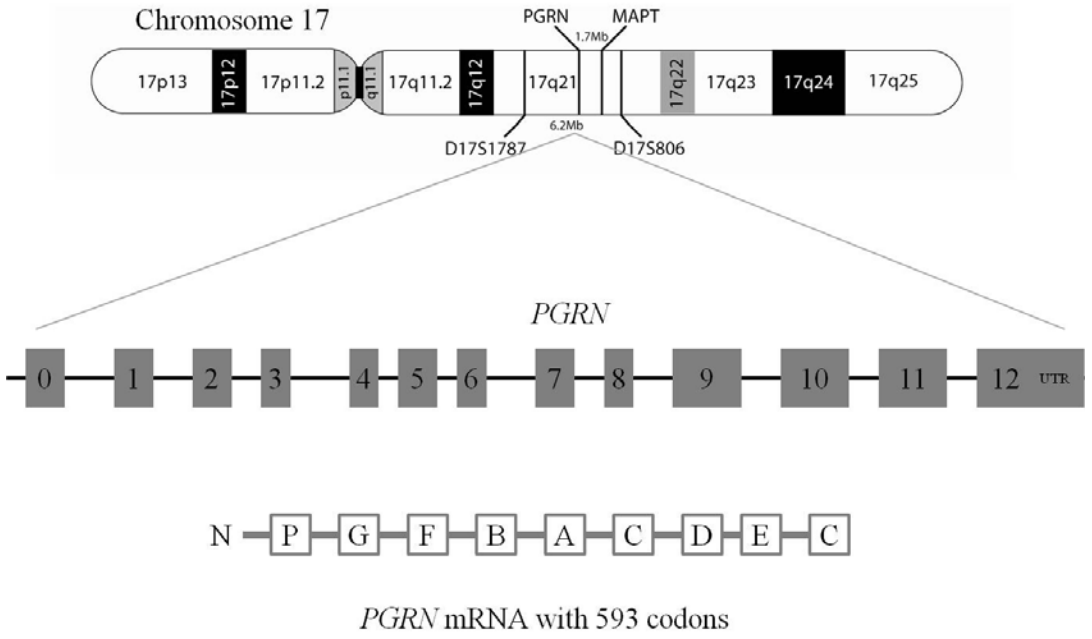
The most common clinical presentation of *MAPT* mutations is bvFTD associated with or without parkinsonism. Some familial cases of PSP and CBD were also shown to harbor *MAPT* mutations (Bugiani et al., 1999; Stanford et al., 2000). The main pathological hallmark is the invariable presence of neuronal and/or glial tau-positive inclusions in affected brain regions. The pathological form of tau is hyperphosphorylated which hamper its physiological functions, thereby disrupting the microtubule assembly and polymerization. These hyperphosphorylated tau proteins can be self-assembled to form the insoluble aggregates in the form of neurofibrillary tangles and filaments (Figure 1) (Goedert, 2004).

#### *II.5.1.2. Progranulin*

The human progranulin gene (*PGRN*) is located on the long arm of chromosome 17q21.3, 1.7 Mb centromeric to *MAPT* and contains 13 exons (Figure 6). It encodes a widely expressed secreted growth factor with 593 amino acids which is a cysteine-

rich protein with a predicted molecular weight of 68.5 kDa. However, by Western blot analysis it migrates around 90 kDa because of glycosylation. The intact progranulin contains 7.5 tandem repeats of a 12 cysteine granulin motif and is cleaved into seven non-identical granulin peptides (granulin A to G) (He & Bateman, 2003) by elastase, which releases the individual granulin peptides; such release is regulated by secretory leukocyte protease inhibitor (SLPI) (Zhu et al., 2002). Progranulin and the granulin peptides have mitogenic functions in regulation of cell growth and cell cycle progression; wound healing, inflammation and tumorigenesis (He & Bateman, 2003; He et al., 2003; Ong & Bateman, 2003).

**Figure 6: Schematic representation of human *PGRN* genomic structure**




*Human PGRN located on chromosome 17 centromeric to MAPT comprises 13 exons, which on transcription produces an mRNA coding for 593 amino acid polypeptide (progranulin). Proteolytic cleavage of progranulin produces granulin peptides designated as A-G. P represents paragranulin.*

Progranulin is expressed in various tissues, especially in mitotically active epithelial and hematopoietic cells (Daniel et al., 2000). In CNS, progranulin is expressed in neurons and microglia of specific brain regions, including the superficial lamina of the cerebral cortex, Purkinje cells of the cerebellum, and pyramidal and granule cells of the hippocampus (Daniel et al., 2000). Although the biological functions of progranulin in CNS have not been very well studied, it is suggested to have a role in neuronal survival and/or inflammatory responses (Eriksen & Mackenzie, 2008).

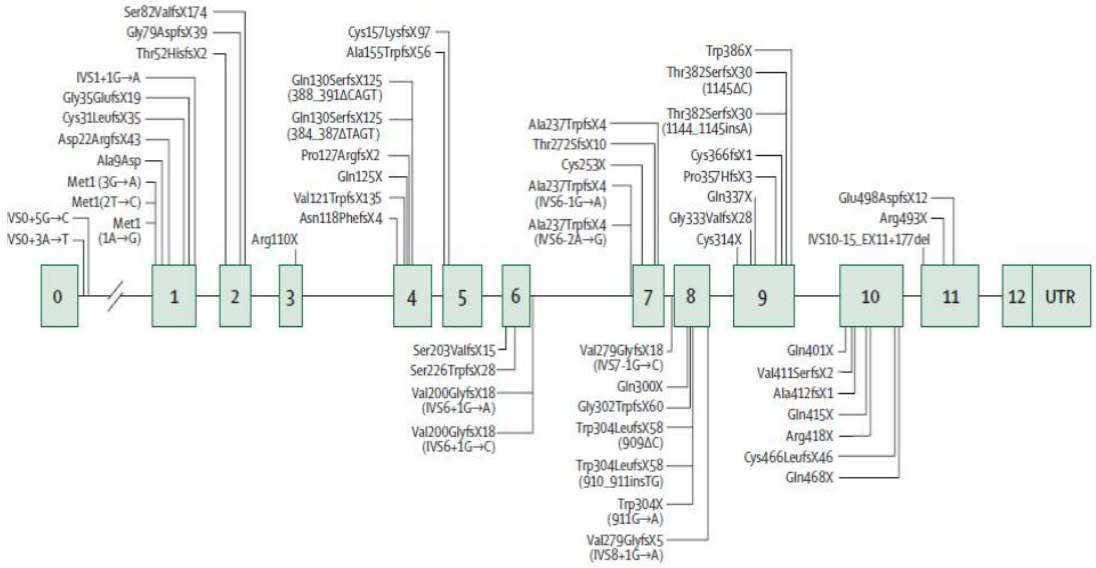
*II.5.1.2.1. PGRN mutations*

Several families with inherited autosomal dominant FTD showing significant linkage to chromosome 17 were found to lack mutations in *MAPT*. These patients were demonstrated to show ubiquitinated protein pathology and were termed as FTDU-17 (Kertesz et al., 2000). In 2006, sequencing of the candidate gene region in three most significantly linked FTDU-17 families: Dutch 1083, Belgian DR8 and Canadian UBC-17 identified mutations in *PGRN* (Baker et al., 2006; Cruts et al., 2006). Subsequently, extensive mutation analyses in other described FTDU-17 families and several FTD patient series identified many additional *PGRN* mutations. To date, 69 different *PGRN* mutations are known worldwide in a total of 163 families (www.molgen.ua.ac.be/FTDMutations) (Cruts et al., 2012).

*PGRN* mutations are located throughout the gene and all of them show an autosomal dominant pattern of inheritance (Figure 7). Almost all pathogenic mutations are null mutations that completely abolish the gene product through introducing a premature termination codon (nonsense mutations) predicted to result in a heterozygous loss of

gene expression and haploinsufficiency. Haploinsufficiency is a condition in which the individual has only one functional copy of a gene and that alone may not produce sufficient gene product to result in the normal physiological activities resulting in a diseased state. The transcripts containing premature termination codons are eliminated by means of nonsense-mediated decay (NMD) which is eukaryotic mRNA quality control mechanism degrading mRNAs containing premature termination codons which arise by the transcription of genes containing nonsense or frameshift mutations, thereby preventing the accumulation of truncated proteins within the cell. Besides the non-sense mutations, splice site mutations and frame-shift mutations also seems to mediate NMD mechanism (Baker et al., 2006; Cruts et al., 2006; Gass et al., 2006; Le Ber et al., 2008). Copy number variations in *PGRN* seem to be rare in causing FTD (Gass et al., 2006; Le Ber et al., 2008; Skoglund et al., 2009). Recently, a heterozygous genomic deletion of entire *PGRN* locus along with two neighboring genes was described in a Belgian FTD patient (Gijselinck et al., 2008b). Another study reported a partial *PGRN* deletion in two siblings, with one presenting with FTD and the other with PD (Rovelet-Lecrux et al., 2008).Overall, the haploinsufficiency mechanism suggests that *PGRN* mutations might lead to neurodegeneration through loss of neurotrophic support and inadequate responses to injury and aging (Ahmed et al., 2010).

**Figure 7: Overview of *PGRN* mutations**




*PGRN mutations are located throughout the gene and most mutations lead to a sequence frameshift that introduces a premature termination codon. The mutations are numbered according to amino acid sequence in the full-length progranulin. del: deletion, fs: frameshift. Ins: insertion. UTR: untranslated region. X: stop codon. (Figure adapted from J. C. van Swieten & Heutink., 2008)*

As a consequence of haploinsufficiency mechanism, plasma progranulin level is significantly lower in both pre-symptomatic and symptomatic *PGRN* mutation carriers than in non-carriers (Coppola et al., 2008; Finch et al., 2009; Ghidoni et al., 2008). Measurement of plasma progranulin levels predicts a pathogenic *PGRN* mutation with almost 100% sensitivity and specificity (Ghidoni et al., 2012).

*PGRN* mutations account for approximately 5-10% of all FTD cases and 10-25% with familial FTD (Goldman et al., 2011; Seelaar et al., 2011). The frequency of *PGRN* mutations varies between different studies ranging from 1.3 to 11.7% in the total FTD group and from 3.4 to 25.6% when only familial FTD patients were considered (Cruts et al., 2006; Gass et al., 2006; Gijselinck et al.,2008a; Le Ber et al., 2008; Pickering-Brown et al., 2008). The most prevalent *PGRN* mutations identified

due to founder effect are p.R493X and IVS1+5 G>C. The highest frequencies were observed in Belgian and US due to the presence of a strong founder effect of the founder mutations, IVS1+5 G>C (Cruts et al., 2006) and p.R493X mutation (Gass et al., 2006) respectively. In contrast, *PGRN* mutations are rare in Finland, Japan and Korea (Kim et al., 2014; Kruger et al., 2009; Ogaki et al., 2013). This disparity in mutation frequencies in different populations may be due to the differences in the sample size, patient recruitment methods, selection criteria, and geographical origin of the study population.

The clinico-pathological aspects of patients with *PGRN* mutations are highly heterogeneous between and within families due to incomplete or age-related penetrance suggesting the impact of other genetic, epigenetic, and/or environmental factors modifying the phenotypic presentation of the disease. Different mutation carriers show variable age at onset, ranging from 35-87 years (mean=57 years) and broad range of disease duration, ranging from 1-22 years (Gass et al., 2006; Le Ber et al., 2008; Le Ber et al., 2007). Approximately 75% of *PGRN* mutation carriers exhibit a positive family history suggestive of autosomal dominant pattern of inheritance (Chen-Plotkin et al., 2011). The other neurodegenerative phenotypes produced by *PGRN* mutations include CBD (Benussi et al., 2008; Le Ber et al., 2008; Le Ber et al., 2007; Masellis et al., 2006; Rademakers et al., 2007; Spina et al., 2007), AD and PD (Brouwers et al., 2007; Rademakers et al., 2007). The neuropathology assisted with *PGRN* mutations often shows the TDP-43 pathology (Mackenzie et al., 2006). The exact pathogenic mechanisms leading to the deposition of TDP-43 is unknown.

### *II.5.1.3. Chromosome 9 open reading frame 72*

In 2011, two independent studies identified a large hexanucleotide (GGGGCC) repeat expansion in the in non-coding region of chromosome 9 open reading frame 72 (*C9ORF72*) as the most common genetic basis for the chromosome 9p-linked FTD and ALS with TDP-43 pathology (DeJesus-Hernandez et al., 2011; Renton et al., 2011). This expansion leads to the loss of one alternatively spliced *C9ORF72* transcript and to formation of nuclear RNA foci (DeJesus-Hernandez et al., 2011). The exact cut-off number of repeats conferring the pathogenesis is yet to be determined (DeJesus-Hernandez et al., 2011). The physiological function of the normal gene product remains unknown, however it is speculated that it may be involved in RNA metabolism.Worldwide, the repeat expansion has been detected in 12% of total FTD and in 25% of cases of familial FTD. The frequency of repeat expansion among patients with co-occurring FTD-ALS is even higher (Boeve et al., 2012; Hsiung et al., 2012; Mahoney et al., 2012; Snowden et al., 2012). The most common clinical presentation of *C9ORF72* carriers has been shown to be bvFTD and its frequent association with mild psychotic symptoms or even florid psychoses, mostly delusions (Boeve et al., 2012; Chio et al., 2012; Snowden et al., 2012). The associated neuropathology of *C9ORF72* expansions is mainly the accumulation of pathologic forms of TDP-43 (Hsiung et al., 2012; Snowden et al., 2012; Stewart et al., 2012).

### *II.5.1.4. Charged multi-vesicular protein 2B*

The gene encoding charged multi-vesicular protein 2B *(CHMP2B)* is located on chromosome 3p11 and the protein encoded is a component of the endosomal

secretory complex required for transport (ESCRT) type III (Momeni et al., 2006; Skibinski et al., 2005). So far, there are 4 different *CHMP2B* pathogenic mutations identified in a large Danish family which are located between exons 5 and 6 (Cruts et al., 2012; Skibinski et al., 2005). No other families have been linked with *CHMP2B* mutations making them a rare cause for FTD.

#### *II.5.1.5. Valosin-containing protein*

Inclusion body myopathy associated with Paget's disease of the bone and/or frontotemporal dementia (IBMPFD) is a rare multi-system disorder linked to chromosome 9p21-12. The gene responsible was identified as valosin-containing protein (*VCP*), which act as a molecular chaperone associated with several cellular functions including ubiquitin-dependent protein degradation, cell cycle regulation and apoptosis (Watts et al., 2004). A total of 17 pathogenic *VCP* mutations have been described (Cruts et al., 2012). Although *VCP* mutation carriers are neuropathologically characterized by the presence of TDP-43 positive inclusions, they are considered as a rare cause of prototypical FTD cases (van der Zee et al., 2009).

#### *II.5.1.6. Trans-active response DNA-binding protein with molecular weight 43 kDa*

In 2006, the trans-active response DNA-binding protein with molecular weight 43 kDa (TDP-43) was identified as the major pathological protein in ALS and FTD-U associated with/without ALS brains (Arai et al., 2006; Neumann et al., 2006). Majority of familial and sporadic FTD-U cases, including those with mutations in

*PGRN*, *VCP* and *C9ORF72*, are characterized by TDP-43 immunoreactive NCIs, NIIs and DNs. Biochemical analysis of these inclusions were shown to contain characteristic profile of disease-specific bands at ~25kDa, ~45 kDa and a smear of high molecular-mass proteins in addition to the normal 43 kDa band due to N-terminal truncation, hyperphosphorylation and ubiquitination of TDP-43 (Neumann et al., 2006). The gene encoding TDP-43 (*TARDBP*) is located on chromosome 1p36.2 and consists of one non-coding and five coding exons. It encodes a 414 amino acid protein which is highly conserved and ubiquitously expressed in various tissues including brain. Under normal conditions, it is localized to the nucleus or shuttles between the nucleus and cytoplasm (Ayala et al., 2008). *TARDBP* mutation screening identified over 30 mutations in familial and sporadic ALS cases (Cruts et al., 2012; Kabashi et al., 2008; Sreedharan et al., 2008).

### II.5.2. Susceptibility loci identified in FTD

Genetic mutations in putative pathogenic genes (*MAPT*, *PGRN*, *C9ORF72* etc.) are the sole causative factors identified responsible for familial FTD. The remaining proportion of FTD is highly sporadic in nature suggesting that other genetic as well as environmental factors are yet to be identified. Many polymorphic nucleotide substitutions found throughout different genes may modulate the function of proteins which could possibly contribute to the population-attributable risk of developing FTD. Regarding the sporadic FTD cases, several genome wide association studies (GWAS) have been conducted and a number of susceptibility loci have been

proposed (Table 2). Large case-control studies of these variants are needed to test this hypothesis.

**Table 2: Genetic susceptibility loci associated with FTD**

| Gene | Chromosome and Locus | Variant | Reference |
|---|---|---|---|
| *MAPT* | 17q21.1 | H1 and H2 haplotypes | (Ghidoni et al., 2006; Verpillat et al., 2002) |
| *PGRN* | 17q21.3 | rs5848 | (Rademakers et al., 2008) |
| *APOE* | 19q13.2 | E2 and E4 allele | (Bernardi et al., 2006; Verpillat et al., 2002) |
| *TMEM106B* | 7p21.3 | rs1990622 | (Van Deerlin et al., 2010) |
| *NOS1* | 12q24.2 | rs2682826 | (Venturelli et al., 2008) |
| *NOS3* | 7q35 | rs1799983 | (Venturelli et al., 2009) |
| *GSK3β* | 3q13.3 | rs13312998 | (Schaffer et al., 2008) |
| *UBAP1* | 9p13.3 | T-G-C haplotype | (Rollinson et al., 2009) |
| *DCUN1D1* | 3q26.3 | rs4859146 | (Villa et al., 2009) |
| *KIF24* | 9p13.3 | rs17350674 | (Venturelli et al., 2010) |
| *CST3* | 20p11.2 | B haplotype | (Benussi et al.) |
| *PRNP* | 20p13 | rs1799990 | (Li et al., 2005) |
| *BAG1* | 9p12 | rs706118 | (Venturelli et al., 2011) |
| *SORT1* | 1p13.3 | rs646776 | (Carrasquillo et al., 2010) |
| *DAPK1* | 9q21.33 | rs4878104 | (Tedde et al., 2012) |
| *TREM2* | 6p21.1 | rs75932628-T | (Giraldo et al., 2013) |

*APOE: Apolipoprotein E, TMEM106B: Trans-membrane protein 106B, NOS: Nitric oxide synthase, GSK3β: Glycogen synthase kinase β, UBAP1: Ubiquitin-associated protein 1, DCUN1D1: Defective in cullin neddylation 1-domain containing 1, KIF24: Kinesin family member 24, CST3: Cystatin C, PRN: Prion protein, BAG1: BCL2-associated athanogene 1, SORT1: Sortilin 1, DAPK1: Death-associated protein kinase 1, TREM2: Triggering receptor expressed on myeloid cells 2.*

#### *II.5.2.1. MAPT haplotypes*

Some proportion of NDDs cases are characterized by tau positive neuropathology without any mutations in the *MAPT*, implicating that some other mechanisms underlying *MAPT* locus might cause the tau-mediated neurodegeneration in such cases. Two examples of such sporadic tauopathies are PSP and CBD. In 1997,

Conrad et al found an association between *MAPT* and PSP with the identification of a polymorphic di-nucleotide repeat marker, TG, found in the intron 9 (A0 allele) that was significantly over-represented in PSP cases compared with normal controls (Conrad et al., 1997). Later, a series of single nucleotide polymorphisms (SNPs) were identified scattered throughout the *MAPT* which are in complete linkage disequilibrium. This led to the elucidation of two extended haplotypes covering the entire *MAPT* locus termed as H1 and H2 (Baker et al., 1999; Poorkaj et al., 1998). These haplotypes are defined by a series of SNPs and a 238 bp deletion in intron 9 found only on the H2 background (Baker et al., 1999). Later it was demonstrated that ~970 Kb inversion of the *MAPT* locus occurred as long as 3 million years ago and resulted in these two major *MAPT* haplotypes, H1 which is directly orientated and H2 with inverted orientation (Baker et al., 1999; Pastor et al., 2002; Pittman et al., 2005; Pittman et al., 2004; Stefansson et al., 2005). H1 haplotype is the most predominant haplotype in all ethnic groups, having an allele frequency of >70% in European populations, while the H2 haplotype has been associated mainly with Caucasian ancestry.

Several independent studies have replicated the association between *MAPT* H1 haplotype and susceptibility to PSP (de Silva et al., 2001; Pittman et al., 2005) and CBD (Di Maria et al., 2000; Houlden et al., 2001; Pittman et al., 2005). However the association between *MAPT* haplotype and susceptibility to FTD lacks consensus. Some reports show an association of FTD with the H1 allele and H1H1 genotype (Hughes et al., 2003; Ingelson et al., 2001; Verpillat et al., 2002b) while others report no association (Bernardi et al., 2006; Laws et al., 2008; Morris et al., 1999;

Panegyres &Zafiris-Toufexis, 2002; Sobrido et al., 2003). Functionally, H2 haplotype carriers have shown significantly severe hypometabolism in frontal lobe (Borroni et al., 2008; Laws et al., 2007). The pathogenic mechanism by which the haplotype could affect the risk for FTD is unknown. Two mechanisms have been suggested: H1 haplotype carriers express higher levels of tau (Kwok et al., 2004) or more of 4R *MAPT* transcripts in their brain (Caffrey et al., 2006).

#### *II.5.2.2. PGRN rs5848 polymorphism*

*PGRN* acts as a heritable genetic causative factor for FTD. Recently, several genetic association studies have identified a common genetic variant, rs5848 located in the 3'-untranslated region (UTR) of *PGRN* as a genetic risk factor for developing FTD. This variant has been predicted as a binding site for the microRNA 659 (miR-659) which plays a role in the *PGRN* translation. microRNAs are a class of non-coding RNAs which plays a vital role in the gene regulation by binding to partially complementary sites in the 3'-UTR of target mRNA transcripts, thereby inducing translational repression. Initially it has been associated as a susceptibility factor for developing FTD characterized by TDP-43 pathology through the suppression of *PGRN* expression (Rademakers et al., 2008). The common genotype of rs5848 is CC whereas the risky genotype is found to be the TT. TT genotype shows a 3.2 fold increased risk of developing the disease when compared with the CC genotype. Functional analyses using both *in vitro* and *in vivo* systems have demonstrated that miR-659 can bind more efficiently to the T allele resulting in the translational inhibition of *PGRN*. Gene expression studies from the diseased brain also confirmed

a 30% reduction of progranulin expression in TT carriers compared to the CC carriers (Rademakers et al., 2008).

#### *II.5.2.3. Apolipoprotein E (APOE) polymorphisms*

The gene encoding apolipoprotein E (*APOE*) is located on chromosome 19q13.2 and it has three different alleles: ε2, ε3 and ε4 that code for three different polymorphic proteins with isoform-specific neurotrophic and antioxidant functions. Through its interaction with the members of low-density lipoprotein (LDL) receptor family, APOE plays a key role in lipid transport both in plasma and CNS. The ε4 allele of *APOE* has been established as a major susceptibility gene for developing late-onset AD (Corder et al., 1993). Several independent studies were conducted to define the association of *APOE* polymorphisms with FTD, however ended up with inconclusive results. Possibly *APOE* ε4 may be a risk factor for both AD and FTD (Gustafson et al., 1997). In 2013, Rubino et al conducted a meta-analysis of *APOE* polymorphisms in FTD through a systematic review of all case-control studies investigating the association between the *APOE* and found an association between the *APOE* ε4 allele and FTD (ε4 carriers vs. non-ε4 carriers: OR, 1.94; 95% CI, 1.43–2.64; ε4 vs. ε3 allele: OR, 1.83; 95% CI, 1.34–2.52) (Rubino et al., 2013). Replication of these studies is essential for establishing the credibility of *APOE* genotype with FTD to resolve this association and would need to be studied in a large study samples.

## II.6. Diagnostic biomarkers

Currently, brain-imaging is the routinely used diagnostic biomarker to predict the underlying pathology in FTD cases.

### II.6.1. Neuroimaging

Magnetic resonance imaging (MRI) is traditionally used diagnostic criteria in order to rule out the treatable and reversible causes of dementia (Sorbi et al., 2012). Structural brain imaging studies demonstrated focal atrophy of frontal and/or anterior temporal lobes which is often asymmetric. Each clinical subtypes display a characteristic pattern of atrophy which can be related to the specific symptoms of each subtype. BvFTD is associated with atrophy of the frontal, insular, and/or anterior temporal lobes, usually symmetrically, although right-predominant atrophy has also been reported (Whitwell et al., 2009). SD cases typically show atrophy of 'knife-edge'-type in the ventral and lateral portions of the anterior temporal lobes bilaterally, although degeneration is usually greater on the left (Gorno-Tempini et al., 2004; Rosen et al., 2002). PNFA is associated with asymmetric, predominantly left hemispheric atrophy of the perisylvian region (Gorno-Tempini et al., 2004; Josephs et al., 2006a). Functional imaging studies such as positron emission tomography (PET) and single-photon emission computed tomography (SPECT) measures the brain activity such as alterations in brain metabolism and/or perfusion. In FTD, PET and SPECT typically show characteristic patterns of hypometabolism or hypoperfusion respectively in frontal and/or anterior temporal cortices. Each clinical variant has its typical profile of hypoperfusion and hypometabolism and it correlates with the structural changes (Diehl et al., 2004; Ishii et al., 1998).

### II.6.2. Biochemical markers

#### *II.6.2.1. Tau as a biomarker*

Tauopathies are neuropathologically characterized by the intracellular aggregation of abnormally hyperphosphorylated fibrillar tau deposits and the definitive diagnosis can be made only after the histopathological examination at autopsy. Increased levels of total and phosphorylated tau in brain and CSF of AD patients have been demonstrated (Shoji et al., 2002). Several studies have investigated the diagnostic value of CSF total tau protein as a biomarker for neurodegeneration in FTD associated with *MAPT* mutations and pathological changes in tau protein (Green et al., 1999) however with inconclusive results (for review see (Hampel & Teipel, 2004)). Since the procedure for CSF collection is invasive (through lumbar puncture), development of blood based biomarker analysis of total tau protein has been evaluated as a non-invasive and easy to perform method by some studies (Ingelson et al., 1999).

#### *II.6.2.2. Progranulin as a biomarker*

Based on the uniform haploinsufficiency disease mechanism associated with *PGRN* mutations, PGRN mRNA and protein levels in blood and CSF of mutation carriers will be reduced up to 50% of that of non-carriers. Reduced progranulin levels in plasma seem to predict the underlying *PGRN* mutation reliably as early as in the pre-symptomatic stage. Recently, several studies have established an assay for measuring the quantity of progranulin in plasma using an ELISA as relatively inexpensive method to predict the *PGRN* mutation status in patients with FTD and the at-risk

asymptomatic family members (Bird, 2009; Coppola et al., 2008; Finch et al., 2009; Ghidoni et al., 2008; Sleegers et al., 2009). To enable reliable detection, an optimal progranulin plasma cut-off value of 61.5 ng/mL has recently been proposed that predicts a pathogenic *PGRN* mutation with almost 100% sensitivity and specificity (Ghidoni et al., 2012). Currently, measuring progranulin levels is a blood based biomarker to detect *PGRN* null mutation carriers.

#### *II.6.2.3. TDP-43 as a biomarker*

TDP-43 is a nuclear protein which is the target protein present in the ubiquitinated cytoplasmic inclusions in FTD caused by mutations in *PGRN*, *C9ORF72* and *VCP* and it is also a major component in familial or sporadic ALS. The pathological modifications of TDP-43 include phosphorylation, N-terminal truncation, ubiquitination, cleavage and redistribution of nuclear TDP-43 to cytoplasm. Some studies have investigated whether the measurement of plasma TDP-43 have utility in detecting the presence of TDP-43 pathology in brain. Increased levels of total or phosphorylated TDP-43 in CSF or plasma have been reported that would correlate with the extent of TDP-43 brain pathology in FTD (Foulds et al., 2008; Foulds et al., 2009; Steinacker et al., 2008).

#### *II.6.2.4. Biomarkers for oxidative stress*

Even if there are several independent hypotheses to explain the pathogenesis, none of them alone is sufficient to explain the multitude of cellular and biochemical alterations observed in FTD. Since FTD is age-related neurodegenerative disorder,

independent of the genetic background, the pathways leading to the aging process are also suggested to be involved in the pathogenesis. Oxidative stress is one such mechanism that has been associated with aging. It is caused by the imbalance between the generation and detoxification of reactive oxygen and nitrogen species collectively termed as free radicals. The free radical species can attack neuronal lipids, proteins and nucleic acids thereby leading to neuronal dysfunction. Among these, increased levels of lipid peroxidation markers such as malondialdehyde, thiobarbituric acid-reactive substances, 4-hydroxynonenal and acrolein have been found to be associated with AD brains (Butterfield et al., 2010). In FTD brains, disease specific oxidative damage and oxidized lipids were found in FTD-tau positive cases (Martinez et al., 2008). However peripheral level analysis of oxidative stress markers has not been performed in FTD cases. So the systemic effect of oxidative stress in FTD is unknown.

# III. OBJECTIVES

## III. Objectives of the study

Most of the genetic etiological factors underlying familial FTD are currently known, that includes mutations in *MAPT* and *PGRN*. Familial occurrence of FTD varies between 10 to 50% of total cases depending on the study population. Identification of the genetic basis of familial FTD cases denotes powerful resources to clarify the molecular bases and neurodegenerative mechanisms of their respective sporadic variants. The principal hypothesis underlying this study is that identifying pathogenic gene mutations, determining the association of known genetic susceptibility loci and evaluating the core biomarkers in both familial/sporadic FTD could elucidate the etiology of FTD in south Indian cohorts.

The specific objectives of the study were as follows,

1. To characterize the patients with FTD and normal controls from the population of Kerala
2. To study the genetic mutations in FTD
    - To determine the prevalence of *MAPT* mutations in the study cohort
    - To determine the prevalence of *PGRN* mutations in FTD
3. To study genetic predisposition to FTD
    - To evaluate *MAPT* haplotypes as a genetic susceptibility factor
    - To evaluate *PGRN* rs5848 as a genetic susceptibility factor for FTD
    - To evaluate *APOE* polymorphism as a genetic susceptibility factor for FTD

4. To search for biomarkers associated with FTD
    - To quantify the plasma levels of tau
    - To quantify the plasma levels of progranulin
    - To quantify the plasma levels of TDP-43
    - To measure the serum levels of lipid peroxides

# IV. MATERIALS AND METHODS

## IV.1. Chemicals

All the routine chemicals used for the preparation of buffers and reagents were purchased from Sigma Aldrich, St. Louis, MO, USA, unless otherwise specified. Plastic wares were supplied by Tarsons products Pvt. Ltd, Kolkata, India and Axygen, MA, USA. Anticoagulant-coated and non-coated vacutainers were supplied by BD Vacutainer, Becton, Dickinson and Company, Plymouth, UK.

DNA isolation kits used were Wizard Genomic DNA Purification Kit from Promega Corporation, Madison, USA and ArchivePure DNA Blood Kit from 5 PRIME, Gaithersburg, USA. PCR reagents were purchased from Promega Corporation, Madison, USA. HhaI restriction enzyme and bovine serum albumin (BSA) were purchased from Invitrogen, CA, USA. *MAPT* and *PGRN* oligonucleotide primers were supplied by Metabion, Martinsried, Germany and primers for *APOE* genotyping were supplied by Sigma Aldrich. MSP1 digested PUC18 marker and 100 bp DNA ladder was purchased from Fermentas, Burlington, Canada. DNA sequencing kit (BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit) was purchased from Applied Biosystems, Foster City, CA, USA.

Human Tau (Total) ELISA Kit (Catalog no. KHB0041) was purchased from Invitrogen, Life Technologies CA, USA, Human *PGRN* ELISA kits (Catalog no. AG-45A-0018EK-KI01) were supplied by Adipogen, Inc., Seoul, Korea, Human TDP-43 ELISA kit (Catalog no. E91951Hu) was supplied from USCN life science, Wuhan, China. Human PGRN polyclonal antibody was purchased from Zymed, Life Technologies CA, USA. Secondary anti-rabbit antibody was from Cell Signalling

Technology, Danvers, MA, USA. Enhanced chemiluminascent (ECL) reagent and nitrocellulose membrane used for blotting were purchased from Thermo Scientific, Rockford, IL, USA. Ethyl alcohol was purchased from Merck, India. A detailed description of reagents employed for the study is given in Appendix II.

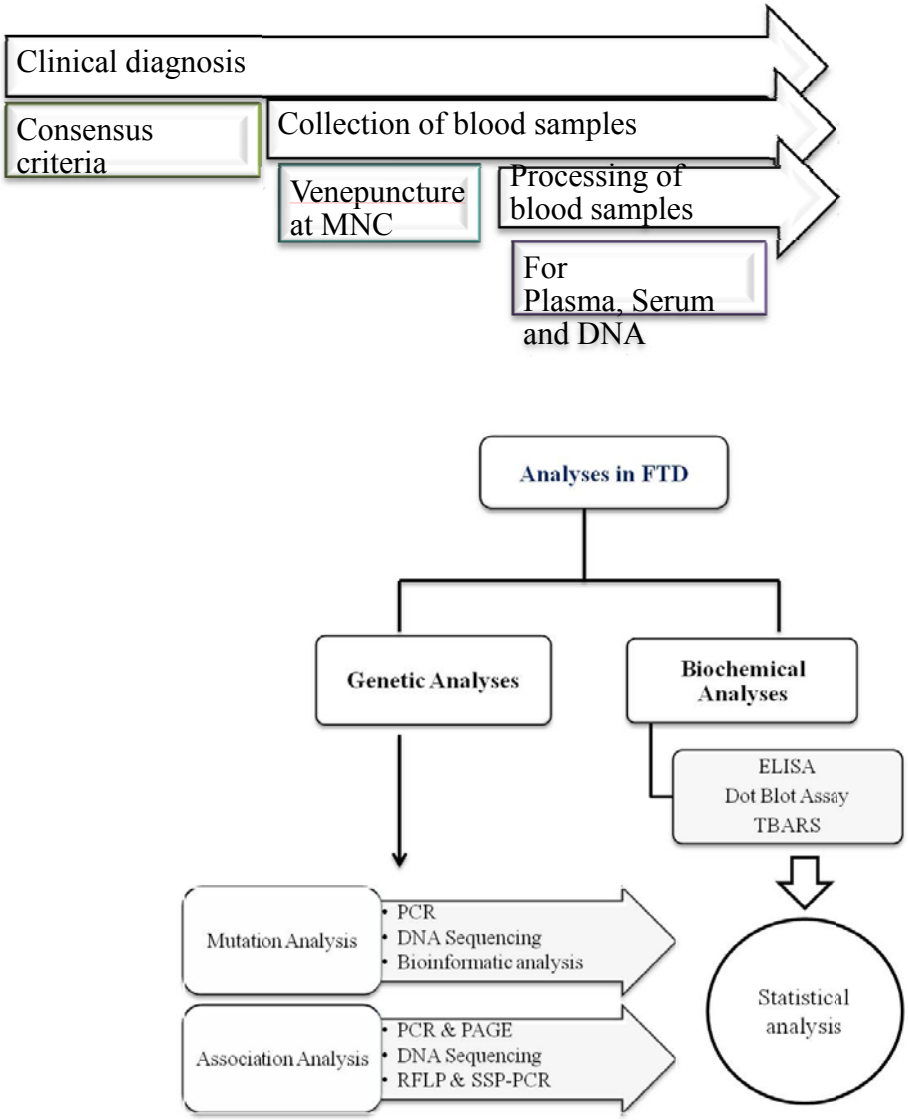
## IV.2. Equipments

PCR Thermal cycler, Electrophoresis unit, Semi dry blot apparatus and Gel documentation apparatus were purchased from Biorad, CA, USA, Applied Biosystems PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA), ELISA plate reader (Bio-Tek instruments, USA), Water bath (Julabo, Germany), Weighing balance (Sartorius, Germany), pH meter (Eutech, USA), Magnetic stirrer (Schott, Germany), $-80^0$C Freezer (New Brunswick Scientific, Edison, NJ), $-20^0$C Freezer (Vestfrost, Falkevej, Denmark), Biophotometer, Cooling centrifuge and Good precision pipettes (Eppendorf, Hamburg, Germany), Centrifuge (REMI, India), Laminar air flow hood (Micro Filt, India), and UV-Visible-Spectrophotometer (Shimadzu, North America).

## IV.3. General study design

The study included genetic analyses comprising mutation analysis and genetic association analysis through candidate gene-based case-control analysis in FTD patients and biochemical analyses in plasma and serum for the identification of disease specific biomarkers in FTD compared with age-matched controls (Figure 8).

**Figure 8: General study design**



## IV.4. Study participants

Study participants were recruited from patients attending the Memory and Neurobehavioral Clinic (MNC) of SCTIMST between 2009 and 2013. The self-centered or referred patients were subjected to longitudinal neuropsychological, neuroimaging and clinical assessment according to the consensus criteria for FTD (Neary et al., 1998), Cambridge criteria for Corticobasal syndrome (CBS) (Mathew et al., 2012), NINDS-SPSP criteria for PSP (Litvan et al., 1996), NINDS-ADRDA criteria for AD (McKhann et al., 1984) and Petersen's criteria for mild cognitive impairment (MCI) (Petersen et al., 1999).

The study cohort comprised 529 participants including patients diagnosed with FTD (n=116), other dementia groups (AD, vascular dementia (VD), PSP, CBS, dementia with Lewy bodies and mixed dementias) (n=205) along with MCI subjects (n=78) and cognitively unimpaired controls (n=130) who were matched for age and ethnicity with the patients. The FTD cohort comprised patients with bvFTD (n=94), PNFA (n=12), and SD (n=7). Three FTD patients had concomitant motor neuron disease (FTD- ALS), the diagnosis of which was carried out following the diagnostic criteria defined by Brooks et al. (Brooks et al., 2000). The presence of family history was investigated through a series of questionnaire in such a way that a first degree relative was suffering from dementia. Cognitively normal, age-matched and healthy control individuals were recruited from the spouses and family members of patients.

## IV.5. Ethical aspects

The research protocols were approved by the Institutional Ethical Committee (IEC) of SCTIMST, Trivandrum (Appendix III). All the participants or caregivers had given a written informed consent in order to take part in the study.

## IV.6. Sample collection and processing

Peripheral venous blood samples (6 mL) were drawn into vacutainers with and without anticoagulants by venepuncture from all recruited participants at MNC of SCTIMST. The blood samples were processed for components such as DNA, plasma and serum.

### IV.6.1. DNA extraction and quantification

Total genomic DNA was extracted from peripheral leucocytes of heparinized blood samples using genomic DNA Purification Kit following the manufacturer's instructions. The quantity and purity of the extracted DNA was determined using known molecular weight DNA marker using Quantity One software/Biophotometer. The ratio of absorbance at 260 nm and 280 nm was used to determine the purity of the DNA and a ratio between 1.7-1.9 was considered as pure DNA without protein/RNA contamination. The absorption of 1 OD (A260) was taken as equivalent to approximately 50 µg/mL of double stranded DNA. The concentration of DNA in 1 µL of the DNA sample was calculated using the following equation.

$$\frac{50 \times A260 \times \text{Dilution factor}}{1000}$$

An average yield of 120 ng/µL DNA was obtained from 300 µL blood sample and the DNA was stored at $4^0$C.

### IV.6.2. Plasma separation

Plasma from heparinized blood samples were separated through centrifugation at 3000 rpm for 15 min at $4^0$C. Plasma samples were aliquoted into cryovials and stored at -$80^0$C until they could be analyzed for ELISA.

### IV.6.3. Serum separation

Blood samples were collected in vacutainers without anti-coagulants and allowed to coagulate for 30 min at room temperature. The coagulated samples were subjected to centrifugation at 1500 rpm for 10 min at $4^0$C. Serum was separated and stored in labeled cryovials at -$80^0$C until they could be analyzed for biochemical assays.

## IV.7. Genetic analyses

### IV.7.1. Mutation analysis

#### *IV.7.1.1. MAPT mutation analysis: Polymerase chain reaction (PCR)*

*MAPT* exons 1, and 9 through 13, in which the pathogenic mutations are reported (Figure 9) were amplified by PCR from genomic DNA using the primers derived from 5' and 3' intronic sequences (Kowalska et al., 2001) (Table 3). Exons 2, 3, 5, 7 in which no mutations were reported as well as Exons 4A, 6, and 8 that are

essentially absent from human tau mRNA were excluded from the analysis. A total of 20 ng of genomic DNA was amplified in a 20 µL reaction volume containing 20 picomols of each primer, 1X reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1.5 units of Taq DNA polymerase. The PCR consisted of 35 cycles of 30 s at $94^0$C, 15 s at annealing temperature (Table 3) and 20 s at $72^0$C, preceded by 4 min at $94^0$C and followed by 10 min at $72^0$C. The reaction products were analyzed on a 5% polyacrylamide gel to verify the size and quantity of the PCR product.

**Figure 9: *MAPT* exons selected for mutation screening**


**Table 3: *MAPT* exonic primers used for PCR and DNA sequencing**

| ***MAPT* Exons** | **Primers** | **Annealing temperature ($^0$C)** | **PCR additive** | **Product size (bp)** |
|---|---|---|---|---|
| Exon 1 | 5'-CTCCTCAGAACTTATCCTCTCC-3'<br>5'-CAGTGATCTGGGCCTGCTGT-3' | 58.5 | Nil | 224 |
| Exon 9 | 5'-TCGAGTCCTGGCTTCACTCC-3'<br>5'-CACGCTCAACCGCGCACC-3' | 58 | DMSO | 407 |
| Exon 10 | 5'-GGTCCAGGGTGGCGCATGTC-3'<br>5'-TCACCCAGAGGTCGCAGCCA-3' | 70 | Nil | 336 |
| Exon 11 | 5'-CTCTCCTCCTCTCTCCCATCTCC-3'<br>5'-TCACCAGGACTCCTCCACCC-3' | 58 | Nil | 169 |
| Exon 12 | 5'-CAGAACCACAGAAGATGATGGC-3'<br>5'-CCAACCACCCTACCCCCT-3' | 61 | Nil | 186 |
| Exon 13 | 5'-ACTTCATCTCACCCTCCCTC-3'<br>5'-CCTCTCCTTCTCCCTCTTCTAC-3' | 59 | DMSO | 597 |

*Exonic primers selected for MAPT mutation analysis. Exon 10 primer pairs were designed through Primer 3 software and all other primers were previously reported (Kowalska et al., 2001). DMSO: Dimethyl sulphoxide*

### *IV.7.1.2. PGRN mutation analysis: PCR*

All coding exons (exons 1-12) as well as the non-coding exon and the flanking intron-exon boundaries of *PGRN* were PCR amplified and sequenced using the oligonucleotide primers listed in the Table 4 (Cruts et al., 2006). The annealing temperature for each primer was optimized and is also listed in the Table 4.

**Table 4: *PGRN* exonic primers used for PCR and DNA sequencing**

| ***PGRN* Exons** | **Primer** | **Annealing temperature ($^0$C)** | **PCR additive** | **Product size (bp)** |
|---|---|---|---|---|
| Exon 1 | 5'-CTGTCAATGCCCCAGACACG-3'<br>5'-CCCCCAAGGAGTTTCAGTAAGC-3' | 60 | DMSO | 499 |
| Exon 2 | 5'-TTGAGAAGGCTCAGGCAGTC-3'<br>5'-GGCCATTTGTCCTAGAAAGACAGG-3' | 60 | Nil | 400 |
| Exon 3+4 | 5'-TGGGTTTTCCCAAAGGGTCA-3'<br>5'-GCACAAGGGCAGGAATCAGG-3' | 60 | DMSO | 516 |
| Exon 5+6 | 5'-GCCACCAGCTCCTTGTGTGA-3'<br>5'-GGCCACTGGAAGAGGAGCAA-3' | 60 | DMSO | 544 |
| Exon 7 | 5'-TGAGGAGGTGGGAGAGCATC-3'<br>5'-CAGGCTCAGTAGCACACAGG-3' | 60 | DMSO | 424 |
| Exon 8 | 5'-TCCCTGTGTGCTACTGAG-3'<br>5'-AAGCAGAGAGGACAGGTC-3' | 58.5 | Nil | 373 |
| Exon 9+10 | 5'-ATACCTGCTGCCGTCTAC-3'<br>5'-GAGGGCAGAAAGCAATAG-3' | 58.5 | DMSO | 457 |
| Exon 11+12 | 5'-TGGACTGGAGAAGATGCC-3'<br>5'-CGATCAGCACAACAGACG-3' | 58.5 | DMSO | 574 |
| Exon 13 | 5'-CAGACCTGCTGCCGAGACAA-3'<br>5'-CGATGTGGGCAGCAGCAAAT-3' | 60 | DMSO | 736 |

*List of primers used for PGRN mutation screening (Cruts et al., 2006). DMSO: Dimethyl sulphoxide*

A total of 20 ng of genomic DNA was amplified in a 20 µL reaction volume containing 20 picomols of each primer, 1X reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1.25 units of Taq DNA polymerase. The PCR consisted of 35 cycles of 30 sec at 94$^0$C, 15 sec at annealing temperature and 20 sec at 72$^0$C, preceded by 4 min at 94$^0$C and followed by 10 min at 72$^0$C. The reaction products were analyzed on a 5% polyacrylamide gel to verify the size and quantity of the PCR product.

### *IV.7.1.3. DNA sequencing: Chain termination method*

Two hundred nanograms of each amplicons were automatically sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions. The final reaction volume was 10 µL containing 200 ng of each amplicons, 1X reaction buffer (1.75µL), ready reaction mix (0.5 µL) containing the four dNTPs, the four fluorescently labeled di-deoxy-ribonucleotides (ddNTPs), and AmpliTaq® DNA Polymerase, 5 picomols of either forward or reverse primer and distilled water to reach the final volume. Fluorescent fragments were generated by incorporation of dye-labeled ddNTPs and each different ddNTPs (ddATP, ddCTP, ddGTP, or ddTTP) will carry a different color of dye. All terminated fragments (those ending with a ddNTP) therefore contain a dye at their 3' end. The thermocycling conditions were 25 cycles of $96^0$C for 30 sec and $60^0$C for 4 min with a thermal ramp rate of $1^0$/sec.

### *IV.7.1.4. Purification of PCR product for DNA sequencing: Ethanol/EDTA/Sodium acetate precipitation method*

Post-sequencing PCR reaction clean-up was done for the removal of un-incorporated terminators, primer dimers and other interfering components form the reaction products. PCR products were purified using Ethanol/EDTA/Sodium acetate precipitation method. For a 10 µL reaction volume of PCR reaction products added 2 µL of 125 mM EDTA, 2 µL of 0.5 M sodium acetate and 50 µL absolute ethanol (chilled) in an eppendorf tube and vortexed for 10 sec. The mixture was incubated at room temperature for 15 min in order to precipitate the reaction products. The

samples were then centrifuged at 12,000 rpm for 20 min at room temperature ($25^0$C). After centrifugation, supernatant was removed completely through blotting to a tissue paper and to the pellet added 250 µL of 75% ethanol and vortexed briefly. The samples were centrifuged at 12,000 rpm for 10 min at room temperature. The supernatant was decanted and pellet was air-dried.

### *IV.7.1.5. DNA sequencing: Capillary electrophoresis*

The sequencing samples were electrophoresed on a capillary electrophoresis-based Genetic Analyzer. To prepare the samples for capillary electrophoresis, 10 µL formamide was added to each sample pellet, vortexed thoroughly, denatured at $95^0$C for 10 min and were snap chilled. The samples were then resolved through the POP-7 polymer and the sequencing data was normalized using the matrix standard.

### *IV.7.1.6. Bioinformatic analysis*

DNA sequencing results after electrophoresis was analyzed using the software package, Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA). Mutation screening was done by Mutation Surveyor software v.3.25 using standard parameters. Sequences from normal healthy control from the general population were aligned as reference sequence and also to rule out the population specific nucleotide changes in the gene sequence.

The SNPs identified through the bi-directional sequencing of exons were compared with those reported in the NCBI Database of Single Nucleotide Polymorphisms (dbSNP) (http://www.ncbi.nlm.nih.gov/snp/), and Alzheimer Disease &

Frontotemporal Dementia Mutation Database (www.molgen.ua.ac.be/FTD Mutations). The genomic reference sequences used for the nomenclature were: NG_007398.1 for *MAPT* and NG_007886 for *PGRN*. The sequence variations can be named based on, genomic (g.), coding DNA (c.) and protein (p.) levels, and the numbering is relative to a reference sequence.

The functional consequences of the sequence variants of unknown significance were predicted *in silico* using PolyPhen-2 and Sorting intolerant from tolerant (SIFT) software. In PolyPhen-2, a mutation is classified as "probably damaging" if it has q probabilistic score greater than 0.15; remaining variants are classified as benign. Using SIFT, scores ranging from 0-1 are obtained to represent the normalized probability that a particular amino acid substitution will be tolerated. SIFT predicts that substitutions with scores less than 0.05 are deleterious.

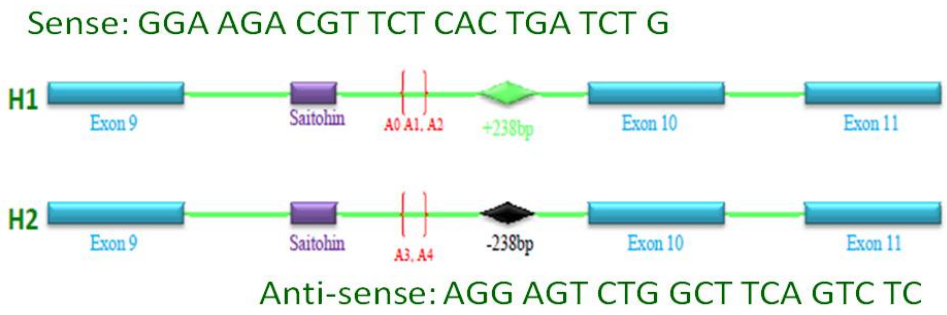
### IV.7.2. Genetic susceptibility factor analysis

#### *IV.7.2.1. MAPT haplotypes association analysis*

To delineate the two distinct *MAPT* haplotypes, all study participants were genotyped through testing for the presence of 238 bp deletion between exon 9 and 10, which discriminate H2 haplotype from H1 through PCR. The primer pairs used were; sense 5'-GAAGACGTTCTCACTGATCTG-3' and antisense 5'-AGGAGTCTGGCTTCAGTCTC-3' (Figure 10). A total of 40 ng of genomic DNA was amplified in a 20 μL reaction volume containing 20 picomols of each primer, 1X reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1.25 units of Taq DNA polymerase and distilled water to reach the final volume. The thermal cycling

conditions were as follows: an initial denaturation at 95$^0$C for 5 min followed by 35 cycles each of 30 sec at 94$^0$C, 15 sec at 60$^0$C and 20 sec at 72$^0$C, followed by 10 min at 72$^0$C. Following the reactions, the genotypes were assessed by visualizing the products on a 5% polyacrylamide gel. The sizes of the products were determined using a 100 bp size marker.

**Figure 10: *MAPT* genomic region encompassing the deletion polymorphism that is used to determine H2 haplotype from H1 haplotype and the primers used for genotyping**




***IV.7.2.2. PGRN rs5848 polymorphism association analysis***

*PGRN* rs5848 genotyping was carried out through PCR and DNA sequencing using specific primers, forward: 5'-CAGACCTGCTGCCGAGACAA-3' and reverse: 5'-CGATGTGGGCAGCAGCAAAT-3'. Briefly, each PCR was carried out in 20 µL total volume containing 25 ng genomic DNA, 10 picomols of each specific primer, 0.6 µM of each dATP, dTTP, dCTP and dGTP, 1unit Taq DNA polymerase and distilled water to reach the final volume. Amplification conditions were as follows; an initial denaturation step at 94$^0$C for 5 min followed by 35 cycles of 94$^0$C for 30 sec, 60$^0$C for 30 sec and 72$^0$C for 45 sec, and a final extension step of 72$^0$C for 10 min.

### *IV.7.2.3. APOE polymorphisms association analysis*

*APOE* genotyping was carried out using restriction fragment length polymorphism (RFLP) PCR and sequence specific primer (SSP) PCR.

*IV.7.2.3.1. Restriction fragment length polymorphism (RFLP) PCR*

RFLP-PCR was carried out using the method standardized by Hixon and Vernier (Hixson & Vernier, 1990). This protocol employs the PCR amplification of *APOE* sequence encoding the amino acids at $112^{th}$ and $158^{th}$ positions followed by the restriction digestion of the PCR product using the restriction enzyme, *HhaI* that generates unique combination of fragments that distinguishes *APOE* isoforms.

The oligonucleotide primers used were, 5'-CAGAATTCGCCCCGGCCTGGTCAC-3' and 5'-TAAGCTTGGCACGGCTGTCCAAGGA-3'. The PCR was performed on 15 ng genomic DNA with 4 picomoles of each primers, 1X reaction buffer containing $MgCl_2$ (25 mM), 0.2 mM each of four dNTPs, 1.25 units of Taq DNA polymerase, 5% DMSO and distilled water to reach the final volume of 10 µL. The thermal cycling conditions were as follows; an initial denaturation at $95^0$C for 5 min followed by 35 cycles each of 1 min at $95^0$C, annealing at $61.5^0$C for 1 min and extension at $72^0$C for 30 sec, followed by a final extension at $72^0$C for 5 min. The size and quality of the PCR products (244 bp) were verified on 1% agarose gel running along with the 100 bp DNA ladder. The reaction mixture for restriction digestion was prepared with 1 unit of *HhaI* restriction enzyme, 1X *HhaI* reaction buffer, bovine serum albumin (BSA) (0.2 µg) and ethidium bromide (EtBr) (0.01 µg). This reaction mixture was incubated at $37^0$C for 30 min for cleavage. After

incubation, the digested alleles were separated on a 5% polyacrylamide gel along with Msp1 digested pUC18 marker. After electrophoresis at 100 V for 1.5 h, the gel was stained using ethidium bromide and visualized on a gel documentation system. The restricted fragments with unique combinations of HhaI fragment sizes in all homozygotic/heterozygotic combinations were determined by comparing with the DNA marker.

*IV.7.2.3.2. Sequence specific primer (SSP) PCR*

In SSP-PCR, sequence-specific forward and reverse primers were combined to raise the three isoforms such as *APOE ε3* (*APOE* Primers 1+2), *APOE ε2* (*APOE* Primers 1+3), and *APOE ε4* (*APOE* Primers 2+4). The protocol employed was as standardized elsewhere (Pantelidis, Lambert-Hammill, & Wierzbicki, 2003) and the oligonucleotide primers employed were,

*APOE* Primer 1: CGGACATGGAGGACGTGT

*APOE* Primer 2: CTGGTACACTGCCAGGCG

*APOE* Primer 3: CTGGTACACTGCCAGGCA

*APOE* Primer 4: CGGACATGGAGGACGTGC

All reaction mixtures were included with the internal control primers for human leukocyte antigen (HLA), forward, 5'-TGCCAAGTGGAGCACCCAA-3' and reverse 5'-GCATCTTGCTCTGTGCAGAT-3'. The final reaction volume was 20 μL containing 8 picomoles each of *APOE* primers and 0.75 picomoles of HLA primers, 1X reaction buffer containing $MgCl_2$ (25 mM), 1.25 units of Taq DNA polymerase,

0.2 mM each of dNTPs, 15 ng genomic DNA and distilled water to reach the final volume. The thermal cycling conditions were, initial denaturation at $96^0$C for 1 min, followed by 10 cycles each of 20 sec at $96^0$C, 45 sec at $70^0$C, and 25 sec at $72^0$C; 21 cycles each of 25 sec at $96^0$C, 50 sec at $65^0$C, and 30 sec at $72^0$C; 4 cycles each of 30 sec at $96^0$C, 60 sec at $55^0$C, and 120 sec at $72^0$C. The multiplex PCR products were analyzed on 5% polyacrylamide gels along with the Msp1 digested pUC18 marker. The PCR products at 173 bp along with the HLA products at 785 bp or 1598 bp indicated the presence of specific *APOE* isoforms.

## IV.8. Biochemical analyses

### IV.8.1. Tau as a biomarker: Enzyme linked immunosorbent assay (ELISA)

To evaluate plasma tau (total) as a biomarker for FTD, the study combined the biochemical analysis of plasma tau (total), through a solid phase sandwich ELISA using Human Tau ELISA kit. The assay recognizes both natural and recombinant human tau proteins. All reagents used in the assay were supplied in the kit including the human tau standards and standard dilution buffer.

#### *IV.8.1.1. Reconstitution and dilution of human tau (total) standard*

Human tau (total) standard used was the recombinant human tau-441 expressed in E. coli. Standards were prepared by diluting the supplied standard with standard dilution buffer (Concentration=2000 pg/mL). Serial dilutions were made into standard dilution buffer to get concentrations of 1000, 500, 250, 125, 62.5 and 31.25 pg/mL (Table 5).

**Table 5: Serial dilution of human tau (total) standard**

| Concentration of standard | Volume of standard | Volume of diluent buffer |
|---|---|---|
| 2000 pg/mL | 0.600 mL reconstituted standard | 0 |
| 1000 pg/mL | 0.300 mL of 2000 pg/mL | 0.300 mL |
| 500 pg/mL | 0.300 mL of 1000 pg/mL | 0.300 mL |
| 250 pg/mL | 0.300 mL of 500 pg/mL | 0.300 mL |
| 125 pg/mL | 0.300 mL of 250 pg/mL | 0.300 mL |
| 62.5 pg/mL | 0.300 mL of 125 pg/mL | 0.300 mL |
| 31.2 pg/mL | 0.300 mL of 62.5 pg/mL | 0.300 mL |
| 0 pg/mL | 0 | 0.300 mL |

***IV.8.1.2. Assay method: Principle, procedure and calculations***

All reagents were allowed to reach room temperature before use. A monoclonal antibody specific for human tau has been coated onto the wells of the microtiter strips provided. About 100 µL of standards of known human tau concentration was added to the appropriate microtiter wells and 100 µL of the standard diluent buffer only to the blank well. The plasma samples were diluted in standard diluent buffer with 1:1 ratio. Briefly, 50 µL of standard diluent buffer along with 50 µL of plasma sample was added to ELISA wells in duplicate and gently tapped to mix. The ELISA plate was then covered with the plate cover and incubated for 2 h at room temperature. During the first incubation, the human tau antigen got bound to the immobilized (capture) antibody on one site. After the incubation the wells were thoroughly aspirated and washed 4 times with the supplied wash buffer concentrate after dilution. After washing, 100 µL aliquot of rabbit polyclonal antibody specific for human tau (total) was added to each well, and the plate was sealed and incubated for 1 h at room temperature. During the second incubation, this antibody got bound

to the immobilized human tau captured during the first incubation. Thereafter, the solution was decanted and the wells were washed 4 times.

After the removal of excess second antibody, 100 µL working solution of horseradish peroxidase (HRP) labeled anti-rabbit antibody (100X, in 50% glycerol concentrate) after dilution with the supplied HRP diluent was added to each well. The plate was sealed and incubated for 30 min at room temperature, during which the HRP-labeled antibody got bound to the rabbit polyclonal antibody to complete the four-member sandwich. After washing 4 times to remove all the excess anti-rabbit HRP,100 µL of stabilized chromogen was added to each well, and the plate was sealed and incubated at room temperature in the dark for 20-30 min till the liquid in the wells began to turn blue. The intensity of the colored product was directly proportional to the concentration of human tau (total) present in the original specimen. The reaction was completed by the addition of 100 µL of provided stop solution to each well. The solution in the wells turned from blue to yellow. The absorbance was read at 450 nm in an ELISA plate reader. A standard curve was plotted using the absorbance of the standards against the standard concentration. Each sample was run in duplicates, averaged and the concentrations of tau in plasma samples were calculated from the graph using GraphPad Prism software. Net absorbance was calculated by deducting the mean value obtained for a duplicate of blank wells containing diluents only. The concentration obtained from standard graph was multiplied by the dilution factorof 2. A total of 23 FTD patient samples were analyzed along with 10 cognitively unimpaired healthy age-matched control samples.

### IV.8.2. Progranulin as a biomarker

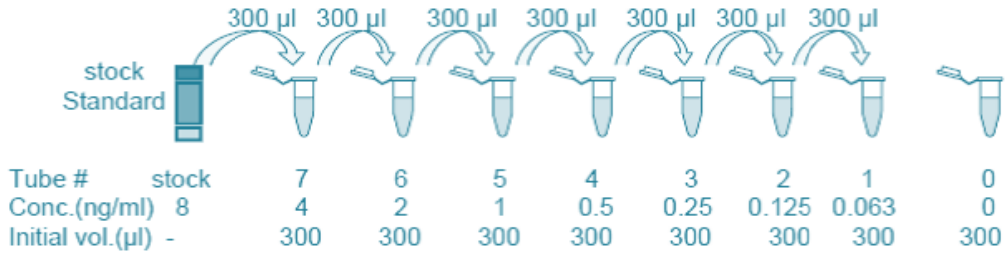
#### *IV.8.2.1. Enzyme linked Immunosorbent assay (ELISA)*

Quantification of plasma progranulin was carried out by means of a solid-phase sandwich ELISA kit according to the manufacturer's instructions. The kit is for the quantitative determination of progranulin in human serum, plasma or cell culture supernatants.

*IV.8.2.1.1 Reconstitution of standard*

Progranulin standard provided was the recombinant human progranulin expressed by HEK 293 cells. Lyophilized standard vial was reconstituted with 1 mL of distilled water to make a stock concentration of 8 ng/mL and mixed well. A serial dilution was performed with 0.3 mL standard diluent to get concentrations of 4, 2, 1, 0.5, 0.25, 0.125, 0.063 ng/mL and blank with diluent only (Figure 11).

**Figure 11: Serial dilutions for human progranulin standards**




*IV.8.2.1.2. Sample preparation*

Plasma samples were diluted into 1:200 with the standard diluent provided (5 µL sample plus 995 µL diluent; dilution factor=200) and mixed well.

*IV.8.2.1.3. Assay procedure*

All samples and kit components were allowed to equilibrate to room temperature (20-25$^0$C). One hundred microlitres of reconstituted standards, the reconstituted quality control (QC) sample and diluted plasma samples were added into the wells coated with polyclonal antibody against human progranulin and incubated at 37$^0$C for 1 h. After incubation, the solution was decanted and each well was washed 3 times with 300 µL of diluted wash solution. Thereafter, 100 µL of secondary antibody (biotinylated polyclonal antibody against human progranulin) was added to each well and incubated at 37$^0$C for 1 h. After incubation the solution was decanted and washed 3 times with 300 µL of diluted wash solution to each well. Then added 100 µL detector (HRP conjugated streptavidin) to each well and incubated at 37$^0$C for 1 h. The solution was decanted and washed 5 times with 300 µL of diluted wash solution to each well. After washing, 100 µL of the substrate solution (chromogenic reagents) was added to each well and incubated at room temperature for 10 min, protected from light till the solution was turned blue. Using the multi-channel pipette, 100 µL stop solution (1M $H_3PO_4$) was added to each well. The solution in the wells changed from blue to yellow. The absorbance was then read at 450 nm. The absorbance of standards and samples were obtained by subtracting the absorbance of the blank from the readings obtained for each. A standard curve was constructed by plotting the known concentrations of standard (on Y axis) versus the absorbance of standard (on X axis). The progranulin concentrations of plasma samples were calculated by interpolation of the regression curve formula as obtained in the graph in a form of a quadratic equation. The values obtained were multiplied by the

dilution factor (200) to get the concentrations of the undiluted samples. A total of 60 FTD patient samples were run along with 36 age-matched control samples.

#### *IV.8.2.2. Progranulin dot blot assay*

To confirm the results from ELISA, dot blot assay was performed with plasma samples. Briefly, a strip of nitrocellulose membrane was spotted with 4 µL of different concentrations of serum and incubated the membrane for 10 min at $37^0$C. After drying the blots, the membrane was blocked with 5% skim milk in TBST for 1 h at room temperature. After incubation, blocking buffer was poured off and the membrane was incubated with the *PGRN* primary antibody (1:125) overnight at $4^0$C. After incubation, the membrane was washed 3 times (5 min each) in TBST on a rocker. In the next step, the membrane was incubated with anti-rabbit HRP-conjugated secondary antibody (1:2000) for 1 h at room temperature in TBST. After incubation, the membrane was washed 3 times (5 min each) in TBST on a rocker. The membranes was developed using Enhanced Chemiluminescence (ECL) reagent. Briefly, the membrane was incubated with ECL reagent (200µL) for 1 min, then covered with a wrap and exposed to X-ray film in the dark room.

### IV.8.3. TDP-43 as a biomarker: Enzyme linked immunosorbent assay (ELISA)

The quantitative measurement of TDP-43 in human plasma samples were performed using the TDP-43 sandwich ELISA kit. The detection range or standard curve concentrations used for the ELISA were 20, 10, 5, 2.5, 1.25, 0.625 and 0.312 ng/mL.

Plasma samples were diluted into 1:10 with the standard diluent provided (dilution factor=10) and mixed well.

***IV.8.3.1. Assay procedure***

The microtiter plate provided was pre-coated with an antibody specific to TDP-43. Standards, blank and plasma samples were prepared as per the recommended dilutions and 100 µL of each was added to the appropriate microtiter plate wells and incubated for 2 h at $37^0$C. After incubation, the wells were aspirated and added 100 µL prepared Detection Reagent A which is biotin-conjugated antibody specific to TDP-43 and incubated for 1 h at $37^0$C. The wells were aspirated and washed 3 times with 350 µL diluted wash solution. Next, 100 µL prepared Detection Reagent B which is the avidin conjugated to HRP was added to each microplate well and incubated for 30 min at $37^0$C. The solution was decanted and wells were washed 5 times with diluted wash solution. After that, 90 µL TMB substrate solution was added to each well and incubated for 15-25 min at $37^0$C during which only those wells that contain TDP-43, biotin-conjugated antibody and enzyme-conjugated avidin were found to exhibit a change in color. Finally, the enzyme-substrate reaction was terminated by the addition of 50 µL stop solution (sulphuric acid) and the color change was measured spectrophotometrically at a wavelength of 450 nm. Net absorbance was calculated by deducting the mean value obtained for a duplicate of blank wells containing the diluent only. The recombinant TDP-43 protein supplied along with the kit was used to create standard curve. The concentration of TDP-43 in the samples was then determined by comparing the optical density of the samples to the standard curve. The values obtained were multiplied by the dilution factor (10) to

get the concentrations of the undiluted samples. A total of 29 FTD samples were run along with 13 age-matched control samples.

### IV.8.4. Thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation in serum was evaluated by the spectrophotometric method based on the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA) (Buege & Aust, 1978). The MDA-TBA adduct formed under high temperature (90-100$^0$C) and acidic condition was measured spectrophotometrically at an excitation wavelength of 532 nm and an emission wavelength of 550 nm.

#### *IV.8.4.1. Assay procedure*

In brief, 1 mL serum and 2 mL TBA reagent (26 mM/L TBA, 0.92 mol/L trichloroacetic acid in 0.25 mol/L HCl) were introduced into 10 mL glass tubes and heated in a boiling water bath for 15 min. After cooling, the flocculent precipitate was removed by centrifugation at 1000 x g for 10 min. The absorbance of the sample was determined at 532 nm against a blank. The breakdown product of 1,1,3,3-tetramethoxypropane was used as a standard. The calibration curve was prepared with MDA standards of 1-5 nM/mL concentrations.

## IV.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism software 5.01. The results showing continuous variables are presented as mean±SD and categorical variables as number of subjects (%). For continuous variables, the difference between two groups

was assessed by using Student's t test after testing for normal distribution. All the p-values were two sided and the minimum significance level was set at p□0.05.

Genotypic and allelic frequencies were calculated and checked for deviation from Hardy-Weinberg equilibrium (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). The frequencies of allele distribution in the study groups were compared using the Fisher's exact test (two-tailed) to analyze contingency tables. The difference in genotype frequencies between patients and controls were calculated using Pearson's $\chi^2$ test. For ELISA, comparison of protein levels in patients and controls was carried out using paired sample t test and multiple comparisons were done by one way ANOVA.

# V. RESULTS

## V.1. Patient characterization

The FTD group comprised 71 males and 45 females with mean age of 63±10 years and mean age of onset as 61±9 years. The demographics and clinical characteristics of patients and age-matched controls included for the study are summarized in Table 6 and 7. Among the 116 FTD patients, 94 (81%) had the clinical presentation of bvFTD, 12 (10%) showed PNFA and 7 (6%) had the diagnosis of SD as per the consensus criteria (Appendix I). Three patients (3%) showed concomitant symptoms of ALS. A positive family history was noticed in 15% FTD cases (17/116), in such a way that at least one first-degree relative was suffering from dementia (A summary of family data is shown in Appendix IV).

**Table 6: Demographics and clinical characteristics of FTD patients**

| Diagnosis | Variables | | | | | | |
|---|---|---|---|---|---|---|---|
| | N | Age (years, mean±SD[a]) | Age at onset (years, mean±SD) | Male, N (%) | Education (years) | MMSE* | Family history, N (%) |
| FTD | 116 | 62.9±10 | 61±9 | 71 (61.2) | 12 | 19.7±7 | 17 (15) |
| bvFTD | 94 | 62±10 | 59±10 | 56 (60) | 11 | 18±7 | 13 (13.8) |
| PNFA | 12 | 63±9 | 61±8 | 8 (66.7) | 14 | 25 | 1 (8) |
| SD[b] | 7 | 65±4 | 63±4 | 5 (71) | 2 | 20.6±7 | 2 (28.5) |
| FTD-ALS | 3 | 61±10 | 58±9 | 2 (66.6) | 12 | NA | 1 (33) |

*N: No of individuals, SD[a]: Standard deviation , FTD: Frontotemporal dementia, bvFTD: Behavioral variant of frontotemporal dementia, PNFA: Progressive non-fluent aphasia, SD[b]: Semantic dementia, ALS: Amyotrophic lateral sclerosis, MMSE: Mini mental state examination, NA: Not available, *Difference on total is due to missing of testing due to disease severity.*

**Table 7: Demographics of controls and other dementia subjects**

| Diagnosis | Variables | | | | | | |
|---|---|---|---|---|---|---|---|
| | N | Age (years, mean±SD) | Age at onset (years, mean±SD) | Male, N (%) | Education (years) | MMSE | Family history, N (%) |
| Controls | 130 | 61±9 | NA | 64 (49%) | 11±3 | 29±1 | Nil |
| MCI | 78 | 66±9 | NA | 56 (72%) | 10±2 | 20±5 | NA |
| AD | 132 | 70±9 | NA | 76 (58%) | 8±3 | 18±8 | 6 (4%) |
| VD | 36 | 67±7 | NA | 30 (83%) | 10±2 | 18±7 | NA |
| Other dementias | 37 | 73±9 | NA | 26 (70%) | 9±4 | 20±7 | NA |

*AD: Alzheimer's disease, MCI: Mild cognitive impairment, FTD: Frontotemporal dementia, VD: Vascular dementia, Other dementias: Comprised those diagnosed as Progressive supranuclear palsy, Corticobasal syndrome, Dementia with Lewy bodies and mixed dementia cases, N: Number of individuals, SD: Standard deviation, MMSE: Mini mental state examination, NA: Not available.*

## V.2. Microtubule-associated protein tau: Genetic and biochemical analyses

### V.2.1. *MAPT* mutation analysis

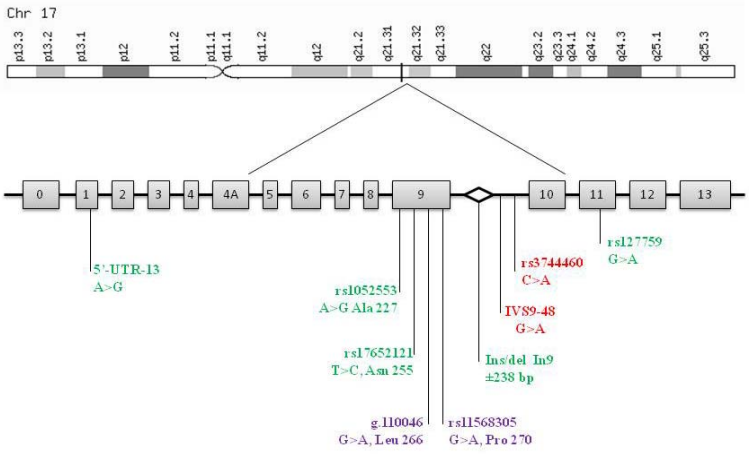
Screening for mutations in *MAPT* was carried out through direct DNA sequencing. *MAPT* exons 1, 9, 10, 11, 12 and 13 along with the respective exon-intron boundaries were PCR amplified separately using the respective primers and the annealing temperatures were optimized for each primer pair. Sequencing analysis was performed in all FTD (n=116) patients. No pathogenic *MAPT* mutations were detected in either familial or sporadic FTD cases.

### V.2.2. *MAPT* gene polymorphisms

The sequencing analysis revealed a list of SNPs in the certain coding exons and flanking introns. A total of eight sequence alterations were identified and among

them, analysis of four polymorphisms confirmed consistent linkage disequilibrium with the 238 bp deletion polymorphism (Baker et al., 1999). They include an A>G transition at 13 bp upstream of exon 1 (5'-UTR-13), an A>G transition in exon 9 (Ala227), a T>C transition in exon 9 (Asn255), and a G>A transition at 34 bp at 3' end of exon 11 (3' Exon 11+34) that are previously reported SNPs shown to be inherited as part of *MAPT* H2 haplotype. The other variants were two silent mutations in exon 9, two G>A transitions (Leu266, Pro270) inherited as part of *MAPT* H1 haplotype. IVS9-47 was one intronic variation located in intron 9 shown to be inherited with the H1 haplotype. A novel polymorphism was identified in intron 9 (IVS9-48) inherited with the H1 haplotype. All of these sequence variants are regarded as non-disease related polymorphisms since they were also prevalent in healthy controls. These non-pathogenic polymorphisms are diagrammatically represented in Figure 12 and their frequency in FTD patients are described in Table 8.

**Figure 12: Schematic representation of *MAPT* genomic region with exons and the sequence variants identified in this study**




*Intronic variants are shown in red, SNPs inherited as part of H2 haplotype in green and exonic variants in purple. All variants except IVS9-48 are previously reported SNPs.*

**Table 8: Frequency of non-pathogenic *MAPT* polymorphisms in FTD**

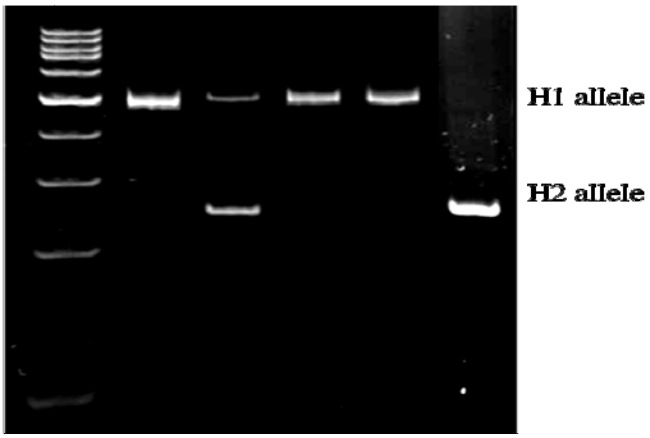
| Exon/Intron | Base | rs ID | Position | Frequency in FTD | References |
|---|---|---|---|---|---|
| Exon 1 | A>G | - | 5' UTR-13 | 12.5% (16) | (Rizzu et al., 1999) |
| Exon 9 | A>G | Rs1052553 | Ala227 | 12.5% (16) | (Poorkaj et al., 2001) |
| Exon 9 | T>C | Rs17652121 | Asn255 | 12.5% (16) | (Poorkaj et al., 2001) |
| Exon 9 | G>A | Rs11568305 | Pro270 | 2% (3) | (Poorkaj et al., 2001) |
| Exon 9 | G>A | g.110046 | Leu266 | 0.8% (1) | (Guerreiro et al., 2010) |
| Intron 9 | C>A | Rs3744460 | IVS9-47 | 1.5% (2) | (Sobrido et al., 2003) |
| Intron 9 | G>A | Novel | IVS9-48 | 0.8% (1) | (Aswathy et al., 2014) |
| Exon 11 | G>A | Rs127759 | 3' Exon 11+34 | 12.5% (16) | (Rizzu et al., 1999) |

*UTR: Untranslated region, IVS: Intron. All the SNPs detected in this study were previously reported except the intronic variation, IVS9-48.*

### V.2.3. *MAPT* haplotype analysis

The PCR amplification of *MAPT* genomic region with the 238 bp deletion polymorphism resulted in the generation of two products. The H1 haplotype produced a 484 bp product and H2 haplotype produced a 246 bp product (Figure 13). The presence of H1H2 genotype produced both PCR bands at 484 and 246 bp.

**Figure 13: *MAPT* haplotypes analyzed on 5% PAGE**




*The gel shows PCR amplified products of different MAPT genotypes. Lane 1: 100 bp DNA ladder, Lane 2: H1H1 genotype at 484 bp, Lane 3: H1H2 genotype and Lane 6: H2H2 genotype at 246 bp.*

### *V.2.3.1. MAPT genotype and haplotype distribution among FTD and other dementias vs. controls*

The *MAPT* H1 and H2 genotype frequencies were determined in 116 FTD patients, 132 AD, 36 VD, 37 other dementia groups (comprising PSP, CBS, Lewy body dementia and mixed dementia patients), and 78 MCI subjects and compared them with 130 controls. The results are summarized in Table 9. The genotype frequencies in the entire study group did not vary significantly from Hardy-Weinberg equilibrium.

The frequency of H1H1 genotype in the whole FTD group was 85% compared to 91.5% in controls, 94.7% in AD, 94% in VD, 96% in MCI, and 86.4% in other dementia groups. However, the chi square ($\chi^2$) analysis revealed no statistically significant differences in the distribution of *MAPT* genotype frequencies between FTD (except SD, p value=0.01) or other dementia groups verses controls (Table 9). In SD, a significant association in the genotype frequency was observed with an overrepresentation of H2H2 genotype when compared to control group (p=0.01).

**Table 9: *MAPT* genotype frequency distribution in FTD and controls**

| **Sample** | | **N** | ***MAPT* genotypes** | | | **$\chi^2$** | **p** |
|---|---|---|---|---|---|---|---|
| | | | **H1H1 N (%)** | **H1H2 N (%)** | **H2H2 N (%)** | | |
| Controls | | 130 | 119 (91.5) | 10 (7.69) | 1(0.76) | NA | NA |
| FTD | Total | 116 | 99 (85.3) | 16 (13.8) | 1(0.86) | 2.43 | 0.29 |
| | bvFTD | 94 | 79 (84) | 15 (15.9) | 0 | 4.4 | 0.11 |
| | PNFA | 12 | 11 (91.66) | 1 (8.3) | 0 | 0.098 | 0.95 |
| | SD | 7 | 6 (85.7) | 0 | 1(14.2) | 8.876 | 0.01* |
| | FTD-ALS | 3 | 3 (100) | 0 | 0 | 0.27 | 0.87 |
| MCI | | 78 | 75 (96.1) | 3 (3.8) | 0 | 1.86 | 0.39 |
| Other dementia patients | AD | 132 | 125 (94.7) | 7 (5.3) | 0 | 1.66 | 0.43 |
| | VD | 36 | 34 (94.4) | 1 (2.7) | 1 (2.7) | 1.99 | 0.36 |
| | Others | 37 | 32 (86.4) | 5 (13.5) | 0 | 1.45 | 0.48 |

*No significant association was found between genotype frequencies of any disease group and controls except in SD in which H2H2 genotype was overrepresented. N: Number of individuals, p: p-value by Pearson's $\chi^2$ test compared to controls, OR: Odds ratio, CI: Confidence interval, AD: Alzheimer's disease, MCI: Mild cognitive impairment, VD: Vascular dementia, Others: Progressive supranuclear palsy, Corticobasal syndrome, Dementia with Lewy bodies and mixed dementia cases, NA: Not applicable. * indicates statistical significance*

The *MAPT* haplotype frequencies were compared using the Fisher's exact test and found that there were no statistically significant differences in either H1 or H2 haplotype distribution between FTD or other dementia groups and controls (Table 10).

**Table 10: *MAPT* haplotype frequency distribution in FTD and controls**

| Sample | | N | *MAPT* haplotypes | | OR | 95% CI | p |
|---|---|---|---|---|---|---|---|
| | | | H1 N (%) | H2 N (%) | | | |
| Controls | | 260 | 248 (95.3) | 12(4.6) | NA | NA | NA |
| FTD | Total | 232 | 214 (92.2) | 18(7.8) | 1.74 | 0.82-3.69 | 0.19 |
| | bvFTD | 188 | 173 (92) | 15(7.9) | 0.55 | 0.255-1.22 | 0.16 |
| | PNFA | 24 | 23 (95.8) | 1 (4.2) | 1.11 | 0.138-8.95 | 1 |
| | SD | 14 | 12 (85.7) | 2(14.2) | 0.29 | 0.058-1.44 | 0.16 |
| | FTD-ALS | 6 | 6 (100) | 0 | 0.65 | 0.034-12.28 | 1 |
| MCI | | 156 | 153 (98.1) | 3 (1.9) | 2.47 | 0.658-8.888 | 0.18 |
| Other dementia patients | AD | 264 | 257 (97.3) | 7 (2.7) | 1.78 | 0.688-4.58 | 0.25 |
| | VD | 72 | 69 (95.8) | 3 (4.2) | 1.11 | 0.305-4.056 | 1 |
| | Others | 74 | 69 (93.2) | 5 (6.7) | 0.67 | 0.227-1.961 | 0.55 |

*No significant association was found between haplotype frequencies of any disease group and controls. N: Number of chromosomes, p: p-value by Fisher's exact test compared to controls, AD: Alzheimer's disease, MCI: Mild cognitive impairment, VD: Vascular dementia, Others: Progressive supranuclear palsy, Corticobasal syndrome, Dementia with Lewy bodies and mixed dementia, OR: Odds ratio, CI: Confidence interval, NA: Not Applicable*

### *V.2.3.2. MAPT genotype and haplotype distribution in FTD vs. other dementias*

When *MAPT* haplotype or genotype frequencies were compared between FTD and other dementia groups, a statistically significant difference was observed when FTD was compared with AD and MCI (Table 11 and 12). There was a significant overrepresentation of H1H2 genotype (p=0.03, $\chi^2$=6.535) and H2 haplotype (p=0.01, 95% CI=0.1327-0.79) in FTD when compared with AD. Similarly, the H2 allelic distribution was significantly higher in FTD when compared with MCI group (p=0.01, 95% CI=0.06746-0.8056). There was no statistical difference in genotype or haplotype frequency between FTD and the other patient groups such as VD or other dementias (Table 11 and 12).

**Table 11: *MAPT* genotype frequency distribution in FTD versus other dementias**

| **Sample** | | **N** | ***MAPT* genotypes** | | | **$\chi^2$** | **p** |
|---|---|---|---|---|---|---|---|
| | | | **H1H1 N (%)** | **H1H2 N (%)** | **H2H2 N (%)** | | |
| FTD | Total | 116 | 99 (85.3) | 16 (13.8) | 1(0.86) | NA | NA |
| MCI | | 78 | 75 (96.1) | 3 (3.8) | 0 | 5.992 | 0.05 |
| Other dementia patients | AD | 132 | 125 (94.7) | 7 (5.3) | 0 | 6.535 | 0.03* |
| | VD | 36 | 34 (94.4) | 1 (2.7) | 1 (2.7) | 4.007 | 0.13 |
| | Others | 37 | 32 (86.4) | 5 (13.5) | 0 | 0.3248 | 0.8 |

*A significant overrepresentation of H1H2 genotype was found in FTD when compared with AD. N: Number of individuals, MCI: Mild cognitive impairment, PSP: Progressive supranuclear palsy, CBS: Corticobasal syndrome, AD: Alzheimer's disease, VD: Vascular dementia, Others: Progressive supranuclear palsy, Corticobasal syndrome, Dementia with Lewy bodies and mixed dementia, NA: Not applicable, * indicates a statistical difference, p: p value by $\chi^2$ test.*

**Table 12: *MAPT* haplotype frequency distribution in FTD versus other dementias**

| Sample | | N | *MAPT* haplotypes | | OR | 95% CI | p |
|---|---|---|---|---|---|---|---|
| | | | H1 N (%) | H2 N (%) | | | |
| FTD | Total | 232 | 214 (92.2) | 18(7.8) | NA | NA | NA |
| MCI | | 156 | 153 (98.1) | 3 (1.9) | 0.2331 | 0.06746-0.8056 | 0.01* |
| Other dementia patients | AD | 264 | 257 (97.3) | 7 (2.7) | 0.3238 | 0.1327-0.7900 | 0.01* |
| | VD | 72 | 69 (95.8) | 3 (4.2) | 0.5169 | 0.1478-1.808 | 0.42 |
| | Others | 74 | 69 (93.2) | 5 (6.7) | 1.161 | 0.415-3.243 | 1 |

*A significant overrepresentation of H2 allele frequency was found in FTD when compared with AD and MCI. N: Number of chromosomes, MCI: Mild cognitive impairment, AD: Alzheimer's disease, VD: Vascular dementia, Others: Progressive supranuclear palsy, Corticobasal syndrome, Dementia with Lewy bodies and mixed dementia, NA: Not applicable, * indicates a statistical difference, p: p value by Fisher's exact test.*

### *V.2.3.3. The effect of MAPT genotypes on age at onset in FTD*

In the FTD patient group, the possession of either H1H2 or H2H2 genotype was not associated with an earlier age at onset of the disease when compared with the H1H1 genotype (58±7 *vs.* 62 *vs.* 60±9) (Table 13).

**Table 13: Association of *MAPT* genotypes with age at onset in FTD**

| Clinical presentation (N) | | *MAPT* genotype Age at onset (mean±SD[a]) (N) | | |
|---|---|---|---|---|
| | | H1H1 | H1H2 | H2H2 |
| FTD | Total (116) | 60±9 (99) | 58±7 (16) | 62 (1) |
| | bvFTD (94) | 60±9 (79) | 56±7 (15) | 0 |
| | PNFA (12) | 61±8 (11) | 68 (1) | 0 |
| | SD[b] (7) | 63±5 (6) | 0 | 62 (1) |
| | FTD-ALS (3) | 58±9 (3) | 0 | 0 |

*N: Number of individuals, SD[a]: Standard deviation, bvFTD: Behavioral variant FTD, PNFA: Progressive non-fluent aphasia, SD[b]: Semantic dementia, FTD-ALS: FTD associated with Amyotrophic lateral sclerosis.*

### *V.2.3.4. The distribution of MAPT genotypes in familial and sporadic FTD cases*

To find whether the H2 allele is clustered with familial FTD, the occurrence of H2 allele and genotype was calculated independently in both familial and sporadic FTD cases. However the H2 allele or H1H2/H2H2 genotypes did not show a significant association with the familial occurrence of the disease (Table 14 and 15).

**Table 14: *MAPT* genotype frequency distribution in familial and sporadic FTD**

| **Samples** | **N** | ***MAPT* genotypes** | | | **$\chi^2$** | **P** |
|---|---|---|---|---|---|---|
| | | **H1H1 N** | **H1H2 N** | **H2H2 N** | | |
| Controls | 130 | 119 | 10 | 1 | NA | NA |
| Familial FTD | 16 | 14 | 2 | 0 | 0.55 | 0.76 |
| Sporadic FTD | 100 | 85 | 14 | 1 | 2.46 | 0.29 |

*No association of MAPT genotypes with either familial or sporadic FTD cases. N: Number of individuals, p: p-value by Pearson's $\chi^2$ test, OR: Odds ratio, NA: Not applicable*

**Table 15: *MAPT* haplotype frequency distribution in familial and sporadic FTD**

| **Samples** | **N** | ***MAPT* haplotypes** | | **OR** | **95% CI** | **p** |
|---|---|---|---|---|---|---|
| | | **H1 N** | **H2 N** | | | |
| Controls | 260 | 248 | 12 | NA | NA | NA |
| Familial FTD | 32 | 30 | 2 | 1.38 | 0.29-6.45 | 0.66 |
| Sporadic FTD | 200 | 184 | 16 | 1.8 | 0.83-3.89 | 0.17 |

*No association of MAPT haplotypes with either familial or sporadic FTD cases. N: Number of chromosomes, p: p-value by Fisher's exact test, OR: Odds ratio, CI: Confidence interval, NA: Not applicable*

### V.2.4. Plasma tau (total) as a biomarker for FTD

In order to investigate the diagnostic value of tau for the identification of tau pathology in FTD, the study combined the evaluation of the plasma total tau and compared them with that in the age-matched controls. Plasma tau (total) levels of 23 patients (mean age at onset=60±11 years) and 10 healthy controls (mean age=65±5 years) were measured using the Human tau (total) sandwich ELISA kit. The demographics of the samples used for tau ELISA are listed in the Table 16. Each standards and plasma samples were run in duplicate and the absorbance of samples was measured at 450 nm. Standard curve was plotted with the absorbance of standards against standard concentration (Figure 14). The tau concentrations for unknown samples and controls were calculated from the standard curve and each value obtained was multiplied by the dilution factor for plasma. The range of plasma tau values varied from 101-397 pg/mL, whereas the control values varied from 147-306 pg/mL. Mean plasma tau (total) concentrations were 210±86 pg/mL in FTD samples and 217±55 pg/mL in controls. However, no statistical difference was observed between FTD and controls in the plasma tau (total) levels ($p=0.82$) (Figure 15). The patient group was then stratified according to age and the variations in mean tau concentrations in plasma were estimated. The age groups were, <50 years (212±42.2 pg/mL), <60 years (276±97.13 pg/mL), <70 years (182.25±50.54 pg/mL), <80 years (161±53.45 pg/mL) and <90 years (169±64.36 pg/mL), but did not find a trend in increasing plasma tau levels with age. However, the highest mean tau levels was detected in the age group <60 years. A gender difference in plasma tau levels

was observed with higher levels in females (254.8±48.5 pg/mL) compared to males (191±68 pg/mL).

**Table 16: Demographics of the samples analyzed for tau ELISA**

| Samples | FTD | Controls |
|---|---|---|
| Total number | 23 | 10 |
| Mean age (years±SD) | 64±12 | 65±5 |
| Male (%) | 69% | 40% |
| Plasma tau concentration (pg/mL) (mean±SD) | 217±86 (101-397) | 183±19 (147-198) |

**Figure 14: Standard curve for the tau (total) ELISA**

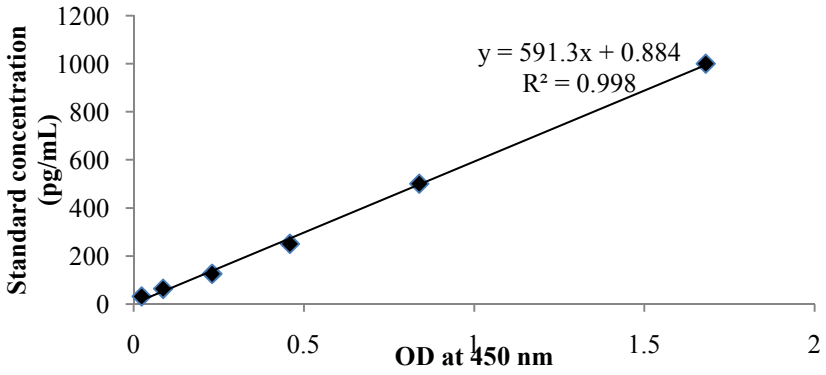
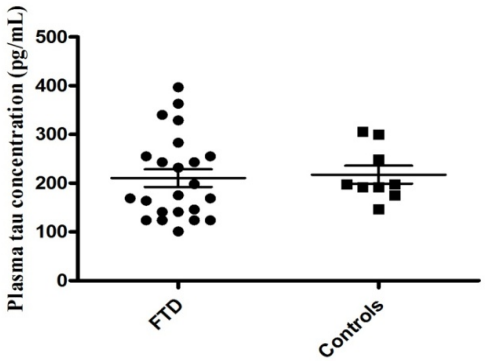



**Figure 15: The scatter plot diagram of the raw values of plasma tau (total) levels in FTD (n=23) and age-matched controls (n=10)**




*Each data point represents an individual. For each group, the median plasma tau level is indicated with a wide horizontal line.*

#### *V.2.4.1. Correlation of MAPT genotypes with plasma tau levels*

To determine whether the plasma tau levels are affected by *MAPT* haplotypes, we have correlated the tau protein levels with the *MAPT* genotypes. The mean values for plasma tau levels in H1H1 carriers were 214±80 and in H1H2 carriers were 203±67 pg/mL. The statistical analysis between either the genotypes or sample groups did not reveal significant difference in the expression of tau protein in plasma (Table 17).

**Table 17: Association of *MAPT* genotypes with plasma tau levels**

| **Samples** | **H1H1** | **H1H2** | **p value** |
|---|---|---|---|
| **Controls, mean±SD (pg/mL)** | 214±58 (n=8) | 249 (n=1) | NA |
| **FTD, mean±SD (pg/mL)** | 214±90 (n=19) | 192±71 (n=4) | 0.64 |
| **p value** | 0.54 | 0.48 | |

*The Student's t test did not reveal a statistical difference in plasma tau protein levels between MAPT genotypes or between sample groups.*

## V.3. Progranulin: Genetic and biochemical analysis

### V.3.1. *PGRN* mutation analysis

The study performed a systematic mutation analysis of *PGRN* through direct sequencing of exonic and flanking intronic regions. All enrolled FTD patients (n=116) were screened for the presence of mutations in whole *PGRN* (exons 0 to 12 along with flanking intronic regions). Extensive *PGRN* mutation analysis in familial as well as sporadic FTD cases has identified one novel heterozygous mutation in exon 12, in 1 out of 116 patients (0.8%). The mutation was confirmed through

sequencing the exon in both directions. The mutation was a C>T transition that occurred in the exon 12 of *PGRN* at position +94$^{th}$ relative to the first coding nucleotide of exon 12 (Figure 16). The SNP position was on chr17:42429802[C/T]. Novelty checking was done with NCBI dbSNP database (Build 137) (http://www.ncbi.nlm.nih.gov/SNP/), 1000 Genomes Project (http://www.1000 genomes.org/), Exome Variant Server of NHLBI GO Exome Sequencing Project (ESP6500SI-V2) (http://evs.gs.washington.edu/EVS/) and found no reports in any database. The functional check was done with PROVEAN (http://provean.jcvi.org/human_protein_batch_submit.php). This mutation was found to introduce a premature termination codon at codon 503 coding for the amino acid glutamine (Figure 17) and would be predicted to terminate the protein prematurely, likely leading to the partial loss of functional progranulin protein. The protein change introduced can be designated as p.Gln.503X. Plasma levels of progranulin was analyzed through ELISA and found significantly reduced levels with 28 ng/mL.

**Figure 16: Electropherogram showing p.Gln.503X mutation in *PGRN* exon 12 (C>T)**

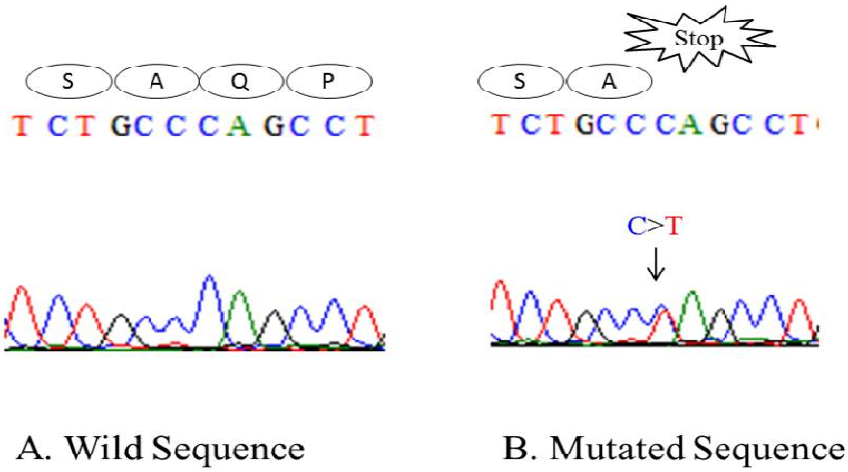


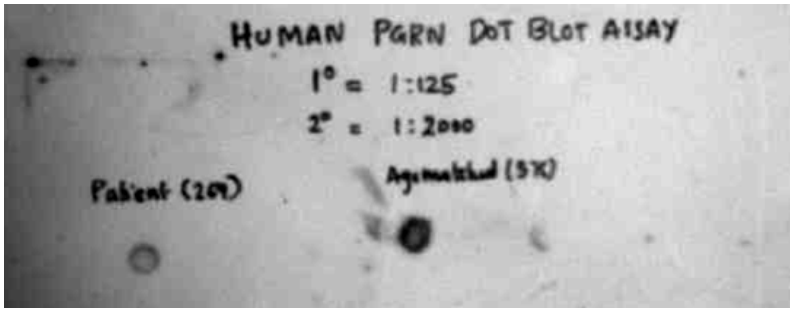
**Figure 17: Protein sequence of human progranulin with altered residue in mutation carrier**


*The protein contains 593 amino acids and the 503rd residue affected by the mutation is indicated in red (q=glutamine).*

### *V.3.1.1. Progranulin dot blot assay*

To confirm the protein analysis in ELISA, a dot blot analysis was done using the plasma sample from mutation carrier (28 ng/mL) and compared with an age-matched control with known plasma progranulin concentration (184 ng/mL). A significant difference was observed between the patient carrying the nonsense mutation and the control individual (Figure 18).

**Figure 18: Representative dot blot showing the haploinsufficiency in *PGRN* mutation carrier**



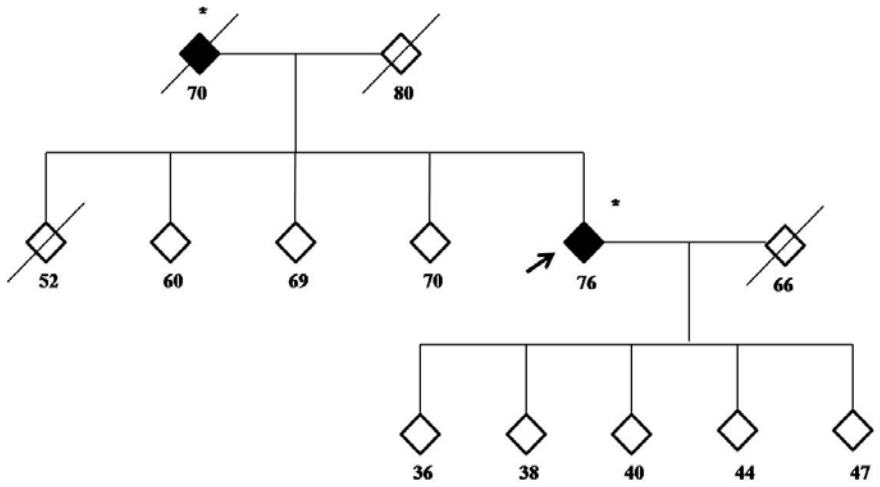

*Plasma progranulin dot blot assay was carried out in PGRN mutation carrier (plasma progranulin concentration through ELISA: 28 ng/mL) and compared with a control sample (plasma progranulin concentration through ELISA: 187 ng/mL) using a polyclonal antibody against human progranulin.*

### *V.3.1.2. Clinical characteristics of the patient carrying PGRN mutation*

A 76 year old woman with a positive familial history for cognitive impairment developed progressive and insidious symptoms of forgetfulness, change in behavior and executive dysfunction at 75 years of age characterized by apathy, social withdrawal, depressed mood and loss of interest in her surroundings, so that she had difficulty in performing daily life activities. Neuropsychological assessment showed MMSE, 17/30, revealed multiple cognitive impairments, especially in visual perceptual and executive control functions. Brain MRI features were consistent with FTD showing perisylvian and frontal lobe atrophy in the right hemisphere. A pedigree analysis was done and a positive family history was reported in the proband's father who developed the symptoms of cognitive impairment after the age of 70 (Figure 19). However samples were not available from other family members to test for segregation of this *PGRN* mutation. The ELISA results showed that

plasma progranulin level in this null mutation carrier to be 28 ng/mL which is reduced up to about one third with respect to non-mutation carriers and controls.

**Figure 19: Family pedigree of the proband carrying the *PGRN* p.Gln.503X mutation**

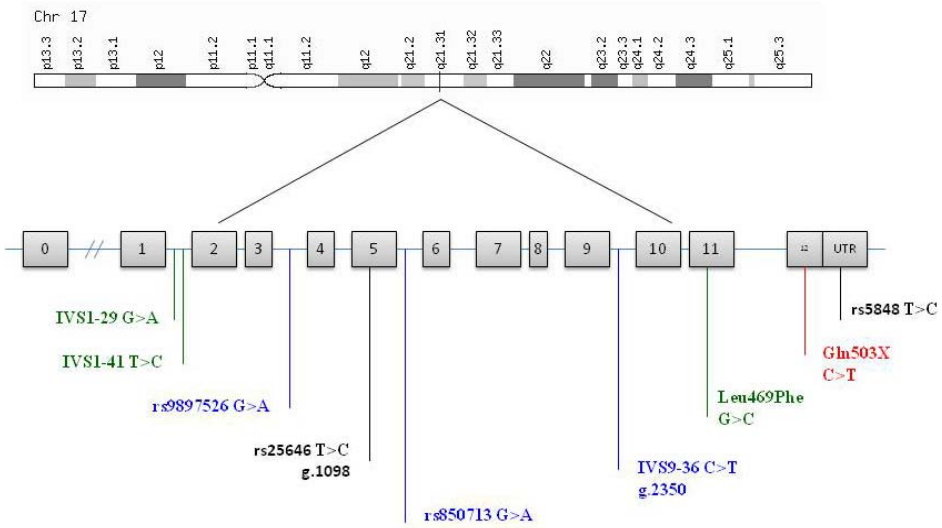



*The arrow indicates the proband. Filled symbols represent affected individuals and open symbols represent unaffected individuals. Age/age at death is given below each individual. A diagonal slash indicates that the person is deceased.*

### V.3.2. *PGRN* polymorphisms

The sequencing of *PGRN* in FTD samples identified eight sequence variants. Of these, three were novel non-synonymous changes occurring within the intron 1 and exon 11. The intron 1 variants include T>C at 41 bp upstream and G>A at 29 bp upstream of exon 1. In exon 11, a point mutation was found in the coding region predicting an amino acid substitution, leucine to phenyl alanine (Leu469Phe). The remaining variants are previously reported ones with non-pathogenic nature. These SNPs are diagrammatically represented in Figure 20 and its frequencies in FTD are listed in the Table 18.

**Figure 20: Diagrammatic representation of the sequence variants identified in *PGRN* through DNA sequencing**




*Known intronic variants are indicated in blue, known exonic variants in black, novel SNPs in green and novel mutation in red.*

**Table 18: Variants identified in *PGRN* and their frequency in FTD identified through this study**

| Intron/Exon | Chromosome position | Genomic variation | Protein change | rs ID | Frequency in FTD |
|---|---|---|---|---|---|
| IVS 1-41 | | T>C | Nil | Novel | 8% |
| IVS 1-29 | | G>A | Nil | Novel | 15% |
| IVS 3+21 | | G>A | Nil | rs9897526 | 42% |
| IVS 5+24 | | G>A | Nil | rs850713 | 50% |
| Exon 5 | g.1098 | T>C | Asp128 | rs25646 | 21% |
| IVS 9-36 | g.2350 | C>T | Nil | | 3% |
| Exon 11 | | G>C | Leu469Phe | Novel | 6% |
| Exon 12 | | C>T | Gln503X | Novel | 6% |
| 5'-UTR | | C>T | Nil | rs5848 | 47% |

*Two SNPs in intron 1 such as IVS1-41, IVS1-29 and one exonic variant in exon 11 are novel non-pathogenic polymorphisms identified through this study. Gln503X is the novel mutation identified. All other SNPs detected were previously reported and are non-pathogenic.*

### V.3.3. Progranulin as a biomarker

The study determined whether the levels of progranulin in plasma could be used to distinguish *PGRN* loss of function mutation carriers from non-*PGRN* mutation carriers in FTD and controls. The demographics of the samples analyzed are given in the Table 19. Each standards and plasma samples were run in duplicate and the absorbance of samples was measured at 450 nm. A standard curve was plotted with the absorbance of the standards against the concentration (Figure 21). The progranulin concentrations for unknown FTD samples and controls were calculated from the standard curve and each value obtained was multiplied by the plasma dilution factor. Plasma progranulin levels were measured in a total of 60 FTD samples (mean age±SD: 64±9 years) and 36 age-matched controls (mean age±SD: 63±7 years). The average progranulin concentration in FTD patient samples was 144±60 (range: 28-293 ng/mL) and in control samples was 150±38 (range: 116-265 ng/mL) (Figure 22). The average values of plasma progranulin levels did not show a statistically significant difference between FTD and controls (p=0.28, 95% CI= -11.34 to 37.48). However, progranulin level was significantly reduced in *PGRN* loss-of-function mutation carrier (28 ng/mL) compared to non-*PGRN* mutation carriers and controls. When the patients were stratified according to age, plasma progranulin levels showed a trend in increasing with age (age group <50 years=129±68.5 ng/mL, <60 years=148±77.8 ng/mL, <70 years=141±43.8 ng/ml, <80 years=167±85.45 ng/mL, <90 years=156±63.27 ng/mL) but did not reach significance.

**Table 19: Demographics of the samples analyzed for progranulin ELISA**

| Samples | FTD | Controls |
|---|---|---|
| Total number | 60 | 36 |
| Mean age (years±SD) | 64±9 | 63±7 |
| Male (%) | 66% | 42% |
| Plasma *PGRN* concentration (ng/mL) (mean±SD) | 144±60<br>(range: 28-293 ng/mL) | 150±38<br>(range: 116-265 ng/mL) |

**Figure 21: Standard curve for the progranulin ELISA**

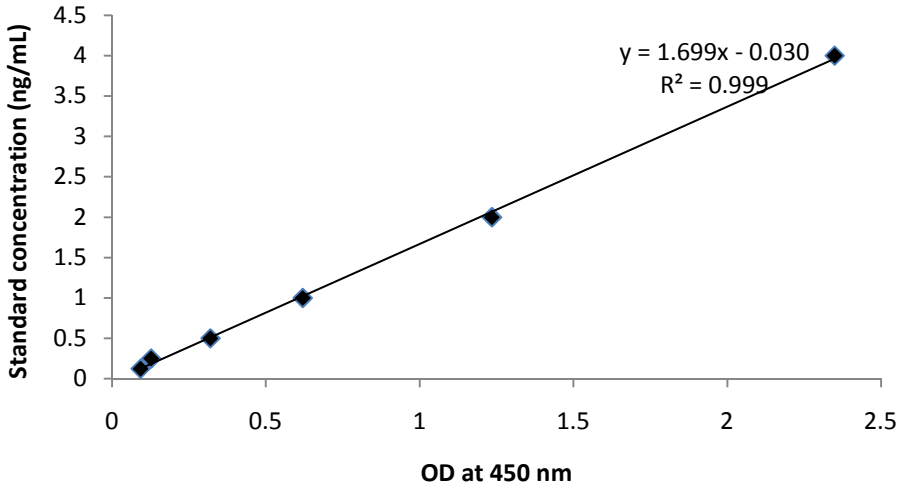
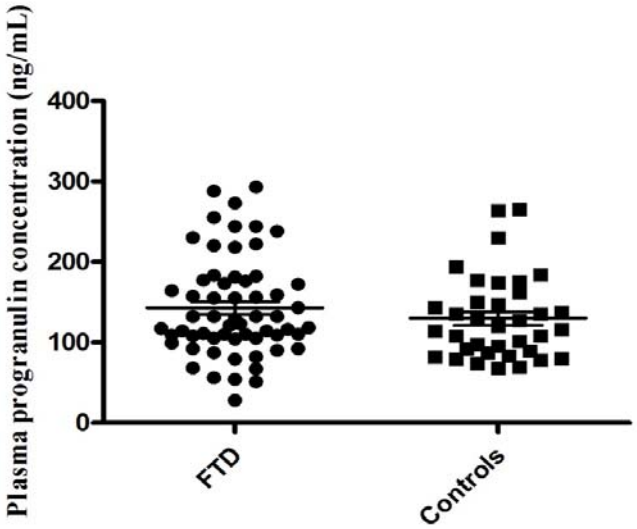



**Figure 22: The scatter plot diagram of the raw values of plasma progranulin concentration in FTD and controls**



*Each data point represents an individual. For each group, the median plasma progranulin level is indicated with a wide horizontal line. The average values of plasma progranulin levels did not show a statistically significant difference between FTD and controls (p=0.28).*

### V.3.4. *PGRN* rs5848 association analysis

To assess whether *PGRN* rs5848 polymorphism contributes to the genetic susceptibility to FTD, a case-control analysis was performed with 116 FTD patients and 130 age-matched healthy controls. The TT genotype or T allele did not show a higher frequency in FTD when compared with controls. The distributions of the genotypes and the minor allele frequency did not differ between FTD and controls (Table 20 and 21). Overall, the frequency of the T allele was 32% in FTD cases and among them 42% FTD cases were heterozygous (CT) and 11% were homozygous (TT).

**Table 20: rs5848 genotype frequency distribution in FTD and controls**

| Sample | | N | rs5848 genotype | | | $\chi^2$ | p |
|---|---|---|---|---|---|---|---|
| | | | CC N (%) | CT N (%) | TT N (%) | | |
| **Controls** | | 130 | 62 (47%) | 56 (43%) | 12 (10%) | NA | NA |
| **FTD** | **Total** | 116 | 54 (47%) | 49 (42%) | 13 (11%) | 0.2625 | 0.87 |
| | **bvFTD** | 94 | 44 (47%) | 38 (40%) | 12 (13%) | 0.7367 | 0.69 |
| | **PNFA** | 12 | 3 (25%) | 9 (75%) | 0 | 4.789 | 0.09 |
| | **SD** | 7 | 4 (57%) | 2 (29%) | 1 (14%) | 0.6321 | 0.72 |
| | **FTD-ALS** | 3 | 3 (100%) | 0 | 0 | 3.211 | 0.20 |

*The rs5848 genotype frequencies did not show a higher frequency in FTD when compared with controls. N: Number of individuals, p: p-value by Pearson's $\chi^2$ test compared to controls, OR: Odds ratio, CI: Confidence interval, NA: Not applicable*

**Table 21: rs5848 allele frequency distribution in FTD and controls**

| Sample | | N | rs5848 allele | | OR | 95% CI | p |
|---|---|---|---|---|---|---|---|
| | | | C N (%) | T N (%) | | | |
| **Controls** | | 260 | 179 (69%) | 81 (31%) | NA | NA | NA |
| **FTD** | **Total** | 232 | 157 (68%) | 75 (32%) | 0.9473 | 0.6476-1.386 | 0.84 |
| | **bvFTD** | 188 | 126 (67%) | 62 (33%) | 0.9196 | 0.6154-1.374 | 0.68 |
| | **PNFA** | 24 | 15 (63%) | 9 (37%) | 0.7542 | 0.3168-1.795 | 0.50 |
| | **SD** | 14 | 10 (71%) | 4 (29%) | 1.131 | 0.3445-3.715 | 1 |
| | **FTD-ALS** | 6 | 6 (100%) | 0 | 5.903 | 0.3284-106.1 | 0.18 |

*The rs5848 allele frequencies did not show a higher frequency in FTD when compared with controls. N: Number of chromosomes, p: p-value by Fisher's exact test compared to controls, NA: Not applicable*

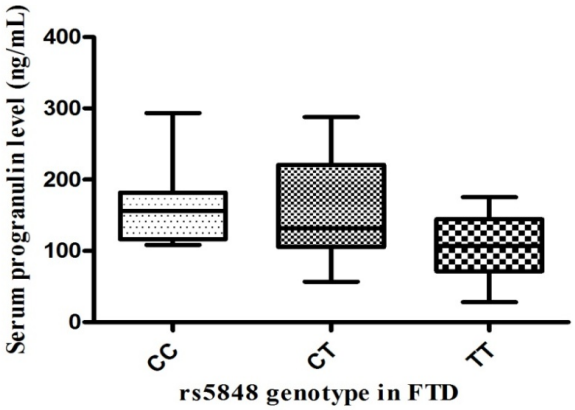
### *V.3.5. PGRN rs5848 polymorphism and plasma progranulin level*

To evaluate whether the rs5848 polymorphism could affect the level of progranulin expression in the study samples, we correlated the plasma progranulin levels with the rs5848 polymorphism. A total of 60 FTD patients with known plasma progranulin levels through ELISA were compared with 36 age-matched controls. The mean levels of progranulin in three different genotypes in FTD and controls are represented in Table 22. The influence of rs5848 polymorphism on serum progranulin in total FTD cohort is shown in Figure 23. TT genotype carriers had significantly lower serum progranulin levels (105±43 ng/mL), than the CT (154±65 ng/mL) and CC genotype carriers (171±77 ng/mL) (overall ANOVA, p=0.01). In age-matched controls, the progranulin levels in three genotypes did not show a statistical difference (Figure 24, overall ANOVA, p=0.8)

**Table 22: rs5848 genotypes and progranulin levels in FTD and controls**

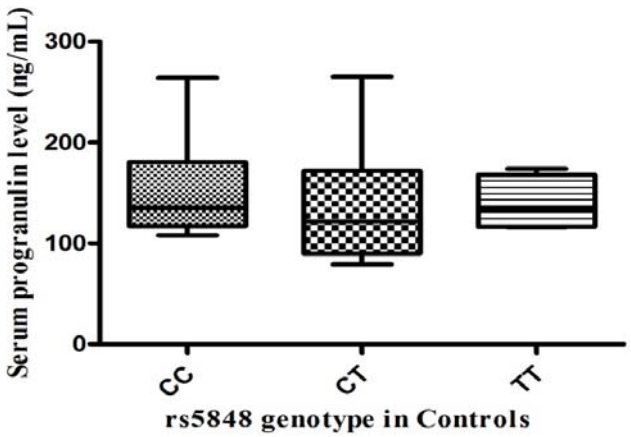
| Mean progranulin levels (ng/mL) | Samples | rs 5848 genotype | | |
|---|---|---|---|---|
| | | CC | CT | TT |
| | Controls (n=36) | 151±44 | 138±60 | 126±43 |
| | FTD (n=60) | 171±77 | 154±65 | 105±43 |

**Figure 23: Box plot diagram for plasma progranulin levels and rs5848 genotype in FTD**




*The progranulin levels in TT genotype carriers were significantly lower than the CC carriers (p=0.01).*

**Figure 24: Box plot diagram for plasma progranulin levels and rs5848 genotype in controls**




*There is no significant association of rs5848 polymorphisms and the plasma progranulin level in controls (p=0.8).*

## V.4. *APOE* genotyping

*APOE* allele and genotype frequencies were determined by the direct counting of alleles (Table 23 and 24). Differences in the distribution of genotype frequencies among groups were tested by Fisher's exact test. The frequency of *APOE* ε4 allele in FTD group was 16% compared with 18% in the control group. The *APOE* ε2 allele was possessed by 2% of FTD cases, while in controls it was significantly higher up to 8% (p=0.001). None of the FTD patients showed ε2ε2 genotype. The *APOE* ε3 allele showed a frequency of 82% in FTD when the corresponding frequency in controls was 74%. The allele or genotype distributions were not significantly different between FTD and controls.

**Table 23: *APOE* genotype frequency distribution in FTD and controls**

| **Samples** | **Age** | **Age at onset** | ***APOE* genotypes** | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | **ε3ε3** | **ε3ε4** | **ε4ε4** | **ε2ε2** | **ε2ε3** | **ε2ε4** |
| Controls (N=130) | 61±9 | NA | 77(59%) | 32(25%) | 6(5%) | 5(4%) | 7(5%) | 3(2%) |
| FTD (N=116) | 62.9±10 | 61±9 | 81(70%) | 28(24%) | 3(2.5%) | 0 | 1(0.8%) | 3(2.5%) |

*ε3ε3 was the most common genotype in FTD and controls. None of the FTD patients showed ε2ε2 genotype. None of the genotypes were significantly different between FTD and controls. N: Number of individuals, NA: Not applicable.*

**Table 24: *APOE* allele frequency distribution in FTD and controls**

| **Samples** | **Age** | **Age at onset** | ***APOE* alleles** | | |
|---|---|---|---|---|---|
| | | | **ε2** | **ε3** | **ε4** |
| Controls (N=130) | 61±9 | NA | 20(8%) | 193(74%) | 47(18%) |
| FTD (N=116) | 62.9±10 | 61±9 | 4(2%) | 191(82%) | 37(16%) |

*The most common allele present in FTD and controls was ε3 and the least common allele was ε2. ε3 and ε4 alleles showed no significant difference between FTD and controls whereas ε2 allele was overrepresented in the control group (0.001). NA: Not applicable.*

## V.4.1. Correlation of *APOE* genotypes with plasma progranulin levels

Correlation of *APOE* genotypes with plasma progranulin levels were carried out in those samples with known progranulin concentrations (FTD, n=60 and controls, n=36). The mean values of plasma progranulin in six different *APOE* genotypes in FTD and controls are represented in Table 25. The association of each genotype with progranulin expression was carried out using the Student's t test (Table 26). In FTD, the average progranulin level showed a statistically significant increase in ε3ε4 carriers compared to ε3ε3 carriers (p=0.02). Moreover, when comparison was done across groups, ε3ε4 carriers in FTD group showed a significant increase in progranulin expression compared with ε3ε4 carriers in control group (p=0.02). The other genotypes were low in frequency to get a statistical difference.

**Table 25: The mean values of plasma progranulin in six different *APOE* genotypes in FTD and controls**

| **Samples** | **ε3ε3** | **ε3ε4** | **ε4ε4** | **ε2ε2** | **ε2ε3** | **ε2ε4** |
|---|---|---|---|---|---|---|
| FTD, mean (ng/mL) | 136 (n=41) | 182 (n=15) | 106 (n=1) | 0 | 108 (n=1) | 273 (n=1) |
| Controls, mean (ng/mL) | 149 (n=15) | 123 (n=16) | 116 (n=1) | 85(n=2) | 87 (n=1) | 78 (n=1) |

*None of the FTD patients showed ε2ε2 genotype.*

**Table 26: Association of ε3ε3 and ε3ε4 genotype with progranulin expression in FTD and controls**

| Samples | ε3ε3 | ε3ε4 | p value |
|---|---|---|---|
| FTD, mean±SD (ng/mL) | 136±57 (n=41) | 183±92 (n=15) | 0.02* |
| Controls, mean±SD (ng/mL) | 149±64 (n=15) | 124±38 (n=16) | 0.18 |
| p value | 0.47 | 0.02* | |

*Based on Student's t test, ε3ε3 is not statistically significant with respect to the sample groups. ε3ε4 is statistically significant with respect to the sample group, with an average protein expression of 183 ng/mL in FTD and 124 ng/mL in controls. In FTD, ε3ε4 carriers showed a statistically significant difference in progranulin expression (p=0.02) with respect to ε3ε3. SD: Standard deviation, * shows statistical significance.*

## V.5. TDP-43 as a biomarker

The demographics of the samples analyzed for TDP-43 ELISA is shown in the Table 27. The standard curve plotted with recombinant TDP-43 protein is shown in Figure 25. A subset of FTD patients (n=29) were analyzed and compared with age-matched controls (n=13) (Figure 26). TDP-43 was detectable in FTD plasma samples and only feebly detectable in controls. Although the comparison did not reveal a statistically significant difference between the groups (p=0.14, 95% confidence interval= -0.6861 to 4.496), 14/29 patients had TDP-43 concentration between 1-18 ng/mL.

**Table 27: Demographics of samples analyzed for TDP-43 ELISA**

| Samples | FTD | Controls |
|---|---|---|
| Total number | 29 | 13 |
| Mean age (years±SD) | 62±7 | 64±9 |
| Male (%) | 58% | 25% |
| Plasma TDP-43 concentration (ng/mL) (mean±SD) | 3±4 | 0.9±0.9 |

**Figure 25: Standard curve for TDP-43 ELISA**

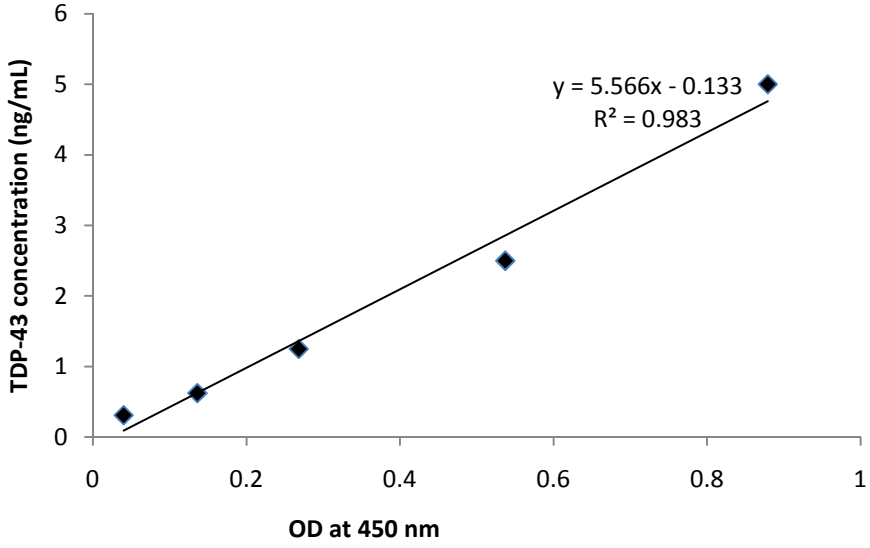
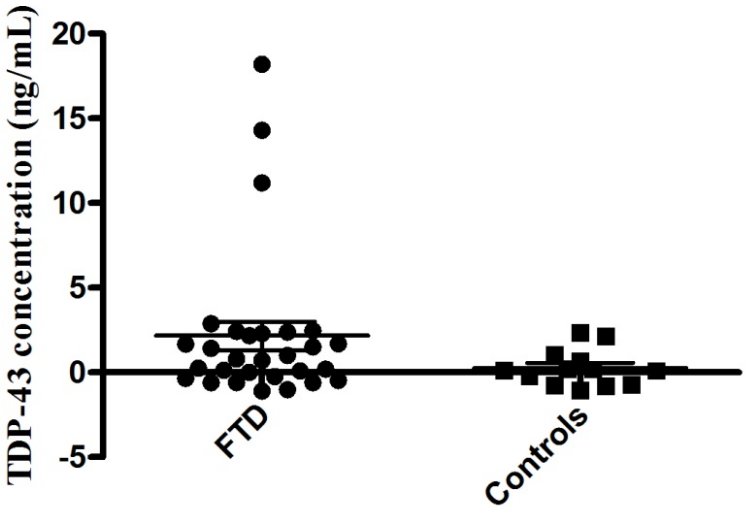



**Figure 26: Scatter plot diagram of the raw values for plasma levels of TDP-43 in FTD and controls**




*Plasma TDP-43 levels in FTD (n=29) and controls (n=13). Each data point represents an individual. For each group, the median plasma TDP-43 level is indicated with a wide horizontal line. There is no significant difference in plasma TDP-43 levels between FTD and controls.*

## V.6. Oxidative stress marker assay: Thiobarbituric acid reactive substances (TBARS) assay

MDA levels were determined by spectrophotometric assay at a wavelength of 532 nm in cryopreserved serum samples from a subset of FTD (n=21) and control (n=20) samples (Table 28). The standard curve plotted with known concentrations of MDA standards is represented in Figure 27.

**Table 28: Demographics of the samples used for TBARS assay**

| Samples | **FTD** | **Controls** |
|---|---|---|
| Total number | 21 | 20 |
| Mean age (years±SD) | 59±8 | 63±8 |
| Male (%) | 53% | 45% |
| Serum MDA concentration (nm/mL) (mean±SD) | 4.8±1.86 | 2.65±0.67 |

*SD: Standard deviation, MDA: Malondialdehyde*

**Figure 27: Standard curve for TBARS assay**

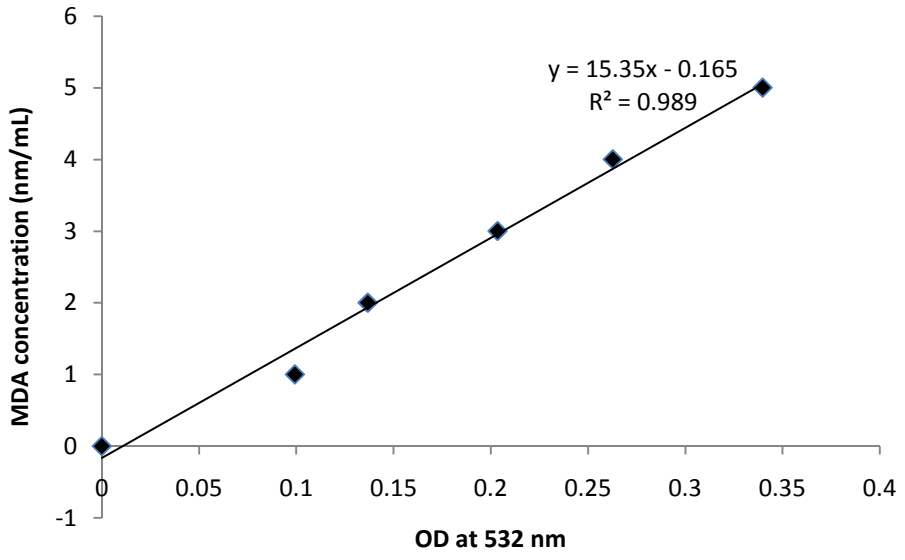
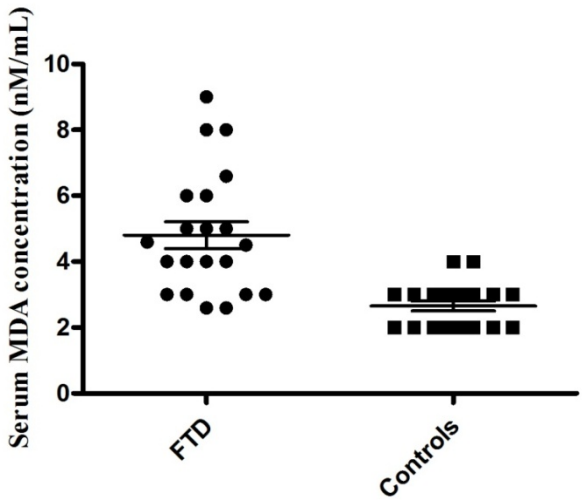


**Figure 28: Scatter plot diagram for TBARS assay plotted with raw values obtained for FTD and controls**




*Each data point represents an individual. For each group, the median serum MDA level is indicated with a wide horizontal line.*

Mean serum MDA levels were found to be increased in FTD patients compared with controls (Figure 28). Student's t test revealed a statistically significant difference in the MDA levels between FTD and controls (p=0.0001).

# VI. DISCUSSION

## VI. Discussion

FTD is a complex disorder with multiple etiological factors contributing to the pathogenesis. Apart from the clinical presentation of the most common dementing disorder, AD which is accompanied by severe memory loss, FTD is the clinical syndrome that is characterized by progressive deterioration in behavior, personality and/or language, with relative preservation of memory. The molecular basis of FTD is heterogeneous; which is the reason behind the clinical and neuropathological heterogeneity observed in FTD. Despite years of intense effort by many research groups worldwide, the genetic heterogeneity in familial cases of FTD is more or less delineated. The role of genetics in FTD has been reported in up to 50% of total cases through independent study groups with the identification of genetic mutations in *MAPT*, *PGRN* and *C9ORF72* (www.molgen.ua.ac.be). The remaining FTD cases are apparently sporadic in nature. In sporadic cases, combinations of genetic variations and environmental factors are likely to be responsible for the pathogenesis. A number of susceptibility loci have been identified associated with sporadic FTD (Table 2). These associations were replicated by several independent study groups. Some of these studies have validated the credibility of these loci while others have invalidated them due to lack of association. However, several GWAS as well as replication studies are underway to identify additional genetic susceptibility loci.

## VI.1. Patient characteristics

This is the first report from south India regarding the clinical and genetic features of FTD. The demography and patient characteristics were similar to that of the previous

reports from other populations. The patient cohort consisted of 116 FTD patients with predominant clinical presentation as bvFTD representing 81% of total FTD cases.Only three cases (2.5%) showed concomitant motor neuron disease which is different from the previous reports (Lomen-Hoerth et al., 2003). A slight predominance of male proportion (61%) was shown by the study population (Ratnavalli et al., 2002). A survival analysis estimating the disease duration was not possible in the study cohort since the patients are alive. The overall frequency of positive family history of dementia in our study cohort was 15%; lower than that of the previous reports from western populations which showed a frequency up to 50% of total FTD cases (Goldman et al., 2005; Ratnavalli et al., 2002; Rohrer et al., 2009; Rosso et al., 2003; Stevens et al., 1998). The remaining 85% of patients had sporadic disease, i.e. without any family history of the disease which is higher compared with other studies. To avoid confounding with other dementia subgroups such as PSP and CBD, the clinical diagnosis followed strict criteria for identifying FTD. Clinical diagnosis was based on the Lund Manchester consensus criteria for FTD, and confirmation of frontotemporal atrophy on MRI. PSP and CBD groups were included separately.

## VI.2. *MAPT* mutations are rare cause of FTD in the study cohort

*MAPT* mutation analysis in both familial and sporadic FTD cases showed absence of known pathogenic mutations in exons 1, 9, 10, 11, 12 and 13 and flanking intronic regions. The non-pathogenic SNPs identified were previously reported and were also

prevalent among the controls in same frequency. The novel polymorphism identified was located in the intron 9 and was also found to be non-pathogenic.

The apparent absence of *MAPT* mutations even in familial cases may be explained by the difference in the geographical distribution pattern of *MAPT* mutations, since higher *MAPT* mutation frequencies have been reported from European countries due to founder effect and lower frequencies in Sweden, Poland and Finland (Kaivorinne et al., 2008). There are very few reports on *MAPT* mutation analysis from Asian populations (Das et al., 2013; Kim et al., 2010; Kowalska et al., 2001; Ogaki et al., 2013). A recent study from Japan reported that *MAPT* mutations are more frequent in familial FTD compared to other loci such as *PGRN* and *C9ORF72* (Ogaki et al., 2013) and another report from Korea showed that *MAPT* mutations are rare in FTD (Kim et al., 2010). Recent study from east India reported the absence of *MAPT* and *PGRN* mutations in FTD patients (Das et al., 2013). To date, no mutation studies on FTD have been reported from south India and this is the first attempt to screen for genetic mutations in *MAPT*. Our study showed that *MAPT* mutations are not a common cause of FTD in the study cohort (Aswathy et al., 2014). The high prevalence of *MAPT* mutations observed in other studies depends strongly on the diagnostic criteria used to select patients since they have recruited patients with strong family history or in pathologically confirmed cases only. Here, in a general population of FTD, we conclude that *MAPT* mutations occur very rarely. Mechanisms other than currently identified *MAPT* mutations might explain the familial as well as sporadic cases of FTD.

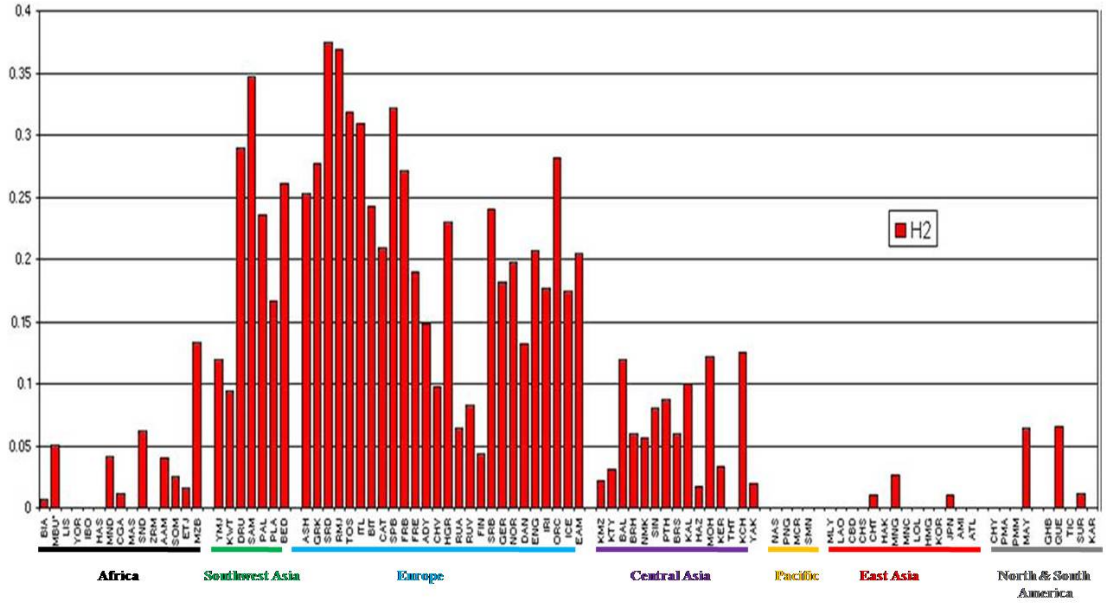
## VI.3. A series of polymorphisms were identified in *MAPT*

Common variations occurring at *MAPT* locus were described and these included eight sequence variants. All of them were substitution polymorphisms and four among them were found to be inherited as a part of an extended haplotype along with the deletion polymorphism that covers the entire *MAPT* locus. We report one novel polymorphism that was detected in one FTD patient located on intron 9. Most of the SNPs (6/8) identified were located in exon 9 or intron 9 (Table 8). Four exonic variants in exon 9 were silent variants which do not cause an amino acid change in tau protein. Also the presence of these variants in controls demonstrates that they are non-pathogenic variants occurring in the general population. However, whether these variants contribute to the etiology through interacting with other genetic or environmental factors remains to be elucidated.

## VI.4. *MAPT* H1 and H2 haplotypes are not associated with susceptibility for FTD

H1 haplotype has been established as the most common with an allele frequency of >70% in European populations (Evans et al., 2004). The H2 haplotype has been associated with Caucasian ancestry and is assumed to be the ancestral one showing only minor variability (Oliveira et al., 2004; Pittman et al., 2005). In Caucasian populations, the frequency of H1 varies between 70 and 80% (Evans et al., 2004). In the Middle Eastern and European populations, the frequency of H2 allele is about 25% whereas in Finnish population it is about 8%, in Central Asian populations it is 5% and found to be non-existent in other populations (Evans et al., 2004) (Figure 29).

**Figure 29: *MAPT* inversion frequencies in different populations**




*This graph shows the frequencies of the H2 (red) haplotype in 90 populations. The populations are grouped by regions: Africa, Southwest Asia, Europe, Central Asia, the Pacific, East Asia, North America, and South America. As we see, the H2 haplotype is found most predominantly in Southwest Asia, Europe, and Central Asia (Figure adapted from Donnelly et al., 2010).*

Since an association between the *MAPT* H1 haplotype and other neurodegenerative disorders has been reported, we investigated whether the H1 or H2 haplotype has any association with FTD cases. In line with other Asian reports, in our study cohort, the control individuals showed an allelic frequency of H1 haplotype about 95% (248/260) and that of H2 was 5% (12/260). Nonetheless, no significant association was observed between either *MAPT* haplotypes or genotypes and the risk for developing FTD in the study population when compared with controls. This is in agreement with previous reports, which also suggested a lack of association between *MAPT* haplotypes and FTD (Borroni et al., 2005; Laws et al., 2008; Panegyres & Zafiris-Toufexis, 2002; Sobrido et al., 2003).

Among the H1H2 carriers, 93.7% were shown to be presenting with the bvFTD and one among the carriers showed a positive family history presenting with PNFA. When the clinical subgroups were compared with controls, bvFTD and PNFA did not show a significant association of either haplotypes or genotypes. A significant overrepresentation of H2H2 genotype was found in SD patients (p=0.01), but it is limited by the relatively small sample size (SD, n=7). Since tau protein has been implicated in a wide spectrum of neurodegenerative disorders collectively termed as tauopathies (including AD, PSP, CBD etc), we have analyzed the association of *MAPT* haplotypes in other dementia subtypes in the same population. However, the comparison of MCI, AD, VD and other dementia groups with controls did not show the evidence of any association with disease risk. The distribution of H2 haplotype and genotype in FTD compared with AD and MCI group showed a significant overrepresentation of H2 haplotype in FTD patients (p=0.01), suggesting that it may aid in the differential diagnosis of FTD from AD and MCI, but needs to be confirmed in larger number of samples.

The H2 haplotype has been found to increase the risk for familial FTD in an Italian study by Ghidoni et al (Ghidoni et al., 2006). However in this study cohort, the analysis did not show an association with familial occurrence of FTD in H2 carriers. The presence of the H2 allele has been associated with a significant decrease in the age of onset (59 in H1 vs. 54 in H2 carriers) in FTD patients and a further decrease in the age of onset was observed in patients carrying two H2 alleles (Borroni et al., 2005). Since the *MAPT* genetic variability does not appear to contribute to the risk for FTD, the study further sought to determine whether the presence of H1 or H2

allele could affect the age at onset in these patient series. Unlike previous reports, the H2 haplotype did not show a significant association with age at onset. These discrepancies may be explained by the relatively smaller size and ethnic difference from other studies.

## VI.5. Plasma tau (total) do not act as a biomarker for FTD

FTD shows a considerable heterogeneity in the neuropathological presentations. A subset of FTD patients with or without *MAPT* mutations are characterized by the abnormal deposition of phosphorylated tau protein as aggregates in the affected brain regions. Total tau is regarded as a general marker for neurodegeneration. Normally, six hyperphosphorylated tau isoforms are located mainly in axons, associated with the cytoskeleton and intracellular transport systems. In FTD, increased CSF tau levels compared to healthy aging have been found by some investigators (Green et al., 1999; Molina et al., 1999), while not in other studies (Hulstaert et al., 1999). However, the plasma tau levels in FTD patients have not been thoroughly investigated. Here we have employed a sandwich ELISA to measure total tau protein in plasma using antibody that detect all six isoforms of tau proteins independent of phosphorylation. We hypothesized that the total tau levels in plasma may probably reflect the general degree of axonal damage and neuronal degeneration in FTD with tau-positive neuropathology. Plasma immunoreactivity for tau could be detected in the analyzed patients and controls and there was a trend towards increase in the tau concentration in FTD patients compared with controls. However, the difference did not show a statistical significance. This might be due to the smaller number of

samples analyzed for the assay. The increased levels observed could probably reflect the tau protein leaked through the blood brain barrier into the peripheral fluids. This needs to be confirmed in larger study cohorts. Moreover, we have correlated the plasma tau levels with the *MAPT* genotype status in FTD and controls. This did not reveal any statistical difference in tau levels either between FTD and controls or between H1H1 and H1H2 genotypes. Increased tau gene expression has been associated with H1 haplotype elsewhere (Myers et al., 2007; Caffrey et al., 2006; Llado et al., 2007). Unlike those reports, H1H1 genotype was not associated with increased tau levels in plasma. This may be explained by the fact that tau gene expression is specific to the CNS and other reports have correlated the *MAPT* haplotypes with tau expression within brain tissue. Moreover, our sample size analyzed for tau ELISA is very small to attain a meaningful interpretation.

## VI.6. *PGRN* mutation analysis revealed one novel variant causing familial bvFTD

*PGRN* mutation screening in a series of 116 independent FTD patients comprising both familial and sporadic FTD cases led to the identification of a novel loss of function mutation in one FTD patient explaining the disease in ~1% (1/116) of FTD cohort and 6% (1/17) of FTD cases with a positive family history. The mutation detected was a typical nonsense mutation in *PGRN* exon 12 in a heterozygous condition which is predicted to result in the introduction of a premature termination codon producing a polypeptide chain terminated at 503$^{rd}$ amino acid residue. This mutation was detected in a familial patient diagnosed with bvFTD at the age of 75

years. This mutation has not been reported before and was absent in the remaining unrelated patients and controls ruling out the possibility of a founder mutation. Most likely the mutant transcript is destroyed by NMD as it was established for several other truncating *PGRN* mutations (Baker et al., 2006; Cruts et al., 2006; Gass et al., 2006). This implies further genetic heterogeneity of FTD, because we detected only one family in which FTD is caused by a novel *PGRN* mutation.

In our FTD cohort, the estimated genetic contribution of *PGRN* mutations is much lower when compared with the other studies (about 10%) (Gass et al., 2006). This discrepancy is likely explained by the fact that the previous reports estimated the frequency of *PGRN* mutations in pathologically confirmed cases with ubiquitin positive inclusions (about 30%), whereas the current cohort did not include such patients. Since *PGRN* dosage alterations appear to be a rare cause of FTD, study of genomic rearrangements in *PGRN* loci may not significantly change the estimated mutation frequency in this patient series.

## VI.7. Several non-pathogenic variants were identified in *PGRN*

The sequencing analysis of *PGRN* identified several common polymorphisms including two novel intronic variations (T>C at -41 bp from intron 1 and G>A at -29 bp from intron 1) and one novel exonic variation (G>C at -7 bp from exon 11). The exonic variation was found in the coding region of exon 11, resulting from a base pair transversion (G>C) and amino acid substitution from leucine to phenylalanine (Leu469Phe). Another base pair transversion occurring at the same locus described elsewhere was G>T (g.3078 G>T); whose pathogenic nature was described unclear

(Beck et al., 2008). The pathologic significance of these novel variants is currently unknown. To detect the effect of these variations on *PGRN* expression plasma progranulin levels were quantified in the carriers. All the variants showed normal progranulin levels suggesting their non-pathogenic nature based on haploinsufficiency. We conclude that *PGRN* mutations in isolated populations such as south India may be lower due to a founder effect in selected studies such as western populations and may explain regional differences in frequency of *PGRN* mutations.

## VI.8. Progranulin serves as a biomarker

Most of the mutations identified in *PGRN* worldwide shows haploinsufficiency as the uniform disease mechanism underlying FTD in *PGRN* mutation carriers (Gijselinck et al., 2008a). Hence we hypothesized that the expression of progranulin in plasma can act as a biomarker to determine the *PGRN* mutation carriers. The plasma progranulin levels were quantified through a sandwich ELISA in a subset of patients in which the plasma samples were available. This revealed that plasma progranulin levels were reduced in 20% (12/60) of FTD cases compared to controls. We observed a wide range in progranulin expression in FTD patients and controls, and the mutation carrier showed significantly reduced progranulin level to about one third of the levels observed in non-*PGRN* mutation carriers and control individuals. However, since there was only one *PGRN* mutation carrier in our FTD cohort, future analysis is necessary to assess the plasma progranulin optimal cut-off value to distinguish the mutation carriers from non-carriers.

## VI.9. *PGRN* rs5848 polymorphism and the risk for developing FTD

A common genetic variation (T allele) located at the 3'-UTR of *PGRN* (rs5848) has been associated with an increased risk for developing sporadic FTD in North American population particularly in pathologically confirmed cases with TDP-43 positive inclusions (Rademakers et al., 2008). The literature showed that the T allele distribution differs significantly by ethnicity. The International HapMap data suggest that the T allele is overrepresented in African population (www.hapmap.org). In Caucasians, the T allele frequency was found to be 34% (42% heterozygous and 13% homozygous). In an attempt to replicate whether rs5848 contribute to the pathogenesis in our study cohort, rs5848 genotyping was carried out in 116 FTD cases and compared them with 130 controls. Overall, the frequency of the T allele was 31% in controls in which 43% were heterozygous (CT) and 10% were homozygous (TT) which is more or less similar with the other reports from Caucasians. Nevertheless, the analysis failed to find any significant association between variation at rs5848 and FTD. This is line with two other reports, one from European clinical FTD cohorts and another one from Netherlands, both of which did not find a significant difference in genotype and allele frequencies of rs5848 either when analyzed by FTD as a whole or for any of the clinical subgroups compared with age-matched controls (Rollinson et al., 2011; Simon-Sanchez et al., 2009). The apparent negative association observed could be due to the heterogeneity underlying FTD, however further replication studies are required to drawn definitive conclusions.

## VI.10. *PGRN* rs5848 polymorphism (T allele) affects progranulin expression

Reduced serum level of the progranulin has been identified in homozygous rs5848 T allele carriers; supporting the hypothesis that rs5848 affects the risk of FTD by regulating *PGRN* expression (Hsiung et al., 2011). In order to examine the effect of rs5848 in the expression of *PGRN*, we compared the plasma progranulin levels in different rs5848 genotype carriers. This revealed that the T allele showed a statistically significant difference in the expression of progranulin in a dose dependent manner in FTD patients but not in controls. TT genotype was associated with the lowest plasma progranulin levels compared with CT and CC genotype carriers. This suggests that *PGRN* rs5848 may affect the risk for developing FTD possibly through decreasing the *PGRN* expression levels in TT carriers.

## VI.11. *APOE* polymorphisms does not act as a genetic risk factor for FTD

*APOE* ε4 allele is an established risk factor for late onset and familial AD (Corder et al., 1993). Several association analyses as well as meta-analysis have examined the potential association of *APOE* polymorphisms with FTD; but ended up with contradictory findings (Verpillat et al., 2002a). In 2013, a comprehensive meta-analysis was done to determine whether *APOE* polymorphism is a risk factor for FTD and found an association between the *APOE* ε4 allele and FTD (Rubino et al., 2013). To determine whether *APOE* could act as a modifier gene in our study cohort,

we conducted an association analysis with 116 FTD and 130 age-matched controls. We did not find a significant difference in the allele or genotype frequencies between FTD and controls. This finding is consistent with previous reports which employed patients with confirmation on autopsy (Geschwind et al., 1998; Minthon et al., 1997; Riemenschneider et al., 2002). In conclusion, we could not replicate the findings of significantly increased ε4 or ε2 allele frequency in our FTD cohort (Lehmann et al., 2000; Rubino et al., 2013). Our results suggest that *APOE* allelic variants do not act as a genetic susceptibility factor for FTD in south Indian population. To find whether *APOE* genotypes influence *PGRN* expression, we have correlated the *APOE* genotype status with plasma progranulin levels. This showed that in FTD, the average plasma progranulin level is significantly increased in ε3ε4 carriers compared to ε3ε3 carriers. Moreover, when the sample groups were compared, the level was significantly increased in FTD group carrying ε3ε4 genotype. However, whether this increase in progranulin expression is related to the primary of secondary effect of pathogenesis is unclear. Since some reports showed the association of ε4 allele with FTD (Rubino et al., 2013) and *PGRN* is anti-inflammatory in function (He & Bateman, 2003), we speculate that the increase in progranulin levels observed may be related to the inflammatory response associated with the pathogenesis.

## VI.12. TDP-43 immunoreactivity detected in plasma

In TDP-43 based brain pathology, phosphorylated and cleaved TDP-43 accumulates in the affected brain regions. Plasma TDP-43 levels were correlated with disease pathology in FTD and has been proposed that it may act as a biomarker to

differentiate FTD with TDP-43 pathology from other neuropathological entities (Foulds et al., 2008; Foulds et al., 2009). In the present study, we determined the plasma levels of TDP-43 in living FTD patients without pathological confirmation and compared them with controls. We detected TDP-43 immunoreactivity in plasma but was present in low concentrations or totally absent in some patients and controls, which is in line with the previous reports. However, in our patient series there was only one *PGRN* mutation carrier which may harbor TDP-43 neuropathology. This patient showed TDP-43 protein levels in between the higher and lower values. Since the patients analyzed for plasma TDP-43 levels were in early or middle stages of disease, these samples should be followed up to deduce whether the disease progression affect the plasma levels of TDP-43. Based on our results, we conclude that plasma TDP-43 cannot act as a predictive test to distinguish TDP-43 based FTD cases from other subtypes. To confirm this finding, this has to be replicated in more number of samples with autopsy confirmation of TDP-43 pathology.

## VI.13. Serum lipid peroxides indicate increased oxidative stress in FTD

The majority of FTD cases are sporadic without any obvious familial inheritance. Hence we are also challenged with the elucidation of molecular basis of sporadic FTD cases. In the present study we selected a subset of patients with sporadic nature (since the volume of serum recovered from all participants was insufficient to determine the lipid peroxidation status) and analyzed whether the oxidative stress marker, lipid peroxides (MDA) are elevated compared with age-matched controls.

The measurement of serum MDA levels (end products derived from the peroxidation of poly-unsaturated fatty acids and related esters) showed a statistically significant difference between the analyzed FTD and controls. Serum MDA levels were found to be elevated in patients with FTD. Some reports have demonstrated the involvement of oxidative stress in the pathogenesis of FTD (Gerst et al., 1999). This may clearly implicate that similar to other neurodegenerative disorders such as AD, oxidative stress may also be involved in the pathogenesis of FTD. However, whether it is a primary or secondary response to pathogenesis remains to be elucidated.

## VI.14. Overall findings

Our study can be considered as the largest report of the *MAPT* and *PGRN* mutation screening in an Asian/Indian FTD cohort. There have been four previous studies on the analysis of *MAPT* and *PGRN* mutations in Asian populations (Das et al., 2013; Kim et al., 2014; Kim et al., 2010; Ogaki et al., 2013). Kim et al. screened *MAPT* and *PGRN* mutations in 45 Korean patients with PSP, CBS, or FTD and did not find any pathogenic mutations (Kim et al., 2010). However, their study was limited by the small number of subjects, especially sporadic FTD patients (n=2). In 2014, another study from Korea reported that *MAPT*, *PGRN*, and *C9ORF72* mutations are rare causes of FTD in 75 Korean patients (Kim et al., 2014). One study from Japan identified five *MAPT* mutations including one novel *de novo* mutation and one novel *PGRN* mutation after direct sequencing analysis in 75 patients comprising bvFTD, FTD-ALS, PPA, PSP and CBS. The number of FTD patients in their study was 38 (50.7%). However, all five patients with *MAPT* mutations were clinically diagnosed

as early-onset PSP with the distinctive eye movement. One patient with the *PGRN* mutation was associated with PPA (Ogaki et al., 2013). Finally, Das et al. from India screened 81 patients comprising FTD (19 bvFTD, SD, 4 PPA, 3 FTD-MND), PSP (n=48) and CBD (n=4) for mutations in *MAPT* and 33 of them for mutations in *PGRN* (Das et al., 2013). They found 11 non-pathogenic SNPs in *MAPT* and 3 in *PGRN* but no pathogenic mutations in either *MAPT* or *PGRN*.

Our study, for the first time analyzed the molecular genetics of FTD in a south Indian clinical cohort. We conclude that *MAPT* and *PGRN* genetic variability plays a less significant role in the etiology of FTD in the analyzed study cohort. One clinically important aspect of our study is the identification of one novel truncating mutation on *PGRN* which adds data on the FTD-associated *PGRN* mutation spectrum. This finding highlight the possibility of occurrence of novel mutations in *PGRN* with same pathogenic mechanism i.e., haploinsufficiency. Also this finding necessitates the importance of genetic counseling and genetic testing in the family members of the mutation carriers. So the screening for genetic variability at the *PGRN* loci should be carried out in south Indian cohorts with first preference other than loci such as *MAPT*. However, the frequency of *PGRN* mutation in our study cohort was low accounting for only 6% of familial and 0.8% of total FTD cases. Our study further confirmed that known mutations in *MAPT* and *PGRN* are relatively rare in Asian populations. These results also highlight the importance of exploring other etiological factors (both environmental and other unknown genetic factors) in causing the pathogenesis in FTD in the study population.

## VI.15. Limitations of the study

FTD patients included in this study were recruited after careful differential diagnosis and extensive follow-up (at least 3 years). However, confounding due to diagnostic misclassification cannot be excluded in the study cohort since the stratification of subjects were based on clinical, neuropsychological and neuroradiological examinations and not confirmed by neuropathology. None of the deceased probands (9%) have undergone autopsy confirmation of FTLD pathology. The collection of family history was based on the retrospective account of the caregivers. However this may not affect the mutation screening since the mutation analyses have been done in the whole study cohort. *MAPT* and *PGRN* dosage alterations have not been analyzed in the patients since they were reported to be rare in FTD worldwide. So copy number variations such as deletion or duplication of *MAPT* locus cannot be ruled out as a cause of FTD in our study cohort. The segregation of the novel *PGRN* mutation was not checked in the family members of the proband, as their DNA samples were not available. Despite the genetic analysis, all assays were performed on only subset of study samples. So we could not perform any multi-variate analysis on the results in order to attain reliable conclusions on the relative contribution of each causative variable for FTD under analysis in the study cohort.

# VII. SUMMARY AND CONCLUSION

## VII. Summary and Conclusion

The present study was aimed at the elucidation of molecular basis of FTD, which is fundamental to the development of disease-modifying and preventive therapies. Here we investigated the role of *MAPT* and *PGRN* mutations, role of known susceptibility loci such as *MAPT* haplotypes, *PGRN* rs5848 polymorphisms and *APOE* polymorphisms in the genetic etiology of FTD in a south Indian clinical cohort. *MAPT* mutation analysis revealed no known pathogenic mutations in our FTD cohort suggesting that they are rare cause of FTD in the study cohort. *PGRN* mutation analysis revealed one novel mutation which explains 6% of familial FTD patients. This mutation invariably showed the proposed uniform disease mechanism of haploinsufficiency which was confirmed through plasma progranulin ELISA. This finding does not rule out the possibility of occurrence of novel mutations in south Indian population of FTD patients. In sporadic FTD, several susceptibility loci have been identified and here we selected *MAPT* haplotypes, *PGRN* rs5848 polymorphism and *APOE* polymorphisms to study their association with the disease. However, the analyses revealed no significant differences in the genotypic or allelic frequencies when compared with controls, negating their role as susceptibility loci in the study cohort. The involvement of pathological proteins in FTD is the pace to develop disease-specific, molecular-based diagnostic tests such as the quantification of total or pathological protein species in biological fluids (Hu et al., 2011). The study combined biochemical analyses evaluating plasma levels of tau, progranulin, TDP-43 and serum levels of lipid peroxides. The plasma distribution of tau and TDP-43 levels did not find to be predictability markers for neurodegeneration. Although there was no statistically significant difference in plasma progranulin levels between FTD

and controls, a proportion of FTD cases were shown the progranulin levels reduced up to 50% of the normal protein levels. Since only one patient harbor the *PGRN* mutation, we speculated that some other pathogenic mechanism at *PGRN* loci might be involved in the reduced expression of *PGRN*. To address this question, we have correlated the plasma progranulin levels with rs5848 polymorphisms. This revealed that the presence of T allele was associated with a significant reduction of progranulin levels in FTD patients but not in controls. This implicates the role of common genetic variants other than mutations acting as genetic susceptibility factors for FTD in the study cohort. In order to confirm this finding it should be demonstrated in more number of both FTD and control samples. Moreover, when progranulin levels were correlated with the *APOE* polymorphism status, there was a significant over expression of progranulin in ε3ε4 carriers compared to ε3ε3 carriers. Since progranulin is anti-inflammatory in function, the increased expression of progranulin in FTD patients observed may be related to the underlying pathology, but needs to be confirmed in more number of samples. A subset of sporadic FTD patients have shown a significantly higher level of lipid peroxides in serum compared with controls which suggests that oxidative stress might be involved in the pathogenesis. More comprehensive genetic/biochemical screening studies are needed to establish the genetic linkage of other putative loci with FTD in the study population. The present study included participants based on strict inclusion criteria of uniform phenotypic definition from Kerala population of south India, thereby reducing the phenotypic disparities and confounding effects contributed by the ethnic differences and population stratification. Through this study it is concluded that known genetic variants plays little role in the etiology and the presence of novel

variants suggest that the population may be peculiar in its phenotypic expression and when studying their genetics novel variants and novel disease mechanisms should be addressed to adopt proper interventional strategies.

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# APPENDICES

# APPENDIX I

## Diagnostic criteria for bvFTD, PNFA and SD

<table>
<tr><th>bvFTD</th><th>PNFA</th><th>SD</th></tr>
<tr><td>Core criteria<br><br>Shows progressive deterioration of behavior and/or cognition</td><td colspan="2">Core criteria<br><br>Difficulty with language that cause the impaired daily living activities, and aphasia should be the most prominent deficit at symptom onset and for the initial phases of the disease</td></tr>
<tr><td>Possible FTD<br><br>At least three of the following features must be present<br><br>A. Early behavioural disinhibition<br>B. Early apathy or inertia<br>C. Early loss of sympathy or empathy<br>D. Early perseverative, stereotyped or compulsive/ritualistic behavior<br>E. Hyperorality and dietary changes<br>F. Neuropsychological profile: executive/generation deficits with relative sparing of memory and visuospatial functions</td><td>At least one of the features must be present<br><br>A. Agrammatism in language production<br>B. Effortful, halting speech<br><br>At least 2 of the following must be present:<br>A. Impaired comprehension of syntactically complex<br>B. sentences<br>C. Spared single-word comprehension<br>D. Spared object knowledge</td><td>Both features must be present<br><br>A. Poor confrontation naming<br>B. Impaired single-word comprehension<br><br>At least 3 of the following must be present:<br>Impaired object knowledge<br>A. Surface dyslexia or dysgraphia<br>B. Spared repetition<br>C. Spared speech production</td></tr>
<tr><td>Supportive imaging features<br><br>Frontal and/or anterior temporal atrophy on MRI</td><td>Supportive imaging features<br><br>Predominant left posterior fronto-insular atrophy on MRI</td><td>Supportive imaging features<br><br>Predominant anterior temporal lobe atrophy on MRI</td></tr>
</table>

# APPENDIX II

## Reagents

**Agarose gel electrophoresis**

**TAE (Tris acetate EDTA) electrophoresis buffer (50X) (1 Liter)**

Tris base: 242 g

Glacial acetic acid: 57.1 mL

Ethylenediaminetetraacetic acid (EDTA) (0.5 M, 1.86 g EDTA in 10 mL distilled water, pH=8):100 mL

Distilled water up to 1 L

Working concentration (1X) (300 mL)

Tris acetate: 40 mM

EDTA: 1 mM

**1% Agarose gel**

Agarose (0.15 g) molten in 1X TAE (15 mL)

**Polyacrylamide gel electrophoresis (PAGE)**

**TBE (Tris borate EDTA) electrophoresis buffer (5X) (1L)**

Trisbase (1.1 M): 54 g

Boric acid (900 mM): 27.5 g

EDTA (25 mM) (0.5 M, 1.86 g EDTA in 10 mL distilled water, pH=8): 20 mL

Distilled water up to 1 L, pH 8.3

Working concentration: TBE (0.5X) (800 mL)

Tris-borate: 45 mM

EDTA: 1 mM

**Acrylamide/bis-acrylamide solution (39:1) (100mL)**

Acrylamide (39%, w/v): 40 g

N,N'- methylene-bis-acrylamide (1%, w/v): 1.379 g

Distilled water up to 100 mL

**5% PAGE compositionfor DNA electrophoresis (10 mL)**

Acrylamide/bis-acrylamide solution: 1.66 mL

TBE buffer (5X): 2 mL

Ammonium per sulphate (APS) (20%): 35 µL

N,N,N',N'-tetramethylethylenediamine (TEMED): 7 µL

Distilled water: 6.3 mL

**DNA ladders: 100 bp ladder and Msp1 digested pUC18 ladder**

Working concentration

Ladder: 1 µL

Loading dye (6X): 5 µL

Distilled water: 12 µL

**Ethidium bromide (EtBr) solution**

EtBr (1%) in distilled water

**Post Sequencing PCR clean-up**

EDTA (125 mM) (1 mL)

EDTA: 46.53 mg

Distilled water: 1 mL

Sodium acetate (3M) (1 mL)

Sodium acetate: 246 mg

Distilled water: 1 mL, pH=5.2 with glacial acetic acid

**Western blotting**

**Running buffer (10X) (500 mL)**

Tris base (0.25 M): 15.15 g

Glycine (1.92 M): 72 g

SDS (1%): 5 g

Distilled water up to 500 mL

Working concentration: 1X running buffer

Running buffer (10X): 100 mL

Distilled water up to 1 L

**Blotting buffer (10X) (500 mL)**

Tris base (0.25 M): 15.15 g

Glycine (1.92 M): 72 g

Distilled water up to 500 mL, pH=8.3

Working concentration: 1X blotting buffer

Blottingbuffer (10X): 100 mL

Methanol: 200 mL

Distilled water up to 1 L

**TBS (Tris buffered saline) (10X) (1 L)**

Tris base: 12.1 g

NaCl: 84.8 g

Dissolved in distilled water and volume made up to 1 L with pH=7.9

**TBST (TBS with Tween-20) (500 mL)**

TBS (1X): 500 mL

Tween-20 (0.05%): 250 µL

**Resolving gel buffer for SDS PAGE (8X) (50 mL)**

Trisbase: 18.15 g

SDS: 0.4 g

Distilled water up to 50 mL, pH=8.8

**Stacking gel buffer for SDS PAGE (4X) (50 mL)**

Tris base: 3.025 g

SDS: 0.2 g

Distilled water up to 50 mL, pH=6.8

**1X SDS gel-loading buffer (10 mL)**

SDS (2% (w/v)): 0.6 g

Tris base (0.067 M, 162.4 mg in 20 mL, pH=6.8): 2 mL

Glycerol (10% (v/v)): 3 mL

Bromophenol blue (0.03%): 9 mg

β-mercaptoethanol (0.3%): 1 µL

**Resolving gel (10%)**

Acrylamide/bis-acrylamide solution: 2.5 mL

Resolving buffer (8X): 1.25 mL

APS (20%): 18 µL

TEMED: 10 µL

Distilled water: 6.25 mL

**Stacking gel (5%)**

Acrylamide/bis-acrylamide solution: 0.625 mL

Stacking buffer (4X): 1.25 mL

APS (20%): 10 µL

TEMED: 10 µL

Distilled water: 3.125 mL

**PGRN primary antibody dilution (1:125, 1 mL)**

TBST (1X): 992 µL

BSA (3%): 30 mg

PGRN primary antibody: 8 µL

**Secondary antibody reconstitution (1:2000, 1 mL)**

TBST (1X): 1 mL

BSA (3%): 30 mg

Secondary antibody: 0.5 µL

**Blocking solution (20 mL)**

Skim milk (5%): 1 g

TBST (1X): 20 mL

**Phosphate Buffered Saline (PBS) (10X) (1 L)**

NaCl (137 mM): 80 g

KCl (2.7 mM): 2 g

$Na_2HPO_4$ (10.14 mM): 14.4 g

$KH_2PO_4$ (1.76 mM): 2.4 g

Distilled water up to 1 L, pH=7.4

Working concentration (1X) (1L)

PBS (10X): 100 mL

Distilled water: 900 mL

**TBARS Assay**

MDA Standard (Stock Solution): 0.5 mM

MDA Standard (Working Concentrations): 1, 2, 3, 4, 5 & 10 nM

Thiobarbituric acid reagent (10 mL)

Trichloroacetic acid: 1.5 g

Thiobarbituric acid: 37.5 mg

HCl (0.25N): 0.21 mL

Distilled water up to 10 mL

# APPENDIX III

# IEC Approval

# APPENDIX IV

## A summary of family data

| Serial No | Sample No | Diagnosis | Age at onset | Parents | Sibs affected | No of relatives affected |
|---|---|---|---|---|---|---|
| 1 | S205 | bvFTD | 53 | NA | 2 brothers | NA |
| 2 | S208 | bvFTD | 49 | NA | NA | 1 second degree relative |
| 3 | S213 | bvFTD | 66 | Affected mother | 1 sister<br>1 brother | NA |
| 4 | S220 | SD | 54 | Affected mother | NA | NA |
| 5 | S226 | SD | 66 | NA | 2 sisters | Uncle |
| 6 | S234 | bvFTD | 59 | Affected mother | NA | 2 maternal aunts |
| 7 | S237 | bvFTD | 59 | Affected father | NA | Father's mother |
| 8 | S242 | PPA | 68 | Affected Father | NA | NA |
| 9 | S264 | bvFTD | 60 | Affected Mother | 1 sister | NA |
| 10 | S268 | bvFTD | 75 | Affected Father | NA | NA |
| 11 | S48 | bvFTD | 62 | NA | 1 sister<br>1 brother | NA |
| 12 | S65 | bvFTD | 68 | NA | 1 brother | NA |
| 13 | S78 | bvFTD | 73 | Affected Father | | NA |
| 14 | S191 | bvFTD | 61 | Affected Mother | Sister | NA |
| 15 | S192 | bvFTD | 80 | Affected Father | Brother<br>Sister | NA |
| 16 | S246 | bvFTD | 49 | Affected Mother | 1 sister | NA |
| 17 | S335 | bvFTD | 59 | Affected Mother | NA | NA |

# APPENDIX V

## List of Publications

**Original Articles**

1. **Aswathy PM**, Jairani PS, Joe Verghese, Srinivas Gopala & PS Mathuranath. "*MAPT* genetic variations are uncommon cause of frontotemporal dementia in south India". Neurobiology of Aging, 2014; 35(2): 443.e23-443.e24.

2. Brief Communication: **PM Aswathy**, PS Jairani, Sheela Kumari R, Joe Verghese, Srinivas Gopala, Priya Srinivas & PS Mathuranath. "Progranulin mutation analysis: Identification of one novel mutation in exon 12". Neurobiology of Aging, 2016; 39: 218.e1–218.e3.

3. Jairani PS, **Aswathy PM**, Joe Verghese, Srinivas Gopala & PS Mathuranath. "Interaction with *MAPT* H1/H1 haplotype increases dementia risk in *APOE* ε4 carriers in a population of southern India". Dementia and Geriatric Cognitive Disorders, 2016, (Accepted).

**Review Article**

**Aswathy PM**, Jairani PS & Mathuranath PS. "Genetics of Frontotemporal lobar degeneration", Annals of Indian Academy of Neurology, 2010; 13:S55-S62.

**Book Chapter**

Jairani PS, **Aswathy PM** & Mathuranath PS. "Emerging concepts in genetics and neurobiology of dementias". In Taly AB & Singh G (Editors), Reviews in Neurology: Neurogenetics and Neuroimmunology, Indian Academy of Neurology, Bangalore. 2009.

# APPENDIX VI

## Reprint of Original Articles