DECLARATION

I, Jairani PS, hereby certify that I had personally carried out the work depicted in the thesis entitled "**GENETIC, BIOCHEMICAL AND CELLULAR CORRELATES ASSOCIATED WITH ALZHEIMER'S DISEASE**". 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 Jairani PS

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The thesis entitled "**Genetic, Biochemical and Cellular Correlates Associated with Alzheimer's Disease**" 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.

Dr. Srinivas Gopala

The thesis entitled

GENETIC, BIOCHEMICAL AND CELLULAR CORRELATES ASSOCIATED WITH ALZHEIMER'S DISEASE

Submitted by

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For the Degree of

Doctor of Philosophy

Of

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
ADL	Activities of Daily Living
ADMM	Alzheimer's Disease Monocytes and Macrophages
ADNI	Alzheimer's Disease Neuroimaging Initiative
ALS	Amylotropic Lateral Sclerosis
ANOVA	Analysis of Variance
APOE ε4	ApolipoproteinE ε4
APP	Amyloid Precursor Protein
Aβ	Amyloid β
BACE-1	β-APP Cleaving Enzyme-1
BBB	Blood-Brain Barrier
BDC	Bis-demethoxy curcumin
CAA	Cerebral Amyloid Angiopathy
CBD	Corticobasal Degeneration
CI	Confidence Interval
CJD	Creutzfeldt Jacob's Disease
CSF	Cerebro Spinal Fluid
CTF	Carboxyl-terminal fragments
DLBD	Diffuse Lewy Body Dementia
DMSO	Di Methyl Sulphoxide
ELISA	Enzyme Linked Immunosorbent Assay
EOFAD	Early Onset Familial Alzheimer's Disease
FBS	Foetal Bovine Serum
FTD	Frontotemporal Dementia
HRP	Horseradish Peroxidase
LMPA	Low melting point agarose

LOAD	Late Onset Alzheimer's Disease
LRP-1	Lipoprotein Related Protein-1
MAPT	Microtubule Associated Protein Tau
MCI	Mild Cognitive Impairment
MFI	Mean Fluorescent Intensity
MGAT-III	β-1, 4-mannosyl-glycoprotein 4-β-N-acetylglucosaminyltransferase
MM	Monocyte/macrophages
MMP	Matrix Metalloproteinases
MMSE	Mini Mental State Examination
MNC	Memory & Neurobehavioral Clinic
NFT	Neurofibrillary Tangles
NIA	National Institute on Aging
NINCDS-ADRDA	National Institute of Neurological and Communicative Diseases and Stroke/ Alzheimer's Disease and Related Disorders Association
OTM	Olive Tail- Moment
PBMNC	Peripheral blood mononuclear cells
PBMNC	peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PET	Positron Emission Tomography
PHF	Paired Helical Filaments
PIB	Pittsburgh Compound B
PSEN	Presenilin
PSP	Progressive Supranuclear Palsy
p-tau	phosphorylated tau
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
sAPPα	secreted APP α
sAPPβ	secreted APP β
SCGE	Single cell gel electrophoresis
SD	Standard Deviation

SEM	Standard Error of Means
SSB	Single Strand Breaks
SSP PCR	Sequence Specific primer PCR
TD	percentage of DNA in the tail
TIMP	Tissue Inhibitors of Metalloproteinases
TL	tail length
TLRs	toll-like receptors
t-tau	total tau
VD	Vascular Dementia

SYNOPSIS

Background and Objectives

Alzheimer's Disease (AD) is a progressive neurodegenerative disease and is the commonest cause of dementia in the elderly. Future epidemiological projections predict the global prevalence of AD to double every 20 years, estimated to reach 65.7 million in 2030 and 115.4 million in 2050. AD is a complex disease in which multiple etiological factors are involved. Two Distinct neuropathological hallmarks for AD are extraneuronal amyloid β (Aβ) plaques and intraneuronal neurofibrillary tangles (NFTs). Age, gender, education and possession of Apo Eε4 allele comprise the well-established risk factors for AD. Despite of several causative theories proposed to explain complex etiopathology of AD, none has been succeeded conclusively in elucidating the complete molecular pathological basis of the disease. Mild Cognitive Impairment (MCI) is the preclinical stage of AD and is the transitional stage between the normal aging and AD. All MCI do not progress to AD and the differentiation of individuals with progressive neurodegeneration is an important clinical problem. Histopathological evidences confirm that appearance of pathological lesions in AD brain may precede symptoms by decades. The current medications modify the symptoms but do not target molecular pathways thereby halting progression. Definite diagnosis of AD is possible only through autopsy. We need interventions that help in early detection of the disease at or before the preclinical stage which help detecting individuals at risk, thus start medications earlier in order to delay progression to AD. A few reports are available on the prevalence/ frequency of genetic risk factors in Indian population. Regardless of the lower prevalence, neither the association of genetic risk factors/ biomarkers in assisting early diagnosis of AD has been investigated in this population. This study has been focused to explore the various Genetic, Biochemical as well as cellular factors with the objective of examining the combination of these biomarkers in order to set a predictability profile of AD at the preclinical stage.

Variations in the distribution of APOE alleles contribute to the differential risk towards developing the disease, since APOE ε4 allele has a gene dose effect on the risk and age of onset of AD. Despite the prevalence studies, none has investigated the association of APOE ε4 with incident cases of AD in our population. Functional variants of MAPT gene- H1/H2 haplotype contribute to susceptibility to different types of dementia. Mutations on PSEN 1 gene contribute to 80.1% of all known mutations associated with EOFAD. On this background, we evaluated the influence of these genetic factors in the study population.

Biomarkers of AD have a promising public health impact since they reflect the neuropathology in AD brain in the pre-symptomatic phase. Aβ-42 and tau are two established core- feasible biomarkers for AD. On the basis of metaanalysis by different study groups across the globe, it is estimated that Aβ-42 levels decrease and tau levels increase in biological fluids such as plasma and CSF as the patient progresses from MCI to AD. There have been reports correlating the differential expression of MMPs in AD. Circulating levels of MMPs could be correlated with AD. This prompted us to investigate the plasma biomarkers in the study population.

Peripheral cellular markers for AD are very significant in their less invasive nature and direct reflection of pathological stage in vivo. The inefficiency of ADMMs in Aβ phagocytosis has been demonstrated by researchers of UCLA. We attempted to correlate this parameter to different stages of AD in an aim of establishing PBMNCs as a peripheral blood based biomarker for AD in the study population. Curcumin, besides its anti-inflammatory and antioxidant properties, is reported to enhance Aβ phagocytosis in cultured monocytes. The bioactive, soluble extract of N-N' bismethoxy curcumin is proposed to serve a tonic for aging brain. However, the study population comprising of Kerala elders are consistently exposed to curcumin as part of their regular diets, might respond differently to curcumin treatment in vitro. We analysed the effect of curcumin in cultured ADMMs in influencing Aβ phagocytosis.

The broad objectives of the study are listed below.

1. To study the frequency of APOE ε4 allele, H1/H2 haplotype and PSEN 1 mutations in AD patients and estimate the risk compared to other dementia subgroups.
2. To determine the plasma levels of Aβ & Tau as a biomarker of disease progression in AD.
3. To study the macrophage amyloid internalisation that could evolve as a peripheral cellular marker for AD in the study population.
4. To identify and develop a combination of Genetic, Biochemical and cellular markers which may prove to be a molecular signature of AD.

Hypothesis

Risk towards development of AD in senile phase is associated with possession of at least one APOE ε4 allele, H1/H1 haplotype, PSEN 1 mutations, lower concentration of Aβ, higher concentration of tau and MMPs in plasma, impairment in Aβ phagocytosis by MMs which is influenced by curcumin supplementation.

Methods

Case control study design was adopted to meet the objectives of the study. Cases were selected from patients attending the MNC, SCTIMST. Cases were stratified into different dementia categories such as AD, MCI, FTD, VD and DLBD based on standard diagnostic criteria. All subjects/first degree relative gave signed informed consent approved by the SCTIMST IEC for Human Studies. Control samples matched for age, sex and ethnicity were collected from the cognitively normal caregivers/spouses of patients and SCTIMST personnel.

Blood samples were collected and separated into plasma, serum and WBCs. DNA was isolated from buffycoat. APOE genotyping was performed employing two standardised protocols viz. Hixon and Vernier Restriction Isotyping and Sequence Specific Primer PCR

(SSP PCR). APOE genotype was determined for all DNA samples and was confirmed by cross-comparison. H1/H2 haplotype was determined using PCR, detecting the deletion on H2. Mutations on PSEN 1 exons 7 & 12 were analysed on early onset familial AD patients by direct sequencing.

Biomarker analyses were performed by ELISA. Plasma tau and Aβ levels concentrations were measured through sandwich ELISA and competitive inhibition ELISA respectively. Serum MMP-2 and MMP-9 activity were assayed through Gelatin Zymography. The presence of immunoreactivity of MMPs was confirmed through western blotting. Monocytes were isolated from freshly collected blood samples. DNA damage due to oxidative stress was assessed by means of Comet assay (SCGE). To analyse the phagocytic potential of monocytes, 1-day old cells were subjected to Flow cytometry analysis after overnight incubation with HiLyte Flour 488-labeled Aβ (Anaspec). For the 'macrophage amyloid internalisation assay', monocytes were cultured in RPMI 1640 medium supplemented with 10% autologous serum for 14 days and were differentiated into macrophages which were exposed to HiLyte Flour 488-labeled Aβ and Lysotracker red (Life Technologies) for 24 h and examined by confocal microscopy for visualising Aβ uptake. Subpopulations of macrophages were treated with varying concentrations of Curcumin C3 complex (Sabinsa Corporation) and analysed the effect in modifying phagocytosis rate. Image analysis was performed using Zeiss LSM imager/ Image J software packages.

Major Findings

Study sample comprised of 557 subjects including AD (156), MCI (87), FTD (127), VD (37), DLBD (12) Controls (138). Cases were compared with controls for every parameter under analysis. APOE genotyping was performed in all samples. The APOE ε3/ε3 was expectedly more frequent in all study groups. APOE ε4 allele was present in 39% of AD patients, 28% MCI and 17% in controls. The APOEε2 allele which is regarded as protective, was significantly more frequent in controls (7.6%) compared to AD (1.28%) or MCI (4.65%). The 'rarest genotype' APOE ε2/ε2 was present in VD (2.7%) and Control (3.62%). The 'at risk

genotype' APOE ε2/ε4 was frequent in AD (2.7%), MCI (2.32%), FTD (2.3%) and DLBD (8.3%) and Controls (2.17%). On Fisher exact test, a highly significant positive association was found to exist between the possession of at least one APOE ε4 allele and having a diagnosis of AD (p=0.001) (OR= 3.015 (95% CI 2.055-4.423)); while MCI (p=.01) (OR= 1.816 (95% CI 1.155-2.855)). H1/H2 haplotype frequencies revealed no significant association with any of the disease groups.H2H2 was present in the FTD, VD and control group at very low frequency. Through Multivariate Logistic Regression analysis, the risk estimates for the possession of all six APOE genotypes with all three H1/H2 haplotypes were determined for every disease group. The H1H1 genotype has found to be having additive effect in contributing to either risk with combinations of APOE ε4 or protective effect with combinations of APOE ε2 or APOE ε3.Mutations on exons 7 & 12 of PSEN 1 gene were screened in 28 subjects (AD (n=15) and MCI (n=7) subjects with age of disease onset at <60 years and EOFAD patients(n=6)).However, no previously characterised or novel mutations were detected on the sequence.

A subpopulation of study subjects comprising MCI (n=10), AD (n=13) and Controls (n=9) were subjected to plasma tau quantification. Plasma tau concentrations were increased in MCI and AD groups compared to controls, however the difference was not statistically significant between groups. Plasma Aβ 1-42 levels were measured in AD (n=15), MCI (n=15) and Controls (n=15). Plasma samples collected during 3 successive annual clinic-visits including AD (n=6)and MCI (n=5) were also subjected to analysis. Mean plasma Aβ 1-42 levels were found to be higher in control group compared to AD/ MCI though the difference did not meet significance. Progressive MCI to advanced AD exhibited marked reduction in plasma Aβ 1-42 levels in follow up samples during 2 years. While, AD cases show either increased (50% of cases analysed) or stable (50%) Aβ 1-42 levels in their plasma. Stable MCI non convertors showed either increase (in 75%) or unchanged (25%) plasma Aβ 1-42 levels. Plasma MMP-2 & MMP- 9 levelswere found to be elevated in AD group compared to controls.

Comet assay on PBMNCs revealed significantly higher extent of DNA damage in AD patients compared to controls with an average tail moment of 2.8 (Control) and 19.4 (AD). Flow cytometry analysis on 1-day old monocytes exposed to fluorescent Aβ revealed the highest MFI in the Control group (47.5±1.5 (n=7)) compared with AD (27±1.9 (n=12)) and MCI (32.9±4.72(n=4). This provides evidence for AD monocyte's inefficiency in internalising Aβ peptide. Macrophage amyloid internalisation assay was performed in AD (n=24), MCI (n=18) andControls(n=20). For each sample, 100 cells from different fields were scored for finding out mean MFI which was significantly higher in the control group compared to AD patients. (MFI: Control (54.46±3.18), MCI (48.84±3.65) and AD (32.87±1.39)).

The influence of immunoenhancing drug curcumin was analysed over a subset of study subjects. Macrophages of AD patients (n=7) and controls (n=4) were treated by curcuminoids *in vitro* and measured Aβ uptake using confocal microscopy and image J software.The MFI of internalisation were 12.2±1.9 (AD macrophages) and 19.72±1.45 (Control macrophages). Treatment with 10µM curcumin enhanced MFI of Aβ uptake by 18.03±3.53 in AD group, while did not significantly improved in controls (20.3±0.8). Curcumin treatment improved the colocalisation of Aβ into macrophage's lysosomes as shown by increase in the colocalisation coefficients (0.72 to 0.93 in AD and 0.87 to 0.92 in Controls).

Implication of the findings

Although the association of APOEε4 as a determinant of AD risk has been established across many ethnic groups, the strength of this association varies greatly. Our findings show that a highly significant genetic association exists for AD and MCI with this locus, which is comparable to other Caucasian groups. The association is the strongest among Asian populations and marginally higher the other Indian reports. No mutations/ SNPs were detected in the EOFAD patients. Even if plasma tau and Aβ 1-42 are indeed amenable for molecular characterisation, ELISA results of the present study fall close to the detection limits of the assays. It is evident that plasma Aβ 1-42 could be used a biomarker to distinguish MCI convertors from non-convertors. Evidence for oxidative stress and impairment in the internalisation and degradation of Aβ clearly portrays the AD brain

subjected to multiple ‘hits’ during pathogenesis. Overall results of the three lines of investigations have led to conclude that association of APOEε4, plasma Aβ concentration and inefficient macrophages serve as risk/predictability factors for development/progression of AD. These studies however, warrant replication. It will be increasingly relevant to have at hand these and other biomarkers that will accelerate the pace of finding interventions to prevent, arrest or reverse AD that definitely bears great implications in the management of AD in future.

I. INTRODUCTION

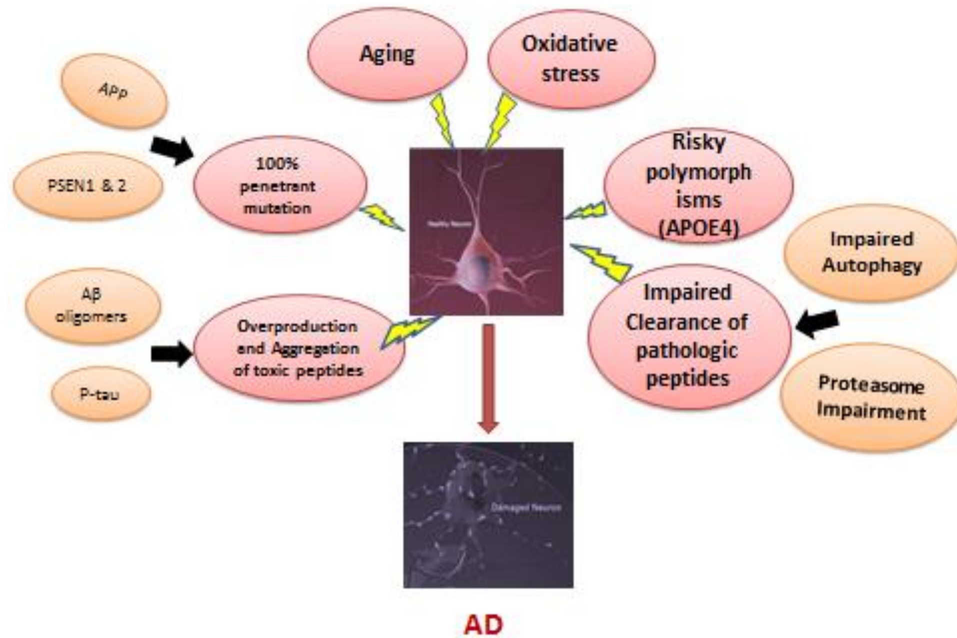
Alzheimer's Disease (AD) has been the most important subject in biomedicine that has aroused the interest of scientific and lay communities alike and is remarked as the 'silent epidemic' of the 20th century. AD is a neurodegenerative disorder characterized by progressive deficit of cognitive function, with greater emphasis on memory loss and interference with occupational and social activities in the elderly. When Alois Alzheimer, a Bavarian Psychiatrist first described the clinicopathological syndrome that bears his name at the 37th German Psychiatrists' meeting in Munich in 1906, the scientific community would not have thought that the disease will become a burgeoning health problem in the elderly in future. We now recognise that the disease originally described almost a century ago is having an incidence which rises almost logarithmically with age.

Global prevalence of AD among those aged ≥60 years has been estimated to be 5% to 7%.35.6 million people lived with dementia worldwide in 2010 and these figures are expected to double every 20 years (Martin Prince et al., 2013). In India, over 3.7 million people aged ≥60 years suffer from dementia (Mathew Verghese et al., 2012) and these numbers are projected to increase to 6.35 million by 2025 (Shaji et al., 2010). The dramatic rise in the life expectancies in the developed as well as in developing countries has aggravated the global epidemic of AD. Any intervention that delays the disease onset or progression by even one year may reduce the prevalence by more than 9 million cases by 2050. Active research for the development of efficient strategies for prevention and cure of AD are therefore urgently needed before this epidemic financially challenges individual families and even cripples national economies.

I.1. Etiology of AD

AD is a complex disease in which multiple genes as well as environmental etiological factors are involved (Nussbaum & Ellis, 2003). Etiological factors include genetic mutations, risky polymorphisms, overexpression or impaired clearance of pathogenic proteins and oxidative stress in the aging brain (Figure 1).

Figure 1: AD is a Complex Disease which is Oligogenic and Multifactorial in Etiology.

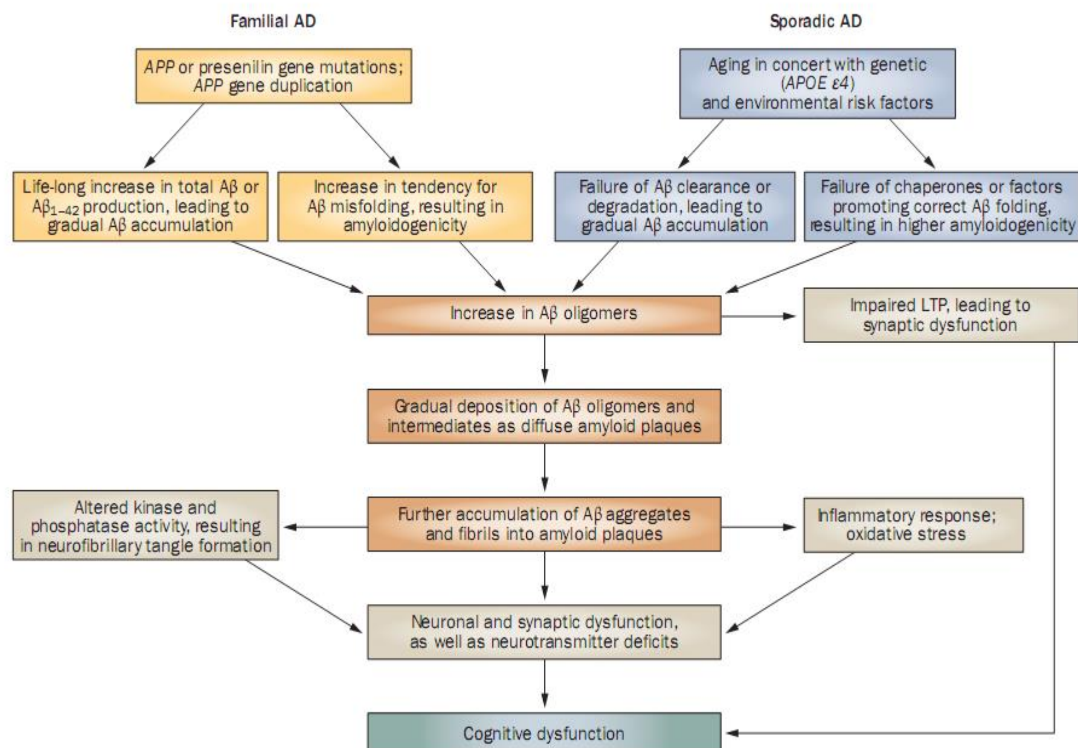


Multiple stresses contribute to pathogenesis in AD. Complex interplay of genetic and environmental factors leading to AD pathology in a healthy neuron.

I.2. Familial and Sporadic forms of AD

Familial aggregation of AD is very strong due to 100% penetrant mutations on genes such as APP, PSEN1 and PSEN2 leading to early onset autosomal dominant familial forms of the disease (EOFAD). Inheritance of apolipoprotein E ε4 allele (APOE ε4) has been known to be the strongest genetic risk factor associated with sporadic late onset AD (LOAD) (Strittmatter et al., 1993). Among the other risk factors of AD, age and a positive history of familial dementia are reported to be definitive and consistent (Breitner, Silverman, Mohs, & Davis, 1988; Lautenschlager et al., 1996). ~95% of AD cases are sporadic in nature (risk age>65 years) and ~5% possess a genetic disposition. Molecular changes leading to neurodegeneration in familial and sporadic AD are presented in Figure 2.

Figure 2: Molecular factors leading to pathogenesis in familial and sporadic AD that culminates in cognitive dysfunction



100% penetrant mutations (EOFAD) or genetic/ environmental risk factors trigger molecular cascades leading to neurodegeneration in AD brain (Adapted from (Hampel et al., 2010)).

I.3. Clinical features and diagnosis of AD

The most important clinical manifestation of AD is the progressive loss of cognitive function, being short term memory impairment/ loss of recent memories as one of the first symptoms. AD patients gradually lose judgement, language and visuospatial skills and the ability to learn new information. Disease progression could be graded based on severity of symptoms as mild, moderate, moderately advanced, advanced and severe AD. Clinical examination and neuropsychological testing combine to make clinical diagnosis of probable AD which is confirmed in 80-100% of cases by neuropathology (Breteler, Claus, van Duijn, Launer, & Hofman, 1992). Neuroimaging is not obligatory for the diagnosis, but indeed helps to differentiate AD from other pathologies such as FTD, PD and DLBD. However, a definite

diagnosis of AD requires histopathological examination which is often made possible by autopsy. The pathological hallmarks of AD are extraneuronal senile plaques, intraneuronal neurofibrillary tangles (NFTs) and neuronal loss in brain (Braak & Braak, 1991). The amyloid β protein is the main constituent of senile plaques whereas neurofibrillary tangles are composed of aggregates of abnormally hyperphosphorylated tau protein. The areas of neuronal loss are mainly hippocampus, neocortex and amygdala, which are the regions associated with memory and cognition. The starting of neuropathological lesions may precede symptoms by decades in an AD patient's brain.

I.4. Genetic risk factors in Early Onset Familial AD (EOFAD)

The rare autosomal dominant presenile dementia families have been crucial for the identification of causative genes for EOFAD. Positional cloning strategy is often employed to identify susceptibility genes for AD which is based on co-segregation analysis of AD with polymorphic genetic markers from known chromosomal loci, without any knowledge of the function or localization of the disease gene prior to the analysis. Three genes have been identified with a strong causative association with EOFAD, amyloid precursor protein (APP) on chromosome 21 at 21q21.1 (Tanzi et al., 1987), presenilin 1 (PSEN 1) on chromosome 14 at 14q24.3 (Sherrington et al., 1995) and presenilin 2 (PSEN 2) on chromosome 1 at 1q42.1 (Levy-Lahad et al., 1995). If one of these genes is mutated, the mutated protein leads to development of AD with a penetrance close to 100%. So far, 49 mutations in the APP gene, 216 mutations in the PS1 gene, and 15 mutations in the PS2 gene have been described worldwide (www.molgen.ua.ac.be/ADMutations). Most of the mutations are substitutions. Only a couple of deletions and insertions and two splicing defect mutations have been reported in the PSEN 1 gene (Cruts et al., 1998).

PSEN1 is the most frequently mutated EOFAD gene with a mutation frequency of 18 to 50% in autosomal dominant forms of the disease. PSEN1 with ten exons (exons 3–12), has been reported to carry 80.1% of mutations causative to AD till date. Almost all PSEN mutations

are missense mutations resulting in a single amino acid substitution that are scattered all over the protein (J. Hardy, 1997).

I.5. Genetic risk factors in Late Onset AD (LOAD)

LOAD shows a multifactorial hereditary pattern caused by complex interactions of genetic and environmental factors. Genome Wide Association Studies have revealed many AD-associated genes, but none of them have been as consistently replicated in all the studied populations as APOE polymorphism. APOE is the apolipoprotein constituent of chylomicron and is the key determinant in the cellular recognition & internalization of cholesterol and phospholipids-rich lipoproteins in the developing brain. The three common isoforms of APOE termed APOE ε2, APOE ε3 and APOE ε4 are defined by two single nucleotide polymorphisms (SNPs) at positions 2059 (T/C) and 2197 (C/T) of the gene, which are located in the codons that code for amino acids 112 and 158 of the mature protein. APOE ε_4 allele has a gene dose effect on the risk and age of onset of the disease (Selkoe, 2001). APOE genotyping has been suggested as a diagnostic adjunct in the work up of dementia (Hixson & Vernier, 1990). This study has intended to analyse the role of this genetic factor in contributing to disease risk and also to evaluate the influence of APOE ε_4 in modifying the predictive values of other AD biomarkers.

I.6. Microtubule Associated Protein Tau (MAPT) H1/H2 haplotype in AD Risk

MAPT gene is primarily expressed in neurons and plays a key role in the organization and integrity of the cytoskeleton. Pathological tau protein inclusions are the characteristic feature of neurodegenerative tauopathies that include AD, FTD (Frontotemporal Dementia), PSP (Progressive Supranuclear Palsy), CBD (Cortico Basal degeneration) and PD (Parkinson's Disease). The region of chromosome 17q21 containing MAPT locus is characterized by the complex genomic architecture, including a large inversion that leads to a bipartite haplotypes

namely H1 & H2. These sub-haplotypes confer susceptibility to tauopathies and this may be due to functional variation at the MAPT locus (Pittman et al., 2004).

I.7. Biochemical markers of AD

Recommendations of promising biochemical markers for AD could have a substantial public health impact through aiding the emergence of next generation therapies. This is achieved by identification of individuals with preclinical AD at the earliest stage and targeting the neuropathological cascade. Amyloid β-42 and phosphorylated or non-phosphorylated forms of tau are two established core- feasible biomarkers for AD. Concentration of amyloid β species such as Aβ1-40 as well as Aβ1-42 in biological fluids serve as a biomarker of amyloidosis in the AD brain which reflects neuropathology. It has been reported that CSF amyloid β protein levels decrease as the individual progress from MCI to clinically overt AD (Andreasen et al., 1999, Hampel et al., 2004). This could be correlated to the increase in amyloid plaques into which the peptide is deposited in brain, thereby reduces efflux into peripheral circulation. Many studies have confirmed that there is an increase in total CSF tau protein concentration in patients with AD compared with controls (Ringman et al., 2008, Shaw et al., 2009).The rationale for p-tau as a predictor is that p-tau is a major component of pre-tangle paired helical filaments and mature neurofibrillary tangles that are present within the brain even before the onset of AD (Hampel et al., 2004). P-tau may be sequestered into the extracellular CSF in a rate proportional to neuronal death/ degeneration. Immunoreactivity of tau in AD plasma had been first demonstrated by Ingelson et al (Ingelson et al., 1999). The plasma levels of tau & Aβ are investigated in patient samples and their role as a biomarker of progression from Mild Cognitive Impairment (MCI) to AD has been evaluated.

I.8. Blood-Brain Barrier dysfunction in AD

Damage of cerebrovasculature is one of the characteristic pathological features associated with AD. This definitely leads to leakage of the Blood- Brain Barrier (BBB) which amplifies the neuropathology. Matrix metalloproteinases (MMPs) are one of the major proteases that

digest the BBB proteins thus contributing to dysfunction of the BBB (Bell et al., 2012). Moreover, the leaky BBB facilitate blood born macrophages to invade neurons to phagocytize amyloid deposits aggregating in AD brain (Fiala, Liu et al., 2007).These factors are becoming the areas of active research for future therapies since they reflect the current neuropathological stage of the affected individuals.

I.8.1. Matrix Metalloproteinases and AD

Matrix metalloproteinases (MMPs) degrade extracellular matrix proteins under a tightly regulated expression mechanism. Correlation between activation of MMPs in AD brain and disease severity has suggested that these proteases play an active role in the pathophysiology of the disease especially in the degradation of Aβ in plaques (Bell et al., 2012). Since MMPs are found in different populations of cells such as neurons, microglia, vascular endothelium and leukocytes, circulating levels of MMPs may evolve as a peripheral biomarker for AD pathology. Differential expression of MMP-2 and MMP-9 in the plasma may be correlated to age, severity of dementia and APOE genotype.

I.8.2. Peripheral cellular marker for AD

According to Amyloid cascade hypothesis, aggregation of amyloid species (amyloidosis) in the brain is the leading cause of neurodegeneration in AD (J. Hardy & Selkoe, 2002). It has been shown that blood-born monocyte /macrophages of AD patients migrate across the blood–brain barrier into AD brain but are defective in clearance of amyloid beta in neuritic plaques (Fiala, Liu, et al., 2007). However, the role of macrophages in amyloid clearance *in vivo* remains unclear and under-investigated. The efficiency of β amyloid uptake by blood born macrophages can be correlated to the state of disease progression. In this study, the amyloid internalisation ability of blood born macrophages is analysed in the patient group and compared with the healthy controls and its potential to serve as a predictor for the progression of MCI to clinically overt AD has been assessed in order to establish these cells as a peripheral cellular marker for AD.

I.8.3. Curcumin as a novel drug candidate in AD in relieving brain oxidative stress

Oxidative stress is one of the most important triggering factors behind neuropathology in AD brain (Morocz et al., 2002). Many investigators have proposed curcumin a potential drug candidate for AD due to its ability in scavenging free radicals thereby reducing oxidative stress (Fiala, Liu, et al., 2007).Thus it effectively counteracts the neuroinflammation and brain oxidative stress thereby reduces neuronal loss. A novel property of curcumin in enhancing the phagocytic potential of brain resident macrophages thereby increasing Aβ clearance is an active area of AD research. Curcumin supplementation was found to be very effective in AD patient's blood born macrophages in increasing Aβ phagocytosis in vitro (L. Zhang et al., 2006). Studies in microglial cells (brain macrophage analogs) demonstrated reduced Nitric Oxide (NO) generation and protection of neurons from oxidative stress following curcumin treatment; hence it may be useful in reducing the neuroinflammation associated with neurodegenerative conditions such as AD (Ray & Lahiri, 2009). The influence of curcumin supplementation on cultured monocytes in activating their Aβ phagocytosis / MMP expression is studied in the population.

It has been proved conclusively that the risk of AD is not likely to be determined in any single time period but results from a complex interplay between genetic and environmental exposures throughout one's life. Therefore an effort is made here to study these genetic, biochemical and cellular changes that are associated with different phases of AD. The study population has been divided into individuals having AD, MCI and age- matched controls on the basis of their clinical examination and scores on the neuropsychological evaluation. Patients from other dementia groups comprising FTD, PSP, DLB and VD are included for comparison and correlation of genetic variables. Before presenting the broad objectives, an overview of the currently available knowledge on these three lines of investigation and associated molecular mechanisms are discussed through the literature review.

II. LITERATURE REVIEW

II.1. Epidemiology

Future demographic projections are predicting the number of people aged 60 years or greater to reach nearly 1.2 billion by 2050 accounting for 22% of the world's population, with 79% living in the world's less developed regions particularly in China, India and Latin America. Each year, 4.6 million new cases of dementia are predicted, with numbers affected nearly doubling every 20 years to reach 81.1 million by 2040 (Martin Prince et al., 2013). Compared to western populations, Indian population is marked with a low prevalence of AD, possibly due to negligence or poor reporting to memory clinics. In North India, AD prevalence ranges between 1.36% (Chandra et al., 1998) to 6.07% (Ramachandran, Menon, & Ramamurthy, 1981). Two South Indian reports have shown the prevalence of 3.19% (in a rural community in Kerala) (Shaji, Promodu, Abraham, Roy, & Verghese, 1996) and 2.64% (in an urban population in Kerala) (Shaji, Bose, & Verghese, 2005). A prevalence of 2.7% has been reported from Tamilnadu (Rajkumar, Kumar, & Thara, 1997). Indian incidence rates of AD were first reported from the Indo-US study in 2001 (Chandra, Ganguli, 2001) on a rural population in Haryana as 3.24 (age>65 years) and 1.74 (age>55 years) compared to 17.5/1000 person years in US. The first South Indian incidence rates for AD were reported by our research group (Mathuranath et al., 2012) at SCTIMST through a 10-year prospective epidemiologic study of community residing subjects carried on Kerala state as 11.67, the incidence rate per 1000 person-years. The incidence rates were much higher than that reported from rural north India, comparable with that reported from China, and marginally lower than that reported from the western world.

Similarly, APOE ε4 allele frequencies have been estimated in North India by three investigators as 0.127 (Hallman et al., 1991), 0.07 (Ganguli et al., 2000) and 0.19 (Chandak, Sridevi, Vas, Panikker, & Singh, 2002) and a single report has been published from Southern India (Bharath S et al., 2010). Hallman et al published the association of APOE allele distribution with cholesterol levels through a large population study comprising of nine populations across the globe (Hallman et al., 1990). However, this group employed APOE phenotyping using serum samples by means of isoelectric focusing. Indian cohort

comprised of 142 samples in which the allele frequencies were 0.046 (APOE ε2), 0.827(APOE ε3) and 0.017 (APOE ε4). The first report of this genetic association with dementia from the Indian subcontinent was that of the Indo-US cross national dementia study (Ganguli M et al., 2000) which has shown that the allele frequency of APOE ε4 was significantly lower in North Indian (Haryana) population compared to Pennsylvania (US); 0.07 versus 0.11. The strength of association in terms of age adjusted odds ratios for AD among carriers of APOE ε4 versus non-carriers were 3.4 (Indian) and 2.3 (US). The association of APOE ε4 polymorphism with AD and VD has been investigated in a rural North Indian population (Luthra K et al., 2004), and reports show that the frequency of APOE ε4 allele was significantly higher in AD (n=29) and VD (n=25) compared to controls (n=76) with $p<0.001$. The risks of developing AD or VD were 4.4 and 3.7 times higher, respectively, even in the presence of a single APOE ε4 allele. These reports show that the increased risk of developing AD or VD is similar among Asian Indians with APOE ε4 compared with Caucasian population.

Kapur S et al reported the correlation of APO E status with lipid profile in AD patients for the first time in India (Kapur S et al., 2006). Although having a small sample size of 14 patients and 46 caregiver controls, the publication reveals an APOE ε4 frequency of 0.11 in controls and 0.43 in AD. Serum cholesterol and LDL levels were significantly higher in APOE ε4 carriers versus non-carriers. Tripathi M et al has investigated the demographic, medical, genetic, dietary, lifestyle and sociocultural protective and risk factors associated with Indian dementia patients through a case- control study (Tripathi M et al., 2011). The reports show that possession of APOE ε4 in homozygous/ heterozygous state significantly increases dementia risk ($p<0.001$) along with other sociocultural risk factors.

However, the first report over interaction effects of two genetic loci in influencing AD risk was published from AIIMS, Delhi (Mansoori N et al., 2010). They evaluated the contribution and interaction of APOE ε4 and IL-6-174 G/C polymorphism among AD and VD patients. The results show that APOE ε4 has a positive association with AD and VD, IL-6-174 G/C has a positive association with only VD, however, the presence of both alleles increased the risks

of having AD 13.75 fold and VD 14.7 fold respectively. This emphasise the role of interaction of genetic risk factors in influencing disease risk in our population.

In Southern India, a positive association of the APOE ε4 polymorphism in dementia was confirmed for the first time in a hospital-based case-control study at NIMHANS, Bangalore (Bharath S et al., 2010). The study comprising of 212 cases (AD (n=137), VD (n=31) & other dementia (n=44)) and 195 controls, reports a significant association of this locus with AD and VD. The risk of AD was 3.72 times and that of VD was 2.72 times in APOE ε4 carriers compared to non-carriers. The latest association study of APOE ε4 in AD with comorbid diabetes mellitus has been evaluated in a hospital based study in Bangalore (Kota LN et al., 2012). The results showed that APOE ε4 frequencies were highest among AD with comorbid DM (0.35) compared to AD without DM (0.25) and Controls (0.08) with a ratio of odds 5.68.

II.2. Clinical features of AD

Clinical features of AD include impairment of recent memory, language disturbances, and alterations of abstract reasoning, concentration and thought sequencing (executive function). The clinical diagnosis of probable AD is made based on criteria from the National Institute of Neurological and Communicative Diseases and Stroke/ Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (Appendix 1). The mean length of life following diagnosis is 8.5 years with a range of 1–25 years (Jost & Grossberg, 1995).

II.3. Clinical features of Mild Cognitive Impairment (MCI)

Initially, AD subjects often present clinically with amnestic mild cognitive impairment (MCI), which is thought to be a transition stage between normal aging and early dementia and at present, likely represents the best opportunity for pharmacologic interventions. The clinical criteria for the diagnosis of amnestic MCI are those described by Petersen et al (Petersen et al., 1999), the features of which are presented in Appendix 1.

II.4. Conversion of MCI to AD

MCI has attained increased attention in the medical community as the phase where medications could be targeted to slow down the progression of neurodegeneration. Current data suggest that conversion from MCI to AD occurs at a rate of 10–15% per year with ~80% conversion by the sixth year of follow-up; although ~5% of MCI subjects remain stable or convert back to normal state of cognition (Petersen et al., 1999). All MCI do not progress to overt dementia. The differentiation of mildly cognitively impaired subjects with and without preclinical AD is an important clinical problem. Research and therapy aims at interventions that delay progression really need to differentiate these subjects at the earliest stage with the help of several genetic, cellular and biochemical markers which are able to track disease progression.

II.5. Other Types of Dementias

Dementia is an umbrella term that is characterised by global deterioration of mental functioning and is comprised of many underlying pathologies such as FTD, LBD, PSP, CBD, VD and AD. Dementia cases are grouped into distinct diagnostic categories based on specific criteria, consensus pathologic criteria for PSP, CBD, LBD (McKeith, 1996), FTD (Neary, 1998), AD (NINCDS-ADRDA) (McKhann, 1984), and VD (Vascular Dementia) (NINDS-AIREN) (Roman, 1993) (Appendix 1).

II.6. GENETIC FACTORS ASSOCIATED WITH AD

II.6.1. Association of Apolipoprotein E with AD

The association of APOE with inherited susceptibility to AD was discovered by the concurrence of three lines of investigations. Genetic linkage studies in pedigrees with predominantly late-onset familial AD provided suggestive evidence for an AD susceptibility locus on chromosome 19q12-q13 near the APOE gene (Pericak-Vance et al., 1991). Second, analysis of CSF proteins having binding affinity to amyloid β peptide revealed that

one of the proteins was apolipoprotein E (apoE) (Strittmatter et al., 1993). Finally, histopathological evidences confirming the presence of apoE in the senile plaques of AD brain. ApoE is a polymorphic 299 amino acid protein (Mr=34,200). In humans, there are three polymorphic forms of apoE: apoE ε2 (Cys-112, Cys-158), apoE ε3 (Cys-112, Arg-158), and apoE ε4 (Arg-112, Arg-158) (Table 1). The amino acid differences at these positions are suggested to be critical as they alter the charge and structural properties of the protein, ultimately influencing the functional properties of apoE isoforms. Of these isoforms, APOE ε4 has been shown to exhibit a gene dose effect on the risk and age of onset of the disease and this association has been robustly confirmed in numerous studies and in several ethnic groups across the globe. ApoE plays a fundamental role in the maintenance and repair of neurons. However, the three isoforms differ in their abilities to accomplish these critical functions.

Table 1: Three allelic forms of APOE gene

Allele	*SNP at Codon 112*	*SNP at Codon 158*
APOE ε2	**TGC** Cys	**TGC** Cys
APOE ε3	**TGC** Cys	**CGC** Arg
APOE ε4	**CGC** Arg	**CGC** Arg

SNP at Codons 112 & 158 on APOE gene resulting in three alleles, APOE ε2, APOE ε3 and APOE ε4.

Following a series of landmark studies identifying the strong association of the APOE ε4 allele with increased AD risk and decreased age of onset and the protective role of the APOE ε2 allele (Corder EH et al., 1993, Saunders EM et al., 1993, Saunders EM et al.,

2000, Roses AD et al., 1996) numerous studies have investigated putative associations of APOE genotype with risk or progression for a wide variety of neurological disorders. Given the various proposed roles of apoE in influencing Aβ metabolism, CNS lipid homeostasis, synaptic activity, response to cellular injury, and neuroinflammation, these investigations were hypothesized to reveal strong associations with several diseases. To date, however, AD and CAA are the only neurological diseases for which the level of evidence for an association between APOE genotype and disease risk and age of onset is compelling (Verghese PB et al., 2011). APOE is believed to be linked to AD and CAA risk through isoform-dependent modulation of Aβ accumulation.

The omnipresence of apoE ε4 in almost every biochemical disturbances associated with AD confers it as the potentially pathogenic isoform while the other isoforms are regarded as rather protective/ neutral. In general population, APOE ε3 is the commonest allele (78%), APOE ε4 (14%) and APOE ε2 being the rarest (8%). Arg 112 in apoE ε4 mediates two key structural properties, domain interaction and molten globule formation (reduced protein stability), that likely contribute to neuropathology and hence predisposed to assume the pathological conformation (Morrow et al., 2002) (Weisgraber, 1990) mediating several neuropathological effects including increase in amyloid β production, potentiation of amyloid β -induced lysosomal leakage & apoptosis, enhanced proteolytic cleavage in neurons etc. However, the exact causative pathway triggered by APOE ε4 is still not completely elucidated. Several hypotheses have been put forward proposing the pathogenic mechanism on APOE ε4 allele. Of these, the most obvious one is that APOE might influence the production, distribution, or clearance of the amyloid β peptide. This has been supported by several observations such as direct interaction of APOE and amyloid β peptide, facilitating a genotype-modulated risk on age of onset of dementia (Hyslop, P& Petit, 2004). Second evidence is that in AD patient group, carriers of APOE ε4 allele have a higher amyloid β plaque burden than non-carriers, which has been proven in transgenic mouse models of AD (Schmechel et al., 1993).

Furthermore, expressing human apoE in animal models results in apoE isoform-dependent differences in Aβ accumulation (E4>E3>E2) (Holtzman DM et al., 2000, Fagan AM et al., 2002, Fryer JD et al., 2005, Bales KR et al., 2009). Whether Aβ toxicity in the brain is due to small Aβ oligomers or larger aggregates such as fibrils, or both, is unclear, though there is evidence that apoE isoforms can influence both Aβ fibril formation and the toxicity of Aβ oligomers (Trommer BL et al., 2005). Converging evidence suggests that the initial pathological feature of AD is Aβ deposition in the brain, which is estimated to begin 10–15 years prior to the onset of any clinical signs and symptoms of cognitive decline (Perrin RJ et al., 2009). Various events appear downstream of Aβ deposition in the AD pathological process, including neurofibrillary tangle formation, neuroinflammation, and neuronal/ synaptic loss. The period of AD pathological changes in the absence of clinically detectable disease has been termed “preclinical” or “presymptomatic” AD. If APOE genotype is linked to AD risk by influencing the probability of onset of Aβ accumulation, it would be expected that cognitively normal individuals at a given age would have greater brain Aβ burden in the order, ε4 >ε3>ε2. In fact, in both CSF biomarker and amyloid imaging studies, isoform dependent brain Aβ pathology (ε4 >ε3>ε2) has been reported in cognitively normal individuals aged 45–90 years (Morris JC et al, 2010). These data suggest that APOE genotype modulates AD risk by affecting the likelihood that Aβ begins to deposit, such that the timing of Aβ accumulation is shifted earlier or later in the preclinical phase depending on APOE genotype.

An alternative hypothesis concerns changes in cholesterol metabolism. Both epidemiological and direct experimental evidence in cell culture models suggest that cholesterol metabolism and APP metabolism are functionally intertwined. Finally there is a good correlation between the severity of dementia and the decrease in synaptic density in AD (Terry et al., 1991) and it has been suggested that apoE may be involved in synaptic plasticity during regeneration and repair; the ε4 allele being less efficient in this role.

According to Farrer, the APOE ε4 allele frequency in AD patients ranges from 35% to 56% and APOE ε4 homozygotes make up 7% to 18% of subjects with AD (Farrer et al., 1997). A

substantial proportion of AD patients do not bear APOE ε4 allele, so it could be stated that possession of APOE ε4 is neither necessary nor sufficient to cause AD pathology in later life. Hence, APOE genotyping may not help in diagnosing such patients. In the context of differential diagnosis AD, the frequency of APOE ε4 allele in other dementing disorders may limit or decrease the value of APOE genotyping in the diagnosis of AD. Frequency of APOE ε4 in Multi infract dementia has been estimated to be 25%, in Diffuse Lewy Body Dementia (DLBD) 35%, in Frontotemporal dementia (FTD) 29%. (Shimano et al., 1989)(St Clair, 1997)(Gustafson, Skoog, Rosengren, Zetterberg, & Blennow, 2007) in Parkinson's disease (PD) 19%, in Creutzfeldt- Jacob disease (CJD) 9% and in Progressive Supranuclear Palsy (PSP) 13%. Many study groups have analysed the diagnostic utility of APOE genotyping in different types of dementia. Clinical utility of this genetic risk factor have been a subject to many investigators around the globe (Genin, E et al., 2011) (Saunders et al., 1993)(Mayeux et al., 1998). Some of these reports are in context of neuropathological findings; correlating the APOE status with Braak Neurofibrillary Staging and amyloid plaque density thus determining the diagnostic sensitivity (Mayeux & Stern, 2012).

II.6.2. Presenilin 1 mutations in EOFAD

Positional cloning strategy has identified two loci at chromosomes 14q24.3 and 1q42.1, consistently associated with Familial AD with onset age below 65 years (presenile AD) that segregate in an autosomal dominant pattern. The genes were named Presenilin 1 & 2 which harbour most of the pathogenic mutations linked to EOFAD (Sherrington et al., 1995). Most of the reported PSEN 1 mutations are base pair substitutions. Only a couple of deletions and insertions and two splicing defect mutations have been reported in the PSEN1 gene (Cruts & Van Broeckhoven, 1998). The pathogenic mutations on all the three genes associated with EOFAD such as APP, PSEN 1 and PSEN 2 lead to increased Aβ-42 production. This may be due to the interconnections of APP metabolism with PSEN mutations. Presenilins probably constitute the active site of γ secretase complex that generate amyloidogenic fragments of Aβ. PSEN1 mutations may disturb protein interactions in the complex through subtle conformational alterations (Kowalska, Florczak, Pruchnik-Wolinska, Hertmanowska,

&Wender, 1998) thereby diverting the APP proteolysis towards more amyloidogenic pathway leading to amyloid plaque neuropathology (Esler & Wolfe, 2001).

II.6.3. Association of MAPT H1/H2 Haplotype with AD

A haplotypic association implies there is a genetic variability either in the amount of expression of the protein or in the alternative splicing of the protein that contribute to disease risk. The microtubule associated protein tau (MAPT) locus has long been associated with most of the sporadic neurodegenerative tauopathies including FTD, PSP, CBD, PD and AD. However, the molecular mechanisms behind the genetic association have only now started to emerge. The genomic architecture in the region spanning MAPT is highly complex, and includes a ~1.8 Mb block of linkage disequilibrium (LD). The region is divided into two major haplotypes, H1 and H2, defined by numerous single nucleotide polymorphisms and a 900 kb inversion which suppresses recombination. Fine mapping of the MAPT region has identified sub-clades of the MAPT H1 haplotype which are specifically associated with neurodegenerative disease conferring disease risk. Despite having tauopathy as a defining lesion, reports of association between AD and genetic variants at the MAPT locus are inconsistent. While MAPT H1 (Gerrish A et al., 2012) haplotype or H1c subhaplotype (Myers AJ., 2005, Myers AJ., 2007) showed association with AD risk in some studies, others failed to detect association with H1 (Abraham R et al., 2009) other MAPT variants (Mukherjee O et al., 2007). In Caucasians, H1 homozygosity has shown robust association with neurodegenerative tauopathies with a frequency of H1 homozygotes being 95% in patients (Pittman et al., 2004). Allen et al conducted the largest study on haplotypic variation at the MAPT locus in 9,814 LOAD cases and 11,550 controls and found robust and replicable association of the MAPT H2 haplotype with reduced risk of LOAD or, equivalently, increased risk of LOAD with the MAPT H1 haplotype- in two independent cohorts from US (Allen et al., 2014).

II.7. BIOMARKERS OF AD

The term 'Biomarker' refers to any measurable indicator that is used to assess the risk or presence of disease. For progressive neurodegenerative diseases like AD, biomarkers facilitate the reliable diagnosis of the disease perhaps in the very early or even in presymptomatic phase. Since the neuropathological changes in the brain of AD patients likely precede symptoms by years or decades, it will be well optimal to treat the disease as early as possible, which may slow down the progression. Hence, biomarkers of AD play a pivotal role in the emergence of next generation therapies. According to the proposed guidelines by a consensus group ("Consensus report of the Working Group on: "Molecular and Biochemical Markers of Alzheimer's Disease". The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and the National Institute on Aging Working Group," 1998) on biomarkers of AD, the key features of an ideal biomarker are,

1. It should detect a fundamental feature of neuropathology.
2. It should have a diagnostic sensitivity and specificity for AD above 80% for distinguishing AD from other dementias.
3. It should be reliable, reproducible, non-invasive, simple to perform and inexpensive.
4. It should be confirmed by at least two independent studies by qualified investigators with the results published in peer reviewed journals, and validated in neuropathologically confirmed cases.

In 2003, A wide range of AD biomarkers were categorised into "Feasible, core", "Feasible, non-core", and "Uncertain feasibility" by a working group commissioned by NIA (National Institute on Aging) as a part of its Alzheimer's Disease Neuroimaging Initiative (ADNI) (Frank et al., 2003) and the biomarker candidates were associated with the key neuropathological features of AD such as β amyloid plaques and neurofibrillary tangles (NFT). Current AD biomarkers comprise of Biochemical (CSF/ blood), Neuroanatomical (CT/ MRI scan), Metabolic (PET), Genetic (APP, PSEN 1, PSEN 2, APOE ε4) and Neuropsychological

(neuropsychological batteries assessing cognitive functioning) markers that track the state of progression of the disease.

II.7.1. Causative pathways- a spectrum of diverse hypotheses for AD pathogenesis

Since AD is a complex disease, several causative pathways have been put forward to explain pathogenesis (Table 2). Each hypothesis portrays a central event as the primary trigger that leading to all other biochemical disturbances and peripheral symptoms comprising neurodegeneration and memory loss.

Table 2: Causative Hypotheses for AD

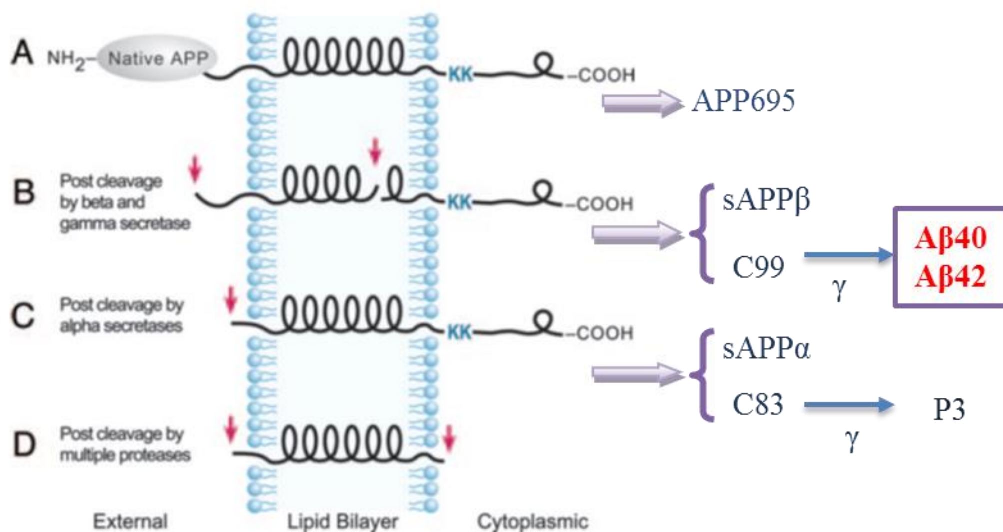
Sl No	Hypothesis	Statement	Reference
1	Neurofibrillary Tangle Hypothesis (Braak Staging hypothesis)	Hyperphosphorylation of tau and aggregation into neurofibrillary tangles inside neurons is the leading event in AD pathogenesis and clinical stages of AD could be correlated with different Braak Stages.	(Braak & Braak, 1991)
2	Amyloid Cascade Hypothesis	Brain amyloidosis is the central event leading to neurodegeneration in AD.	(J. A. Hardy & Higgins, 1992)
3	ApoE/ Lipid Recycling Cascade Model	Alterations in lipid homeostasis could serve as the common denominator for apoE and amyloid β dysfunctions in AD.	(Poirier, 1994)
4	Oxidative Stress Hypothesis	Increased lipid peroxidation and decreased polyunsaturated fatty acids in the AD brain lead to increase in oxidative stress which triggers the pathogenesis of neuronal death in AD.	(Markesbery, 1997)
5	Immune Hypothesis	The neurons are stimulated by Aβ deposits, oxidative stress, hyperphosphorylated tau or pathogenic microorganisms to signal for help and to attract MM's across BBB through chemokines and cytokines.	(Fiala et al., 1998)
6	Modified Amyloid Hypothesis	Oligomeric amyloid-*β* (A*β*) accumulation and chronic inflammation lead to neuronal demise.	(J. Hardy & Selkoe, 2002)
7	Cell Cycle Re-entry Hypothesis	The cell cycle hypothesis proposes that either mitogenic signalling, or cell cycle control, or both, are deranged with respect to neurons within the brain of individuals with AD which make the terminally differentiated neurons to reenter into cell cycle and undergo cell death termed aposklesis.	(Bowser & Smith, 2002)
8	Two Hit Hypothesis	Neurons are damaged by oxidative stress in combination with cell-cycle dysregulation.	(Zhu, Raina, Perry, & Smith, 2004)
9	Oligomeric A*β* Hypothesis	Soluble oligomeric aggregates of A*β* rather than A*β* monomers or insoluble fibrils may be responsible for neuropathology in AD brain.	(Glabe, 2006)
10	GSK3β Hypothesis	Over-activity of GSK3β accounts for memory impairment, tau hyper-phosphorylation, increased β-amyloid production and local plaque-associated microglial-mediated inflammatory responses; all of which are hallmark	(Hooper, Killick, & Lovestone, 2008)

		characteristics of AD.	
11	AD is a form of type III diabetes	AD represents a form of diabetes that selectively involves the brain and has molecular and biochemical features that overlap with both Type 1 & Type 2 diabetes mellitus.	(de la Monte & Wands, 2008)
12	BBB Dysfunction Hypothesis	APOE ε4 first damages cerebrovasculature, leading to the leakage of Blood-Brain Barrier.	(Zlokovic, 2011)

II.7.2. The Amyloid Cascade Hypothesis

Of the several hypotheses put forwarded on AD pathogenesis, the Modified Amyloid Cascade Hypothesis (J. Hardy & Selkoe., 2002) has gained much attention as well as support by the scientific community which states that an imbalance between the production and degradation/ clearance of amyloid β in the brain is the initiating event in AD, subsequently followed by several downstream events such as NFT formation, oxidative damage, synaptic dysfunction and ultimately leading to neurodegeneration. Aβ is generated from the type I transmembrane glycoprotein APP by concerted proteolysis by a group of enzymes comprising α, β & γ secretases (Selkoe, 1999) (Figure 3). β-secretase generates carboxyl-terminal fragments (CTFs) of APP, which then get cleaved by γ-secretase to release Aβ monomers.

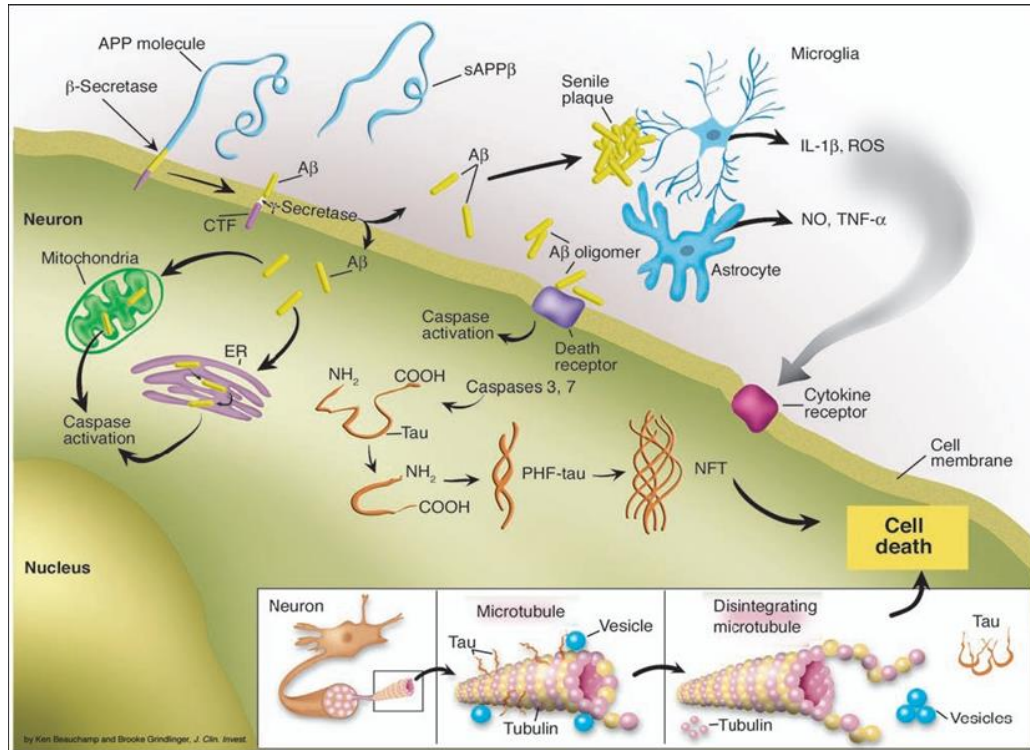
Figure 3: APP Proteolysis by Secretases



Native APP (A) is subjected to proteolytic cleavage by β secretase results in C99 fragment (B) which is cleaved by γ secretase to generate amyloidogenic fragments of Aβ (shown in Red). However, APP cleavage by α secretase (C) results in non amyloidogenic C83 and P3 (Figure adopted and modified from (Blennow & Hampel, 2003)).

The mechanisms that drive pathogenic misfolding and aggregation of these Aβ species are still not completely understood. Soluble native Aβ monomers aggregate and misfold giving rise to oligomers, protofibrils, fibrils and eventually insoluble plaques (Walsh, Klyubin, Fadeeva, Rowan, & Selkoe, 2002). It has been well evidenced that Aβ42 variant (which makes up only less than 10% of the total Aβ) is the most amyloidogenic form that is most prone to aggregate compared to other more abundant species such as Aβ40. The extracellular Aβ oligomers may activate caspases through activation of cell surface death receptors (Blennow K, 2004). Alternatively, intracellular Aβ may activate caspases through a process that involves ER (Endoplasmic Reticulum) stress or mitochondrial stress (Ken Beuchamp and Brooke Grindinger, 2008). One of the consequences of caspase activation is cleavage of tau, which favours conformational changes characteristic of paired helical filaments (PHF-tau). Progressive accumulation of tau leads to cytoskeletal disruption, failure of axoplasmic and dendritic transport, and subsequent loss of trophic support that culminates in neuronal death (Figure 4).

Figure 4: Downstream pathogenic events initiated by amyloidosis in neurons



Proteolytic processes contribute to the amyloidosis in brain. Toxic Aβ oligomers trigger several downstream pathological processes such as activation of caspases, overload stress on endoplasmic reticulum (ER), mitochondrial dysfunction, hypometabolism, hyperphosphorylation of microtubule associated protein tau and cytoskeletal disruption leading to neuronal death. Figure adopted from (Ken Beuchamp and Brooke Grindinger, 2008).

The extracellular amyloid deposits in senile plaques also trigger reactive glial changes and neuroinflammation that can also contribute to neuronal loss through production of reactive oxygen species (ROS), NO, and pro-inflammatory cytokines such as TNF-α and IL-1β.

II.7.3. Biochemical markers of progression in AD based on brain amyloidosis

II.7.3.1. Amyloidogenesis in brain

Amyloid plaques are relatively insoluble dense cores of 5-10 nm thick amyloid fibrils with a surrounding 'halo' of dystrophic neurites, reactive astrocytes and activated microglia. The main proteinaceous component of plaques is Aβ peptide. Once released, the Aβ peptide may exist extraneuronally in solution and can be detected in CSF and plasma which reflects the amyloidogenesis in brain. This makes different Aβ species highly promising candidate biomarkers for AD (Blennow & Hampel, 2003). On the basis of brain amyloidosis, several core candidate biomarkers for AD have been proposed and they include APP isoforms, BACE1 (β-APP Cleaving Enzyme-1) protein, Aβ isoforms (Aβ42 & Aβ40) and autoantibodies against Aβ peptide. Autoantibodies against Aβ have been detected in serum and were found to be significantly different between AD patients and controls (Gustaw et al., 2008). However, their potential as biomarker is still need to be explored.

II.7.3.2. Aβ isoforms in CSF

CSF is in direct contact with brain interstitial fluid and therefore being the most obvious source of AD biomarkers. CSF Aβ42 has been reported to the most important feasible-core biomarker for AD and has been confirmed through more than 30 studies published so far, most of which have found nearly 50% decrease in CSF Aβ42 levels in AD patients compared to controls. The mean sensitivity and specificity to discriminate between AD and normal aging were >85% (Blennow, 2004). The decreased CSF Aβ42 level in AD is caused by the increased deposition of Aβ42 as senile plaques in the brain (Blennow, 2004). Plaque burdening in the brain can be correlated to decreased diffusion of Aβ42 into CSF; accordingly, high plaque density in the neocortex and hippocampus on autopsy (Strozyk, Blennow, White, & Launer, 2003) and high retention of Pittsburgh Compound B (PIB) in PET scans (Fagan et al., 2006) being associated with marked reduction in CSF Aβ42 levels well supports the statement.

However, some reports have also found a marked reduction in diseases without amyloid pathology such as CJD (Otto et al., 2000), ALS (Sjogren et al., 2002), and multiple system atrophy (Holmberg, Johnels, Blennow, & Rosengren, 2003). This suggests that there may be other reasons for low CSF Aβ42 in addition to plaque burdening. These factors may include formation of Aβ42 oligomers that escape ELISA detection (Stenh et al., 2005); association with other molecules that blocks access to epitopes recognised by detection antibodies (Kanekiyo et al., 2007). CSF level of Aβ42 together with total tau (t-tau) is very useful as predictive biomarkers of preclinical AD. A summary of studies on CSF biomarkers in AD is given in Table 3.

Table 3: Summary of studies on CSF biomarkers in AD

Reference	***Study design***	***Change reported with progression***	***Comment***
(Andreasen et al., 1999)	Longitudinal MCI-control study	Low CSF Aβ42, high CSF T-tau	Sensitivity 88%, specificity 80%
(Zetterberg, Wahlund, & Blennow, 2003)	Longitudinal MCI study	Low CSF Aβ42, high CSF T-tau, high CSF P-tau181	Sensitivity 68%, specificity 97%,
(Skoog et al., 2003)	Population-based longitudinal cohort study	Low CSF Aβ42	Low levels of CSF Aβ42 predicted progression to dementia
(Hampel et al., 2004)	Longitudinal MCI-AD-control study	Low CSF Aβ42, high CSF T-tau	Sensitivity 59–83%, specificity 90–100%
(Herukka, Hallikainen, Soininen, & Pirttila, 2005)	Longitudinal MCI-study	Low CSF Aβ42, high CSF T-tau, high CSF P-tau181	Low levels of CSF Aβ42 predicted progression to AD
(Hansson et al., 2007)	Longitudinal MCI-study	Low Aβ42/Aβ40 ratio	Sensitivity 87%, specificity 78%
(Li et al., 2007)	Longitudinal control study	High T-tau/Aβ42 ratio	Individuals with high ratio had higher APOE ε4 allele frequency and higher risk of progression to MCI
(Bouwman et al., 2007)	Longitudinal MCIstudy	Low CSF Aβ42, high CSF T-tau	Patients with abnormal values at baseline had higher risk of developing AD
(Gustafson et al., 2007)	Population-based longitudinal cohort study	Low CSF Aβ42	Low levels of CSF Aβ42 predicted cognitive decline
(Stomrud, Hansson, Blennow, Minthon, & Londos,	Longitudinal cohort study of healthy controls	Low CSF Aβ42	Low levels of CSF Aβ42 predicted cognitive decline

2007)			
(Ringman et al., 2008)	Genetic case–controlstudy	Low CSF Aβ42, low Aβ42/Aβ40 ratio, high CSF T-tau, high CSF P-tau181	Asymptomatic FAD mutation carriers had abnormal CSF biomarkers alreadyin their 30 years of age
(Shaw et al., 2009)	Longitudinal multi-center study	Low CSF Aβ42, high CSF T-tau, high CSF P-tau181	CSF T-tau/Aβ42 had a sensitivity of 89% for MCI cases with progression toAD
(Brys et al., 2009)	Longitudinal MCI-control study	Low CSF Aβ42, low Aβ42/Aβ40 ratio, high CSF T-tau, high CSFP-tau231	Sensitivity 68–86%, specificity 60–91%
(Mattsson et al., 2009)	Longitudinal multi-center study	Low CSF Aβ42, high CSF T-tau, high CSF P-tau181	Sensitivity 83%, specificity 88%

Adopted data from (Hampel et al., 2010)

II.7.3.3. Aβ Isoforms in Plasma (Brain- Plasma Aβ Flux)

Plasma biomarkers are especially useful due to their minimally invasive nature. However, in the case of Aβ isoforms, the results are quite contradictory in the plasma of AD patients. Normal proteolytic processing generates Aβ in neurons which is secreted about 12 h later into the CSF, and then excreted through the blood-brain barrier 24 h later into blood; the process termed 'Aβ clearance', and finally degraded in the reticulo-endothelial system (Shoji et al., 1992). Aβ levels are regulated in strict equilibrium among the brain, CSF, and blood. However, CSF Aβ levels do not appear to be correlated with plasma Aβ levels in individual patients (Vanderstichele et al., 2000). Animal studies indicate that Aβ can pass between the CSF and plasma compartments, and get deposited in the walls of blood vessels as congophilic angiopathy (Ghersi-Egea et al., 1996). Some patients with AD may also incidentally have Cerebral Amyloid Angiopathy (CAA). This is further evidenced by studies employing APP transgenic mice reporting that the cerebral amyloid load did not correspond to variations in plasma Aβ levels (DeMattos, Bales, Cummins, Paul, & Holtzman, 2002) and that peripheral administration of high affinity binding compounds to Aβ led to increased Aβ efflux from the brain into plasma (DeMattos et al., 2002).

Some investigators have reported high concentrations of plasma Aβ species in AD, although with a broad overlap between patients and controls; whereas most of the reports show no

change (Irizarry, 2004). Studies have shown that plasma Aβ (1–42) and Aβ (1–40) levels can be elevated, reduced or even unchanged in AD versus control patients (Cedazo-Minguez A. et al, 2010, Zetterberg H et al, 2010). Some longitudinal studies have reported high plasma Aβ42 in the very early phases of AD (Mayeux et al., 2003)(Pomara, Willoughby, Sidtis, & Mehta, 2005). In contrary, van Oijen and co-workers have reported high Aβ40 and low Aβ42 in AD patients' plasma (van Oijen, Hofman, Soares, Koudstaal, & Breteler, 2006) which is on strong agreement with the later findings of Graff Radford (Graff-Radford et al., 2007) stating that the association between low plasma Aβ42/Aβ40 ratio and preclinical AD is weak. This discrepancy among results may in part, may be due to some analytical artefacts. Aβ being a highly hydrophobic peptide exhibits binding affinity to plasma albumin (Kuo et al., 1999), LRP1 (Sagare et al., 2007) and even polypropylene walls of test tubes/ ELISA wells. This may reduce the availability of the peptide on measurement. Additionally, Aβ oligomers escape the classical ELISA detection in which the antibodies bind only to monomers (Stenh et al., 2005). These factors act as possible confounders that may vary from assay to assay and this could explain some of the contradiction in the results.

II.7.4. Biochemical markers of progression in AD based on tau pathology

II.7.4.1. Mechanism of tau mediated neuropathology

Neuronal microtubules functions in axonal transport and maintenance of structural and synaptic integrity of neurons and are critically involved in supporting neuronal viability. Tau protein stabilises microtubules; however detach from the microtubular network when they are hyperphosphorylated. Tau involve in neurodegeneration when its normal intracellular location is altered. Unlike Aβ, which is partly hydrophobic, tau is hydrophilic and highly soluble protein and is natively unfolded. Aggregation of hyperphosphorylated forms of tau results from complex interaction of lipid-derived poly anionic molecules at the core microtubule binding domain, and this initiates PHF assembly at a site on plasma membrane, named nucleation site (Karla, I, 2009). The abnormal localization of tau in the plasma membrane could be a triggering event in AD.

II.7.4.2. CSF tau as biomarker

The disintegration of axonal microtubules is a hallmark of AD, and a longstanding question has been whether the accumulation of insoluble tau paired helical filaments (PHF) or Aβ plaques initiates neurodegeneration in AD brain. Although initial research had focused on the role of Aβ and tau individually, recent evidence, including data demonstrating that amyloid pathology can up-regulate tau pathology (Gotz, Chen, van Dorpe, & Nitsch, 2001)(Lewis et al., 2001) defines a signalling pathway that leads from Aβ through tau directing pathogenesis in AD brain (Lee, Goedert, & Trojanowski, 2001). It has been demonstrated that microtubules are primary, tau-dependent targets of Aβ, and suggest that oligomeric Aβ and p-tau underlie the detrimental neurodegeneration observed in AD before the accumulation of fibrillar forms in senile plaques and neurofibrillary tangles. CSF total tau levels increase slightly with aging. However, a large scale multicentre study has reported that CSF tau levels show a 3-fold greater increase in AD patients than in normal controls (Shoji et al., 2002). It is thought that the rise in CSF total tau is related to degeneration of axons and neurons and to the degree of severity of the disease. Tau levels might also be a prognostic marker with a good predictive validity for conversion from MCI to AD, because high CSF tau level has been found in 90% of MCI cases that later progressed to AD, but not in cases with stable MCI (Blennow K et al, 2004).

Mostly, investigations on CSF tau levels as AD biomarker have been done in combination with CSF Aβ42. The first large-scale longitudinal study of CSF Aβ40, Aβ42, and total tau as biomarkers of AD was reported by a Japanese study group in 1998 in which AD index was evaluated as CSFAβ40/Aβ42 X total tau, which showed a diagnostic sensitivity of 71% and specificity of 83% in AD (Kanai et al., 1998). It has been evidenced that the combination assay of these biomarkers improved biochemical discrimination of AD patients from other dementias (Hulstaert et al., 1999). Most of the combination studies reported decreased Aβ42 and increased tau in CSF from AD patients. A metaanalysis of 17 reports on CSF Aβ42 and 34 reports on CSF total tau has showed a final diagnostic sensitivity of 92% and specificity of 89% in AD (Sunderland et al., 2003). In the case of phosphorylated tau, assay systems for p-

tau231, p-tau181, and p-tau199 have been internationally standardized and measures of p-tau 181 is used widely used as a biomarker of progression from MCI to AD.

II.7.4.3. Plasma tau as biomarker

Compared to CSF tau levels, circulating tau levels are less studied. Methods to analyze tau in blood are only at the experimental beginning (Hampel et al., 2010). Many research groups have recently finished validating and standardising the outputs of Blennow's research group on the predictable changes in CSF levels of tau and amyloid beta (Aβ) and the risk/ progression of AD as part of ADNI (Sparks et al., 2012)(de Leon et al., 2007). However, none of these reports have focused on circulating tau levels as an AD biomarker. There is one report of high levels of circulating antibodies to tau, but not to p-tau in human blood (Rosenmann, Meiner, Geylis, Abramsky, & Steinitz, 2006). Initial studies focused on measuring tau in the blood of individuals with AD were discouraging. It was demonstrated that plasma has immunoreactivity to tau by means of sandwich ELISA (Ingelson et al., 1999). However, it was quantifiable in only 1 of 16 individuals with AD and 7 of 15 normal controls. It was concluded that plasma tau levels were not increased in AD and circulating tau levels cannot be utilized diagnostically as a biomarker. As part of the validation of employed ELISA methods to quantify circulating tau, Larry sparks et al concluded that substantial levels of tau are found in circulation and they are significantly lower in plasma of AD patients compared with controls (Sparks et al., 2012). Based on their data, the circulating levels of tau in elderly normal controls are 2-3 times greater than encountered in the CSF. Reduced tau in plasma of AD patients would be the result of reduced clearance of excess central tau into peripheral circulation. An individual progressing through MCI to AD may bear low levels of tau in plasma. There are reports showing that decline in plasma tau levels could be correlated with the decline in cognitive performance in AD.

II.7.4.4. Matrix Metalloproteinases and AD

Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-containing, calcium dependent endoproteases capable of degrading components of the extracellular matrix. They are

secreted from different cell types including astrocytes, neurons, microglia, leukocytes and macrophages, and their target compounds include extracellular matrix constituents such as collagen, gelatin, fibronectin, laminin, elastin and proteoglycans. MMPs are reported to have the property to degrade Aβ protein *in vitro* (Backstrom, Lim, Cullen, & Tokes, 1996). MMP expression has been investigated in postmortem brain tissue from AD patients and was shown that MMP-9 is localized to neurons of the hippocampus which is capable of degrading Aβ (Backstrom et al., 1996). Its expression is elevated in neurons, neurofibrillary tangles, senile plaques and in the vascular wall in postmortem AD brain tissue (Asahi et al., 2001). Significant increases in MMP-9 expression in plasma of AD patients has been detected which were not shown to be associated with APOE genotypes (Lorenzl et al., 2003). It has been reported that APP fragments can stimulate MMP-9 expression in cultured microglia cells (Gottschall & Deb, 1996) and induce MMP-9 release from human monocytes (Chong, Sung, Shin, Chung, & Suh, 2001). However, plasma MMP-2 levels were unchanged and this reflects its more constitutive expression, whereas MMP-9 levels were found to be more responsive to reactive oxygen species and inflammatory cytokines (Paul et al., 1998). The activity of MMPs is dependent on activation of latent proforms, and additionally by endogenous tissue inhibitors of metalloproteinases (TIMPs) (Brew, Dinakarpandian, & Nagase, 2000). Nevertheless, there was a significant correlation of both TIMP-1 and TIMP-2 levels with MMP-9 in the AD subjects. However, the association of TIMP1 levels with different types of dementia varies inconsistently. Expression of TIMPs was reported to be increased in AD postmortem brain tissue (Peress, Perillo, & Zucker, 1995). It is therefore possible that increased circulating levels of MMP-9 in AD may contribute to the endothelial pathology of AD patients.

II.8. PERIPHERAL CELLULAR MARKERS OF AD

A blood-based peripheral cellular marker for AD is a 'Holy Grail' for early diagnosis and management of the disease since, plasma based markers may not be specific for AD pathology because of the multiple mechanisms that are common to almost every neurodegenerative disease including inflammation, oxidative stress and aberrant lipid metabolism. Markers of oxidative stress are reported to be reflected in peripheral blood mononuclear cells of AD patients as single stranded breaks (SSB) on cellular DNA (Morocz et al., 2002). Oxidative damage can originate from endogenous sources, as a result of leakage of reactive oxygen species (ROS) from mitochondria during respiration. The relatively neutral superoxide anion radical is converted by superoxide dismutase into H_2O_2, which can take part in the Fenton reaction with copper or iron ions as catalysts, to produce the very reactive hydroxyl radical (.OH). Major (.OH)-induced oxidative products of purines and pyrimidines are detected in AD neurons. Reflections of oxidative stress on peripheral cells may evolve as a blood-based marker for the disease.

AD patients are reported to have functional defects in phagocytosis of Aβ in peripheral blood mononuclear cells (PBMNCs) (Fiala, Cribbs, Rosenthal, & Bernard, 2007). Monocyte/macrophages (MM's), the effector cells of the innate immune system in AD brain were reported to have the ability to disrupt the tight junction protein ZO-1 in microvessel's endothelium and penetrate the blood-brain barrier (BBB), infiltrate perivascular spaces and neuritic plaques and phagocytize Aβ (Fiala et al., 2002). Immunohistochemistry on brain sections revealed CD68-positive macrophages infiltrated AD frontal lobe and hippocampus. However, this was not found to be associated with reduction in Aβ plaques density, and was suggested that Alzheimer's Disease Monocytes and Macrophages (ADMM) were inefficient in clearing Aβ deposits *in vivo*. Milan Fiala's research group at UCLA have conducted experiments on cultured monocytes exposed to Aβ and immunostained with CD 68 and evidenced that ADMM have impairments in either internalisation or clearance of Aβ and that ADMM had poorer differentiation and more degree of apoptosis compared to MMs of cognitively normal controls on treatment with exogenous Aβ. Control monocytes ingested

significantly more Aβ and were localised into lysosomal compartments for degradation. However ADMM showed only weak surface binding of Aβ. Moreover, these cells do not differ in their efficiency in engulfing bacteria, proving that phagocytic dysfunction is specific for Aβ. In elucidating the molecular basis of the innate immune response, they analysed differential expression of two target genes that play important roles in Aβ phagocytosis such as β-1,4-mannosyl-glycoprotein 4-β-N- acetyl glucosaminyl transferase (MGAT-III) and toll-like receptors (TLRs) (Fiala, Liu, et al., 2007). Microarray analysis revealed that both the genes were strongly down regulated during Aβ phagocytosis in mononuclear cells of AD patients compared to cognitively normal control subjects.

Immunomodulatory properties of curcuminoids have been tested in ADMM and they have shown that a specific defect of Aβ phagocytosis by AD macrophages may be improved in approximately 50% of AD patients by curcuminoid treatment *in vitro* (L. Zhang et al., 2006). Immunomodulation on improvement in Aβ internalisation had been shown to be mediated through upregulation of MGAT3 gene transcription in AD patients on treatment with Bis-demethoxycurcumin (BDC) (Fiala, Liu, et al., 2007) Thus, BDC has been proposed to correct immune defects of AD patients and provide a previously uncharacterized approach to AD immunotherapy.

The dynamically growing field of biomarker assignment to progressive neurodegenerative diseases such as AD demands additional data on added value of combinatorial assay systems on different ethnic groups. Population based prospective studies can contribute much to this prospect and thus lead to establish the utility of these biomarkers in aiding early diagnosis and start interventions that arrest/ delay disease progression. No curative treatments are available for Alzheimer's disease now. The medications provided may either modify or alleviate the symptoms, but not halt the progression of the disease. Regardless of the lower prevalence, neither the association of genetic risk factors/ biomarkers in assisting early diagnosis of AD has been investigated in our population. This study has been focused to explore the various Genetic, Biochemical as well as cellular factors with the objective of examining the combination of these biomarkers in order to set a predictability profile of AD.

III. RATIONALE AND OBJECTIVES OF THE STUDY

III.1. RATIONALE OF THE STUDY

Dementia is seriously disabling for those who have it and is often devastating for their caregivers and families. AD is the commonest form of neurodegenerative dementia in the elderly. Slow pace of disease progression and diverse heterogeneity in overlapping symptoms make early diagnosis quite difficult and this results in delaying the phase at which medications actually start for a patient. Further, current medications that target only the peripheral symptoms, but not the causative pathways and can only delay/ arrest the progression, making it impossible to revert back to normal cognition. Such a devastating disease affecting an individual collapses his family economically and emotionally. The high global prevalence, economic impact of dementia on families, caregivers and communities, and the associated stigma and social exclusion present a significant public health challenge. These factors place dementia a public health priority.

The genetic background of AD is much heterogenous and strongly depends on the form of the disease such as familial or sporadic. The descendants/ relatives of patients coming to memory clinic are in panic as they also get affected by this disease in future. Since the penetration of pathogenic mutations is 100% in familial cases, identification of EOFAD cases at the earliest is critical to start medications. It is equally important that to give family counselling to make them aware on genetic risk which help them to be prepared for the awaiting future. The risk conferred by APOE polymorphism on sporadic cases needs to be delineated in every ethnic group since it is subjected to variations with ethnic diversity.

Even if the complete molecular pathogenesis of AD has not been definitively elucidated, approaches for establishing disease-specific biomarkers are successful in detecting the stage of disease progression. Since AD is a disease with is no cure by therapeutics, early detection and starting of medication is the only way to halt progression. CSF Amyloid β-42 and tau are the two core biomarkers for AD that reflect the stage of progression. However, less invasive, blood based biomarkers need to be established for easy screening and identification of affected individuals. Among these markers, plasma Amyloid β-42 and tau are

extensively studied, and contradictory associations are reported worldwide. Peripheral cellular markers are established based on the Immune Hypothesis of AD. A combinatorial approach using different biomarkers may lead to more effective differentiation of AD patients from subjects with normal cognition even in their preclinical phase. This study is aimed at finding out correlations of genetic, biochemical and peripheral cellular factors of AD/ MCI that serve as markers to distinguish diseased individuals from normal controls.

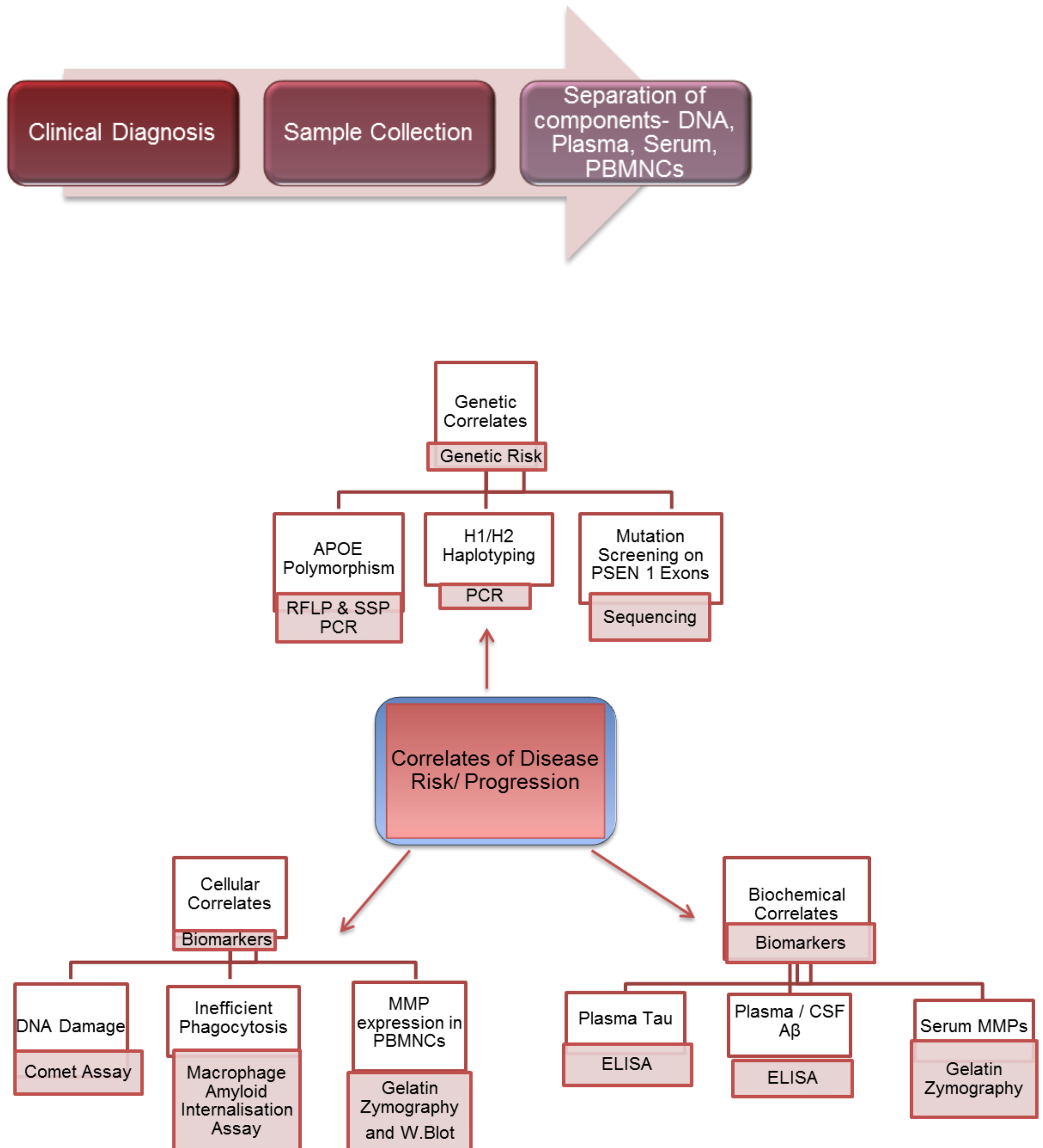
III.2. THE BROAD OBJECTIVES OF THE STUDY

1. To analyse the distribution of genetic risk factors in the study cohort
    - APOE polymorphism
    - MAPT (H1/H2) haplotypes
    - Mutations on PSEN 1 (exon 7 and 12)
2. To determine less invasive, blood-based biochemical markers of AD
    - Plasma Aβ
    - Plasma Tau
    - Serum MMPs
3. To study the peripheral cellular markers of AD in the study population
    - DNA damage on PBMNCs
    - Phagocytic dysfunction on PBMNCs and differentiated macrophages

4. To develop a combination of Genetic, Biochemical and Cellular markers based on their respective significance in contributing to disease risk which may prove to be a molecular signature of AD in the South Indian population.

IV. MATERIALS AND METHODS

IV.1. Design of Study



IV. 2. Materials

IV.2.1. Study samples

Blood samples (6 ml) were separated into plasma and sera. DNA was isolated from buffycoat. The study materials were properly labelled and stored at -80^{0}C till use.

IV.2.2. Chemicals

Oligonucleotide primers for RFLP & SSP PCR (APOE), cell culture media RPMI1640, antibiotics, Trypan blue, Giemsa stain, Low Melting Point Agarose, Ethidium Bromide, DiMethylSulphoxide (DMSO) and all the routine chemicals used for the preparation of buffers and for experiments were purchased from Sigma Aldrich (St. Louis, MO, USA). Consumables were purchased from Tarsons and Axygen, India. Heparinised vacutainers and cryovials were used for blood collection and storage of biological samples. DNA isolation kit was purchased from ArchivePure (5 PRIME), GoTaq Flexi PCR reagents were purchased from Promega Corporation (Madisson, USA). HhaI Restriction Enzyme and BSA were purchased from Invitrogen, CA, USA. Oligonucleotide primers for PCR (PSEN & H1/ H2) were designed and supplied by Metabion (Martinsried, Germany). MSP1 digested PUC18 marker and 100 bp DNA Ladder were purchased from Fermentas (Burlington, Canada). DNA sequencing kit was purchased from Big dye terminator (Applied Biosystems, Foster City, CA, USA).

Human Total Tau ELISA Kit (KHB0041), Aβ 1-42 ELISA kit (KHB 3441) and lysosomal tracker dye Lysotracker Red were bought from Life Technologies (CA, USA), Plasma Aβ 1-42 ELISA kit was purchased from USCN Life Science Inc. (China). Gelatin, Sodium azide and calcium chloride were purchased from Merck (India) for Gelatin Zymography. Antibodies for MMP-2 and MMP-9 were purchased from Abcam (Cambridge, MA, USA). Secondary anti rabbit antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). Chemiluminescent reagents used for western blots and Nitrocellulose membrane were purchased from Thermo Scientific (Rockford, IL, USA).

Culturewares were ordered from GenAxy (Solan, HP, India) and BD Falcon (BD Biosciences, US). Rosettesep Human Monocyte Enrichment Cocktail was purchased from Stem Cell Technologies (Vancouver, Canada). HiLyte Flour 488-labeled Aβ was bought from Anaspec (CA, USA). Comet Slides were ordered from Bluestar (India). Curcumin C3 complex was supplied generously from Sabinsa Corporation (Piscataway, NJ).

IV.3. Equipments

PCR Thermal Cycler (Biorad, USA), ELISA plate reader (Bio- Tek instruments USA), Electrophoresis unit (Biorad, USA)), Waterbath and Rocking Waterbath (Julabo, Germany), Weighing balance (Sartorius, Germany), pH meter (Eutech, USA), Magnetic Stirrer (Schott, Germany), Good precision Pipettes (Eppendorf, Hamburg, Germany), $^{-}80^{0}$C Freezer (New Brunswick Scientific, Edison, NJ), $^{-}20^{0}$C Freezer (Vest frost, Falkevej, Denmark), Cooling centrifuge (Eppendorf, Hamburg, Germany) Centrifuge (REMI, India), CO_2 incubator (Sanyo, Japan), Laminar Air Flow Hood (Micro Filt, India), Semi Dry Blot Apparatus (Biorad, USA), BD FACS Aria bench top flow cytometer (Becton and Dickinson, USA), Fluorescent Microscope (Olympus, Japan), Confocal Microscope (Carl Zeiss, Germany), Gel Documentation apparatus (Biorad, CA, USA).

IV.4. Media, Buffers and Reagents

IV.4.1. PCR reagents

IV.4.1.1. Restriction Isotyping –RFLP PCR

IV.4.1.1.1. Oligonucleotide Primers

F4 (5'-CAGAATTCGCCCCGGCCTGGTCAC-3')

F6 (5'-TAAGCTTGGCACGGCTGTCCAAGGA-3')

IV.4.1.1.2. PCR mix

F4 & F6 (4 pico moles), Go Taq Buffer (1X) in which $MgCl_2$ (25 mM) is incorporated, DNTP mix (0.2 mM), Taq DNA Polymerase (1.25 U), DNA template (15 ng) DMSO (5%) and deionized water.

IV.4.1.1.3. HhaI Restriction enzyme reaction mix

HhaI RE (1 unit), HhaI buffer (1X), BSA (0.2 µg) and EtBr (0.01 µg)

IV.4.1.2. SSP PCR

IV.4.1.2.1. Oligonucleotide Primers

MDL Primer 1 (CGG ACA TGG AGG ACG TGT)

MDL primer 2 (CTG GTA CAC TGC CAG GCG)

MDL primer 3 (CTG GTA CAC TGC CAG GCA)

MDL primer 4 (CGG ACA TGG AGG ACG TGC)

MDL primer 8 (HLA) (TGC CAA GTG GAG CAC CCA A)

MDL primer 9 (HLA) (GCA TCT TGC TCT GTG CAG AT)

IV.4.1.2.2. PCR Mix

Specific MDL (8 picomoles) & HLA primers (0.75 picomoles), Go Taq Buffer (1X) in which $MgCl_2$ (25 mM) is incorporated, Taq DNA Polymerase (1.25 U), DNTP mix (0.2 mM), DNA template (15 ng) and deionized water.

IV.4.1.3. H1/H2 PCR

IV.4.1.3.1. Primers

F 5’-GAAGACGTTCTCACTGATCTG-3’

R 5’-AGGAGTCTGGCTTCAGTCTC-3’

IV.4.1.3.2. PCR Mix

Primer (8 picomoles), Go Taq Buffer (1X) in which $MgCl_2$ (25 mM) is incorporated, Taq DNA Polymerase (1.25 U), DNTP mix (0.2 mM), DNA template (15 ng) and deionized water.

IV.4.1.4. PCR for PSEN exon 7 & 12

IV.4.1.4.1. Primers

PS 07F (5'GGAGCCATCACATTATTCTAAA3')

PS 07R (5'AACAAATTATCAGTCTTGGGTTT3')

PS 12F (5'GTCTTTCCCATCTTCTCCAC3')

PS 12R (5'GGGATTCTAACCGCAAATAT3')

IV.4.1.4.2. PCR Mix

Forward and reverse primers (8 picomoles), Go Taq colourless Buffer (1X) in which $MgCl_2$ (25 mM) is incorporated, DNTP mix (0.2 mM), Taq DNA Polymerase (1.25 U), DNA template (15 ng) and deionized water.

IV.4.2. Reagents for electrophoresis

IV.4.2.1. Agarose Gel Electrophoresis

IV.4.2.1.1. TAE Buffer 50X

Tris Base (242g), Glacial Acetic Acid (57.1 ml) and 0.5 M EDTA (100 ml) dissolved in distilled water and the volume is made up to 1 L. Working concentration (1 X) comprised of 0.04 M Tris – Acetate and 0.001 M EDTA

IV.4.2.1.2. 1% agarose gel

Agarose (1%) molten in 1X TAE

IV.4.2.2. Polyacrylamide Gel Electrophoresis

IV.4.2.2.1. TBE Buffer 5X

1.1 M Tris, 900 mM Borate & 25 mM EDTA, pH 8.3. TBE (0.5X) is used as working buffer

IV.4.2.2.2. Acrylamide/ Bis-acrylamide solution (29:1)

29% (w/v) Acrylamide and 1% (w/v) N, N'- Methylene Bis-acrylamide in deionized water.

IV.4.2.2.3. 5% PAGE gel composition

Acrylamide/ Bis-acrylamide solution (1.66 ml), 5X TBE buffer (2 ml), 20% APS (20 µl), TEMED (7 µl) mixed in deionized water (6.3 ml).

IV.4.2.3. DNA ladder

100 bp ladder (Fermentas) and Msp1 digested pUC18 ladder

Dilution = 1µl ladder: 5µl 6X loading dye: 12 µl deionized water

IV.4.2.4. Ethidium bromide solution

EtBr (1%) in distilled water

IV.4.3. REAGENTS FOR DNA SEQUENCING

Sequencing reaction mixture composition:

Amplicons (200 ng), unlabeled primer (5 picomoles), 1X reaction buffer (1.75 µL), Ready reaction mix (containing four dNTPs, four fluorescently labeled di-deoxy-ribonucleotides (ddNTPs), and AmpliTaq® DNA Polymerase) (0.5 µL) mixed in distilled water.

IV.4.4. REAGENTS FOR GELATIN ZYMOGRAPHY

IV.4.4.1. Resolving buffer

Trizma (1.5 M) in distilled water, pH 8.8

IV.4.4.2. Chamber buffer

Trizma (3.027 g), Glycine (14.4 g) SDS (1 g) dissolved in 1 L distilled water

IV.4.4.3. SDS (10%)

SDS (10%) in distilled water

IV.4.4.4. Gel composition (10%)

Gelatin (30 mg), Acrylamide/ Bis-acrylamide solution (1.65 ml), Resolving Tris buffer (2.5 ml), 10% SDS (100 µl), 20% APS (20 µl), TEMED (8 µl) mixed in deionized water (5.65 ml).

IV.4.4.5. 2X Non reducing loading buffer

TrisHCl (0.5 M, pH-6.8), Glycerol (0.8 ml), 10 % SDS (3.2 ml), 0.2% Bromophenol blue (0.2 ml) mixed in distilled water (2.8 ml).

IV.4.4.6. Renaturation buffer

Triton X-100 (2.5%) in deionized water, prepared fresh.

IV.4.4.7. Incubation Buffer

$CaCl_2$ (dihydrate) (4.41 g), Sodium Nartimuzide/sodium azide (60 mg), Trizma (3.63 g) in 300ml distilled water. pH adjusted to 7.5 with glacial acetic acid.

IV.4.4.8. Stain CBB R-250

0.5% Coomassie Brilliant Blue R-250 in 13% acetic acid solution, filtered.

IV.4.4.*9. Destainer solution*

7.5% acetic acid solution.

IV.4.5. AEBSF Protease inhibitor solution

AEBSF (MW 239.69) working solution- 10 mM in distilled water.

IV.4.6. REAGENTS FOR COMET ASSAY

IV.4.6.1. Alkali Lysis Buffer

NaCl (2.5 M), EDTA (100 mM), Trizma Base (10 mM),Triton X-100 (1%), DMSO (10%), dissolved in distilled water, pH adjusted to 10, refrigerated 30 min prior to use.

IV.4.6.2. Electrophoresis buffer (alkaline)

Mixed 9 ml of NaOH (10N) and 1.5 ml of EDTA (200mM); volume made upto 300 ml ensured that pH>13 (strictly alkaline).

IV.4.6.3. Neutralisation buffer

Tris (0.4 M) in deionized water, pH 7.5

IV.4.7. REAGENTS FOR WESTERN BLOTTING

IV.4.7.1. Running Buffer (10X)

Tris base (0.25 M), Glycine (1.92 M), SDS (1%) in deionized water.

IV.4.7.2. Blotting Buffer (10X)

Tris base (0.25 M) & Glycine (1.92 M) in deionized water. 1X blotting buffer constitutes 100 ml 10X buffer & 200 ml methanol, made up the volume to 1 L.

IV.4.7.3. TBS (Tris Buffered Saline) (10X)

Tris base (12.1 g) & NaCl (84.8 g) dissolved in deionized water (1 L), pH 7.9

IV.4.7.4. TBST (Tris Buffered Saline with Tween-20)

TBS (1X) containing Tween-20 (0.1%).

IV.4.7.5. Resolving Gel Buffer for SDS PAGE (8X) (10%)

Trizma (18.15 g) and SDS (0.4 g) in 50ml deionized water, pH 8.8

IV.4.7.6. Stacking Gel Buffer for SDS PAGE (4X) (5%)

Trizma (3.025 g) and SDS (0.2 g) in 50 ml deionized water, pH 6.8

IV.4.7.7. Loading Dye for SDS PAGE

SDS (2% (w/v)), Bromophenol blue (0.03%), β-mercapto ethanol (0.3%), Glycerol (10% (v/v)) and Tris (0.067 M) in distilled water. pH 6.8.

IV.4.7.8. Resolving Gel (10%)

Acrylamide/ Bis-acrylamide solution (2.5 ml), 8X buffer (1.25 ml), 20% APS (18 µl), TEMED (10 µl) mixed in deionized water (6.25 ml).

IV.4.7.9. Stacking Gel (5%)

Acrylamide/ Bis-acrylamide solution (0.625 ml), 4X buffer (1.25 ml), 20% APS (10 µl), TEMED (10 µl) mixed in deionized water (3.125 ml).

IV.4.7.10. TBST for antibody dilution

BSA (3%) in 1X TBST

IV.4.7.11. Blocking solution

Skim milk (5%) in 1X TBST

IV.4.8. REAGENTS FOR MONOCYTE CULTURE

IV.4.8.1. Phosphate Buffered Saline (PBS) (1X)

NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (10.14 mM), KH_2PO_4 (1.76 mM) dissolved in distilled water, pH 7.4

IV.4.8.2. Lymphoprep (Ficoll- histopaque) density gradient medium

IV.4.8.3. RPMI 1640 Medium

RPMI 1640 was reconstituted with sterile water, supplemented with 10% autologous serum, penicillin-streptomycin (100 mg/L) and gentamycin (50 mg/L), pH adjusted to 7.4 using $NaHCO_3$. Autologous sera samples were collected by centrifugation of coagulated blood (10 ml) filtered through Millipore 0.22 µm filters and were complement-inactivated and stored at 4^0C.

IV.4.8.4. β Amyloid 1-42, HiLyte Flour 488-labeled working solution

0.1 mg peptide reconstituted with 50 µl NH_4OH (1%) and diluted with 1X PBS to substocks (1 µg/µl), stored at -20^0C till use.

IV.4.8.5. Lysotracker Red working solution

Lysotracker red dye (0.1 mg/1ml) in 1X PBS.

IV.4.8.6. Curcumin C3 Complex working solution

Fresh curcuminoid stock solution (100 mM) was prepared by dissolving 36.8 mg curcuminC3 complex in 1 ml DMSO. Substocks (1 mM) were prepared with sufficient quantity of sterile deionised water.

IV.5. METHODS

IV.5.1. Inclusion of study subjects

Case control study design was adopted to meet the objectives of the study. Patients were recruited from those attending the Memory & Neurobehavioral Clinic (MNC) at our hospital, SCTIMST. All subjects recruited into the study were subjected to a structured interview which included inquiry into neurological symptoms and also for the presence of vascular risk factors such as hypertension, hyperlipidaemia, hypercholesterolemia, thyroid dysfunction, diabetes, cardiopathy or any previous history of cranial trauma. All subjects also underwent a series of blood biochemical tests of Vitamin B12, thyroid function tests, VDRL etc, and detailed neuropsychological tests as detailed in an earlier report from us (Mathuranath PS et al., 2010).

Based on a detailed collection of family history of dementia, the familial cases were detected and for which pedigrees were established. All subjects/ first degree relative gave signed informed consent approved by the SCTIMST Institute Ethics Committee for Human Studies. IEC Approval and patient's consent form are presented in Appendix 3. Control samples were collected from the cognitively unimpaired caregivers/ spouses of patients (strictly non-consanguineous) and healthy volunteers. Inclusion/ exclusion criteria for patients and controls are presented in Appendix 2.

Diagnosis

Patients were stratified into different dementia categories based on standard diagnostic criteria (Appendix 1). The NINCDS–ADRDA (McKhann G et al., 1984), McKeith (McKeith IG et al., 1996), Lund-Manchester Group criteria (Brun A et al., 1994) and NINDS–AIREN criteria (Roman GC et al., 1993) were used to establish diagnosis of AD, DLBS, FTD and VD respectively. Severity of AD cases was determined according to their performance in neuropsychological tests and Clinical Dementia Rating Scale (CDR). Preclinical AD cases were grouped into MCI, on the basis of their MMSE (Mini Mental State Examination) scores and performance on the Addenbrook's Cognitive Examination (ACE) (Mathuranath PS et al.,

2000) and using Petersen’s criteria (Petersen RC et al., 1997). The local language, Malayalam adaptation of the ACE (Mathuranath PS et al., 2004) was used that comprised of tests to analyse all major cognitive functions and other tests detailed elsewhere (Mathuranath PS et al., 2010). Blood samples were collected through venupuncture from all participants. For selected AD/MCI cases, for which markers of disease progression were analysed, three repeated blood collections were performed on three annual follow-up visits to the clinic from 2009-2013.

IV.5.2. Separation of blood into components for assays

Plasma and sera were separated from freshly collected blood samples through centrifugation. Blood samples drawn into heparinised vacutainers were centrifuged at 3000 rpm for 15 min and the supernatants (plasma) were collected in labelled cryovials as aliquots of 100µl. Centrifugation of blood samples after coagulation (2500 rpm for 10 min) yielded sera collected in labelled cryovials. Plasma and sera samples were stored at $^{-}80^{0}$C until analysis. CSF samples were stored in the same method. DNA was isolated using standardised protocol using kit reagents, properly labelled and stored at $^{-}20^{0}$C.

IV.5.3. APOE GENOTYPING PROTOCOLS

IV.5.3.1. Hixon- Vernier Restriction Isotyping

Restriction Isotyping (Hixson & Vernier, 1990) relies on PCR amplification of the APOE sequence encompassing the codons 112 and 158 followed by restriction digestion of the PCR product by the restriction enzyme, HhaI that generates unique combination of pattern of fragments that distinguishes APOE isoforms.

Each reaction mixture is subjected to PCR cycles- 95^{0}C for 5 min for initial denaturation, 30 cycles of amplification by primer annealing (61.5^{0}C for 1 min), extension (72^{0}C for 30 sec), denaturation (95^{0}C for 30 sec) and a final extension at 72^{0}C for 5 min. The size of PCR product was verified to be 244 bp by running on a 1% agarose gel with 100 bp ladder. Restriction reaction mixture was prepared and incubated at 37^{0}C for 30 min for digestion.

The restriction digest was loaded on to a 5% poly acrylamide gel along with Msp1 digested pUC18 marker and electrophoresed at 100V for 1.5 h in order to visualize the APOE fragments. Isoforms were distinguished by unique combinations of HhaI fragment sizes in all homozygous/ heterozygous combinations.

IV.5.3.2. Sequence Specific Primer (SSP) PCR

In this method, sequence- specific forward and reverse primers were combined to raise three haplotype-detecting reaction mixtures such as APOE ε2 (MDL 1+3 & 8+9), APOE ε3 (MDL 1+2 & 8+9) and APOE ε4 (MDL 2+4 & 8+9). Here, presence of APOE band indicates that sample is positive for that allele. Hence a pair of control primers (HLA) was also incorporated. PCR mixtures were subjected to initial denaturation for 1 min at 96 °C; followed by 10 cycles of 20 sec at 96 °C, 45 sec at 70 °C, and 25 sec at 72 °C; 21 cycles of 25 sec at 96 °C, 50 sec at 65 °C, and 30 sec at 72 °C; 4 cycles of 30 sec at 96 °C, 60 sec at 55 °C, and 120 sec at 72 °C. The PCR products are analyzed by agarose gel electrophoresis. For all PCR reactions, the presence of a 173 bp band indicates the presence of specific APOE haplotype along with any of the HLA bands at 785 bp or 1598 bp.

IV.5.4. MAPT H1/H2 Haplotyping

To delineate the two distinct MAPT haplotypes, we genotyped all study participants through testing for the presence of a 238 bp deletion between exon 9 and 10, which discriminate H2 haplotype from H1 through PCR amplification comprising 35 cycles of 30 sec at 94^0C, 15 sec at 60^0C, 20 sec at 72^0C, preceded by 4 min at 94^0C followed by 10 min at 72^0C. Following the reactions, the genotypes were assessed by visualizing the products on a 5% polyacrylamide gel.

IV.5.5. MUTATION SCREENING ON PSEN1 EXON 7 & 12

IV.5.5.1. Amplification of PSEN exons

PCR mixtures were subjected to initial denaturation for 1 min at 96 °C; followed by 10 cycles of 20 sec at 96 °C, 45 sec at 70 °C, and 25 sec at 72 °C; 21 cycles of 25 sec at 96 °C, 50

sec at 65 °C, and 30 sec at 72 °C; 4 cycles of 30 sec at 96 °C, 60 sec at 55 °C, and 120 sec at 72 °C. Product sizes were verified over agarose gel electrophoresed along with 100 bp ladder.

IV.5.5.2. Sequencing and Purification

IV.5.5.2.1. Sequencing:

The two amplicons were automatically sequenced in both directions using the Big-Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions. Fluorescent fragments were generated by incorporation of dye-labeled ddNTPs. Each different ddNTP (ddATP, ddCTP, ddGTP, or ddTTP) carried a different colour of dye. All terminated fragments (those ending with a ddNTP), therefore, contained a specific colour at their 3' end. The thermocycling conditions were 25 cycles of 96^0C for 30 sec and 60^0C for 4 min with a thermal ramp rate of 10/sec.

IV.5.5.2.2. Purification of PCR product:

The PCR products were purified using Ethanol/EDTA/Sodium Acetate precipitation method. To 10 µL PCR products, added 2 µL of 125 mM EDTA, 2 µL 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 (25^0C). 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. Decanted the supernatant and pellet was air-dried which was subjected to DNA sequencing in automated sequencer.

IV.5.6. BIOMARKER ANALYSIS

IV.5.6.1. ELISA for Plasma Aβ1-42

In vitro quantitative measurement of Aβ1-42 in plasma samples was done using ELISA kits.

IV.5.6.1.1. Sandwich ELISA (Invitrogen KHB3441)

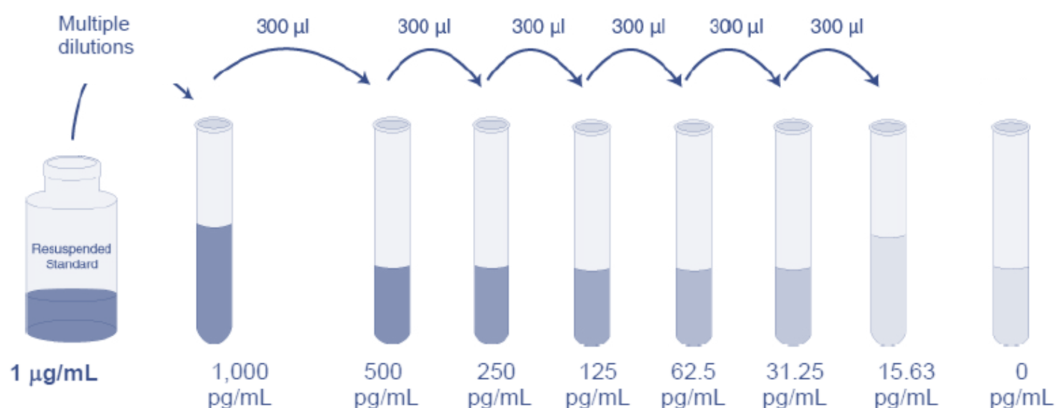
The assay employs a solid phase-sandwich ELISA protocol to estimate the amount of Aβ42 peptide in the samples. During the first incubation, known Aβ42 standards, controls and unknown samples are pipetted into the wells and co-incubated with a rabbit antibody specific for the COOH-terminus of the Aβ 1-42 sequence. Bound rabbit antibody is detected by the use of a horseradish peroxidase-labeled anti-rabbit antibody. After a second incubation and washing to remove the entire unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce colour. The intensity of this coloured product is directly proportional to the concentration of Aβ42 present in the original sample.

Step 1:

Generation of standard curve

Reconstituted standard to 1.0 µg/mL with Standard Reconstitution Buffer (55 mM sodium bicarbonate, pH 9.0). Standard dilutions were performed serially using standard diluent buffer (Figure 5).

Figure 5: Serial Dilution Method

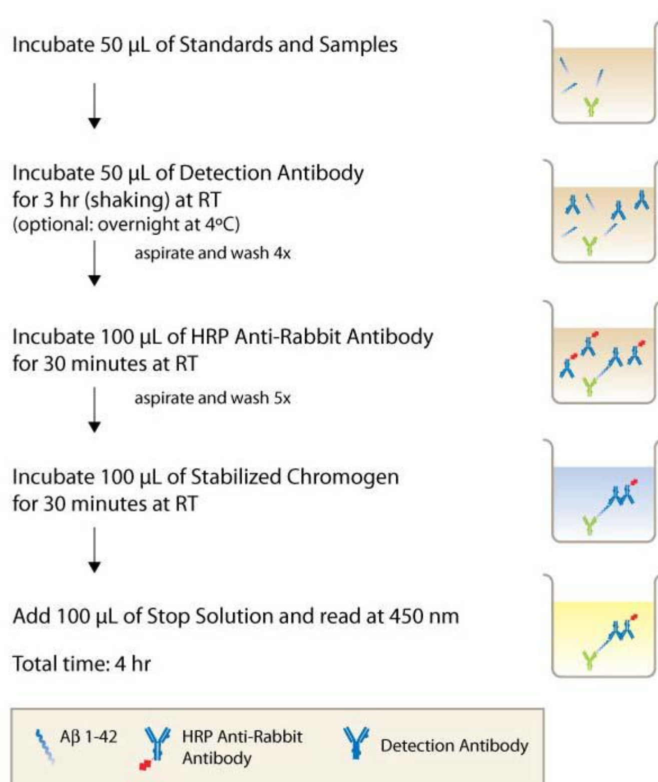


Reconstituted standard (1.0 µg/ml) was serially diluted to obtain multiple dilutions ranging from 1000 pg/ml to 15.63 pg/ml by directly transferring 300 µl serially using standard diluent buffer.

Step2: Procedure

Plasma samples were diluted 2X and were incorporated with 1 mM AEBSF protease inhibitor cocktail. The assay summary is given in Figure 6.

Figure 6: Assay summary of Sandwich ELISA protocol



IV.5.6.1.2. Competitive Inhibition assay (USCN)

The assay employed competitive inhibition principle to quantitate Aβ1-42 in plasma samples. A competitive inhibition reaction was launched between biotin labelled Aβ1-42 and unlabelled Aβ1-42 (standards or samples) with the pre-coated antibody specific to Aβ1-42. After incubation the unbound conjugate was washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. The amount of bound HRP conjugate was reverse proportional to the concentration of Aβ1-42 in

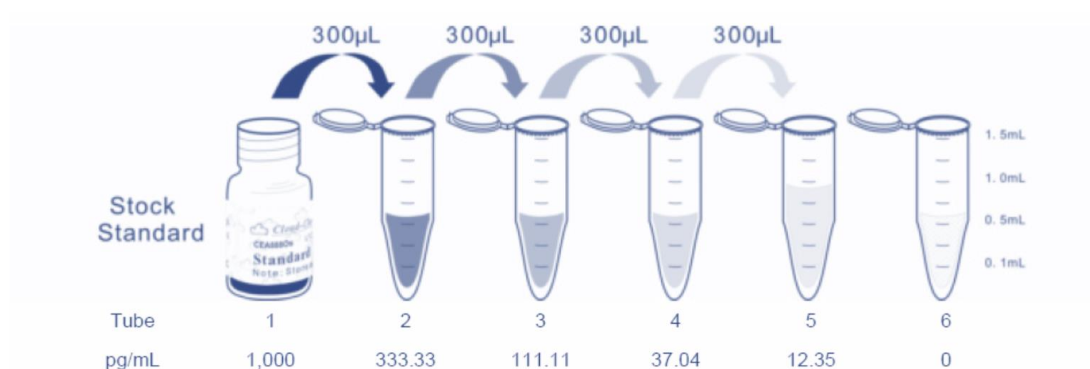
the sample. After addition of the substrate solution, the intensity of colour developed was reverse proportional to the concentration of Aβ1-42 in the sample.

Step1:

Generation of standard Curve

Reconstituted standard to 1.0 µg/mL with standard diluent buffer. Standard dilutions were performed serially using standard diluent buffer generating standard solutions of Aβ1-42 at 333.33, 111.11. 37.04 and 12.35 pg/ml concentrations (Figure 7).

Figure 7: Standard Dilution Method



Reconstituted standard (1.0 µg/ml) was serially diluted to obtain triple dilutions ranging from 1000 pg/ml to 12.35pg/ml by directly transferring 300 µl serially using standard diluent buffer.

Step2: Procedure

Samples were diluted 3X using standard diluent. 50 µl of each sample was added to antibody-coated wells along with 50 µl of detection reagent A, mixed by gentle shaking and incubated at 37^0C for 1 h. After the incubation, aspirated the liquid and washed 3 times with wash buffer and decanted the remaining liquid by blotting the wells against absorbent paper. Second incubation was done for 30 min at 37^0C after adding detection reagent B. Repeated the aspiration/ wash step 5 times and added 90 µl substrate solution, and incubated 15 min at 37^0C, protected from light. The solution turned yellow from blue, on the addition of 50 µl stop solution. The absorbance readings were taken immediately on ELISA reader at 450 nm.

IV.5.6.2. ELISA for Plasma Total Tau

In vitro quantitative determination of total tau in plasma samples was performed using a sandwich ELISA kit.

IV.5.6.2.1. Sandwich ELISA for Human Tau

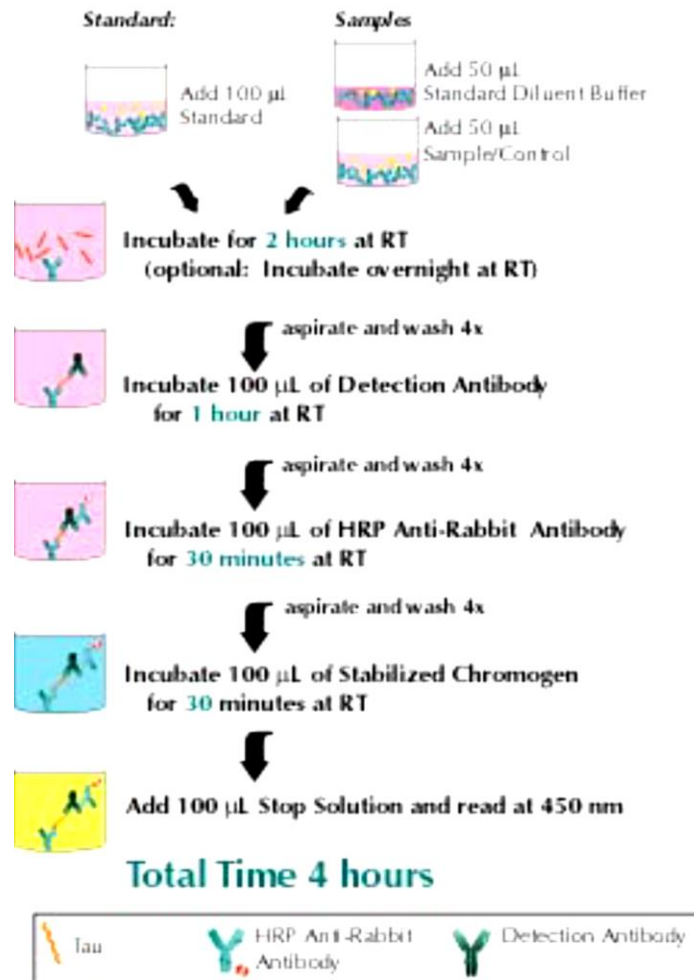
This is a sensitive method to measure human total tau levels (both phosphorylated and non-phosphorylated) in biological samples. Samples & standards were pipetted into the wells pre coated with monoclonal antibody specific for tau protein. During the first incubation, the tau antigen bound to the capture antibody on one site. After washing, a rabbit polyclonal antibody specific for tau was added. During the second incubation, this antibody bound to the immobilized tau captured during the first incubation. After washing of excess second antibody, HRP-labelled anti-rabbit antibody was added. This bound to the rabbit polyclonal antibody to complete the four-member sandwich. After a third incubation and washing to remove all the excess anti-rabbit HRP, a substrate solution was added, which was acted upon by the bound enzyme to produce colour. The intensity of this coloured product was directly proportional to the concentration of tau present in the original sample.

Step 1: Generation of standard curve

Reconstituted standard to 2000 pg/ml with standard diluent buffer. Standard dilutions were performed serially using standard diluent buffer into 1000, 500, 250, 125, 62.5, and 31.2 pg/ml concentrations.

Step2: Procedure: Plasma samples were diluted 2X with standard diluent buffer and added into microtitre wells. Assay summary is presented in Figure 8.

Figure 8: Assay protocol for Human Tau ELISA



IV.5.6.3. Estimation of Serum MMP Activity

Circulating MMP activity was determined using Gelatin Zymography and the results were confirmed through western blotting.

IV.5.6.3.1. Gelatin Zymography

MMP-2 and MMP-9 were measured in serum samples by gelatin-substrate zymography as described earlier. In brief, samples were loaded on 10% sodium-dodecyl sulfate (SDS) polyacrylamide minigels copolymerized with gelatin. The gels were run with 1X tris-glycine-SDS running buffer according to the standard running conditions viz 125 V for 120 min. The

gels were separated from the cassette, and kept in the zymogram renaturation buffer for 30 min at RT on gel rocker. Decanted the renaturation buffer, which is replaced with zymogram developing buffer, equilibrated the gel with gentle shaking for 30 min at RT and fresh incubation buffer was added and incubated at 37^0C in a shaking water bath for 24-36 h. After incubation, the gels were stained with 0.05% CBB R-250 stain for 30 min, and then destained with water overnight. Areas of protease activity appeared as clear bands against a dark blue background. A computer-based imaging system Quantity One was used to measure relative lysis areas and the values were compared.

IV.5.6.3.2. Western Blot for MMP-2 & MMP-9

Serum samples were subjected to a western blot analysis to detect MMPs.

IV.5.6.3.2.1. separation and transfer of serum proteins on to Nitrocellulose Membrane

Sera were diluted 100X using 1X PBS, mixed with gel loading dye at a ratio of 6:1 and were heated for 2 min at 92^0C on a dry bath. The constituent proteins were electrophoretically fractionated on a 10% SDS PAGE gel. Separated proteins were electophoretically blotted onto a Nitrocellulose membrane for 45 min at 20 V using a Semi dry blotting apparatus. Membranes were rinsed with Ponceau S solution for 30 sec to visualise and confirm the transferred bands.

IV.5.6.3.2.2. Development of specific bands

The membranes were blocked in 5% non-fat skim milk for 1 h and incubated with primary antibodies (MMP-2 & MMP-9) at 1:1000 dilution in 1X TBST overnight at 4^0C. After incubation, primary antibody was removed and the membranes were washed in 1X TBST (3 X 5 min) and then incubated with HRP conjugated secondary antibody for 1 h. the membranes were blocked with skim milk and again washed with 1X TBST (3 X 5 min), subjected to the Enhanced Chemiluminescence (ECL) detection system to detect the antigen- antibody complex. The ECL substrate was applied on the membrane and the

signals thus developed were exposed to an X-ray film for 30 sec in the dark room to develop corresponding bands. MMP bands were quantified using Quantity One software.

IV.5.6.4. PERIPHERAL CELLULAR MARKERS FOR AD

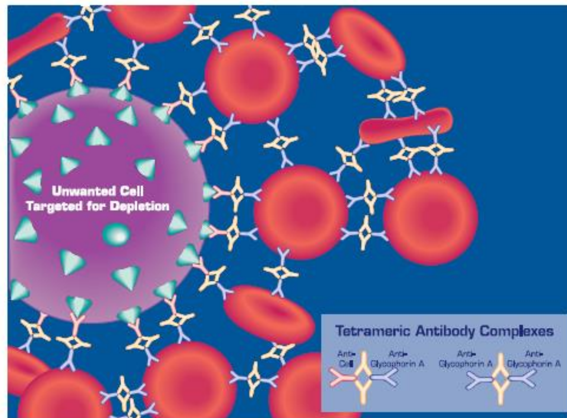
IV.5.6.4.1. Comet assay

Single cell gel electrophoresis (SCGE) or Alkaline Comet assay was performed as described previously. PBMNCs were isolated from fresh heparinised blood samples, washed with 1X PBS and resuspended in 0.5% LMPA (low melting point agarose). The cells were encapsulated in a layer of LMPA covered with a coverslip, spread over a frosted glass slide which is pre-coated with 1.5% agarose. This was allowed to solidify over a tray placed on ice-packs for 10 min. The coverslip was gently removed and a 3rd layer of 0.5% LMPA was added, and a coverslip placed on top again to spread the layer evenly. After solidification by placing the slide over ice-packs, the cells were subjected to alkali lysis by immersing the comet slide in lysis buffer for 2 h. The slide was then subjected to electrophoresis, after equilibrating the slide in running buffer for 20 min. Electrophoresis was conducted for 24 min at 25 volts/ 300 mA constant current. DNA with strand breaks will move extensively from 'head' through the gel like a 'comet tail'. After run, slides were neutralised and stained with EtBr solution and were visualised using fluorescent microscope. Image analyses were performed using Comet Score software. The extent of DNA damage was calculated as tail moment and that of cases and controls were compared.

IV.5.6.4.2. Isolation of Monocytes- RosetteSep protocol

The RosetteSep antibody cocktail crosslinks all cells in human whole blood other than monocytes to multiple red blood cells (RBCs), forming immunorosettes (Figure 9).

Figure 9:Immuno Rosette of Unwanted Cell and RBCs Formed by RosetteSep Tetrameric Antibody Complexes (TAC)

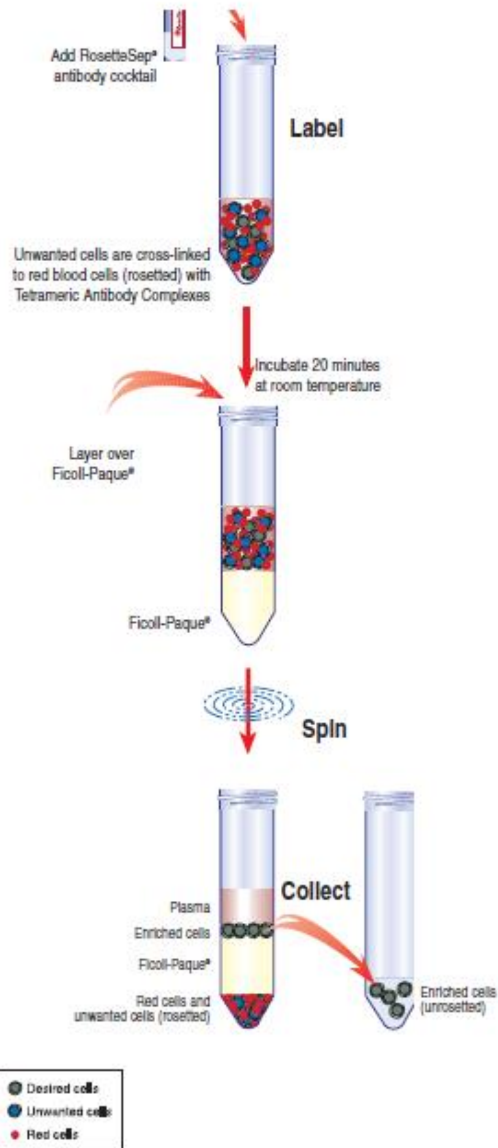


This increases the density of the unwanted (rosetted) cells, such that they pellet along with the free RBCs when centrifuged over a buoyant density medium such as Ficoll-Paque. Monocytes are never labelled with antibody and are easily collected as a highly enriched population at the interface between the plasma and the buoyant density medium.

Protocol:

RoseteSep Cocktail was mixed at 50 µL/mL of whole blood and was incubated for 20 min at RT. Diluted sample with an equal volume of PBS + 2% FBS and 1 mM EDTA. Mixed gently and layered the diluted sample on top of the Ficoll-Paque medium, centrifuged for 20 min at 1200 Xg at RT. The enriched cells, monocytes were collected from the Ficoll-Paque- plasma interface and washed in 1X PBS, and subjected to culture. The assay protocol is presented in Figure 10.

Figure 10: Protocol for monocyte isolation



Isolation of Monocytes through RosetteSep protocol involving separation of unwanted cells in the form of immunorosettes that are sedimented during centrifugation. Monocytes are enriched at the Ficoll-Paque-plasma interface which are collected, washed and subjected to culture.

IV.5.6.4.3. Flow cytometry on 1-day old monocytes

Monocytes were washed twice with culture media, RPMI 1640 and were seeded at a density of $1.6X10^5$ cells/ml of serum-free media in 35 mm culture dishes. To test Aβ phagocytosis property of freshly isolated monocytes, the cells were exposed to HiLyte Flour 488-labeled Amyloid β 1-42 (1 µg/ml) overnight at 37^0C in a 5% CO_2 humidified incubator, washed with 1X PBS and 100,000 monocytes per sample were subjected to flow cytometry to determine FITC fluorescence. BD FACS Aria benchtop flow cytometer was employed for the analysis. Monocytes were gated according to SSC/FSC and at least 10,000 cells were analyzed in FL1 (FITC) and FL2 (PE) using BD FACScan with a 488 nm argon laser and the FL1 filter 530 nm±15 and the FL2 585 nm±21. Aβ phagocytosis rate was calculated as the MFI in upper right corner times % cells upper right corner.

IV.5.6.4.4. Culture and Differentiation of Monocytes

In order to estimate the Aβ phagocytic potential of macrophages, monocytes were cultured for 14 days and were differentiated into macrophages with characteristic fried egg morphology. ~200,000 monocytes per sample were suspended in RPMI 1640 medium with 10% autologous human serum in 8 chamber polystyrene vessel TC treated glass slides.

IV.5.6.4.5. Macrophage amyloid internalisation assay

Differentiated macrophages were exposed HiLyte Flour 488-labeled Amyloid β 1-42 (1 µg/ml) and incubated overnight, washed with 1X PBS and examined by fluorescence or confocal microscopy for visualising Aβ uptake. Lysosomal marker Lysotracker Red was used to find out the extent of intra-lysosomal localisation of phagocytosed Aβ. Carl Ziess Confocal Microscope was used for acquiring images. Image analysis was performed using Zeiss LSM imager/ Image J software packages. Mean fluorescent intensity (MFI) over six different fields per sample were subjected to analysis. For colocalisation analysis of the two signals, Image J Colocalisation finder was employed and means of values of colocalisation coefficient were calculated for all study samples.

IV.5.6.4.6. Treatment of curcumin on macrophages

Differentiated macrophages were treated with varying concentrations of curcuminoids (Curcumin 3 complex) ranging from 2 μM to 20 μM in the medium overnight and were then exposed to FITC- Aβ 1-42, incubated for 24 h and examined by fluorescence or confocal microscopy post exposure to Lysotracker Red in order to visualise the influence of curcuminoids on improving the phagocytic potential of macrophages. Cell culture supernatants were collected and subjected to Gelatin Zymography to determine the effect of curcuminoids in macrophages' MMP activity.

IV.5.7. Statistical analyses

Cases were compared with controls for every parameter under analysis. The 2-tailed *t* test, Fisher exact test and Multivariate logistic regression analysis were used to compare the features of patients and controls, APOE ε4 carriers and non-carriers. Bonferroni correction (BC) was applied for multiple comparisons. Results were represented as mean ± SD. The effects of MAPT haplotypic variability on influencing the risk in each study group were independently assessed and the interaction between APOE and MAPT haplotype variability was analysed through Multivariate logistic regression analysis. GraphPad Prism software was employed to plot standard curve for ELISA. Difference between two group means were considered statistically significant at $p<0.05$.

V. RESULTS

V.1. STUDY POPULATION

The study population comprised of 557 individuals, including 138 cognitively normal controls. APOE polymorphism was analysed on all study subjects in order to derive predictive values for AD and MCI and to compare with other dementia groups. All the other analyses were confined only on the major study groups comprising AD and MCI. The subject demographics are represented in Table 4.

Table 4: Demographics of study groups

Variables	AD	MCI	FTD	VD	DLBS	Controls
No of individuals	156	87	127	37	12	138
Age (years ± SD)	70.65±9.40	64.5±8.75	64.65±9.8	67.42±8.2	73.35±8.8	68.7±8.2
Gender, Male, N (%)	88 (56.4%)	50 (57.5%)	71 (56%)	18 (48%)	5 (45%)	74 (54%)
Education in Years (mean ± SD)	7.75±3.20	9.5±2.25	8.45±2.5	10.2±2.45	8.6±3.5	10.62±3.15
MMSE Score (mean ± SD)	17.7±8	20.2±5	19.7±7	18±7	NA	29±0.8
Family history, N (%)*	6 (3.8%)	NA	17 (13.4%)	NA	NA	Nil

AD = Alzheimer's disease; MCI, Mild Cognitive Impairment; FTD, frontotemporal dementia; VD, Vascular dementia; DLBS, Diffuse lewy body syndrome; N, number of individuals; SD, Standard Deviation; MMSE, Mini Mental State Examination; N (%)* = Number of patients with Familial form of the disease. NA, Not Available.

All study groups were matched for age, sex and ethnicity. The subject stratification was based on clinical and neuropsychological examination and diagnosis was established according to standard diagnostic criteria.

V.2. APOE GENOTYPING

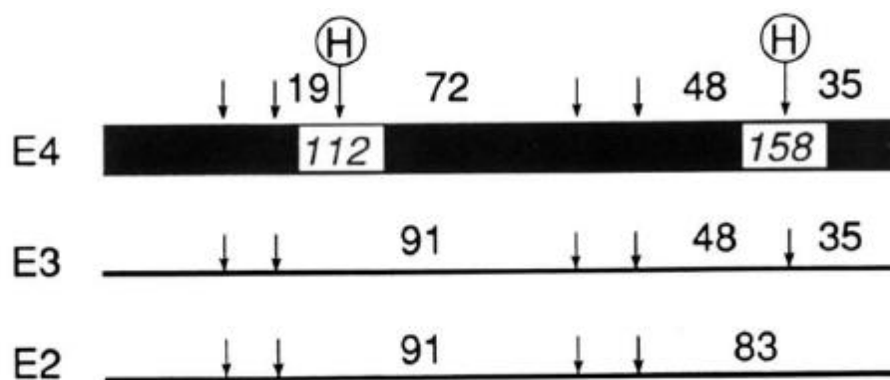
A total of 557 individuals were genotyped for APOE polymorphism, of which 243 had received a clinical diagnosis of MCI and AD (at their baseline) comprised the major study cohort. Family history of dementia was present in 12 patients. 138 cognitively normal individuals were included as control subjects who were matched for age, sex and ethnicity with patient groups. 176 subjects comprised the 'other dementia' category including FTD, VD

and DLBD. All participants were natives of Kerala state, South India. Hixson and Vernier Restriction isotyping protocol (RFLP) was performed on all samples and the results were confirmed through SSP-PCR on the same samples.

V.2.1. RFLP Analysis

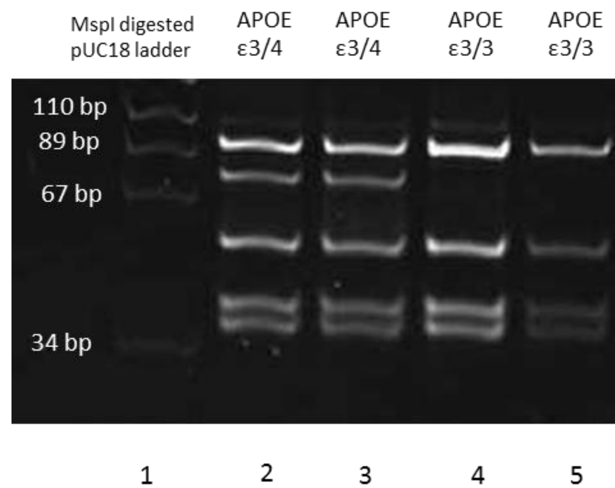
RFLP using HhaI Restriction Enzyme revealed all homozygous/ heterozygous combinations of APOE genotypes. HhaI digestion of amplified APOE gene generated unique fragments corresponding to distinct genotypes (Figure 11). The sizes of fragments were estimated by comparison with known size marker, MspI-digested pUC18 DNA Ladder (Figure 12).

Figure 11: Cleavage map/ Pattern of Restriction digestion of APOE amplicon by RE HhaI.



APOE ε4 is shown as a filled box containing codons 112 and 158, APOE ε3 and APOEε2 maps are shown below E4. The distances (in bp) between polymorphic HhaI sites (circled H) that distinguish isoforms are shown for each cleavage map. (Figure adopted from (Hixson & Vernier, 1990)).

Figure 12: Electrophoretic separation of HhaI-digested APOE fragments

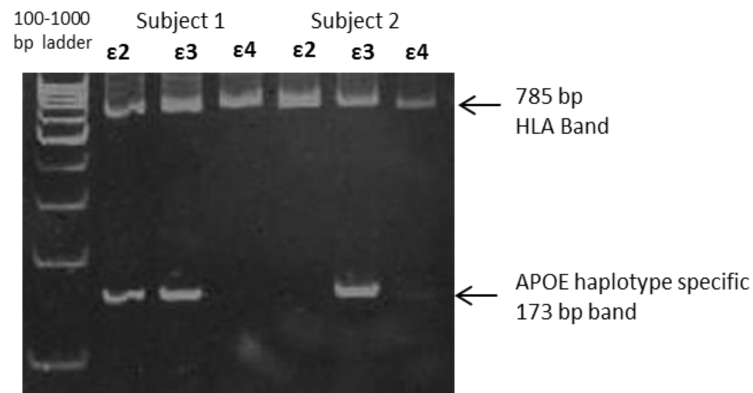


Electrophoretic separation of HhaI-digested APOE fragments showing bands at 91, 72, 48 & 35 bp corresponding to APOE ε3/ε4 genotype (Lanes 2 &3) and at 91, 48 and 35 bp size corresponding to APOE ε3/ε3 genotype (Lanes 4 & 5). MspI digested pUC18 DNA ladder and corresponding band sizes are shown in Lane 1.

V.2.2. Sequence-Specific Primer PCR (SSP PCR)

For each sample, 3 PCR reactions were carried out and the presence of a173-bp band indicated the presence of the specific APOE haplotype. HLA primer pair generated two products at 785 and 1598 bp of which the 785-bp band was always present when PCR amplification occurred, the1598-bp product was often missing in those samples with poor-quality DNA or excessive APOE haplotype-specific amplification.

Figure 13: Electrophoretic separation of SSP PCR products



Visualisation of APOE haplotype specific bands at 173 bp and HLA bands at 785 bp on separation of SSP PCR products on a 2% agarose gel. Subject 1 is identified to be having APOE ε2/ε3 genotype and subject 2 with APOE ε3/ε3 genotype.100-1000bp ladder is resolved on lane 1.

V.2.3. APOE Allele and Genotypic frequencies in the study groups

Allele and genotypic frequencies were calculated for patients and controls, and for gender in each group (Table 5). The APOE ε3/ε3 genotype was most frequently found in all diagnostic categories. The rarest genotype APOE ε2/ε2 was present in Controls and VD. The thrifty genotype APOE ε4/ε4 was present in disease groups at a higher frequency compared to controls. The allele frequencies for men and women did not differ significantly either in controls or patients. The differences in allele frequencies in AD, MCI patients and controls were found to be statistically significant.

Table 5: APOE Allele and Genotype frequencies in all diagnostic groups under study

Study group	Number	Age at inclusion± SD	APOE Allele frequencies (%)			APOE Genotypes					
			ε2	ε3	ε4	ε2/ ε2	ε2/ ε3	ε2/ ε4	ε3/ ε3	ε3/ ε4	ε4/ ε4
AD	Total 156	70.32±8.91	1.28	59.30	39.42	-	1.28	1.28	34.63	48.07	14.74
	Females 68	69.86±8.25	2.94	59.56	37.5	-	2.9	2.9	33.82	48.62	11.76
	Males 88	70.67±9.42	-	59.1	40.9	-	-	-	35.24	47.72	17.04
MCI	Total 87	64.51±8.31	4.65	66.87	28.48	-	6.89	2.29	40.24	45.98	4.60
	Females 37	64.69±6.57	5.77	65.38	28.85	-	11.53	-	38.47	42.31	7.69
	Males 50	64.44±9.0	4.1	67.22	28.68	-	10.53	2.20	38.48	46.52	2.27
FTD	Total 127	64.08±9.45	1.89	82.28	15.83	-	1.55	2.31	70.02	23.82	2.3
	Females 56	62.88±7.98	-	80.0	20.0	-	-	-	64.0	32.0	4.0
	Males 71	64.64±10.1	3.1	83.7	14.28	-	1.85	3.71	72.21	20.38	1.85
VD	Total 37	67.02±7.7	4.05	78.38	17.57	2.7	2.7	-	62.15	29.75	2.7
	Females19	67.83±8.84	8.3	75.0	16.7	-	16.6	-	66.7	-	16.7
	Males 18	67.06±7.50	3.22	79.04	17.74	3.24	-	-	61.24	35.52	-
DLBS	Total 12	69.90±9.7	8.33	70.84	20.83	-	8.33	8.33	58.33	16.68	8.33
	Females 7	74.50±8.75	14.28	57.15	28.57	-	14.28	14.28	42.88	14.28	14.28
	Males 5	72.32±6.45	-	90.0	10	-	-	-	80.0	20.0	-
Control	Total 138	65.51±7.4	7.6	74.64	17.76	3.62	5.79	2.17	59.43	24.64	4.35
	Females 64	64.55±6.45	6.95	70.84	22.21	2.79	2.79	5.57	50.05	38.8	-
	Males 74	71.47±9.42	9.8	61.77	28.43	5.88	5.88	2.96	41.65	31.87	11.76

Number of patients, age at inclusion, allele frequencies and genotypes in patient groups and controls.SD- Standard Deviation.

V.2.4. Subjects (AD, MCI and Control) stratified with possession of APOE ε4

While 64.1% of the AD patients had at least one APOE ε4 allele, only 31.15% of the control individuals carried an APOE ε4 allele ($p < 0.0001$). In MCI subjects, 52.8% possessed at least one APOE ε4allele ($p<0.01$) (Table 6).

Table 6: Major Study Groups Stratified According to the Possession of At least One/ Two/ No APOE ε4 allele

Diagnostic group	With at least one APOE ε4 allele (No. of subjects)	With two APOE ε4 alleles (No. of subjects)	Without APOE ε4 allele (No. of subjects)
AD	100	23	56
MCI	46	4	41
Control	43	6	95

V.2.5. APOE ε4 allele frequencies in AD, MCI and Controls stratified by age and sex

Allele frequencies were also calculated for patients and control individuals according to age and sex (Table 7). The frequency of the APOE ε4 allele in AD patients was highest in those with age >80 years (50%) in males and 70-79 years (41.6%) in females. However in MCI, this was observed in 70-79 years age group for both males (70%) and females (35.71%). The oldest controls with age 70-79 years had the lowest proportion of the APOE ε4 allele (12.5%).

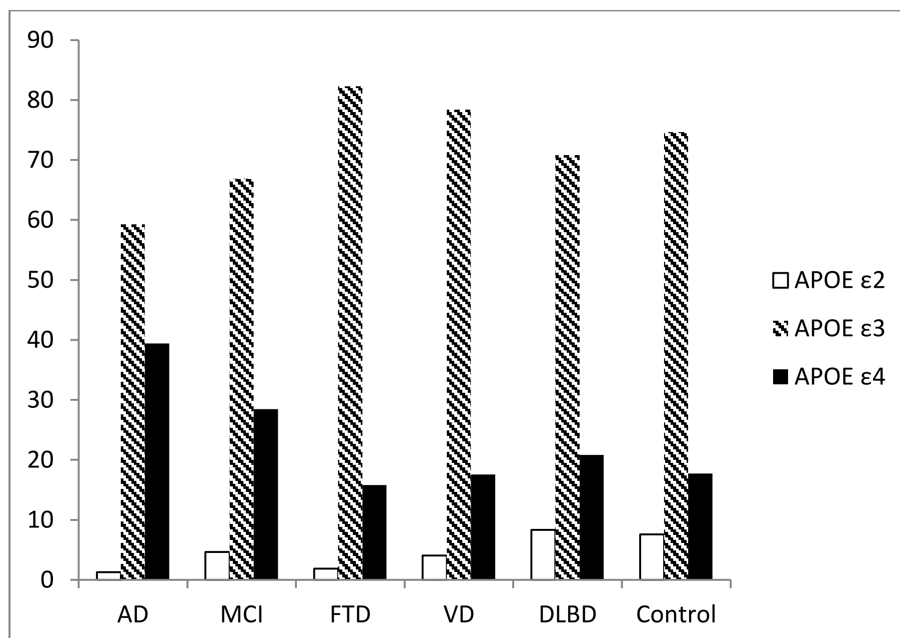
Table 7: APOE ε4 Frequencies Stratified by Age and Sex

	APOE ε4Frequency (%)								
Age	AD			MCI			CONTROL		
	Total	**M**	**F**	**Total**	**M**	**F**	**Total**	**M**	**F**
<60	36.84	38.46	33.3	30.95	33.33	28.57	19.04	16.66	21.42
60-69	37.77	39.13	36.36	23.075	21.15	25	41.19	30	52.38
70-79	39.16	36.66	41.66	52.85	70	35.71	6.25	-	12.5
>80	40	50	30	18.75	37.5	-	-	-	-

V.2.6. APOE allele frequencies in all diagnostic groups

It is evident that all study groups possess the commonest allele APOE ε3 at maximum frequency. However, the frequency of the 'thrifty allele' APOE ε4 is highest in AD, followed by MCI supporting the strong genetic association with disease. Frequency of APOE ε2, the 'protective' allele is highest in DLBD and Control groups.

Figure 14: Graphical representation of APOE allele frequencies in all diagnostic groups



APOE allele frequencies of all disease groups comprising the study cohort are graphically represented, showing the highest APOE ε4 frequency in the AD group.

V.2.7. Correlations of APOE alleles in different diagnostic groups compared to controls

We compared AD/MCI with controls on the APOE ε4 frequency by means of Fisher exact test. The APOE ε4 allele was significantly over-represented in the AD group (P<.001) compared with normal subjects. Likewise, the APOE ε2 and APOE ε3 alleles were under-represented in the AD group (APOE ε2, P=<.001; APOE ε3, P<.001). The strongest association was found between AD and possession of APOE ε4 (OR- 3.015) followed by that of MCI (OR-1.816).

Table 8: Estimates of OR and p value of APOE alleles and genotypes for all groups compared to controls

	APOE alleles								
	APOE ε2			APOE ε3			APOE ε4		
Study group	OR	95% CI	p value	OR	95% CI	p value	OR	95% CI	p value
AD	0.158	0.053-0.465	<0.001	0.495	0.348-0.704	<0.001	3.015	2.055-4.423	<0.001
MCI	0.5852	0.2532-1.352	0.2405	0.662	0.4374-1.003	0.0546	1.816	1.155-2.855	0.01
FTD	0.2381	0.088-0.64	0.0022	1.624	1.066-2.472	0.0269	0.842	0.533-1.33	0.48
VD	0.5131	0.1487-1.770	0.4363	1.232	0.6650-2.282	0.5468	0.987	0.5033-1.937	1
DLBD	1.104	0.2427- 5.021	0.7045	0.82	0.3285-2.073	0.63	1.219	0.434- 3.424	0.78

V.2.8. OR for AD and MCI in all genotypes

The association of AD and MCI with all six possible APOE genotypes were analysed through Fisher's Exact test and that is shown to be highest in AD group with the APOE ε4/ε4 genotype (OR 3.8). For MCI, an OR of 2.6 was observed with APOEε3/ε4 genotype.

Table 9: The OR for developing AD/MCI for all genotypes compared with controls

	APOE genotypes					
	ε2/ ε2	**ε2/ ε3**	**ε2/ ε4**	**ε3/ ε3**	**ε3/ ε4**	**ε4/ ε4**
OR for AD	0.0775	0.2110	0.5844	0.3615	2.832	3.8
95% CI	0.0042-1.416	0.0440-1.012	0.09617-3.551	0.2251-0.5806	1.720-4.663	1.5-9.647
p value	0.0219	0.0498	0.6683	<0.001**	<0.001**	0.003*
OR for MCI	0.1387	1.204	1.059	0.4597	2.603	1.060
95% CI	0.0075-2.542	0.4029-3.596	0.1733-6.4	0.2660-0.7943	1.468-4.615	0.2904-3.871
p value	0.1592	0.7811	1.00	0.0061*	0.0013*	1.00

V.2.9. Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV)

The specificity and PPV of possession of two APOE ε4 alleles in AD cases were found to be higher than that of possession of at least one APOE ε4 allele. The observed NPV was high since majority of AD cases had either one or both APOE ε4 alleles.

Table 10: Sensitivity, Specificity, PPV and NPV of possession of one or two APOE ε4 alleles with AD

	AD cases with at least one APOE ε4 allele	AD cases with two APOE ε4 alleles
Sensitivity (%)	64	15
Specificity (%)	69	96
PPV (%)	47	59
NPV (%)	82	72

V.3. MAPT (H1/H2) HAPLOTYPING

MAPT H1/H2 haplotyping was performed in all study groups, and the allele and genotypic frequencies were calculated (Table 11). Association of H1 homozygosity with the disease groups was evident from the results showing higher H1H1 genotypic frequency in AD (96.15) compared to Controls (92.03). However, since the allelic distribution of the H2 allele was very low in the study population (Case/Control- 1.6/ 4.35), a statistically significant association could not be established between the possession of H1 allele and disease group.

Table 11: Haplotypic and genotypic frequencies of H1 and H2 in all study groups

Study group	Number	H1/H2 Haplotype frequencies (%)		H1/H2 Genotype Frequency (%)		
		H1	**H2**	**H1H1**	**H1H2**	**H2H2**
AD	Total 156	98.4	1.60	96.15	3.85	---
	F 68	97.8	2.20	95.60	4.40	---
	M 88	98.8	1.20	97.73	2.27	---
MCI	Total 87	97.12	2.88	94.25	5.75	---
	F37	96.2	3.80	92.30	7.70	---
	M 50	97.5	2.50	95.08	4.92	---
FTD	Total 127	92.4	7.60	86.60	12.60	0.78
	F 56	91.96	8.04	83.92	16.07	---
	M 71	93.66	6.34	88.75	9.85	1.40
VD	Total 37	93.24	6.75	91.90	2.70	5.40
	F19	100.00	---	100.00	---	---
	M 18	86.11	13.89	83.35	5.55	11.10
DLBS	Total 12	95.83	4.16	91.67	8.33	---
	F 7	100.00	---	100.00	---	---
	M 5	90.00	10.00	80.00	20.00	---
CONTROL	Total 138	95.65	4.35	92.03	7.25	0.72
	F 64	94.45	5.55	91.67	5.56	2.77
	M 74	96.1	3.90	92.15	7.85	---

AD = Alzheimer's disease, MCI = Mild Cognitive Impairment, FTD = frontotemporal dementia, VD = Vascular dementia, DLBS = Diffuse lewy body syndrome

H2H2 was present in the FTD, VD and control group at very low frequency.

V.3.1. Logistic Regression analysis

The interaction between MAPT H1/H2 haplotypes and APOE genotypes in the study group was determined statistically through Multivariate Logistic Regression analysis. The risk estimates for the possession of all six APOE genotypes with all three H1/H2 haplotypes were determined for every disease group (Table 12).

In AD, the presence of APOE ε3/ ε3 with MAPT H1/H1 haplotype increased the protective effect significantly (OR= 0.396 with 95% CI= 0.247-0.636, p<0.001) whereas no significant interaction was observed in APOE ε2/ ε2 or APOE ε2/ ε4 with any of the H1/H2 combinations. In this group, possession of H1H1 along with APOE ε4 allele as APOE ε3/ ε4 (OR= 3.086 with 95% CI= 1.848-5.152, p<0.001) and APOE ε4/ ε4 (OR= 3.61 with 95% CI= 1.419-9.193, p<0.01) significantly found to increase disease risk. Again, no significant interaction was observed with any of the H2 allele combinations in AD patients.

In MCI, a significant protective interaction was observed between H1H1 and APOE ε3/ ε3 (OR= 0.484 with 95% CI= 0.280-0.838, p<0.01). Risk of disease when having H1H1 with APOE ε3/ ε4 was found to be significant (OR= 2.792 with 95% CI= 1.554-5.015, p=0.001). However, no significant interaction was obtained either for APOE ε4/ ε4 with H1H1 or between any of the H2 allele combinations. In FTD, VD and DLBD groups, none of the APOE- H1/H2 combinations were found to be significantly influencing disease risk.

Table 12: Interaction between MAPT H1/H2 haplotypes and APOE genotypes in the study group determined by Logistic Regression analysis

		APOE genotypes										
		APOE ε2/ ε2			APOE ε2/ε3			APOE ε2/ε4			APO	
Study group	MPT Haplotype	OR	95% CI	p-value	OR	95% CI	p value	OR	95% CI	p-value	OR	95
AD	HIHI	IN	IN	IN	0	IN	IN	0.584	0.096-3.55	0.560	0.396	0.24
	H1H2	IN	IN	IN	0	IN	IN	0.764	0.310-1.884	0.560	0.345	0.06
	H2H2	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	I
MCI	HIHI	IN	- IN	IN	1.341	0.397-4.536	0.637	1.059	0.173-6.468	0.951	0.484	0.28
	H1H2	IN	IN	IN	0.791	0.071-8.853	0.849	IN	IN	IN	0.626	0.11
	H2H2	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN
FTD	HIHI	IN	IN	IN	0.282	0.033-2.386	0.245	1.169	0.191-7.149	0.866	1.366	0.77
	H1H2	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN
	H2H2	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN
VD	HIHI	0.739	0.084-6.526	0.785	0.611	0.071-5.240	0.653	IN	IN	IN	1.040	0.5-
	H1H2	IN	IN	IN	IN	IN	IN	IN	IN	IN	0.739	0.08
	H2H2	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN
DLB	HIHI	IN	IN	IN	2.000	.221-18.13	0.538	IN	IN	IN	1.109	0.33
	H1H2	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN
	H2H2	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN

OR = Odd's Ratio, CI = Confidence Interval, IN = Insufficient numbers available for calculation, *p

V. 4. SCREENING FOR MUTATIONS ON PSEN EXONS 7 & 12

The mostly reported mutations on Exons 7 & 12 of PSEN 1 gene were screened in 28 subjects (AD (n=15) and MCI (n=7) subjects with age of disease onset at <60 years and EOFAD patients (n=6)) through direct sequencing of amplicons. However, no previously characterised or novel mutations were detected on the sequence.

V.5. BIOMARKER ANALYSES

V.5.1. Quantification of plasma Aβ1-42

Sandwich ELISA and Competitive inhibition ELISA yielded information on Plasma Aβ1-42 concentration on a subset of AD, MCI and Controls. The demographics of subjects selected for Aβ1-42 quantification is presented in Table 13.

Table 13: Subject demographics for plasma Aβ1-42 quantification

	MCI	**AD**	**Control**	**Follow up (3 Years)**
Number	15	15	15	11 (MCI =5, AD= 6)
Age	63±1.63	65.86±7.78	62.87±1.96	61.8±8.25
Male: Female ratio	9M: 6F	11M: 4 F	5M: 10F	6M: 5 F
MMSE	24.6±2.1	18.4±5.3	27.1±2.5	20.8±5.4 (at baseline)
APOE ε4 frequency(%)	26.6	53.33	23.33	36.36

A total of 56 plasma Aβ determinations were performed using two independent ELISA protocols, and the results were pooled together to derive conclusions. For sandwich ELISA, a standard curve was obtained by plotting OD against known concentrations of Aβ standard. Semi log standard curve was constructed for competitive inhibition ELISA and the antilogarithms of the respective sample concentrations were calculated. Invalid values/out of detection range (n=6) were not considered for the analysis. Data were compiled and are represented as Mean±SE for every group (Table 14). Student *t* test and F test were performed to find out the difference in means and variance between groups.

Figure15: Semi-log standard curve for competitive inhibition ELISA

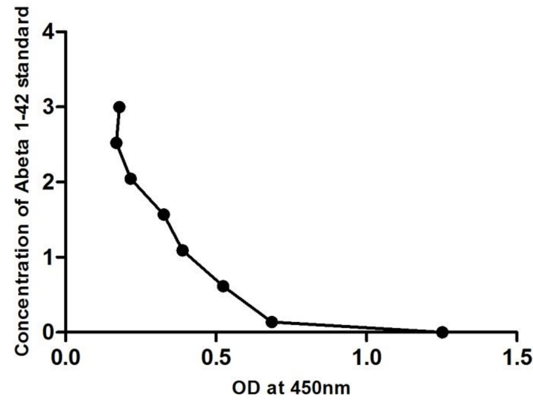
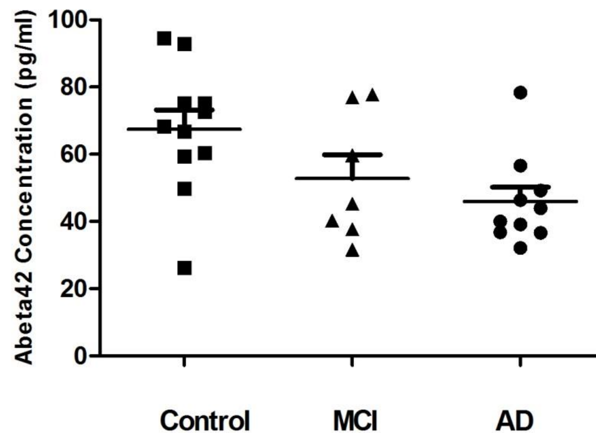


Figure 16: Scatter plot for plasma Aβ concentrations in Control, MCI and AD samples



AD patients showed statistically significant reductions in mean Aβ levels in plasma compared to controls. Error bars are shown on respective lanes.

The plasma Aβ concentrations showed evidences of association with progressive stages of AD. AD patients have shown statistically significant reductions in mean Aβ levels compared to Controls (p=0.0081); however, the difference between MCI and Controls was not statistically significant. AD when compared to MCI, p=0.3947 was obtained with 95% CI=-23.49 to 9.8, R2 =0.04870 F test p=0.3417, which was not found to be statistically significant (Table 14).

Table 14: Correlations of plasma Aβ concentrations in AD, MCI and Control

Study Group	Plasma Aβ concentration pg/ml (Mean±SEM)	t test p value	95% CI	R^2	F test p value
Control	67.43±5.7				
AD	45.92±4.2	(AD Vs Control) 0.0081	-36.75 –6.272	0.3148	0.3027
MCI	52.77±7.148	(MCI Vs Control) 0.1307	-4.848-34.18	0.1369	1

V.5.1.1. Correlation between APOE genotype and Aβ 42 concentrations

Plasma Aβ 42 concentrations did not vary consistently with the APOE genotype (Table 15). No remarkable difference was observed in plasma Aβ 42 concentrations between subjects possessing APOE ε2/ε3 and APOE ε4 alleles. However, in AD group, plasma Aβ 42 level was lower with the possession of one or two APOE ε4 alleles compared to controls.

Table 15: Plasma Aβ 42 concentrations stratified with respect to APOE genotype

Genotype	***plasma Aβ 42 concentrations (pg/ml)***		
	Control	**MCI**	**AD**
APO E ε3/ε3	67.88	75.55	58.7
APO E ε3/ε4	80.905	83.63	39.24
APO E ε4/ε4	68.3	182.01	40.02
APO E ε2/ε2	40.3	--	--
APO E ε2/ε3	109.68	83.39	56.64
APO E ε2/ε4	--	187.56	--

(--- = out of detection range)

V.5.1.2. Variations in plasma Aβ 42 concentrations with disease progression

The follow up data (Table 16) shows evidence of association with disease progression either from MCI or early AD to severe forms of the disease. Among AD, 80% progressed to moderate/ advanced status who exhibit reductions in levels, however the difference in variance among patients and controls have not shown statistical significance by one way ANOVA test (p=0.87). MCI group with 33.33% convertors, also exhibited non-statistically significant difference in variance between annual measurements in subjects when analysed by one way ANOVA (p=0.98).

Table 16: Plasma Aβ 42 concentrations in 3 successive years; the follow up data in AD and MCI

Study Group	Sl no	Plasma Aβ 42 Concentrations (pg/ml)			APOE genotype	Remarks*
		visit 1	visit 2	visit 3		
AD	1	53.73	49.8	low	APOE3/4	Progressed from early AD to moderate AD
	2	131.55	184.11	59.43	APOE3/3	Stable AD
	3	105.96	49.44	66.84	APOE4/4	progressed from early AD to moderate AD
	4	100.95	138.06	123.33	APOE4/4	Moderate AD progressed to Advanced AD
	5	156.36	133.38	75.18	APOE3/3	progressed from early AD to moderate AD
MCI	1	83.19	119.16	110.5	APOE4/4	progressive MCI to early AD
	2	37.68	24.66	20.8	APOE3/3	Stable MCI
	3	182.01	92.6	87.5	APOE3/4	Stable MCI
	4	45.3	high	135.54	APOE4/4	progressive MCI to early AD
	5	76.92	114.3	high	APOE3/4	Stable MCI
	6	113.52	119.7	high	APOE3/4	Stable MCI

**Based on clinical diagnosis on disease stage/ progression from year 1 to final follow up.*

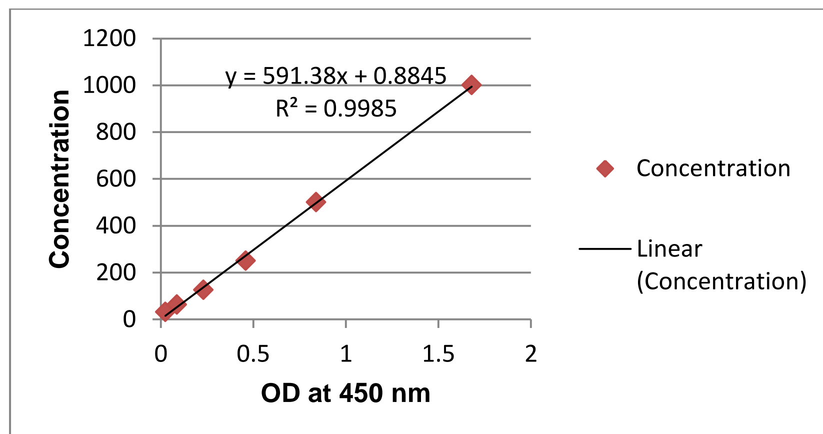
V.5.2. Human tau total immunoassay

A total of 32 plasma tau determinations were performed using a sandwich ELISA protocol. The demographics of subjects selected for plasma tau quantification is presented in Table 17. A standard curve was obtained by plotting known concentrations against OD and unknown values were calculated using Graphpad Prism software.

Table 17: Subject demographics for plasma-tau ELISA

Demographics	MCI	AD	Control
Number	10	13	9
Age	65.3±2.8	71.08±8.16	65.55±5.15
Male: Female ratio	8M: 2F	7M: 6 F	4M: 5F
MMSE	24.8±2.1	17.4±4.2	28.2±1.8
APOE ε4 frequency (%)	25	30.7	22.2

Figure 17: Standard curve for tau ELISA quantification

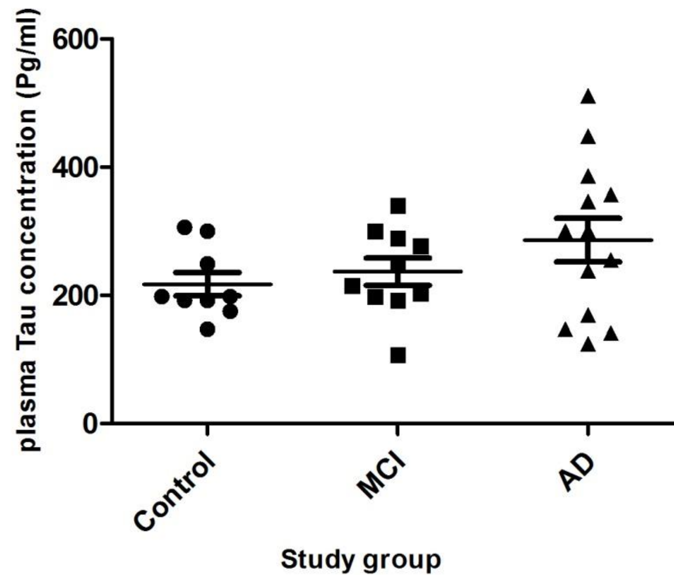


Data were compiled and are represented as Mean ± SE for every group (Table 18). Student *t* test and F test were performed to find out the difference in means and variance between groups.

Table 18: Results of statistical analysis on plasma-tau ELISA

Study Group	Plasma Tau concentration (Mean±SEM)	t test p value	95% CI	R^2	F test p value
Control	217.4±18.43				
AD	289.2±33.7	(AD Vs Control) 0.1148	-162.6 –19.03	0.1197	0.0329
MCI	237.0±21.4	(MCI Vs Control) 0.5029	-79.82-40.72	0.0268	0.5801

Figure18: Scatter plot for plasma Tau concentrations in Control, MCI and AD samples



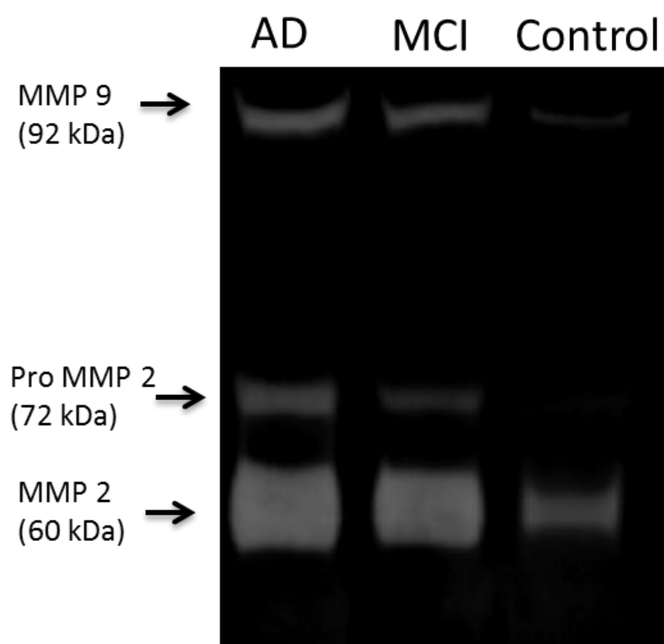
Plasma tau levels were found to be higher in Patients compared to Controls. Error bars are shown on respective lanes.

The plasma tau concentrations showed evidences of association with progressive stages of the disease. AD and MCI patients have shown elevations in mean tau levels compared to Controls; however this did not meet statistical significance (p=0.1148, 0.5029). Mean plasma tau were lower in MCI compared to AD (p=0.2367).

V.5.3. Analysis of activity of Gelatinase A&B (MMP-2 & MMP-9) by Gelatin Zymography

Activities of MMPs in sera were determined by means of Gelatin Zymography. Zymogram gel, consisting of 10% polyacrylamide co-polymerized with gelatin developed clear zones of gelatine lysis by gelatinase species A & B (MMP-2 & MMP-9) present in the samples. Quantitative zymography data was obtained by calculating the lysis units per sample. Intensities of respective bands were measured using a Biorad gel documentation system using Quantity One software.

Figure19: MMP Activity in Serum Samples of AD, MCI and Controls Represented as Clear Bands on Gelatin Zymogram



Clear bands of gelatinase activity of serum samples. Zymogram showing variations in MMP-9 and MMP-2 activity in AD, MCI and Controls. Pro MMP-2 was active in patient samples, but not in Controls.

Serum samples were analysed for MMP activity. Results show significantly increased MMP-2, MMP-9 and pro MMP-2 activity in disease groups (AD>MCI) compared to Controls. *t* test results are summarised in Tables 19 & 20.

Table 19: Results of Fisher Exact Test for the Association of MMP-2 Activity on AD, MCI and Controls

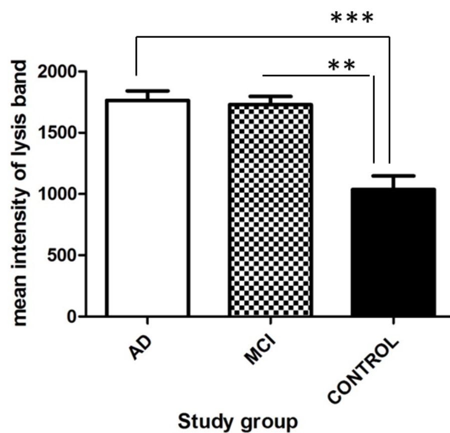
Study Group (No)	Mean intensity (Mean±SEM)	t test p value	95% CI	R^2	F test p value
Control (6)	1038±109.4				
AD (7)	1764±76.32	(AD Vs Control) 0.0002	439.8 to 1013	0.7386	0.5098
MCI (4)	1729±66.98	(MCI Vs Control) 0.0015	353.1 to 1029	0.7353	0.2832

Table 20: Results of Fisher Exact test for the association of MMP-9 Activity on AD, MCI and Controls

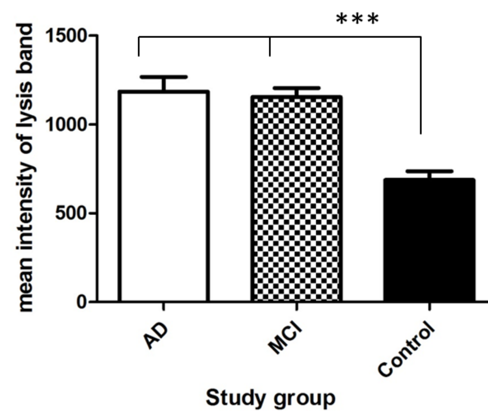
Study Group (No)	Mean Intensity (Mean±SEM)	t test p value	95% CI	R^2	F test p value
Control (6)	688±48.58				
AD (7)	1186±83.24	(AD Vs Control) 0.0004	282.8 to 712.2	0.7271	0.2623
MCI (4)	1154±52.05	(MCI Vs Control) 0.0002	296.8 to 635.2	0.8346	0.8792

Figure 20: Graphical representation of serum MMP-2 & MMP-9 activity in AD, MCI and Control groups

A. Serum MMP-2 levels



B. Serum MMP-9 levels

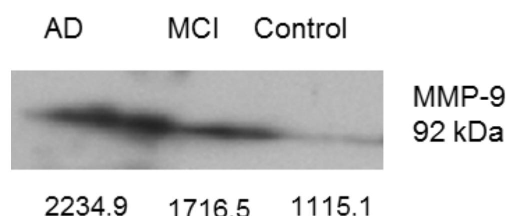


*Serum MMPs showed statistically significant elevations in patient groups (AD & MCI) compared to Controls. A. MMP-2 levels in sera samples. B. MMP-9 levels in sera samples. The three study groups are represented on the X- axis and the mean intensity of gelatin lysis area on the zymogram measured through Quantity One software are represented on the Y-axis. ** (p<0.01), *** (p<0.001).*

The results of Gelatin Zymography show that both MMP-2 and MMP-9 levels are elevated in AD and MCI compared to Controls. On Student's *t* test, highly significant variations were observed between AD/ MCI and Controls for both MMPs. However, the F test results show that the difference in variances between these groups is not statistically significant.

V.5.3.1. Confirmation of serum MMP activity through Western Blotting

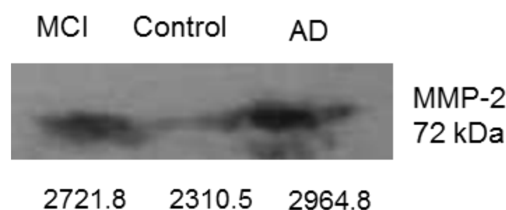
Figure 21: MMP-9 activity is increased in patient sera compared to that of control



Serum samples of AD, MCI and Control immunoblotted against monoclonal antibody against MMP-9. The bands were quantified using Quantity One software, Biorad and are presented as mean intensity/ mm^2 below each corresponding band.

Serum samples of Advanced/ Moderately Advanced AD, MCI and cognitively normal control were immune blotted against monoclonal antibody against MMP 9. Serum samples were not undergone albumin stripping, albeit a dilution of 1:20 in 1X PBS was done. The MMP-9 levels were highest in the patient sera compared to control as shown in Figure 21.

Figure 22: MMP-2 activity in AD patient' sera are higher compared to that of controls



Serum samples of AD, MCI and Control immunoblotted against monoclonal antibody against MMP-2. The bands were quantified using Quantity One software, Biorad and are presented as mean intensity/ mm^2 below each corresponding band.

Compared to controls, AD and MCI sera showed elevations in MMP-2 activity on western blot analysis

V.5.3.2. Association of APOE genotype with serum MMP levels in patients and controls

Mean activity of MMP-2 & MMP-9 were stratified for AD, MCI and controls with possession of APOE ε4 allele (Table 21).

Table 21: Correlation of serum MMP activity with APOE status of AD, MCI and Controls

	AD		**MCI**		**CONTROL**	
MMP Species	**with APOE ε4**	**without APOE ε4**	**with APOE ε4**	**without APOE ε4**	**with APOE ε4**	**without APOE ε4**
MMP2 (Mean Intensity)	1706.5±52.9	1616.6±70.2	1649±41.3	1602±55.2	1044±53	1025±44.2
MMP 9 (Mean Intensity)	1293.3±56.2	1077.6±74.3	1192±66.2	1103±55.5	716±73.2	632±49.35

Although the mean MMP levels in AD, MCI and Controls were higher in subjects who possess APOE ε4, remarkable/ statistically significant variations were not obtained on Student's *t* test.

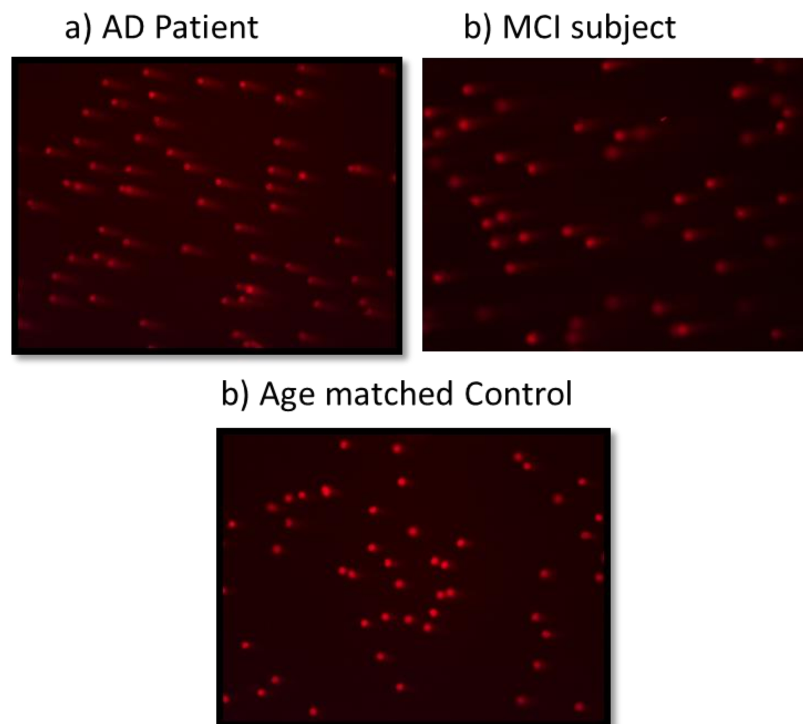
V.6. Analysis on Peripheral cellular markers of AD

V.6.1. Comet assay for analysing basal level DNA damage in PBMNCs

Comet assay is the microgel electrophoresis technique which permits the measuring of DNA damage cell by cell. Cells undergone immobilisation and alkali-denaturation of DNA when subjected to electrophoresis, cellular DNA with damage migrates from the position of the nucleus in the gel towards the positive electrode. The migrated DNA fragments were visualised as comets and the extent of DNA damage could be quantified using Cometscore analysis software that measures several damage parameters. The most useful are tail length

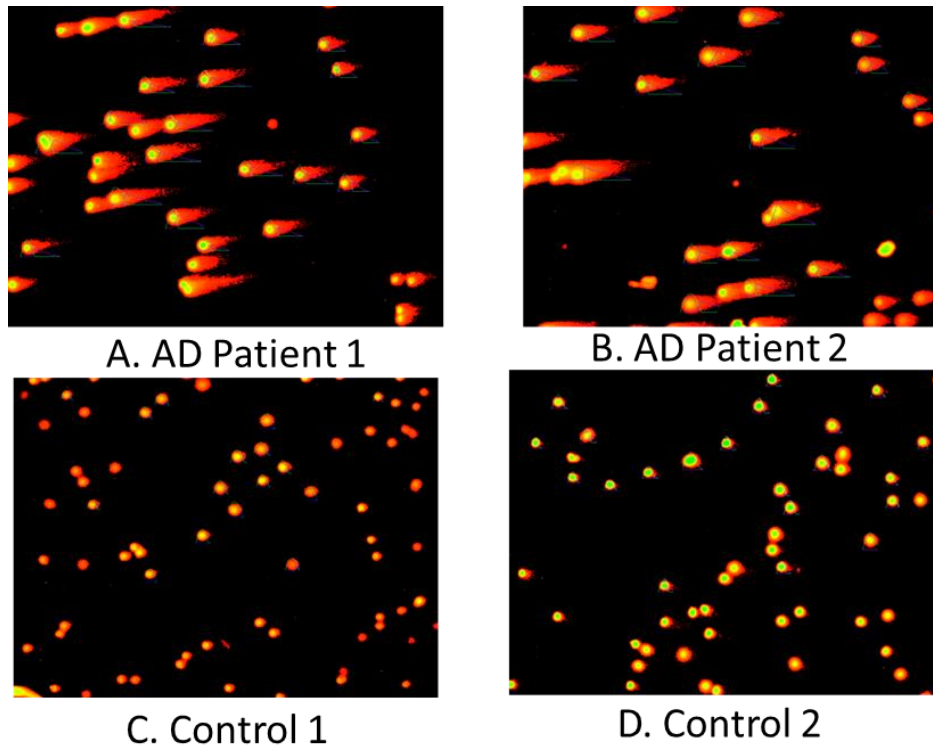
(TL), measured from the centre of the nucleus towards the end of the tail, the percentage of DNA in the tail (TD), and Olive Tail- Moment (OTM = TL X TD). Comet analysis was performed in a subset of the study population comprising 5 AD and 2 MCI (on their visit 1 to the memory clinic, SCTIMST) and 5 controls and comet scores were compared. Visualisation of comets representing the three study groups is presented in Figure 23.The scoring of comet tail lengths on Cometscore software and corresponding Tail Moments are given in Figure 24 and Table 22 respectively.

Figure 23: Fluorescent microscopic observation of Comet Assay of Peripheral Blood Mononuclear Cells.



Representative images of comets on peripheral blood cells of AD patient (a) MCI subject (b) and age matched control (c) after electrophoresis under UV microscopic observation.

Figure 24: Visualisation of Comets on CometScore Software



Visualisation of two AD patients versus two Control subject's comets on CometScore. Top panel showing comets with longer tails (AD Patients- A& B) compared to controls (bottom panel C & D).

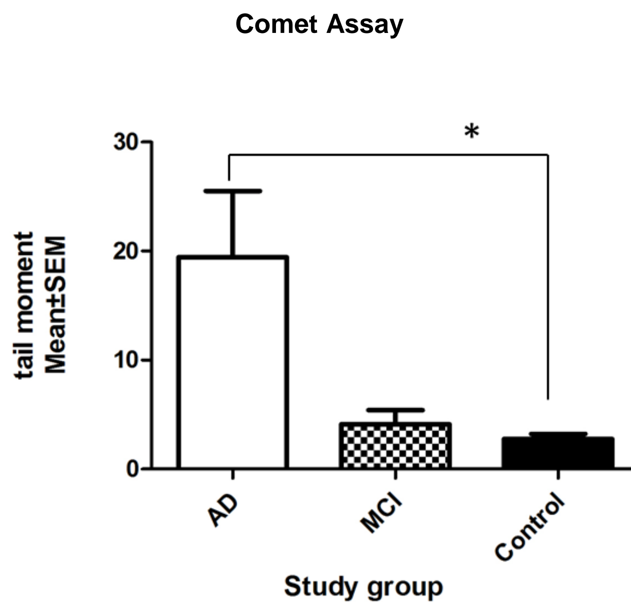
Table 22: Results of Comet Assay

Patients AD				Age Matched Controls			
Sl No	**% of DNA in tail**	**Tail moment**	**tail moment (Mean±SEM)**	**Sl No**	**% of DNA in tail**	**Tail moment**	**tail moment (Mean±SEM)**
1	27.42	6		1	19.1	2.71	
2	27.75	8.51		2	17.88	4.34	
3	29.16	16.32		3	10.28	1.37	**2.766±0.4711**
4	44.21	38.18	**19.43±6.071**	4	15.55	2.73	
5	35.09	28.16		5	13.25	2.68	
		MCI subjects					
1	19.9	2.8	**4.1±1.305**				
2	26.8	5.41					

Results of comet assay, represented as means of 100 cells per sample from different fields

The tail moments were correlated to the APOE status of the subjects. On stratification of mean tail moments according to APOE genotype, AD patients homozygous for APOE ε4 scored higher tail moments than the other alleleic types. The tail moments for AD with APOE ε4/ε4 (28.16), APOE ε3/ε4 (22.32) and APOE ε3/ε3 (11.6) were obtained. The two MCI subjects were having APOEε3/ε3 genotype. For age matched controls, the mean tail moments did not vary consistently with possession of APOE ε4, APOE ε3/ε3 (2.8) and APOE ε3/ε4 (2.7).

Figure 25: Graphical representation of Tail Moment with respect to study group on Comet Assay



Graphical representation of mean tail moments of AD, MCI and Controls on Comet Assay.
** represents p<0.05.*

Student *t* test (2 tailed) was performed to compare means of two groups under comparison, and obtained a p value= 0.0256 with CI= -30.71 to-2.627, showing a statistically significant difference between AD and Control group. F test yielded a p value of 0.0002, with a statistically significant difference in variance.

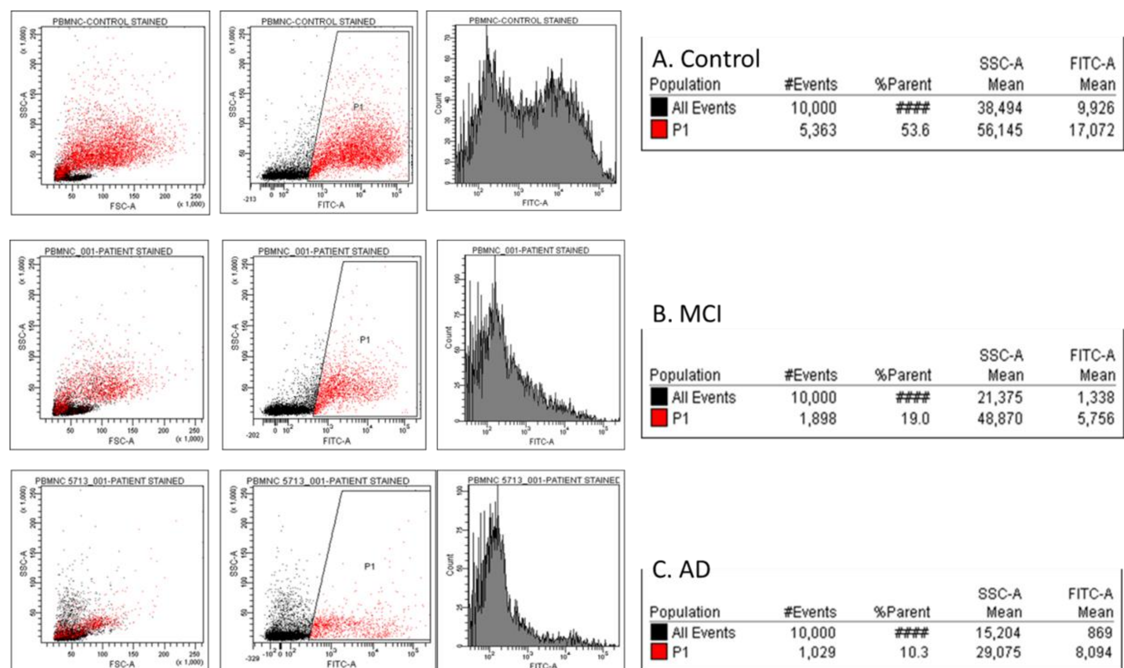
V.6.2. Flow cytometry analysis of Aβ phagocytosis by monocytes

Phagocytic potential of freshly isolated monocytes for internalising Aβ-42 *in vitro* was determined by means of flow cytometry. Monocytes isolated through Rosettesep protocol on overnight incubation with HiLyte Flour 488-labeled Amyloid β 1-42 were washed and subjected to FACS as described in the methods section. Blood samples of 12 AD patients, 4 MCI and 7 age matched controls were subjected to FACS. Monocytes were gated according to SSC/FSC and at least 10,000 cells were analyzed in FL1 (FITC) and FL2 (PE) using BD FACScan analyser.

Table 23: Subject Demographics and Mean MFI of their Monocytes Exposed to FITC-Aβ *in vitro*

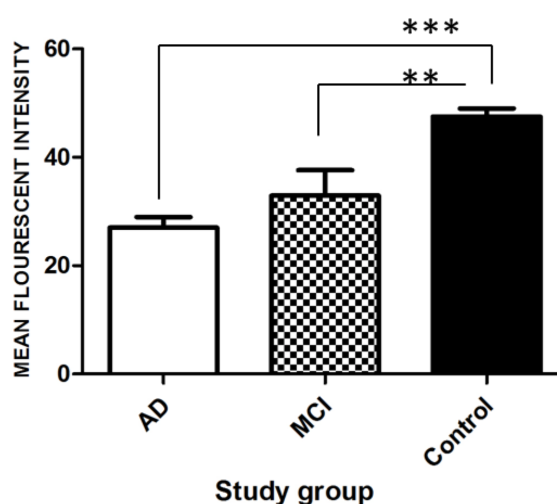
Study group	No of samples	M:F ratio	MFI
AD	12	6:6	27±6.4
MCI	4	1:3	32.9±9.4
Control	7	1:6	47.5±3.9

Figure 26: Representative Flow Cytometry Histograms



Representative flow cytometry histograms of cognitively normal control, MCI and AD patient. Phagocytosis of Aβ = Mean fluorescence intensity (MFI) of FITC Aβ in upper right corner times % cells upper right corner. Top panel A (Control) showing higher proportions of fluorescent cells compared to that of MCI and AD patient (B & C). This further illustrates that the impairment of Aβ phagocytosis is evident in peripheral cells supporting the hypothesis in different clinical stages of AD.

Figure 27: Graphical representation of MFI of monocytes with respect to study groups- AD, MCI and Controls



*Monocytes of disease groups (AD & MCI) showed statistically significant reductions on mean fluorescent intensity compared to controls. ** represents p<0.005 and *** representsp<0.0001 on Student's t test.*

Table 24: Results of statistical analysis on MFI on monocytes exposed to Aβ 42

Study Group	MFI (Mean±SEM)	t test p value	95% CI	R^2	F test p value
Control	47.5±1.5				
AD	27±1.9	(AD Vs Control) 0.0001	-26.30 – -14.70	0.7781	0.2459
MCI	32.9±4.721	(MCI Vs Control) 0.0052	-23.58—5.595	0.5993	0.0701

On Student *t* test, AD patients as well as MCI subjects have shown statistically significant reductions in mean MFI on monocytes compared to Controls (p=0.0001, 0.005). AD when compared to MCI, p=0.1868 was obtained with 95% CI=$^{-}$15.06 to 3.249, R^2 =0.1300 F test p=0.3186, which was not a statistically significant difference between these groups.

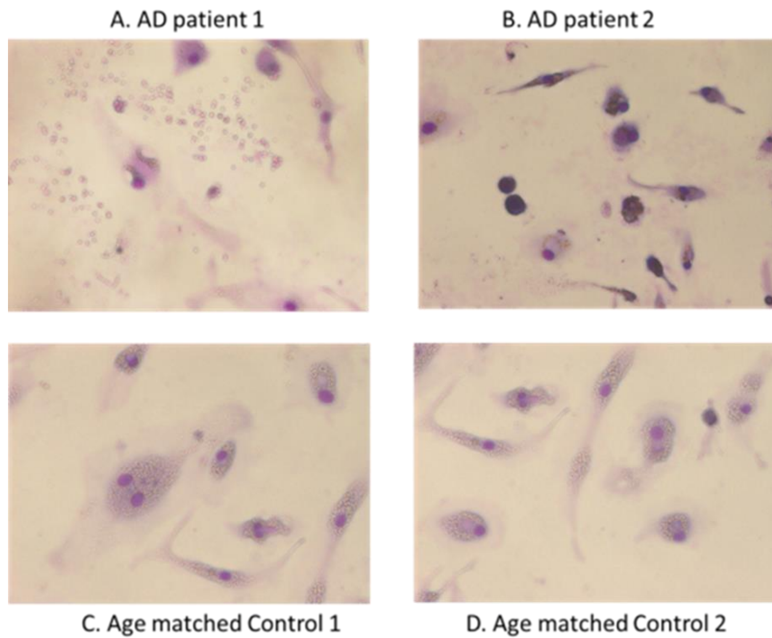
V.6.3. Differentiation of monocytes into macrophages by a 14 day culture

Monocytes were given a 2 h adherence time over 8 well culture plates and then supplemented with RPMI1640 with autologous serum (10%). The cells attained the characteristic "fried egg morphology" of mature macrophages with processes in 14 days; however differed in size and adherence properties between AD and control samples.

Monocytes of control subjects appeared enlarged by the 7th day of culture with excellent adherence over the culture dish, whereas monocytes of AD patients showed uniformly small size and poor differentiation/ adherence properties. While washing and changing of fresh media, a substantial proportion of cells were lost from patient cell cultures. Culture characteristics of MCI were similar to that of AD. The cells were supplemented with RPMI medium containing autologous serum (10%). To analyse the morphological details of differentiated macrophages, Giemsa staining was performed, that made it possible to differentiate the number and pattern of nuclei and active processes on mature macrophages. Oil red staining revealed the lipid vesicles found within healthy macrophages. These two staining methods helped to differentiate patient' and control' cell cultures morphological features and differentiation potential *in vitro.*

Figure 28: Representative images of differentiated macrophages of AD and Control on 14th day of culture

Giemsa Staining



Oil Red Staining

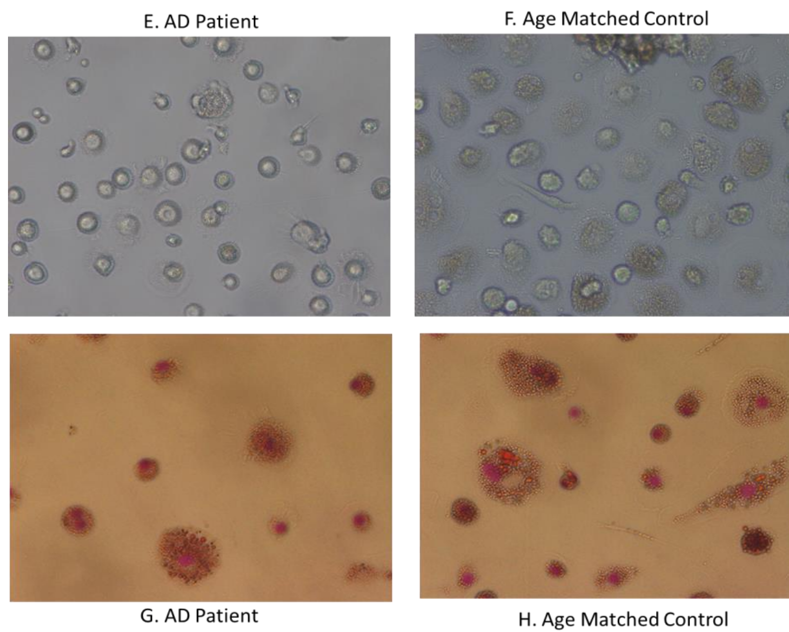


Figure 28. Giemsa stained images of control (C & D) and AD (A &B) macrophages on a 14-day old culture. Unstained (E & F) and oil-red stained (G &H) fields in cultured macrophages.

The figure indicates the morphological difference between cells and AD patients' macrophages were poorly differentiated on culture.

V.6.3.1. Macrophage Amyloid β internalisation assay

Differentiated macrophages from AD, MCI and controls were subjected to amyloid β internalisation assay and their phagocytic potential were estimated and compared between groups. The subject demographics are presented in Table 25.

Table 25: Subject demographics and MFI for macrophage amyloid β internalisation assay

Study group	No of samples	M:F ratio	MFI
AD	24	16:8	32.87±1.39
MCI	18	9:9	48.84±3.65
Control	20	7:13	54.46±3.18

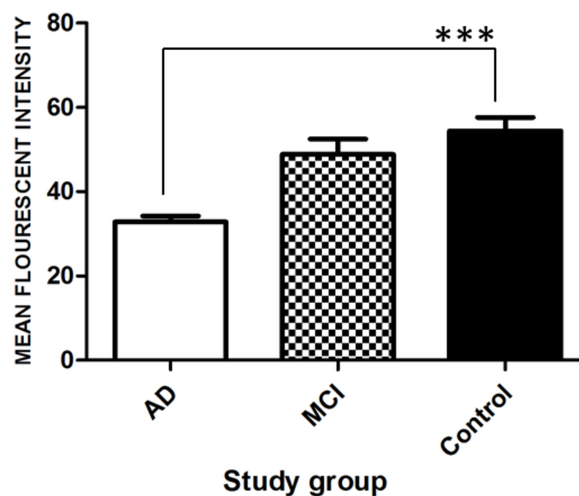
On the 14th day of culture, mature macrophages were exposed overnight to fluorescent labelled A*β.* Fluorescent microscopy and Confocal Imaging were performed over cells after treatment and images were analysed through image analysis softwares such as LSM and Image J. For each sample, 100 cells from different fields were scored for finding out mean MFI.

Table 26: Results of statistical analysis for macrophage Amyloid β internalisation assay

Study Group	MFI (Mean±SEM)	t test p value	95% CI	R^2	F test p value
Control	54.46±3.18				
AD	32.87±1.39	(AD Vs Control) 0.0001	-28.13 to -15.15	0.5203	0.0018
MCI	48.84±3.65	(MCI Vs Control) 0.2540	-15.44 to 4.217	0.03701	0.6462

On Student *t* test, AD patients have shown statistically significant reductions in mean MFI on differentiated macrophages compared to that of Controls (p=0.0001). However, the difference in MFI between the macrophages of MCI and control has not met statistical significance (p= 0.2540). AD when compared to MCI, p=0.0001, was obtained with 95% CI= (23.15 to -8.812). On F test, significant difference in variance was obtained between AD & Control and AD & MCI groups.

Figure 29: Graphical representation of MFI on Aβ internalisation by macrophages

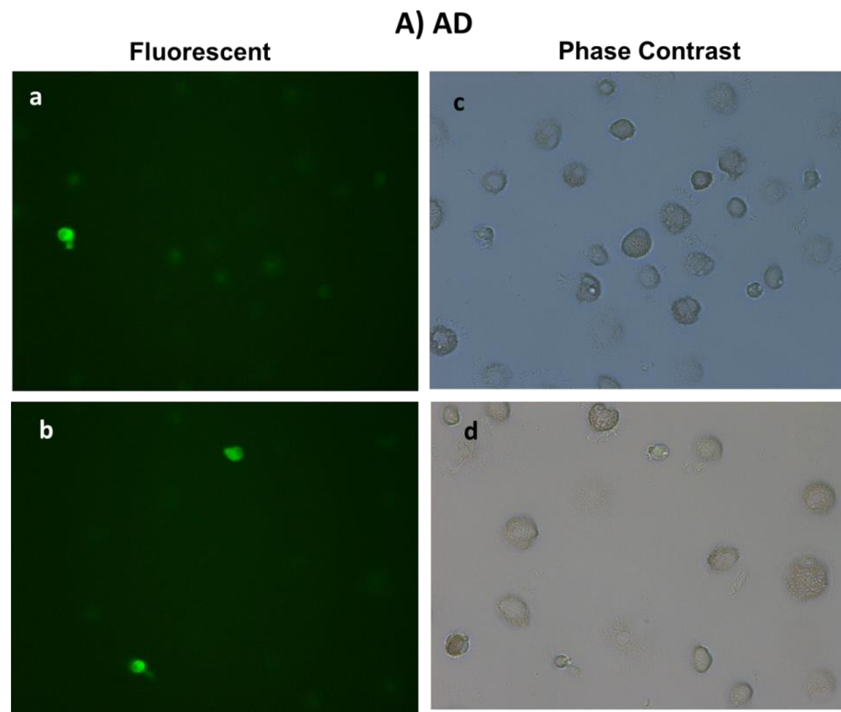


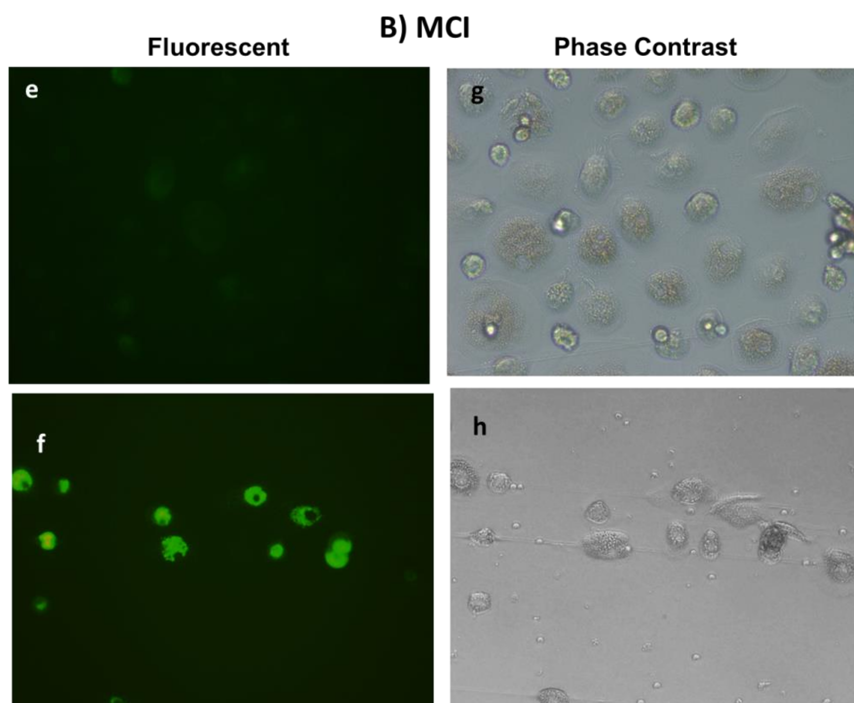
*Graphical representation on MFI (Mean ± SEM) for the three groups, showing highest phagocytic potential in control group compared to patients.***p<0.0001*

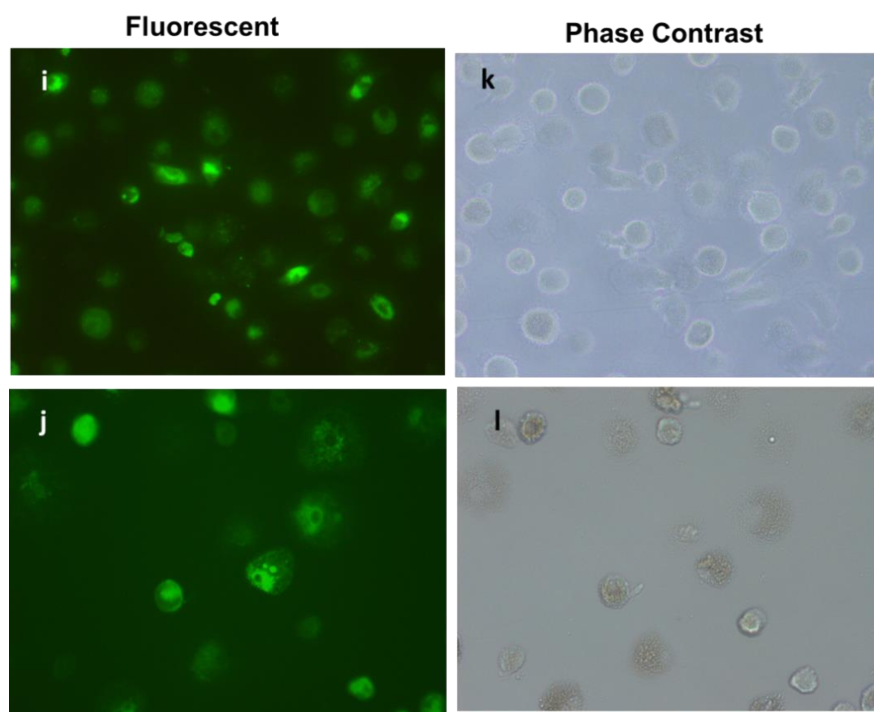
V.6.3.2. Fluorescent microscopic observations

Mature macrophages, after overnight exposure to FITC- Aβ, were observed by fluorescent microscope. Representative images at 40X magnification on fluorescent microscope are given along with respective phase contrast images of the same fields (Figure 30 A, B & C).

Figure 30: Fluorescent and phase contrast microscopy of macrophages after exposure to FITC-Aβ

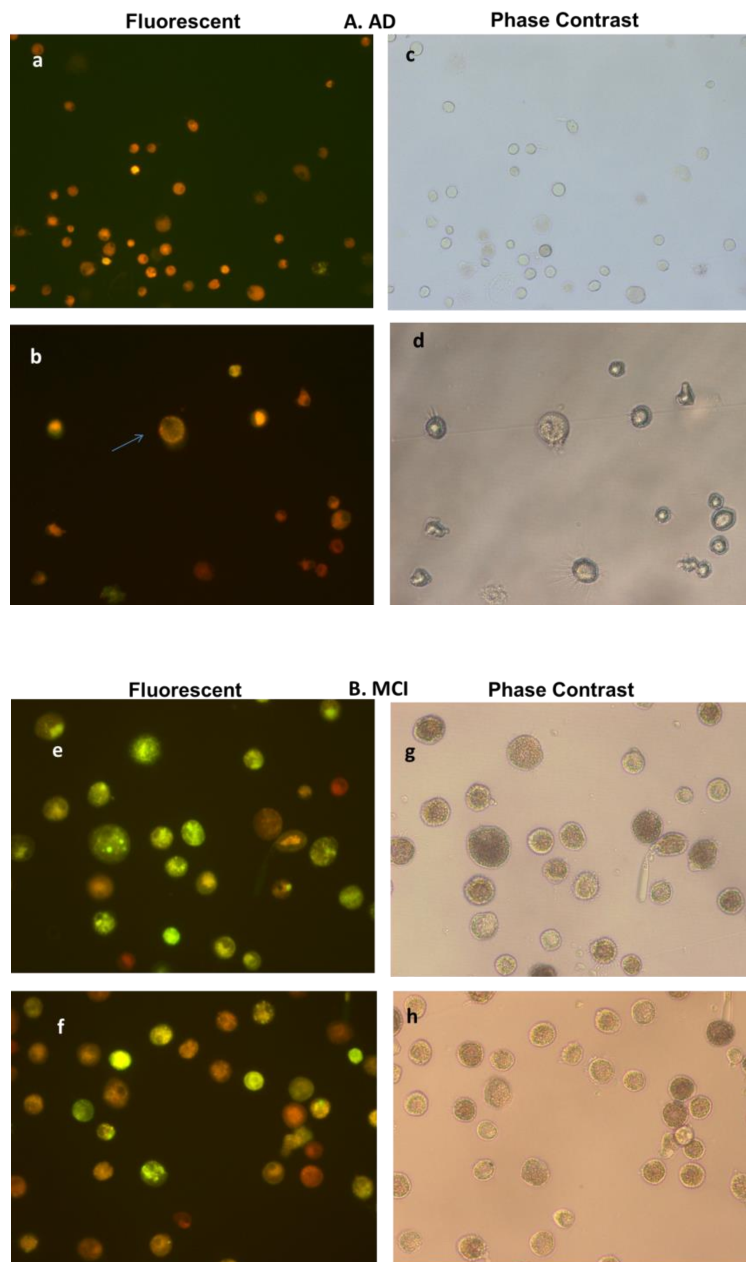


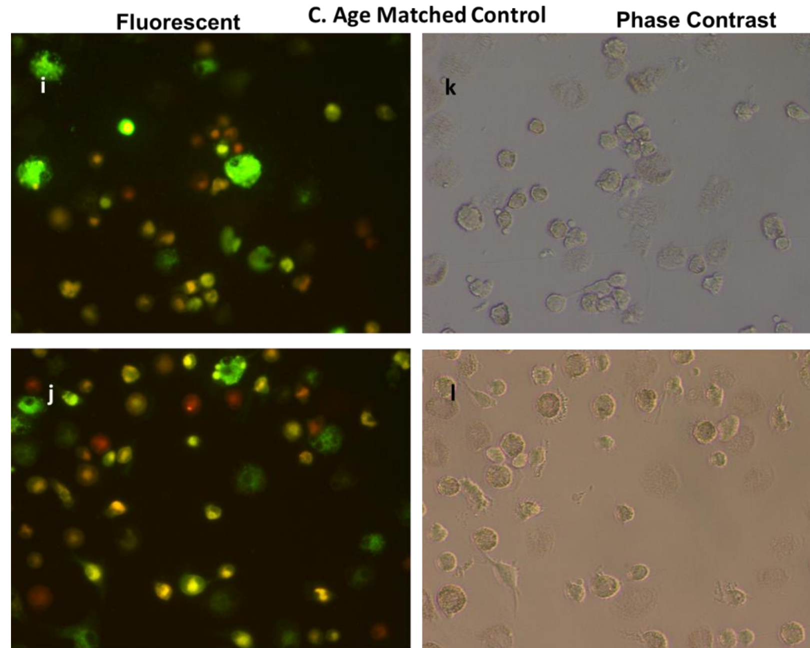


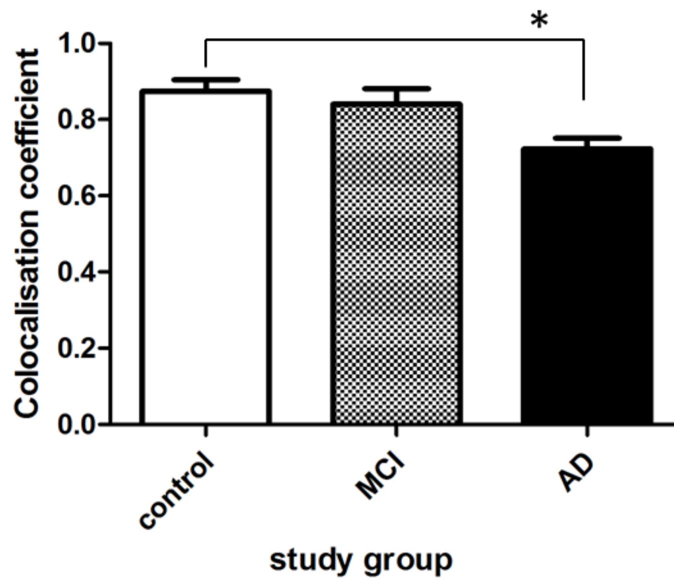


Control macrophages showed vigorous phagocytosis of fluorescent Aβ as seen by fluorescence microscopic images (left panels in Fig Ci and Cj). In comparison, AD macrophages showed least degree of internalisation of Aβ (left panels in Fig Aa and Ab). MCI macrophages showed wide variations in mean uptake, as some subjects showed good to excellent internalisation (Fig Bf) (which was similar to controls) and in others, weak internalisation potential was observed (Fig Be).

Figure 31: Flourescent and phase contrast microscopy on macrophages after exposure to FITC-Aβ and Lysotracker Red







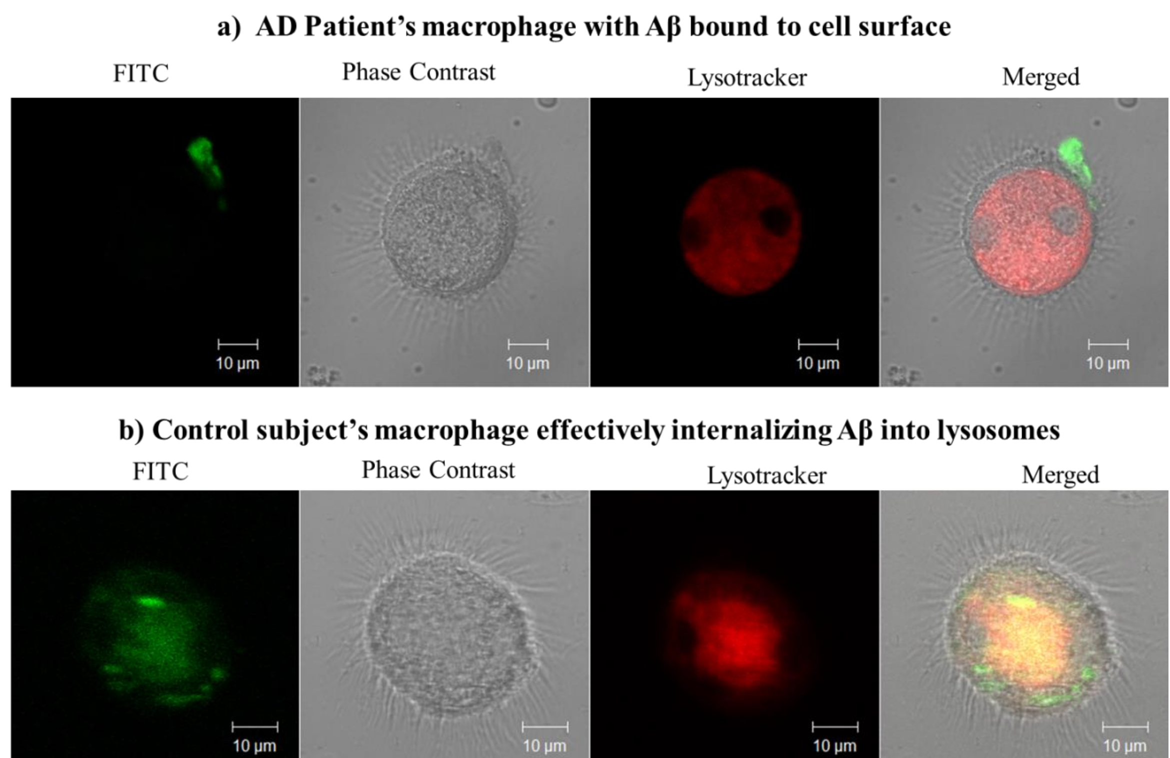
*Differentiated macrophages of an AD patient (Aa, Ab), MCI subject (Be, Bf) and a control subject (Ci, Cj)) were undergone overnight exposure to Aβ (1µg/ml) and stained by Lysotracker red, examined by fluorescent microscopy (left panels of A, B &C). D. Degree of colocalisation was determined using image J colocalisation finder software and results are presented in graph. * represents $p<0.05$.*

Localisation of amyloid beta into cellular compartments was studied in detail using the lysosomal tracker dye, Lysotracker Red. Compared to AD cells, MCI and Control macrophages showed good to excellent lysosomal trafficking of internalised Aβ, as shown in Figure 31. Student *t* test was performed to compare the means of colocalisation coefficients between groups. The difference in mean colocalisation between AD and Controls were found to be statistically significant ($p=0.0134$). However, MCI and Control were not significantly different ($P=0.53$) in localisation of intracellular Aβ into lysosomes.

V.6.3.3. Confocal Microscopy to differentiate surface binding and uptake

Further evidence for the inefficiency of Aβ phagocytosis, internalisation and colocalisation into periplasmic vacuoles/ lysosomal compartments in AD macrophages were obtained through confocal imaging. Z imaging and overlays of slices clearly distinguished phagocytic properties of AD and MCI from controls. Representative confocal captures of macrophage culture are given in Fig 32a &32 b.

Figure 32: Phagocytic properties of AD and Control macrophages



Confocal images of AD and Control' macrophages after overnight exposure to fluorescent tagged Aβ. a) Activated AD macrophage (with processes) being unsuccessful in internalising Aβ, magnified image (100X) split XY into 3 channels & merged view showing only surface binding. b) Control subjects' macrophage efficiently internalised Aβ and transported into inner lysosomal compartment where the two colour channels red and green merges to yellow.

V.6.3.4. Curcumin treatment enhances Aβ uptake by macrophages in vitro

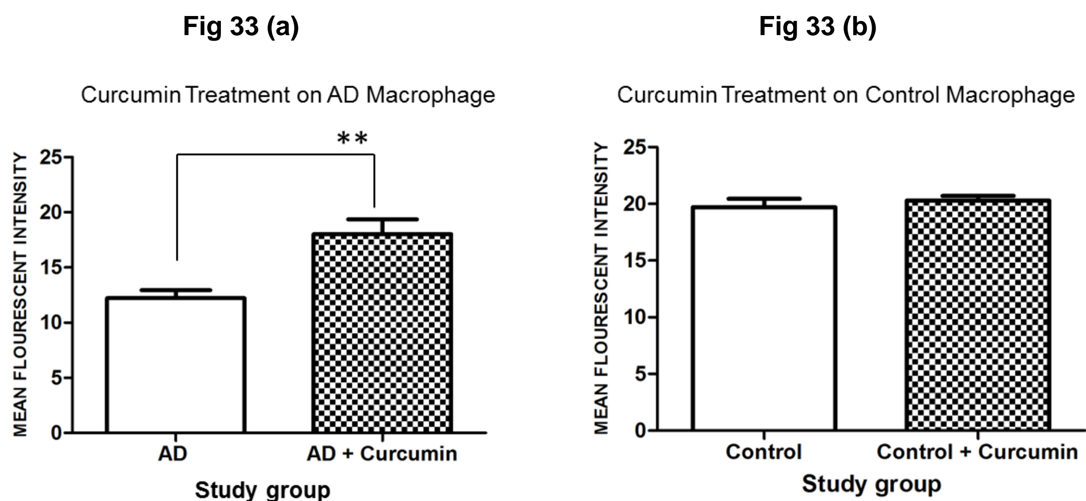
As an extension to macrophage amyloid internalisation assay, the influence of immunoenhancing drug curcumin was analysed over a subset of study subjects. Macrophages of AD patients (n=7) and controls (n=4) were treated by curcuminoids *in vitro* and measured Aβ uptake using confocal microscopy and image J software. Replicate macrophage cultures of AD and Controls were established on 8 chamber culture slides. At baseline, the intensity of Aβ uptake by AD macrophages was significantly lower in comparison to control macrophages and involved surface binding but no intracellular uptake. After treatment of macrophages with curcuminoids, Aβ uptake by macrophages of four of the seven AD patients was significantly (P <0.0001) increased. In the other patients, this treatment was found to be ineffective. Confocal microscopy of AD macrophages responsive to curcuminoids showed surface binding in untreated macrophages but co-localization with lysotracker red in an intracellular compartment after curcumin treatment. Curcumin C3 complex had intense orange colour, however, macrophages exposed to this compound at the concentration used in the assay (2 to 20 μM) did not show any background fluorescence in the green or red emission spectrum. MFI Variations on curcumin treatment are presented in Table 27 and representative bar graphs are given in Figure 33.

Table 27: Mean MFI and colocalisation coefficients in untreated versus treatment croup

	AD Patient (7)		Age Matched Control (4)	
	Without curcumin treatment	Treatment with 10µM curcumin	Without curcumin treatment	Treatment with 10µM curcumin
MFI (mean± SD)	12.2±1.9	18.03±3.53	19.72±1.45	20.3±0.8
Colocalisation Coefficient	0.72	0.93	0.87	0.92

Unpaired, 2-tailed *t* test revealed that a statistically significant difference exists in the AD macrophages between the MFI's of curcumin treated and Untreated cells on Fluorescent Aβ exposure. P value=0.0025, 95% CI=-9.138 to -2.490. However, in the macrophages of Controls, curcumin treatment did not result in statistically significant improvement on internalisation of Aβ.

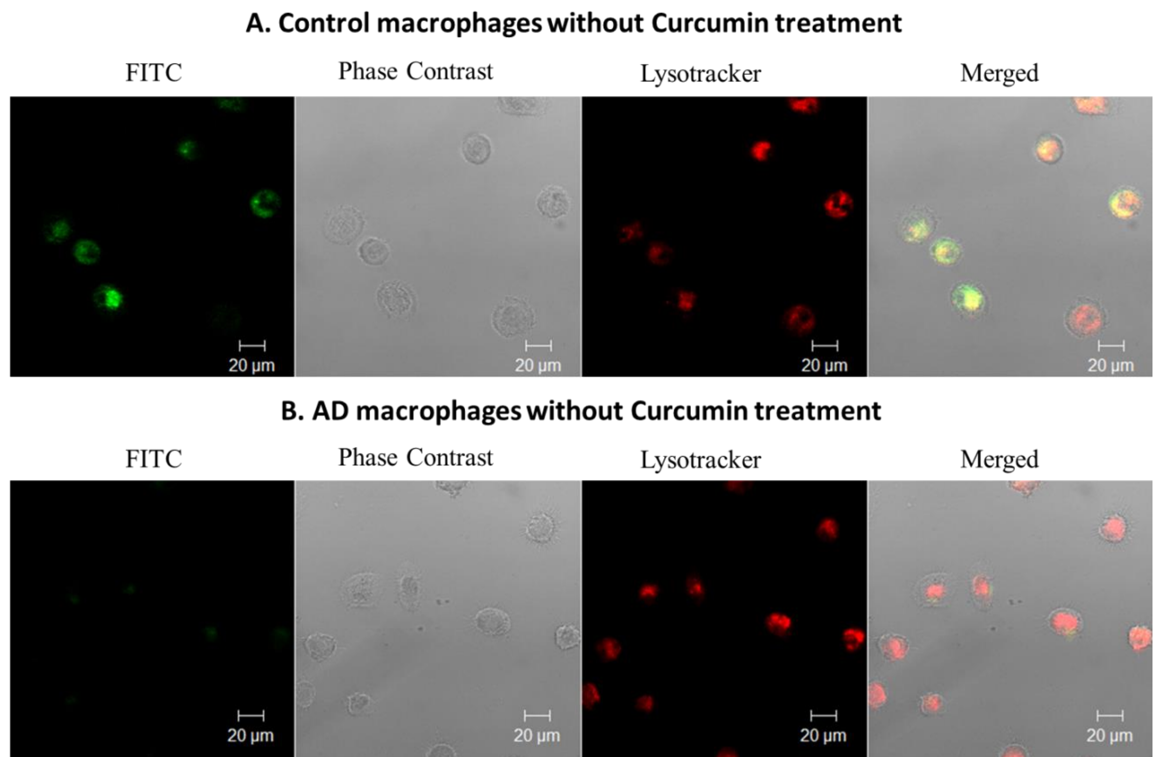
Figure 33: Graphical representation of difference in Aβ uptake on curcumin treatment by AD (Fig 33 (a)) and control (Fig 33 (b)) macrophages



*Curcumin treatment on AD macrophages resulted in statistically significant increase in mean flourescent intensity on exposure to FITC- Aβ. ** represents p<0.005.*

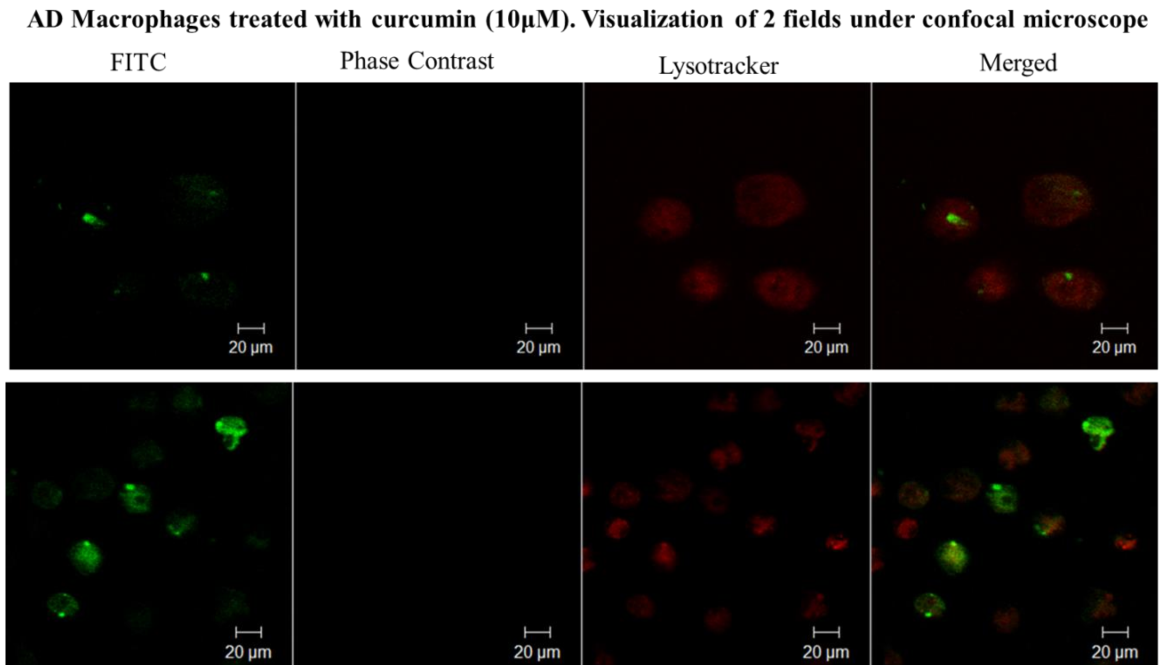
V.6.3.5. CONFOCAL IMAGING OF MACROPHAGE Aβ INTERNALISATION

Figure 34: AD and control macrophages showing baseline internalisation of fluorescent Aβ



Confocal images of control (34 A) macrophages showing baseline fluorescence on amyloid β internalisation. Untreated AD macrophages (34 B) showing least degree of green fluorescence.

Figure 35: AD macrophages on Curcumin Treatment



AD macrophages with enhanced phagocytic efficiency on treatment with curcumin.

Figure 36: Control Macrophages on Curcumin Treatment

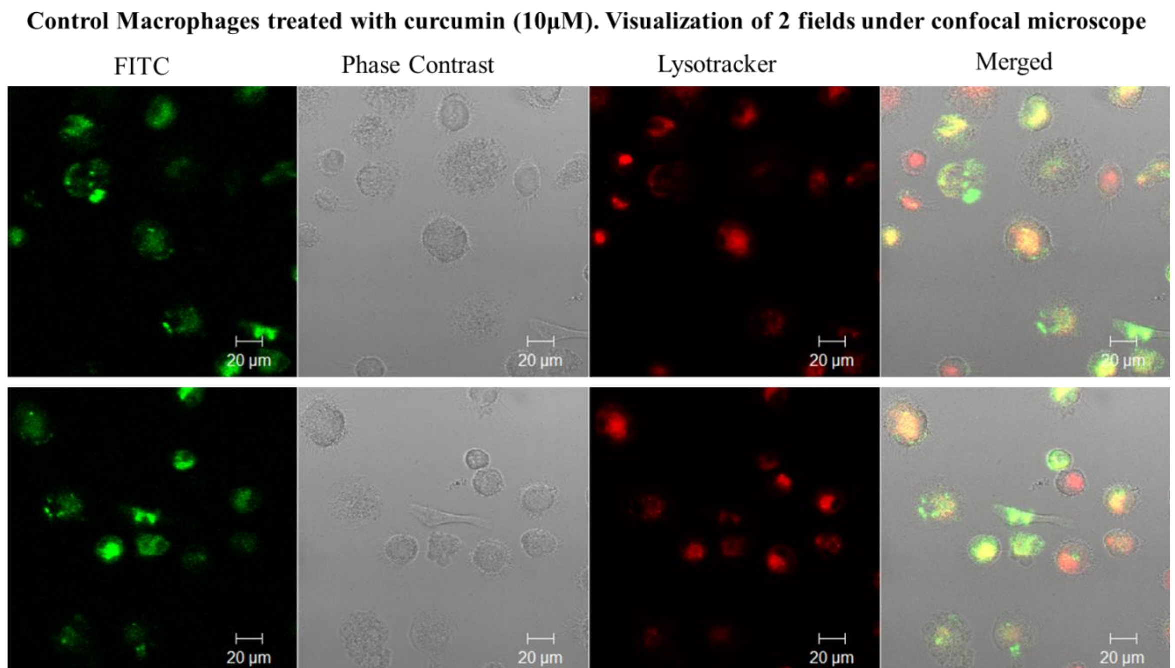
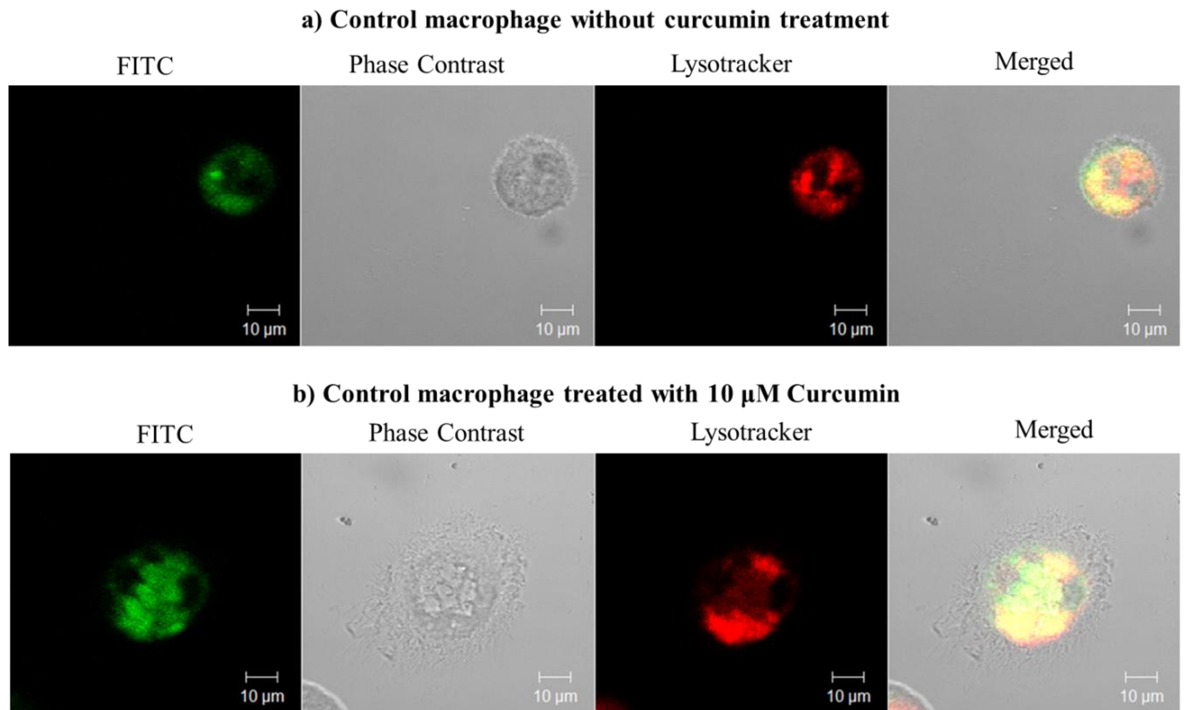


Figure showing excellent phagocytic potential in two of the four Controls undergone curcumin treatment.

Figure 37: Control subjects seem to be unresponsive to curcumin treatment

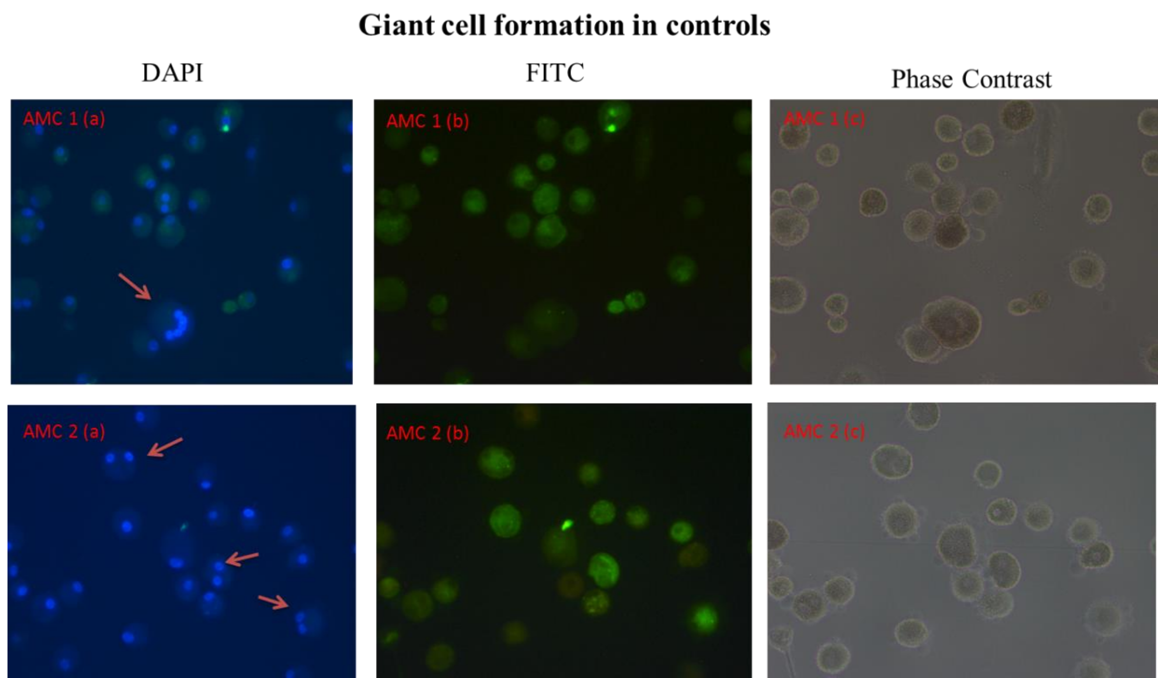


Unresponsive control macrophages without (a) and with (b) and Curcumin treatment. 37 a. Untreated cells, 37 b. Replicate culture treated with 10µM curcumin. Results showing no remarkable improvement in Aβ internalisation efficiency.

V.6.3.6. Control cultures showed characteristic giant cell morphology on 72 h post exposure to amyloid β

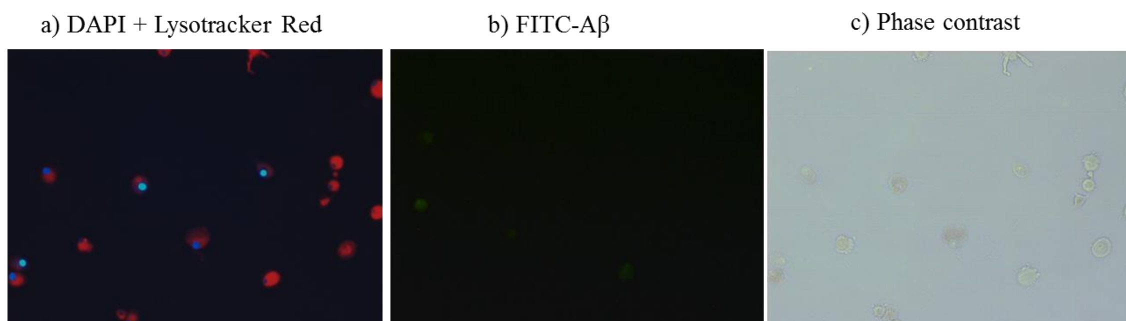
It was confirmed that control cells have the increased potential to actively phagocytise exogenous Aβ and translocate it to inner lysosomal compartments. The control cells were followed up after 72 h post exposure and analysed by fluorescent microscopy. The characteristic giant cell formation was observed in a percentage of control cells. In an attempt to clear the internalised peptide, two or more activated macrophages happened to fuse together. This cell fusion leads to giant cell morphology in culture bearing more number of nuclei in giant cells. This was visualised by staining cells with nuclear stain DAPI. Resulting images are represented in Figure 38.

Figure 38: Giant cell morphology acquired by controls, not AD macrophages



Upon 72 h exposure to Aβ control macrophages showed aggregation into giant cells in an attempt to clear Aβ without any sign of apoptosis (Arrows pointing to giant cells Figure 38. AMC (Age Matched Control) 1(a) & AMC 2 (a)); whereas macrophages of AD subjects showed cell rounding and poor internalisation (Fig 39).

Figure 39: AD macrophages not showing giant cell formation



AD macrophages 72 h post exposure to Aβ did not show giant cell morphology. Fig 39 a. Cells stained by nuclear stain DAPI and Lysotracker Red. No macrophages with 'double nuclei' are visible in the field. Fig 38.b. Cells under fluorescence Fig 39 c. Phase contrast.

V.6.3.7 Internalisation of Aβ is influenced by APOE genotype

The subjects were stratified according to the possession of APOE ε4. Although the mean MFI for total samples showed an expected gradation in phagocytic potential, as AD<MCI<Control, there were wide variability in this property within group. In order to analyse whether this is influenced by their APOE genotype, APOE ε4 frequency and MFI were correlated as shown in Table 28.

Table 28: Presentation of MFI on study groups as per possession of APOE ε4.

	AD (n=24)	MCI (n=18)	CONTROL (n=20)
MFI for subjects with at least one E4	30.12±3.7	47.76±19.17	44.74±7.88
MFI for subjects with no E4	34.5±7.8	50.54±8.01	58.93±13.96

On student *t* test, the difference in MFI for AD group was not found to statistically significant (p=0.1322, 95% CI= -1.431 to 10.20). For MCI, a p value of 0.7227 was obtained (with 95% CI=-13.55 to 19.11). However in the control group a statistically significant difference was found between the MFIs (p=0.03, 95% CI=1.18 to 27.19). In AD and MCI subjects the mean

MFI is lower with possession of at least one APOE ε4 allele, and in Controls the difference meets statistical significance.

V.6.3.8. Enhancement of amyloid uptake by curcumin is influenced by APOE genotype

A subset of samples chosen for curcumin treatment study was proved to be unresponsive to treatment. The actual MFI values and APOE genotypes are represented in Table 29.

Table 29: Presentation of MFIs of selected AD patients and controls with their APOE status

Study subject	MFI of Aβ internalisation without curcumin	MFI of Aβ internalisation with curcumin	change in MFI (Mean ±SD) for responsive patients	APOE Genotype
AD Patient 1	10.4	**20**		E3E3
AD Patient 2	12.8	**22.4**	12.21±1.9 to	E3E3
AD Patient 3	10.2	**21.5**	18.02±3.53	E3E4
AD Patient 4	10	**19**		E3E4
AD Patient 5	14	14.4	NR*	E3E4
AD Patient 6	13.9	14	NR	E4E4
AD Patient 7	14.2	14.9	NR	E4E4
Control 1	20.5	20.5	NR	E3E4
Control 2	19.7	**21.3**	19.72±1.45 to	E3E3
Control 3	17.7	**19.4**	20.3±0.8	E3E3
Control 4	21	20	NR	E3E4

*Statistically significant improvement in MFI on curcumin exposure is represented in bold letters. *NR-Not responsive to curcumin treatment*

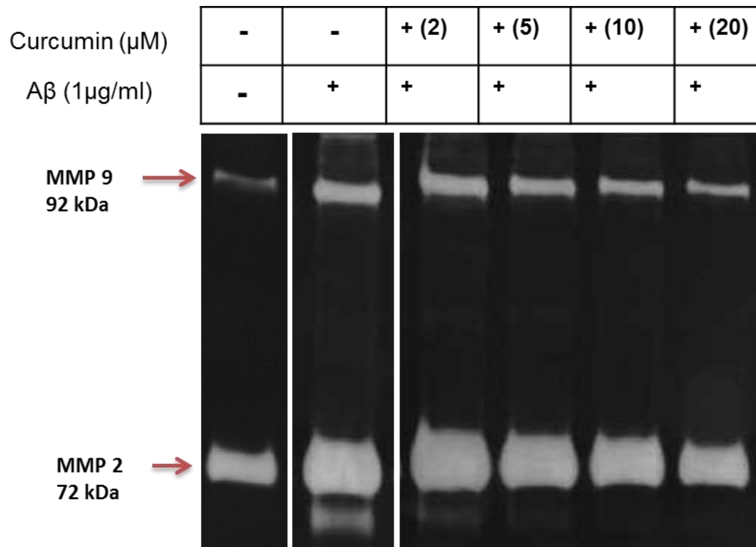
The property of responsiveness toward curcumin treatment is probably correlated well to the subject's APOE status. For AD patient's macrophages, excellent improvement in Aβ internalisation on curcumin exposure is confined in 4 subjects out of 7, who are bearing APOE ε3 rather than APOE ε4. Similarly, in Controls, highest MFI variations were observed in individuals bearing APOE ε3 compared to APOE ε4. However, with this data, we cannot

substantially state that APOE ε4 negatively regulates Aβ internalisation even in the presence of an inducer (curcumin) due to lack of effective sample size for the analysis.

V.6.3.9. Analysis of MMP activity on cell culture supernatant of AD and Control

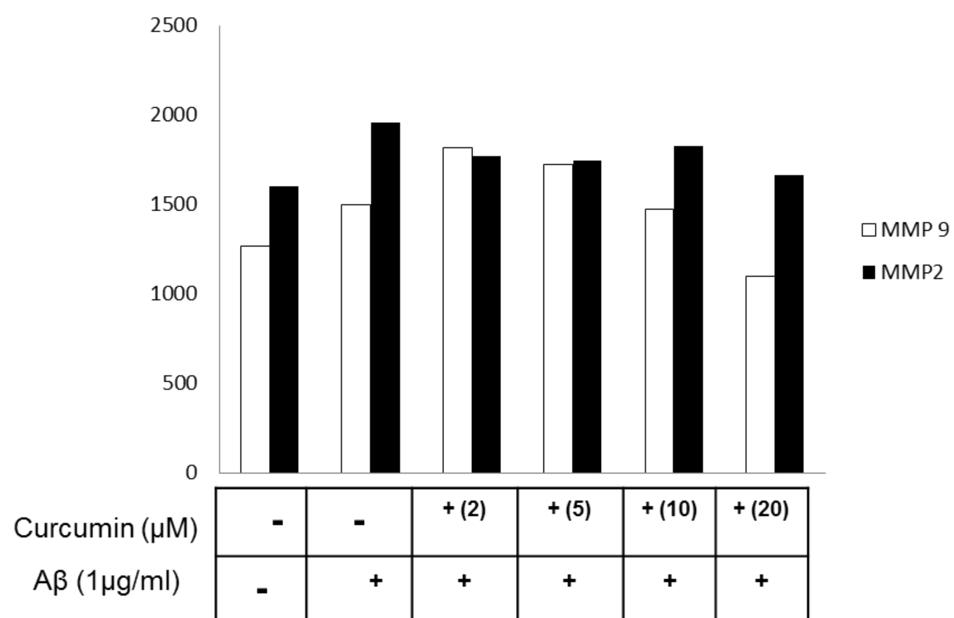
Since MMPs are markers of inflammation and reported to be capable of degrading Aβ species, it is imperative to analyse the change in expression of MMP-2 & MMP-9 in blood borne macrophages of AD patients and Controls. As an extension to our search for disease correlates in the biological samples, we analysed the MMP activity on cell culture supernatants to find out differential responses of AD and Control cells to Aβ treatments and curcumin exposure. Curcumin, being an anti-inflammatory agent readily reduced MMP levels in cells. AD and control macrophages were initially subjected to treatment with Aβ (1µg/ml) overnight and subsequently exposed to a range of varying concentrations of Bis Demethoxy Curcumin (BDC) (2, 5, 10 & 20 µM) in serum free media. 24 h post exposure to BDC, the cell culture supernatants were collected and subjected to Gelatin zymography in order to analyse relative activity of MMP species. The results derived from a single experiment are summarized in figures 40, 41 (AD patient) and 42, 43 (Control).

Figure 40: Activities of MMP-2 and MMP-9 in AD macrophage culture supernatants subjected to exogenous stress by Aβ (1µg/ml) and varying concentrations of BDC



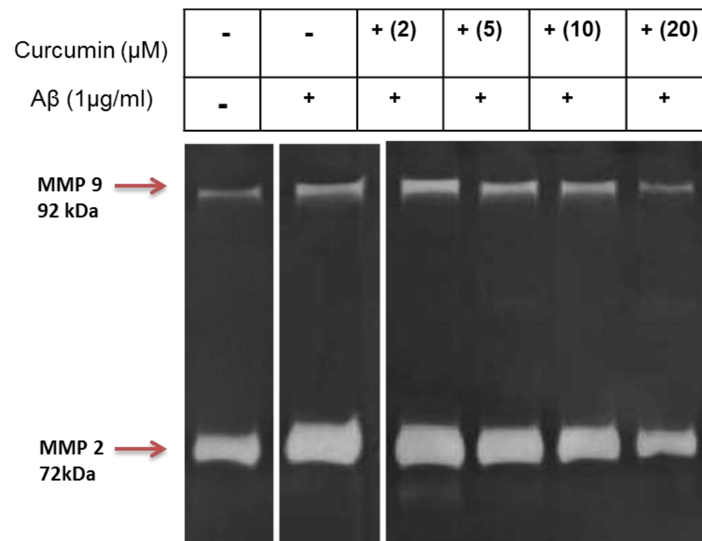
Representative zymogram showing response of AD macrophages towards treatment of Aβ, and varying concentrations of curcumin as shown in the top of each lane. The marked reduction in MMP levels were obtained only at the treatment of highest concentration of curcuminoides (20µM) on AD macrophages. Results derived from a single experiment are presented.

Figure 41: Graphical representation of AD macrophages MMP activity in response to BDC



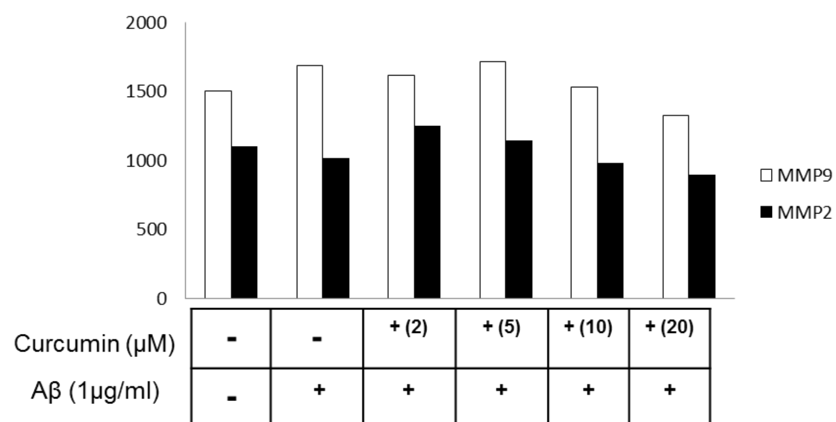
MMP-2 & MMP-9 expression on cultured AD macrophages under treatments with Aβ and Curcumin. X axis– (AD macrophages without any treatment), +(AD macrophages after treatment) Bottom panel shows variations in curcumin concentrations (2, 5, 10 and 20 μM)given post exposure (overnight)to exogenous Aβ. Y axis- Quantity of MMP lysis bands (quantified using Quantity One software, Biorad).

Figure 42: Activities of MMP-2 and MMP-9 in control macrophage culture supernatants Subjected to exogenous stress by Aβ (1μg/ml) and varying concentrations of BDC



Representative zymogram illustrating serial reductions on MMP expression of cultured Control macrophages upon BDC treatment as shown in the top of each lane. The reductions in both MMP-2 & MMP-9 expression were achieved with a lower concentrations of BDC compared to AD patients.

Figure 43: Graphical representation of control macrophages MMP activity in response to BDC



MMP-2 & MMP-9 expression on cultured Control macrophages under treatments with Aβ and Curcumin. X axis– (AD macrophages without any treatment), +(AD macrophages after treatment) Bottom panel shows variations in curcumin concentrations (2, 5, 10 and 20 μM)given post exposure (overnight) to exogenous Aβ. Y axis- Quantity of MMP lysis bands (quantified using Quantity One software, Biorad).

The MMP lysis bands were quantified using Quantity One software (Biorad). The results show that in control cells, lower doses of BDC efficiently reduce MMP expression. 2-5 μM BDC were able to reduce MMP levels up to normal physiological level (intensity of band at 1100 (MMP-2) and 1600 (MMP-9)) in control' macrophages. However, in AD cells, very high doses of BDC were required to bring down the elevated MMP levels (upon Aβ stress) to normative condition (20 μM). The results show that differential responses of phagocytic properties of peripheral cell are reflected in their differential expression of anti-inflammatory defence mechanism. Even in the presence of an immune enhancer, the patient cells could not counteract the inflammatory stress.

VI. DISCUSSION

The urge to associate markers with human diseases has been around for centuries. Especially for AD having an oligogenic and multifactorial etiology, investigations on predisposing genetic and environmental factors are very crucial in different populations across the globe for effective management of the disease. This is important in that the relative distributions of these factors vary among different ethnic groups. Associations of various genetic, biochemical and immunologic markers with susceptibility to AD have been reported, many of which have not been replicated, most of which are not widely accepted, and the rest remain controversial. Indian studies regarding AD biomarkers are very few except genetic association with APOE ε4 allele (Bharath S et al, 2011).Studies on the markers for prediction and early diagnosis are cornerstone approaches to the management of dementia.

Our study is focused on correlates on three distinct lines- Genetic, Biochemical and Cellular – associated with AD, which could be evolved as markers that can distinguish patients with AD and its preclinical phase MCI from healthy controls. We have analysed patterns of differences for each variable/ factor independently and the deviations between disease and control groups were statistically analysed. The study group was a clinic-based cohort comprising of patients attending the Memory Neurology Clinic, SCTIMST from 2009 to 2013, hence represents the relative frequency of reported dementia cases in the place such as AD (37.2%), MCI (20.8), FTD (30.3%), DLBD (2.8) and VD (8.8%). All study groups were matched for age at inclusion into the study and sex and ensured that control group was not having any genetic relation with patient group. The spouses selected as controls were non consanguineous, and the disease groups were ethnically matched with controls.

VI. 1. GENETIC CORRELATES

VI.1.1. APOE ε4 is over represented in AD patients compared to other diagnostic groups and controls

APOE is a well-known genetic and anthropological marker showing wide geographical and ethnic variation across the world (Table 30). Recent research on assessments on AD lifetime

risks associated with possession of APOE ε4 has proposed to shift the position of this factor into "Major gene" from the status of a "risk factor" (Genin et al., 2011). In Caucasian populations, roughly 2% of the population bears the APOE ε4/ ε4 genotype. Considering the major risk conferred by this genotype (~30% by age 75 and >50% by age 85), it would be appropriate to target in priority these individuals, in clinical trials of disease preventing therapies. Globally, the APOE locus shows substantial allelic variation with ranges 0–20% for ε2, 60–90% for ε3 and 10–20% for ε4 alleles (P. P. Singh, Singh, & Mastana, 2006). In Indian populations, these values vary with ranges 3.3- 9.4% (ε2), 85.1-88.2% (ε3) and 3.0 to 8.8% (ε4) (P. Singh, Singh, Gerdes, & Mastana, 2001)(Chandak et al., 2002). The highest proportions of ε3 allele are reported from Asian and Indian populations (Table 30).

Table 30: Gene Diversity Analysis- distribution of APOE alleles across various populations

Populations	APOE ε2 frequency	APOE ε3 frequency	APOE ε4 frequency
Africa	0.099±0.083	0.690±0.110	0.209±0.090
Europe	0.077±0.033	0.790±0.056	0.127±0.049
Asia	0.063±0.030	0.847±0.054	0.090±0.043
North America	0.049±0.041	0.824±0.060	0.127±0.057
South America	0.046±0.069	0.767±0.129	0.187±0.132
Oceania	0.111±0.052	0.667±0.162	0.221±0.149
India	0.051±0.017	0.881±0.039	0.068±0.030
All Populations	0.073±0.047	0.790±0.088	0.133±0.074

Data adopted from (P. P. Singh et al., 2006)

In this clinic based prospective case-control study involving 557 individuals, we have estimated the allelic and genotypic distribution of APOE isoforms (APOE ε2, APOE ε3 and APOE ε4) and examined the correlations with the development of disease. This study confirms the work of different groups in India pertaining to strong association of ApoEε4 in dementia, AD in particular. Firstly, the present work reports a frequency of 0.18 for APOE ε4in South India which is higher than the single, previous report (0.08, (Bharath S et al.,

2010)). The epidemiological study from Ballabgarh (Ganguli M et al., 2000), the largest in the country (n = 4,450), reported a frequency of 0.07 for APOE ε4 in their cohort. Other Indian studies have quoted an APOE ε4 frequency range of 0.00 (specific communities) (Singh PP et a.l, 2006) to 0.09 (Luthra K et al., 2004). Secondly, the current study reiterates a high APOE ε4 frequency in the dementia group. In AD the frequency is 0.40 which is also higher than other Indian studies, yet these ranges fall very close to the reports from Caucasian population (Quiroga P et al., 1999). With a very low sample size, two North Indian reports show similar figures; one from Panjab (APOE ε4 frequency 0.47 and 0.13 for AD and controls) (Kaur and Balgir, 2005) and the other from Delhi (ApoE4 frequency 0.43 and 0.11 for AD and controls) (Kapur et al., 2006). Also, the frequency is higher than the largest community-based study from Ballabgarh (Ganguli M et al., 2000) (0.15 in all dementias), the hospital-based study from Lucknow (0.13) (Pandey et al., 2007), and a rural community study from North India (0.29) (Luthra K et al., 2004).

Individuals carrying the APOE ε4 allele had an increased risk for developing AD, both in homozygous (OR=3.8) and heterozygous condition (OR=2.8 for ε3/ε4, 0.5 for ε2/ε4) (Table 10). The frequency of the protective allele APOE ε2 was found to be higher in Control group (0.07) compared to disease groups- AD (1.28%), MCI (4.65%), FTD (1.89%) and VD (4.05%). However the highest APOE ε2 frequency was detected in the LBD patient group (8.33%). This warrants further confirmations on the association despite having the lowest sample size in the study population.

Again, the APOE ε2 frequencies are lower than the other South Indian report (0.08% in AD and Controls) (Bharath S et al., 2011) and marginally lower than all the North Indian reports. As a prodromal stage of AD, MCI subjects also have a high frequency of APOE ε4 allele (0.28). For developing MCI, individuals homozygous for APOE ε4 alleles had an OR of 1.06 and heterozygotes had 2.603 (ε3/ε4) and 1.059 (ε2/ε4) times risk.

Caucasians, as a distinct ethnic group, are reported to have APOE ε4 frequency of 0.36 in AD cases and 0.13 in cognitively healthy controls by a meta-analysis by Farrer et al in 1997

as part of the APOE and Alzheimer Disease Meta-Analysis Consortium contributed by 40 research teams worldwide (Farrer et al., 1997). The present study reports similar figures in the Asian population as 0.40 in AD and 0.18 in controls. Our reports show an age dependent variation in APOE ε4 frequencies. In AD patients, the oldest old (age 70-79 &>80 years) had the highest frequency of APOE ε4. However, in Controls, median range of age for the group had the highest APOE ε4 frequency. The present study confirms that individuals carrying the APOE ε4 allele are at increased risk for developing AD compared to other forms of dementia. In our study, the risk of AD was significantly increased for people with genotypes ε2/ ε4 (OR=0.58, 95% CI=0.09617-3.551), ε3/ ε4 (OR=2.8, 95% CI=1.720-4.663), and ε4/ ε4 (OR=3.8, 95% CI= 1.5-9.647); whereas, the association was found to be weaker (with low ORs) for people with genotypes ε2/ ε2 (OR=0.07, 95% CI=0.0042-1.416) and ε2/ ε3 (OR=0.21, 95% CI=0.0440-1.012) (Table 9).

Table 31:Studies on the association of APOE gene and AD in different populations showing the strength of genetic association in comparison with the current study.

Country	Population	Cases/ Controls	Age	APOE Allele frequencies in cases (row 1)/controls (row 2)in each population			OR (95% CI)	Reference
				ε2	ε3	ε 4		
USA	Hispanics	61/ 90	76±9.4	0.06 0.03	0.70 0.84	0.12 0.24	2.6 (1.6-6.4)	(Maestre G et l, 1995)
Carribbea	Negroid	145/ 516	75.3±5.8	0.083 0.088	0.77 0.77	0.15 0.14	1.1 (0.7-1.6)	(Tang MX et al, 1996)
Cuba	Hispanics	188/ 84	76.0±8.1	0.02 0.04	0.72 0.82	0.26 0.14	3.5 (2.3-5.5)	(Harwood DG et al, 1999)
Columbia	Caucasian	83/ 44	68.1±8.5	0.019 0.09	0.75 0.83	0.23 0.08	5.1 (1.9-13.6)	(Jacquier M et al, 2001)
Chile	Caucasian	95/ 187	79.2±3.2	0.084 0.072	0.52 0.74	0.40 0.19	12.8 (3.9-47.6)	(Quiroga Pet al, 1999)
Brazil	Latin American	55/ 56	65.9±5.1	0.064 0.063	0.73 0.85	0.21 0.09	---	(Almeida OP et al, 1997)
Argentina	Latin American	45/ 45	74.7±5.5	--	--	--	3.3 (1.2-9)	(Morelli L et l, 1996)
India	Asian	142	----	0.046	0.827	0.127	---	(Hallman DM et al, 1991)
India	Asian	44/ 50	67.1±7.2	0.04	0.89	0.07	3.4 (1.8-9.3)	(Ganguli M et l, 200)
India	Asian	78/ 100	---	0.03 0.033	0.78 0.88	0.19 0.085	2.59 (1.2- 5.6)	(Chandak GR et al, 2002)
India	Asian	137/195	67.5±8.3	0.08 0.08	0.70 0.84	0.21 0.08	3.72 (2.20–6.27)	(Bharath S et al, 2011)
India	Asian	156/138	70.32±8.9	0.01 0.07	0.59 0.75	0.40 0.18	3.468 (2.052-5.862)	Current Study

OR = Odd's Ratio; 95% CI = Confidence Interval

The ORs found in the current study are higher than that of the three previous north Indian reports (Table 31) and slightly lower than that published from Southern India. The association between AD and possession of APOE ε4 is in this population is one of the strongest among Asian populations reported so far. Moreover, our study can be considered as the second largest study in India, including 156 AD patients, 87 subjects at the preclinical phase of AD (MCI) and 138 cognitively normal age-matched controls compared to the other reports. The positive predictive value was higher for APOE ε4 homozygosity compared to possession of at least one allele in the AD group. Also, the specificity (96%) and negative predictive value (72%) were higher as per our estimations for APOE ε4 homozygotes, adding more credibility to a very strong positive association of this locus in the patient group. Thus, it might be useful as an adjunction to clinical diagnosis, although we do not use APOE genotyping as a diagnostic tool.

Several investigators have suggested that APOE ε4 is the ancestral allele (Fullerton et al., 2000, Seixas, Trovoada, & Rocha, 1999); which is associated with increased absorption of dietary lipids and fat-soluble vitamins and this was advantageous when food supplies were scarce or irregular. With long established agricultural economy, allelic divergence resulted in increase in proportions of APOE ε3 allele, due to its selective advantage for some infectious diseases and immunoregulation. Whereas APOE ε2 allele is regarded as protective that is the lastly evolved form of APOE alleles that specifically transports HDL and perform normative cholesterol homeostasis functions in the body. Allele frequencies of APOE ε2 is highest in our reports compared to other Indian studies. It could be attributed to the homogeneity of our study population, without any admixtures. In addition, stratification of our data by age- groups clearly demonstrates that APOE ε4 is a risk factor not only for late-onset but for early- onset AD as well. Together, these results urge a reappraisal of the impact of APOE ε4 in AD.

The inclusion of other dementia categories (FTD, VD & DLBD) into the genetic analysis was mainly aimed to investigate the relative strength of genetic markers in them in the south Indian population. In all these groups, the APOE ε4 frequency is found to be 0.15 (FTD),

0.17 (VD) and 0.21 (DLBD) and none of these figures were found to be significantly different when compared to controls (Table 5). The results of APOE ε4 association with VD is in extreme contrast with the findings of Luthra K et al (Luthra K et al., 2004).

In FTD patients, no significant association with disease risk was observed in APOE ε4 allele carriers. This observation is in extreme contrast with the earlier reports on genetic associations of FTD with this locus (Verpillat P et al., 2002, Bernardi L et al., 2006). We had analysed the frequency of pathogenic mutations on selected exons of MAPT gene earlier in the same FTD study cohort (Aswathy PM et al., 2014) and found low frequency. It is possible that associations with some previously uncharacterised genetic factors may influence FTD pathogenesis in this population. In VD and DLBD groups, none of the APOE variants showed significant associations towards influencing disease risk (Table 5). This is in contrast with the earlier reports from northern India that had shown that the increased risk of developing AD or VD is similar among Asian population with APOE ε4 compared with Caucasian populations (Luthra K t al., 2004) which reported a frequency of ApoE 4 in VD (0.34) higher than in AD (0.29). This might be attributed to the smaller size of the VD and DLBD group which is a limitation of this study

VI.1.2. MAPT H1 is not a risky allele associated with AD in the Indian population

It has been reported that Amyloid pathology and tau pathology are interlinked, and are quite complementary to one another leading to neurodegeneration in AD hence variations on MAPT locus are equally contributing factors to pathogenicity (J. Hardy, Duff, Hardy, Perez-Tur, & Hutton, 1998; J. Hardy & Selkoe, 2002). Experiments in which mice with APP mutations have been crossed with those with MAPT mutations have shown that the major route by which Aβ triggers neurodegeneration involves alteration of tau phosphorylation, dislodging of tau from neuronal microtubules, destabilisation of neuronal cytoskeleton and NFT formation (Lewis et al., 2001). Furthermore, it has been confirmed through *ex vivo* experiments that tau expression is needed for Aβ toxicity (Oddo et al., 2003). In the presence of an amyloid load, those individuals with MAPT loci which are either highly

expressing or prone to express a more pathogenic species of tau through alternate splicing, may be more prone to AD.

MAPT H1 haplotype has been reported to be over-represented in patients with neurodegenerative diseases such as AD, FTD, PD, CBD and PSP (Zabetian et al., 2007). This variation within a single gene (MAPT) conveys risk for a number of seemingly distinct neurodegenerative dementias. This could be attributed to two causes. The first one being the H1 variant of MAPT gene imparts a functional effect on MAPT that include varying gene expression by means of alternative splicing thereby altering the ratios of six major tau isoforms in adult brain (Pittman, Fung, & de Silva, 2006). Secondly, risk conveyed by MAPT H1 is determined by its interaction with other genetic and environmental factors. Evidences from *in vitro* experiments and post mortem brain tissues indicate that variation within the H1 and H2 clades might influence both overall levels of MAPT expression (Kwok et al., 2004; Rademakers et al., 2005). Studies on the effects of two risky loci, APOE ε4 and MAPT H1 on MCI subjects in Spain has suggested that these two genetic risk factors had an additive effect in progression to AD, and that MCI subjects homozygous for MAPT H1 had shown a faster degree of progression (Samaranch et al., 2010).

However, unlike PD, PSP and CBD (Myers et al., 2005), the reports on the association between MAPT haplotypic variability and risk to AD have been inconclusive, with largely negative results (Crawford et al., 1999; Green et al., 2002; Russ et al., 2001). Recent reports have shown that H1 allele has protective effects on Alzheimer’s pathology through an autopsy confirmed study (Wood, 2012) revealing that brains from individuals with the H1 haplotype had lower neurofibrillary tangle and amyloid plaque counts than those from individuals with other haplotypes at this locus.

In our study, H1 homozygosity was assessed among all dementia groups and Controls. Haplotype H2, with European ancestry was least observed in the study population at a frequency of 1.6 (AD) to 4.35 (Control). No significant variations in frequencies were noted on segregation with respect to gender. In FTD, no significant haplotypic associations could

be established when compared to controls; which was in agreement to our earlier observation (Aswathy PM et al., 2014). No significant interaction was observed for any of the H2 allele combinations with APOE alleles in influencing disease risk in our cases. This led us to conclude that the transcriptional difference among these haplotypes have little role in the Indian dementia patients, and that H1 is not an over-represented marker of neurodegenerative tauopathies in Indian population. The H1H1 genotype has found to be having additive effect in contributing to either risk with combinations of APOE ε4 or protective effect with combinations of APOE ε2 or APOE ε3.

VI.1.3. No mutations/ Single Nucleotide Polymorphisms were detected on PSEN exons 7 and 12 in the EOFAD patients

PSEN1 is the gene harbouring most of the reported mutations associated with EOFAD (185) compared to PSEN2 (13) and APP (33). Mutation Statistics per exon reports that 42 of these mutations are located on Exons 7 and 15 on exon 12 comprising 30.81% of reported mutations on this gene (www.molgen.ua.ac.be/ADMutations). Mutation screening at this locus is particularly important since it affects at an age of onset less than 60 years, secondary identification of at risk family members become possible. Recently, a report on neuropathological and biochemical characterisation of a PSEN1 mutation has suggested that the pathogenicity of the mutation may be validated by radiological and biochemical markers in the patients (Ringman et al., 2011). Most of the pathogenic PSEN1 mutations reported so far interfere with either the α helical structure in the transmembrane domain of the protein or normal proteolytic processing of APP leading to increased Aβ production (Cruts, Hendriks, & Van Broeckhoven, 1996). A population based study in Netherlands has shown that PS-1 and PS-2 mutations are rare genetic causes of presenile AD, and that the frequency of PS-1 mutations in autosomal dominant AD families (18%) was less than initially estimated (Cruts et al., 1998).

PSEN 1 mutations are the most common genetic markers of 100% penetrance associated with EOFAD. Initial estimates based on linkage analysis studies suggested that 70% of the

presenile AD families were linked to chromosome 14 (Schellenberg et al., 1992). Later, a mutation in PS-1 was found in each chromosome 14-linked AD family (Sherrington et al., 1995). This locus has been a subject of genetic association with AD cases in India in terms of a polymorphism located within the intron 3' of the exon 8 of PSEN-1 gene (Chandak et al., 2002). We performed mutation screening through directly sequencing PSEN exons 7 and 12 (n=28)in order to determine its direct contribution to mutations on EOAD in our cohort. However, we could not detect any previously characterised/ novel mutations or pathogenic/ non-pathogenic polymorphisms on these two exons. While considering the APOE genotypes of these subjects, 85% were not bearing at least one E4 allele. The over-representation of 'safe and protective' APOE isoforms and absence of PSEN mutations in these EOFAD subjects warrant other causative factors contributing to AD pathogenesis. Comprehensive mutation analysis on the subjects may lead to identification of associated genetic loci such as PSEN2, APP or other PSEN 1 exons.

VI.2. BIOCHEMICAL CORRELATES

VI.2.1. Reduction on Plasma Aβ 1-42 was detected correlating to disease progression in MCI and AD

Latest scientific findings suggests that a blood test would be predictive of cognitive decline a decade later in life, and this approach have profound implications on slowly progressive dementia that affect only elderly. Along with core feasible CSF biomarkers, identification and validation of disease specific markers in blood and peripheral tissues are an active area of AD research. If blood reflects the pathologies affecting brain, it could be adopted as a less invasive source of AD biomarkers. To our knowledge, this is the first attempt to find out AD biomarkers in Indian population. We investigated the quantities of plasma Aβ 1-42 and total tau in patient's plasma and compared to that of controls. Plasma Aβ 1-42 levels were very low in plasma samples, and the first set of ELISA experiments resulted in values which were not quantifiable. We experimented empirically with a range of plasma dilutions, and

concluded that dilution of plasma samples expose antigenic sites of Aβ 1-42 and giving results in quantifiable range. Again the ELISA was performed using kits, which was not sensitive to the samples that were freeze-stored for a long time. Hence, every year, the follow up samples were collected in a month and ELISA was performed at a single run to get a maximum quantifiable protein in the samples. This made us restrict the analysis in small subset of samples.

However, our study revealed statistically significant reductions in mean plasma Aβ 1-42 levels compared to Controls which is in agreement with previous reports on this association (Blennow et al., 2009). However, this was not found to vary consistently with the possession of APOEe4 allele. It has been reported that as an individual progress through advanced stages of AD, Aβ 1-42 levels get reduced in CSF and plasma since the Aβ oligomers are deposited in the brain lesions (Blennow, 2004). When taking into account of the 3 year follow up estimations done in our study on 5 MCI and 6 AD patients, 3/5 of AD and 2/6 of MCI patients showed serial reductions of plasma Aβ 1-42 on successive years. Remarkably, correlating to their clinical status, AD patients were progressive. However, MCI were rather stable over these 3 years. We cannot explain the discrepancy in plasma Aβ 1-42 of MCI with this available data with very low sample numbers. In two AD patients, plasma Aβ 1-42 levels increased during visit-2 and subsequently decreased on visit 3. This trend was observed in progressive MCI patients as well (Table 16). This may be explained by increased leakage of Aβ 1-42 into brain interstitial fluid due to overproduction in brain which in turn released to plasma during progressive course of the disease.

VI.2.2. Plasma tau levels were found to be elevated in AD & MCI patients compared to controls

Immunoreactivity of tau proteins was detectable in plasma samples which was higher in patient groups (AD & MCI) compared to cognitively healthy controls. However, the elevations did not meet statistical significance among the patient and control groups; which might be attributed to the low sample size. This increase could be explained by the fact that tau

concentrations in CSF and brain ISF reflects the neuronal demise in brain, and that is expelled into peripheral fluids such as plasma. Plasma tau levels vary among AD and MCI, however, the window of variation for these two stages is detected to be very narrow, which could be attributed to the very small sample size for this analysis. Our finding on plasma measures of total tau is in fact, not in agreement with the previous reports stating that tau levels are significantly lower in plasma of AD patients compared with controls (Sparks et al., 2012).

VI.2.3. Serum MMP activity was detected to be higher in patients compared to controls

The multifaceted role of APOE ε4 has been the subject of active research to elucidate molecular pathways leading to neurodegeneration in AD. This protein is found to be omnipresent with almost all the biochemical disturbances associated with AD, the most recent hypothesis being a 'two hit' mechanism proposed by Zlokovic, (Zlokovic, 2011). It states that APOE ε4 first damages cerebrovasculature, leading to leakage of Blood-Brain-Barrier (BBB). Next, it gets associated with amyloid plaques and amplifies the neuropathology. This leaky BBB in turn favours blood born macrophages to invade brain cells trying to phagocytise Aβ. However, AD macrophages are less efficient in this role. Matrix Metalloproteinases (MMPs) are one of the important proteases that play a major role in the leakage of BBB. APOE ε4 induces a pro-inflammatory cytokine Cyclophilin A (CypA) which activates Nuclear Factor κB (NFκB) that in turn triggers the production of MMP-9 (Bell et al., 2012). This protease digests capillary basement membrane proteins and tight junction proteins which efficiently damage the BBB. Moreover, neurons and astrocytes secrete MMPs and Neprilysin to degrade the aggregates of oligomeric Aβ species (Bell et al., 2012). Since there is profound neuroinflammation associated with AD, MMP secretion is increased that converts pro-inflammatory molecules into mature forms. Hence there is a net elevation of MMPs in AD brain. We investigated whether we could use serum MMP levels as a biomarker of AD in our study subjects and whether this could be correlated to their APOE status.

In our study, elevated serum MMP levels were detected in AD and MCI samples compared to Controls. There are reports showing the presence of MMP-9 mRNA and latent protease in Aβ laden AD hippocampal sections (Backstrom et al., 1996). The immunoreactivity of MMP-9 detected in close proximity to plaques raise the possibility that this enzyme is synthesised by neurons in response to stress generated by Aβ species. This implies that an oversecretion of MMPs in AD patients is indicative of inflammation and increased Aβ aggregation in the brain. Our results have also provided supportive evidence to increase in MMP expression with respect to disease state. In the preclinical phase (MCI), MMP levels elevate and increase with disease progression. Both MMP-2 and MMP-9 showed statistically significant increase in patients compared to controls. However, no significantly positive association could be established between possession of APOE ε4 and increased MMP expression in either patients or controls.

VI.3. PERIPHERAL CELLULAR MARKERS

VI.3.1. Peripheral Blood Mononuclear Cells (PBMNCs) of AD patients were subjected to DNA damage by endogenous oxidative stress

There are enough evidences suggestive of the causative role of brain oxidative stress in the development of AD. With aging, the imbalance between reactive oxygen species and the opposing antioxidant and oxidation repair mechanisms culminates in neuropathology. These changes are however, reported to be reflected in the peripheral tissues (Morocz et al., 2002). AD lymphocytes bear significantly higher concentrations of oxidised purine bases in their DNA (Mecocci et al., 1998).

Results of comet assay are in agreement to these findings, as mean tail moments of AD patients are significantly higher than that of age matched controls. However, we have analysed only basal level damage to DNA, but not the relative repair capacity of DNA damage by either groups. We have just demonstrated the elevation of oxidative damage in freshly isolated peripheral lymphocytes of AD patients and could be explained by the

presence of endogenous oxidative stress. Evidences of Oxidative stress are reported in AD brain lesions (Good, Werner, Hsu, Olanow, & Perl, 1996) and cultured fibroblasts of sporadic AD patients (Curti et al., 1997). These findings all show a generalized defective cellular defence mechanism in AD cells, leading to the accumulation of oxidized macromolecules such as DNA, proteins and lipids. The changes identified in peripheral blood cells indicate that concerning the oxidative stress, AD imparts a generalized systemic damage in the patients. In order to minimise confounding with the action of drugs on cellular oxidative stress, the samples for Comet assay were collected on the first visit of subjects to the Memory Clinic before they start medications.

VI.3.2. Phagocytic Dysfunction of Peripheral Blood Mononuclear Cells (PBMNCs) was established as a Cellular Correlate of Disease Progression

The immune hypothesis of AD strongly propose that pathologic aggregates of Aβ peptides trigger immune system, however the immune cells are defective in clearing such aggregates. This defective clearance is not only found in AD, but also reported in Parkinson's Disease (PD), where microglia fail to phagocytise aggregated α-synuclein (W. Zhang et al., 2005). Immune hypothesis of AD pathogenesis is gaining increased attention since AD patients have been proved to have an impairment of anti-amyloid-β (Aβ) innate immunity and a defect in immune gene transcription (Avagyan et al., 2009). Early diagnosis using disease-specific biomarkers is a cornerstone of preventive approaches to AD. The classical AD biomarkers, tau, phospho-tau and Aβ 1-42 in biological fluids are useful markers of neurodegeneration but not of a process leading to neurodegeneration. More specifically, these are markers of the stage of neurodegeneration (van Oijen et al., 2006). Plasma assays of chemokines, interleukins and TNF-α have been proposed to monitor progression of AD since these molecules reflect the extent of inflammation. Recent reports show that blood monocyte chemokine markers could discriminate AD from healthy elders (Hochstrasser et al., 2011). However, these factors may be influenced by other confounders such as recurrent infections, traumatic/ metabolic/ autoimmune insults which lead to misinterpretation of results. However, impairment in phagocytic properties of peripheral cells which has an

important role in AD immunopathology might be less affected by other physiological confounders. Impairment in phagocytic properties has been established on AD macrophages by Fiala et al, (Fiala et al., 2005). However, no reports are available on the phagocytic properties of peripheral monocytes/ macrophages of MCI subjects. We therefore examined whether the immune dysfunction could be detectable in the preclinical phase (MCI) of our patients.

As a first phase, we analysed the phagocytic potential of freshly collected monocytes exposed to fluorescent labelled Aβ1-42 overnight. Results of flow cytometry show that AD and MCI monocytes have significantly lower phagocytic potential compared to cognitively normal controls. This is definitely a marker at peripheral cellular level, as shown by the flow cytometry histograms (Figure 26). The declining immune function in flow cytometric test may be a warning to future neurodegeneration. This may evolve as an early predictor of dementia in future. This is further supported by the observation that mean MFI of MCI group was intermediate between that of AD and Controls.

Aβ1-42 peptide secretion increases in brain with age. When stressed by these noxious peptide and age-associated oxidative stress, neurons secrete chemo attractants to make MMs (Monocyte- Macrophages) migrate into brain crossing the Blood-Brain-Barrier (BBB) which get 'leaky'. In normal subjects, MM's migrate and phagocytize Aβ at a physiologic pace and thus forestall accumulation of Aβ. However, in AD patients, these MM's are defective and, instead of providing help, disrupt BBB, produce neurotoxic cytokines, invade but only ineffectively phagocytize Aβ deposits and suffer apoptotic cell death with release of Aβ. Release of Aβ at the vessel wall could be one of the factors in congophilic angiopathy (Fiala et al., 1998). Thus in AD brain, the physiological balancing of Aβ production and clearance is disturbed resulting in neuropathology.

While analysing the macrophage amyloid internalisation assay, we could distinctly differentiate AD and Control macrophages in culture, in their morphology, adherence properties and relative survival up to 14th day differentiating into macrophages. On exposure

to Aβ, different types of responses were observed among patient's macrophages, such as either no surface binding, weak surface binding, strong surface binding forming a halo around reactive macrophages, lesser degree of internalisation or persistence inside cells after internalisation. In macrophages of healthy controls, cells effectively internalised Aβ, and actively translocated the peptide into lysosomal compartments as visualised through fluorescent or confocal imaging after staining with Lysotracker Red. This internalisation and starting of localisation into intracellular vesicles was evident even at 1 h post exposure in control's cells (unpublished observation).

Macrophages undergo fusion with other macrophages to form the hallmark multinucleated "Giant Cells" at the sites of chronic inflammation. However, the distinct morphological subtypes, signalling pathways or molecular mechanisms behind their formation and the significance of macrophage multinucleation at chronic inflammatory sites are least understood. In the nervous system, Giant cells have been previously characterised with microglial neuroinflammatory response in a rodent model of ALS (Fendrick, Xue, & Streit, 2007). It was demonstrated that multinucleated giant cells were formed by abnormal aggregations of microglia in the CNS even in presymptomatic phase of the disease. However, this is followed by extensive fragmentation of their cytoplasm (cytorrhexis), indicative of widespread microglial degeneration on neuroinflammation (microgliosis). It is therefore conceivable that neurodegeneration occurs as a consequence of glial cell deterioration in ALS (Fendrick et al., 2007). Neuropathological studies have provided supporting evidence for microglial giant cell formation in AD brain sections in which the Giant cells surrounding plaques were found to be filled with dense Aβ42 and Aβ40 (Ferrer, Boada Rovira, Sanchez Guerra, Rey, & Costa-Jussa, 2004).

In our study, the characteristic "Giant Cell morphology" was observed on a number of Control macrophages, which is an indication of active phagocytosis and trial of more macrophages fusing together to clear the internalised peptide resulting in multinucleated giant cells. However, such a giant cell response was absent in patient's macrophages. Our current findings provide a perspective on the pathogenesis of Alzheimer's disease that is

different from the neuroinflammation theory which claims that control macrophages, on exposure to Aβ assumes Giant cell morphology in order to efficiently clear the phagocytosed Aβ peptide. We propose that, AD macrophages, being deficient in Aβ phagocytosis do not form Giant cells on Aβ exposure. The giant cells found on control macrophage cultures did not show any signs of cytorrhexis.

The results of phagocytic rate of MCI macrophages were much heterogenous, showing wide range of variations in MFI (Figure 30 B). This could be explained by the clinical course of the MCI subjects, who comprised of converters, stable at MCI or may revert back to normal cognition in future. On staining with Lysotracker Red and FITC-Aβ, MCI macrophages often stained heterogeneously even in a single field, having cells fluorescing green only, red only and a small percentage of yellow (colocalisation of two signals) (Figure 31 B). This implies the heterogenous response of peripheral cells of a subject towards a stress in his prodromal phase to clinically overt dementia. Due to this heterogeneity, we could not get statistically significant reductions in colocalisation of MCI subjects when compared to Controls, whereas AD patients were shown to have significant reduction in Aβ internalisation. However, confocal microscopy Z imaging could differentiate the surface bound FITC-Aβ signal from internalised signals which get localized to Lysotracker (Figures 32 (a) and 32 (b)).

Our data provides supporting evidence to the Immune Hypothesis of AD, and is in agreement with Fiala's findings (Fiala et al., 2005) over the phagocytic dysfunction of AD blood-derived macrophages. We have provided evidences for the heterogenous phagocytic dysfunction in MCI. The possibility for correlation of this dysfunction with pathogenic isoform of APOE locus has been analysed through student's t test, however a statistically significant difference in MFI values was not found between MCI subjects with and without possession of APOE ε4 allele. In controls, a positive correlation was obtained in contrary to that of patients. In controls, mean MFI is significantly lower with possession of even one APOE ε4 allele compared to possession of no APOE ε4 allele at all. Perhaps, these results need to be reproduced on a larger sample to establish that the possession of APOE ε4 allele influences the phagocytic functioning of macrophages in AD/MCI patients.

As an extension to the studies on cellular phagocytic dysfunction as a peripheral cellular marker for AD in the study population, we analysed the response of these macrophages towards the treatment of the immune-enhancing drug, Bis-demethoxy curcumin. Studies by Zhang et al had revealed that curcuminoids enhance Aβ uptake by AD macrophages (L. Zhang et al., 2006). In our study, the AD and Control subjects selected for this analysis had significant difference in MFI at baseline. However, on analysing the MFI after curcumin treatment, significant improvement in MFI was observed in a number of AD patients ($p<0.0001$), but not in Controls. In 3/7 AD patients and all the 4 controls analysed, no significant improvements in Aβ uptake was observed, which could be termed as "unresponsiveness" to treatment. The responsiveness to curcumin treatment was correlated to their APOE genotype in responsive subjects. An over representation of APOE ε3allele was detected in this group; 75% in AD patients and 100% in controls. However, this data may not be sufficient to make a conclusion that APOE ε4 is a negative regulator towards responsiveness on curcumin treatment, largely due to lack of effective sample size.

Recent research on immune hypothesis of AD has reported the two distinct types of macrophages, Type I and Type II based on the degree of regulation of phagocytosis related genes such as β-1,4-mannosyl-glycoprotein 4-β-N-acetyl glucosaminyl transferase (MGAT-III) and toll-like receptors (TLRs) (Fiala, Liu, et al., 2007). These genes were found to be strictly down-regulated in AD patients, and conversely, up-regulated in healthy controls. However, upon treatment with Bis-demethoxy curcumin (BDC), MGAT-III transcriptional activation and enhancement of phagocytic potential were observed in Type I AD patients. Type II AD patients were not sensitive to curcumin, and down-reglulation of MGAT-III and non-responsiveness to curcumin treatment in terms of phagocytic activation were observed in this group (Avagyan et al., 2009). Therefore, we could conclude that our patient group too comprised of Type I and Type II classes, having 57% Type I patients. Vast numbers of people in India and other countries take curcuminoids in their diet either as a dietary habit or with preventive or therapeutic goal not only against AD but also against cancer (Aggarwal & Shishodia, 2006). The differences in phagocytic responses between Type I and Type II

patients observed in this study are important for the design of immunotherapeutic studies with curcuminoids. However, more detailing on complex transcriptional defect in patient macrophages is beyond scope of this study.

An inverse association of anti-inflammatory treatments and the onset of AD has been published in a co-twin control study (Breitner et al., 1994). Thus, it is conceivable that the cascades triggered by inflammatory mediators and their cross talk with pathogenic isoforms such as apoe ε4, and noxious aggregates of abnormally functioning proteins such as Aβ species and p-tau, ultimately results in neuronal demise. Hence the role of anti-inflammatory molecules in combating disease is getting more attention. Since we had analysed the role of curcumin, an immunoenhancer in modifying the phagocytic properties of MM's, and have concluded that it is beneficial in improving the phagocytic efficiency in patient' cells, it is imperative to study the effect of curcumin treatment on influencing MMP expression in cultured macrophages. Supernatants collected from cultured macrophages of AD and Control subjects were analysed for MMP expression. Cells were stressed with Aβ overnight and then exposed to varying concentrations of BDC ranging from 2 µM to 20 µM. The trend in reduction of MMPs by alleviating inflammatory molecules was observed in both patients and controls. However, lower doses of curcuminoids were sufficient to bring down MMP levels in controls compared to patients. This implies that therapeutic implementation of curcuminoids in AD patients should make sure that bioavailability of these active compounds reach optimum to give appreciable outcomes.

VI.4. LIMITATIONS OF THE STUDY

There were a few of limitations for our study; first we could not follow the same group/number of patients (the sample numbers enough to derive significant results on association study calculated through Power analysis) for the analysis of multitude of markers. Secondly, assessment of risk factors/ collection of family history were based on retrospective account of the caregivers. Thirdly, the problem due to diagnostic misclassification cannot be completely ruled out. In order to avoid confounding due to

diagnostic misclassification, ‘Mixed Dementia’ category was excluded completely from the analysis. And, those who were first diagnosed as AD and later progressed to Vascular Dementia cases/ Mixed Dementia cases were completely excluded. Finally, for analysing the cellular correlates, repetitive analyses on cultured samples were not possible to perform again, and enough blood volume was not available to perform FACS and macrophage phagocytosis assay simultaneously for a sample. This is mainly due to the fact that we could not simultaneously perform the different lineages of investigations simultaneously on our study cohort. Despite of the genetic analysis, all assays were performed on separate subpopulations of the study samples. And hence, we could not perform any multivariate analysis on results and come into reliable conclusions on the relative contributions of each causative variable under analysis to AD pathology in the study population.

VII. SUMMARY AND CONCLUSION

We present here the reports on biomarkers associated with Alzheimer's disease which is the first of its kind in India. The study was designed to explore the risk contributed by genetic, biochemical and cellular factors on AD in the population. This is the second largest study comprising 156 AD patients and 138 age matched controls in India. Of the genetic factors analysed in the cohort, a highly strong positive association was established between the APOE ε4 allele and AD which is in concordance with previous reports worldwide. Estimations of allelic frequencies of this genetic risk factor resulted in similar figures as reported from the large meta-analysis (Farrer et al, 1997); 0.40 in AD and 0.18 in controls. The Odds Ratio of 3.8 obtained for the association of APOE ε4 homozygosity with AD is the one of the highest among the available Indian reports. We speculate that the strength of this association might be attributed to the ethnic homogeneity of the study population which was not having any admixtures. APOE ε3 was the commonest allele in all the study groups, and APOE ε2, regarded as 'protective allele' being over represented in the Controls. Neither an association with pathogeneic mutations on Presenilin (Exons 7 & 12) nor a risk with the possession of MAPT H1 haplotype was established which might be in part, due to the small sample size comprising only early onset familial AD patients.

Search for less invasive biomarkers of AD based on blood led us to estimate plasma Aβ 1-42 and tau in the study subjects. There were significant correlations between the degree of reduction in plasma Aβ 1-42 and advancing disease severity in progressive AD and MCI cases even though having a very small sample size. We could not find any statistically significant elevations in plasma tau levels with disease progression, albeit a trend in increase was observed among the patients compared to controls (AD>MCI>Control). Serum MMP expression was analysed in the subjects and have shown statistically significant increase in both MMP-2 and MMP-9 activity in patients compared to controls (AD>MCI>Controls). This is an indicator of increased neuroinflammation, oxidative stress and loss of integrity of the Blood-Brain-Barrier. An intermediate level of MMP activity was observed in the MCI subjects, which implies that response to aggravated neuropathology is evident in the preclinical stage.

Peripheral blood cells were analysed at multiple dimensions to derive a cellular marker for AD. Investigations on basal level DNA damage on PBMNCs revealed that AD monocytes are subjected to high endogenous oxidative stress that contributes to their increased tail length compared to the cells of cognitively normal individuals on Comet assay. Possible confounding by influence of drugs was negated from the study by taking blood samples for comet assay in the first visit of patients to clinic with memory complaints. Studies on phagocytic dysfunction of blood born macrophages accumulated supportive evidences to the immune hypothesis of AD (Fiala et al., 1998) and have proved that this could distinguish patients with AD from Controls. In MCI, the phagocytic properties were found to be much heterogenous in binding, uptake, internalisation or lysosomal localisation. Treatments with immunoenhancing drug Bis-demethoxy curcumin provided evidences that it could improve the phagocytic properties of AD macrophages, better than that of controls. This could be a matter of concern while designing immunotherapeutic/ neutraceutical formulations based on curcuminoids.

Despite the infinite degrees of complexity in the molecular mechanisms behind AD pathogenesis which have not been completely elucidated, the scientific community still try to make the causative pathways tractable through multitude of biomarkers, and our study recommends that these biomarker screening should be practically implemented at the initial/ preclinical stage of the disease or at-risk individuals identified by genetic testing in order to benefit the antecedent subjects of AD. This cohort, showing similar strength of APOE ε4 association to Caucasian populations may serve as an ideal study group for further explorations into additional genetic risk/ protective factors, environmental factors and survival factors contributing to AD which are promising directions of future research. Together, the investigations resulted in data which clearly state that reflections of neurodegeneration are evident in peripheral cells and body fluids and that AD imparts a systemic damage to the affected individuals.

VIII. BIBLIOGRAPHY

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IX. THE ANNEXURE

IX.1. APPENDIX 1:

DIAGNOSTIC CRITERIA

a) NINCDS-ADRDA (AD)

Based on criteria from the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) ("Consensus report of the Working Group on: "Molecular and Biochemical Markers of Alzheimer's Disease". The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and the National Institute on Aging Working Group," 1998), the diagnosis of probable AD is made when patients demonstrate

(i) Dementia established by clinical examination and documented by mental status tests
(ii) Deficits in two or more areas of cognition
(iii) Progressive worsening
(iv) No disturbance in consciousness
(v) Onset between age 40 and 90 years
(vi) No systemic or other brain diseases that could account for the progressive deficits.

b) **Petersen's Criteria for diagnosis of MCI**

The clinical criteria for the diagnosis of amnestic MCI are those described by Petersen et al. (Petersen et al., 1999) and include the features given below.

(i) Memory complaints
(ii) Objective memory impairment for age and education
(iii) Intact general cognitive function
(iv) Intact activities of daily living (ADLs)
(v) The subject is not demented

c) Diagnostic Criteria for Dementia with Lewy Bodies (DLB) (McKeith IG et al, 1996)

Central feature

- Progressive dementia: deficits in attention and executive function are typical. Prominent memory impairment may not be evident in the early stages.

Core features:

- Fluctuating cognition with pronounced variations in attention and alertness.
- Recurrent complex visual hallucinations
- Spontaneous features of Parkinsonism.

Suggestive features:

- REM sleep behaviour disorder (RBD), which can appear years before the onset of dementia and Parkinsonism.
- Severe sensitivity to neuroleptics occurs in up to 50% of DLB patients who take them.
- Low dopamine transporter uptake in the brain's basal ganglia as seen on SPECT and PET imaging scans.

Supportive features:

- Repeated falls and syncope (fainting).
- Transient, unexplained loss of consciousness.
- Autonomic dysfunction.
- Hallucinations of other modalities.
- Visuospatial abnormalities like depth perception, object orientation, directional sense and illusions
- Other psychiatric disturbances like systematized delusions, aggression and depression.

A probable DLB diagnosis requires either:

- Dementia plus two or more core features, or
- Dementia plus one core feature and one or more suggestive features.

A possible DLB diagnosis requires:

- Dementia plus one core feature, or
- Dementia plus one or more suggestive features.

d) NINDS – AIREN criteria for the diagnosis of vascular dementia

The criteria for the clinical diagnosis of *probable* vascular dementia include *all* of the following:

1. *Dementia* defined by cognitive decline from a previously higher level of functioning and manifested by impairment of memory and of two or more cognitive domains (orientation, attention, language, visuospatial functions, executive functions, motor control, and praxis), preferable established by clinical examination and documented by neuropsychological testing; deficits should be severe enough to interfere with activities of daily living not due to physical effects of stroke alone. *Exclusion criteria*: cases with disturbance of consciousness, delirium, psychosis, severe aphasia, or major sensorimotor impairment precluding neuropsychological testing. Also excluded are systemic disorders or other brain diseases (such as AD) that in and of themselves could account for deficits in memory and cognition.
2. *Cerebrovascular disease*, defined by the presence of focal signs on neurologic examination, such as hemiparesis, lower facial weakness, Babinski sign, sensory deficit, hemianopia, and dysarthria consistent with stroke (with or without history of stroke), and evidence of nof relevant CVD by brain imaging (CT or MRI) including *multiple large vessel infarcts* or a *single strategically placed infarct* (angular gyrus, thalamus, basal forebrain, or PCA or ACA territories), as well as *multiple basal*

ganglia and *white matter lacunes*, or *extensive periventricular white matter lesions*, or combinations thereof.

3. *A relationship between the above two disorders*, manifested or inferred by the presence of one or more of the following: (a) onset of dementia within 3 months following a recognized stroke; (b) abrupt deterioration in cognitive functions; or fluctuating, stepwise progression of cognitive deficits.

e) Lund-Manchester Group criteria for diagnosis of FTD

Character change and disordered social conduct are the dominant features initially and throughout the disease course. Instrumental functions of perception, spatial skills, praxis, and memory are intact or relatively well preserved.

1. Core diagnostic features
2. Insidious onset and gradual progression
3. Early decline in social interpersonal conduct
4. Early impairment in regulation of personal conduct
5. Early emotional blunting
6. Early loss of insight
7. Supportive diagnostic features
8. Behavioral disorder

IX.2. APPENDIX 2:

INCLUSION/ EXCLUSION CRITERIA

A) INCLUSION CRITERIA FOR AD PATIENTS:

1. Subject is diagnosed with probable AD according to the NINCDS-ADRDA criteria for Alzheimer's disease.
2. Onset age ≥60 years.
3. Subject or subject's relative has signed the informed consent form.

B) EXCLUSION CRITERIA FOR AD STUDY GROUP:

1. Subject is diagnosed with possible AD according to the NINCDS-ADRDA criteria for Alzheimer's disease.
2. Subject is diagnosed with dementia due to other diseases, mixed dementia or pseudo dementia, severe depression, head trauma, Vitamin B12 or folic acid deficiency, Down syndrome, HIV dementia.

C) INCLUSION CRITERIA FOR MCI:

1. Subject is diagnosed with MCI according to the Petersen's diagnostic criteria
2. Onset age ≥60 years.
3. Subject or subject's relative has signed the informed consent form.

D) INCLUSION CRITERIA FOR OTHER DEMENTIA GROUP:

1. Subject is diagnosed with one of the dementia categories such as DLBS, FTD and VD according to the standard diagnostic criteria, McKeith, Lund-Manchester Group criteria and NINDS–AIREN criteria.
2. Onset age ≥60 years.
3. Subject or subject's relative has signed the informed consent form.

E) EXCLUSION CRITERIA FOR MCI AND OTHER DEMENTIA GROUP:

Subject is diagnosed with dementia due to other diseases, mixed dementia or pseudo dementia, severe depression, head trauma, Vitamin B12 or folic acid deficiency, Down syndrome, HIV dementia.

F) INCLUSION AND EXCLUSION CRITERIA FOR NORMAL CONTROLS

Inclusion criteria:

1. Individual is conformed to be cognitively normal as per neuropsychological testing and MMSE scores.
2. Age ≥60 years
3. Included healthy volunteers, caregivers and the spouses of patients (strictly non-consanguineous).
4. Individual has signed the informed consent form to participate in the study as control.

Exclusion criteria:

1. Presence of vascular risk factors such as hypertension, hyperlipidaemia, hypercholesterolemia, thyroid dysfunction, diabetes, cardiopathy or any previous history of cranial trauma.
2. Age <60 years
3. Subjects of different ethnic origin.
4. Consanguineous spouses of patients/ first degree relatives/ having genetic relation with the patient.

IX.4. APPENDIX 4

Abstract of Poster Presented on International Conference on Biological Sciences (ICABS), Kannur, Kerala, 2012

Genetic risk factors of Alzheimer's Disease comprise APOE epsilon 4 allele and H2 haplotype frequency in South Indian Population

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Alzheimer's Disease (AD) is the most prevalent form of neurodegenerative dementia in the elderly. Mild Cognitive Impairment (MCI) is the preclinical stage of AD. Being a complex disease, there is no known definite cause for AD and there are several risk factors predisposing to the development of the disease. Epsilon 4 allele of the apolipoprotein E gene (APOE) is considered as the major genetic risk factor which has a gene dose effect on the age of onset and the rate of progression of the disease. The human APOE gene shows polymorphism with 3 alleles (epsilon 2, epsilon 3 and epsilon 4). Most of the populations exhibit a positive association of the epsilon 4 allele with disease onset; there are controversies on the relative protective roles of epsilon 2 and epsilon 3. Hence it becomes important to confirm the potential susceptibility of these genetic variants in different populations in order to establish a genetic profile of the disease in specific communities. The present study evaluates the association of APOE polymorphism with the onset of AD compared to MCI and normal age-matched controls. The study analyzed the APOE polymorphism regarding susceptibility to AD in a sample of 120 Kerala elders comprising of 64 patients (mean age68 ± 6) with AD, 36 patients with MCI (mean age66±8) and 20 Age-matched Controls (mean age63 ± 7).Blood samples were collected from AD patients and subjects with MCI at the Memory Clinic, SCTIMST. Genomic DNA was isolated from the blood samples and subjected to Sequence Specific Primer Polymerase Chain Reaction (SSP-PCR). Hixson-Vernier PCR-RFLP protocol confirmation was performed in all cases. Allele frequencies were determined.

APOE ε4 was more prevalent in the AD & MCI group (Allele frequency: MCI 0.32, AD- 0.31) compared to AMC (0.24). APOE ε2 allele was not found in the AD group and it was the rarest allele in the cohort. APO E ε3 was found to be more prevalent in the AMC group (0.76) compared to the patient groups (MCI- 0.64, AD- 0.69). The positive association of the epsilon 4 allele with the disease group confirms the role of this polymorphism in development of the disease, and it is inferred that the ε3 allele is protective in the studied population. It can be concluded that in Kerala population APOE ε4 allele is a risk factor for AD.

Key words: Alzhiemer's Disease, Apolipoprotein E, Mild Cognitive Impairment, SSP-PCR

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IX.5. APPENDIX 5
List of Publications

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.

Review Article

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

Original Articles

- **P S Jairani**, P M Aswathy, Srinivas Gopala, Joe Verghese, P S 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***, (Accepted) 2016.
- P M Aswathy, **P S Jairani**, Joe Verghese, Sheelakumari R, Srinivas Gopala, Priya Srinivas, P S Mathuranath. Progranulin mutation analysis: Identification of one novel mutation in exon 12 associated with frontotemporal dementia. ***Neurobiology of Aging*** 39: 218e1-218.e3, 2016.
- P M Aswathy, **P S Jairani**, Joe Verghese, Srinivas Gopala, P S Mathuranath. *MAPT* genetic variations are uncommon cause of frontotemporal dementia in south India. ***Neurobiology of Aging*** 45: 443.e23–443.e24, 2014.