# Antibody-based Diagnostics and Therapeutics for Alzheimer's disease and Frontotemporal Dementia

by

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ABSTRACT

Alzheimer's Disease (AD) and Frontotemporal Dementia (FTD) are the leading causes of early onset dementia. There are currently no ways to slow down progression, to prevent or cure AD and FTD. Both AD and FTD share a lot of the symptoms and pathology. Initial symptoms such as confusion, memory loss, mood swings and behavioral changes are common in both these dementia subtypes. Neurofibrillary tau tangles and intraneuronal aggregates of TAR DNA Binding Protein 43 (TDP-43) are also observed in both AD and FTD. Hence, FTD cases are often misdiagnosed as AD due to a lack of accurate diagnostics. Prior to the formation of tau tangles and TDP-43 aggregates, tau and TDP-43 exist as intermediate protein variants which correlate with cognitive decline and progression of these neurodegenerative diseases. Effective diagnostic and therapeutic agents would selectively recognize these toxic, disease-specific variants. Antibodies or antibody fragments such as single chain antibody variable domain fragments (scFvs), with their diverse binding capabilities, can aid in developing reagents that can selectively bind these protein variants. A combination of phage display library and Atomic Force Microscopy (AFM)-based panning was employed to identify antibody fragments against immunoprecipitated tau and immunoprecipitated TDP-43 from human postmortem AD and FTD brain tissue respectively. Five anti-TDP scFvs and five anti-tau scFvs were selected for characterization by Enzyme Linked Immunosorbent Assays (ELISAs) and Immunohistochemistry (IHC). The panel of scFvs, together, were able to identify distinct protein variants present in AD but not in -FTD, and vice versa. Generating protein variant profiles for individuals, using the panel of scFvs, aids in developing targeted diagnostic and therapeutic plans, gearing towards personalized medicine.

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Chapter 1

# Introduction

## 1.1 What is dementia?

Dementia is an umbrella term that describes progressive disorders of the brain that affects various aspects of an individual such as memory, language, and behavior. There are 10 million individuals affected with dementia worldwide every year [1]. Symptoms include memory loss, confusion, mood swings, issues with visual perception and orientation, and lack of judgement. While there is a decline in cognitive abilities with aging, there is severe cognitive decline in dementia typically accompanied by changes in behavior that affect an individual's ability to perform everyday tasks. There is an extensive overlap of symptoms among the dementia subtypes, especially during the initial phase, making early diagnosis difficult [2–4]. There are currently no options to prevent, cure or slow down the progression of dementia. With a lack of effective diagnostic/therapeutic intervention, the caregiving costs for individuals with dementia has been estimated to reach 1.1 trillion dollars by the year 2050 [5].

## 1.2 Types of dementia

Dementia has been classified into subtypes based on the region of the brain affected and the clinical symptoms observed in the individual. Types of dementia include Alzheimer's Disease, Frontotemporal Dementia, Lewy Body Dementia, Vascular dementia, and Mixed Dementia.

Alzheimer's Disease accounts for a large fraction of individuals suffering from dementia. It can be classified as either early onset (30-50 years of age) or late onset (60-75 years of age) (which is more common) depending on the age of onset of symptoms. AD has a

genetic component and individuals with early onset AD typically have a family history. Lifestyle and environmental factors also play a role in AD. Symptoms include memory loss, repetitive behavior, delusions, apathy and mood swings. A characteristic pathological feature is atrophy of brain hemispheres with presence of extracellular plaques and intracellular neurofibrillary tangles (NFT) [6,7].

Frontotemporal Dementia is the second leading cause of early onset dementia following AD[3]. It primarily affects the frontal and temporal regions of the brain. Depending on the region of the brain affected and symptoms of the individual, FTD can be classified as behavioral variant (bvFTD) and language variant - progressive non-fluent aphasia (PNFA) and semantic dementia (SD). bvFTD constitutes a majority of the FTD cases and includes individuals who display apathy, mood swings, overeating and impulsive/repetitive behavior. Individuals with the language variant have trouble communicating with others. They are unable to express themselves clearly either because they are unable to understand the meaning of words or they retain full comprehension but have trouble pronouncing words. In all the FTD subtypes memory deficits typically follow the behavioral and language changes and are not observed initially.

Lewy Body Dementia (LBD) is cognitive decline that occurs along with the presence of Lewy bodies in the brains of affected individuals. It is the result of abnormal buildup of α-synuclein protein which in turn affects effective communication between neurons. LBD can be classified as Dementia with Lewy Bodies (DLB) and Parkinson's disease dementia. Symptoms include motor issues such as tremors, shuffling movement, visual hallucinations, lack of alertness, lack of regulation of autonomic functions such as bowel movement and temperature regulation, apathy, and sometimes depression. In DLB cognitive decline is followed by motor issues. However, in Parkinson's disease dementia, motor issues are followed by cognitive decline.

Vascular Dementia occurs later in life and is caused by vascular diseases that affects blood flow in brain. This leads to decreased supply of oxygen and nutrients to the brain. Vascular dementia typically follows a series of cardiac events, cerebrovascular disease, and strokes that leads to brain infarcts. Symptoms vary depending on the region of the brain with decreased blood flow. The onset of symptoms can be immediate (acute onset) or it might slowly progress, following cardiac events that occurred over time.

Mixed Dementia occurs in individuals who suffer from two or more types of dementia. In aged individuals (> 80 years), a large fraction of them suffer from dementia caused by more than two types. Most common in the mixed dementia category is vascular dementia which occurs along with AD in individuals. Since there is an overlap of symptoms in dementia subtypes, it is difficult to recognize the type of dementias involved in mixed dementia without a pathological examination. AD with vascular dementia presents with changes in blood vessel, and occurrence of plaques and NFTs in brains of affected individuals. Mixed dementia occurs often and is clinically relevant than a pure dementia subtype like Alzheimer's or vascular dementia. Studies indicate that occurrence of brain infarcts seems to determine the severity of cognitive decline and any Alzheimer's symptoms associated with it [8,9].

### 1.3 Causes

Although the exact causes of dementia are unknown, there are several factors that have been associated with the risk of dementia – cardiovascular factors, lifestyle factors, family history and genetics (Fig 1.1).

#### *1.3.1 Risk factors*

Cardiovascular factors including obesity in midlife have been shown to increase the risk of dementia development in later stages [10–12]. Hypertension or high blood pressure for an

extended time damages the arteries and blood vessels, especially in the brain. This affects supply of nutrients to the brain which in turn results in vascular dementia. Apart from blood pressure, high levels of Low-Density Lipoprotein (LDL) and total cholesterol levels are not only a risk factor for cardiovascular diseases but also for dementia. Studies have shown that statins taken to control cholesterol levels also help lower risk of dementia [13–15]. Type 2 Diabetes Mellitus and associated obesity increases the risk of dementia in individuals [16]. Impaired glucose tolerance and insulin sensitivity are potential risk factors for dementia [15,17]. Effect of drugs like Metformin which is used to treat type 2 Diabetes have been explored in the treatment of dementia [18].

Lifestyle factors include excessive consumption of alcohol, smoking, sedentary lifestyle, and diet with high intake of red meat. These are high risk factors not only for dementia but also for cardiovascular diseases, certain types of cancer and other diseases. These factors affect the vasculature and blood flow increasing the risk of dementia. Diet rich in red meat, processed foods rich in saturated fats and sugars result in weight gain and plaque buildup in arterial walls which is a dementia risk. High intake of salt also results in hypertension which is another factor. A sedentary lifestyle with lack of physical activity further increases risk for cardiovascular diseases, diabetes and dementia [19]. There are physical and chemical changes that occur in the brain which correlate to an inactive lifestyle [11,19].

Depression also correlates with risk of developing dementia [20,21]. It is possible that depression could also be a very early symptom of dementia rather than a risk factor. Another factor associated with dementia is obstructive sleep apnea [22,23]. Disturbances in sleep can increase plaque buildup in brains of individuals [24]. During sleep, brain consolidates memories and experiences and repairs itself. Disturbances in sleep can affect the homeostasis increasing the risk of dementia.

*1.3.2 Genetics*

Genetics is one of the important factors to consider in dementia. Individuals with a family history of dementia are at higher risk [25,26]. There are several genes that have been linked with different dementia subtypes.

ApoE which codes for Apolipoprotein E is a major predictor of late onset Alzheimer's disease (E4). Mutations in presenilin 1 and 2 (PS1 and PS2), and amyloid precursor protein (APP), located on chromosome 21 are associated with early onset familial AD [27,28]. Individuals with Down's syndrome have an extra copy of chromosome 21. These individuals are hence prone to increased Amyloid-Beta (Aβ) which increases their risk of AD [29].

Genetics plays a very important role in familial Frontotemporal dementia. Individuals with FTD have mutations in MAPT (tau), C9orf72, and GRN (progranulin) which is associated with familial FTD [25,30,31]. It is inherited in an autosomal dominant fashion which means an individual carrying a single copy of the affected gene gets FTD. Mutations also occur in other genes like TARDBP (TDP-43), FUS, CHMP2B [32]. These genes are involved in the production of proteins like TDP-43 and tau. These proteins are responsible for regulating transcription, translation, alternate splicing, formation of stress granules, and microtubule stability. However, mutations in these genes cause aggregated TDP-43 and tau, a hallmark feature in FTD brains.

Lewy body dementia is associated with mutations in SNCA and SNCB genes which are involved in the production of α-synuclein and β-synuclein proteins [33]. While α-synuclein is responsible for maintaining supply of synaptic vesicles and regulating dopamine (neurotransmitter) and movement of microtubules; β-synuclein is involved in maintaining neural plasticity and preventing α-synuclein aggregation.

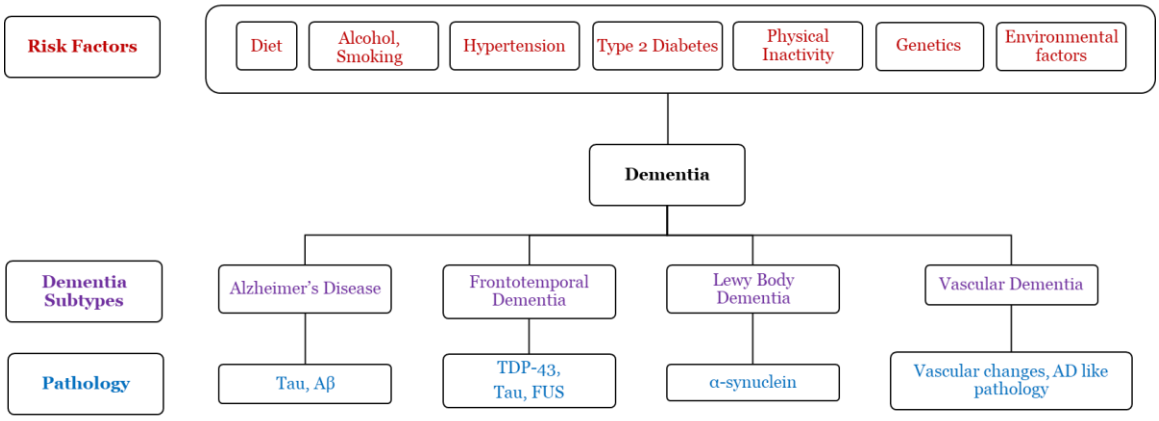



Figure 1.1 Risk Factors and Pathology Associated with Dementia Subtypes

Another gene implicated in Lewy body dementia is GBA which codes for the enzyme β-glucocerebrosidase. This enzyme is involved in breaking down abnormally accumulated proteins like α-synuclein in the lysosome. Mutations in this gene leads to a modified β-glucocerebrosidase which is unable to break down α-synuclein leading to the formation of Lewy bodies.

Unlike AD which has several genetic factors linked, vascular dementia is primarily influenced by lifestyle factors like blood pressure and cholesterol levels [11]. Genetics of vascular dementia hence involves mutations in genes that result in elevated blood pressure or cholesterol. A rare form of vascular dementia called Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is caused due to mutations in a single gene called NOTCH3 [34]. This gene is responsible for maintaining function of vascular smooth muscle cells which regulate blood vessels. Several mutations in this gene have been shown to cause apoptosis of the vascular smooth muscle cells. This in turn affects vasculature which in turn affects blood supply to the brain, causing vascular dementia.

### 1.4 Current treatment

Dementia currently has no cure but there are several Food and Drug Administration (FDA) approved drugs that have been used to improve symptoms associated with dementia. A class of drugs called cholinesterase inhibitors are used to treat symptoms associated with cognitive decline in Alzheimer's disease and related dementias. These drugs include rivastigmine, galantamine, and donepezil which are involved in inhibiting breakdown of the chemical neurotransmitter acetylcholine [35]. Acetylcholine functions as a neurotransmitter and neuromodulator in the brain and is involved in learning, memory and motivation. These drugs provide temporary relief for individuals in early stages of

dementia. Another drug called memantine, which was recently approved, regulates glutamate, a neurotransmitter involved in brain function and memory. Memantine helps improve memory and cognition in individuals with moderate to severe AD [35].

Unlike AD, individuals with vascular dementia are treated with drugs that can help maintain their blood pressure and cholesterol levels like statins and aspirin to prevent blood clots. In Lewy body dementia, the monoamine oxidase inhibitor (MAO-B), Rasagiline, prevents the breakdown of dopamine [36]. This drug helps maintain levels of dopamine despite a decrease in dopaminergic neurons. Dementia is also treated with anti-anxiety and anti-depressant medications, especially in FTD, to help cope with mood swings, aggressive behavior and restlessness.

Apart from pharmacological intervention, there are several non-pharmacological therapies that help individuals with dementia. These include physical therapy including exercise to help with movement issues associated with dementia; cognition-oriented therapy that focuses on learning and recall; sensory stimulation therapy that includes music, creating arts etc.; and emotional therapy to aid individuals cope with their behavioral changes and mood swings.

Several clinical trials that focus on treating/curing neurodegenerative diseases like AD have failed in Phase II or Phase III trials [37,38]. There are several reasons – inability to translate the data obtained with an animal model into humans, time taken to recruit participants, and lack of studies focusing on very early stages of dementia. The focus needs to be on treating individuals even before the onset of any symptoms, and possibly prolonging trials to see any noticeable effect on the participants. The biggest challenge comes down to selecting participants that are in really early stages of dementia.

## 1.5 Diagnosis

There is a lack of imaging/biomarker candidates that can predict different dementia subtypes especially in the early stages. Currently most of the diagnosis relies on imaging, neurological and mental state exam.

### *1.5.1 Imaging*

Imaging primarily involves Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) scan. Several tracers (florbetapir, flutemetamol and florbetaben) for amyloid PET scans have been approved by the FDA and they can show plaque buildup in the brain[39]. Elevated levels of tau in cortex found by PET scan is also used to identify potential NFT location in the brain. Another characteristic feature utilized in imaging is the decreased glucose metabolism found in dementia patients. This is visualized by Fluorodeoxyglucose - Positron Emission Tomography (FDG-PET) scan. MRI is used to visualize brain atrophy and structural changes associated with dementia. A major drawback of these imaging techniques is their lack of specificity despite sensitivity. Although these techniques show amyloid deposits, tau tangles and brain atrophy, they are not capable of distinguishing within dementia subtypes and with other neurodegenerative diseases [40]. They are not very effective in identifying changes at the molecular level in the brain.

### *1.5.2 Neurological and mental state exam*

Neurological exams are performed by doctors to examine reflexes, overall coordination, speech patterns and sensation to eliminate other probable causes of memory loss such as tumors, movement related disorders etc. Apart from neurological exam, several tests have been designed to assess various aspects of cognitive function affected by dementia. These tests include Mini Mental State Examination (MMSE), and mini-cog test. Other tests

include Montreal Cognitive Assessment (MCA), Saint Louis University Mental Status Exam (SLUMS), Informant Questionnaire on Cognitive Decline in the Elderly (IQCODE), Self-Administered Gerocognitive Examination (SAGE), Abbreviated Mental Test Score (AMTS). These tests assess various mental abilities such as memory and recall, spatial orientation, calculations, language fluency, completing complex tasks and attention. Since there is an extensive overlap of symptoms between the dementia subtypes, a neurological and mental state exam might not be conclusive [41–43]. Hence there is an imminent need for a biomarker capable of distinguishing different dementias.

### *1.5.3 Protein variants in Dementia*

Several neurodegenerative diseases have been associated with a key protein with altered folding, resulting in protein aggregates. This includes Aβ and tau in AD, α-synuclein in Parkinson's, prion protein in prion disease, and TDP-43 in Amyotropic Lateral Sclerosis (ALS) and FTD[44]. In the aggregated state, these proteins usually tend to have a rich β-pleated structure as opposed to random coil or α-helical conformation in the native state. This could either lead to a loss of physiological function or a toxic gain of function or both disrupting several downstream processes. This protein build-up along with decreased clearance rates of aggregated protein could lead to extensive neuronal loss and neurodegeneration.

For decades, aggregated proteins (E.g.: plaques, tangles, aggregated TDP-43) were thought to be responsible for causing dementia. However, the intermediate conformation in which these proteins exist (oligomers) play a crucial role [45–48]. These intermediate protein variants tend to correlate much better with cognitive decline associated with dementia [49]. The occurrence and progression of these protein variants is unique. Effective diagnostic and therapeutic agents would selectively recognize toxic disease specific

variants of these proteins. Because of their diverse binding capabilities, antibodies or antibody fragments such as scFvs are a good starting point to develop reagents that can selectively bind the protein variants. The reagents can be screened for various specificities such as to identify different protein variants present in dementia subtypes like AD and FTD (Fig 1.2).

A combination of phage display library and AFM was used to obtain scFvs against these intermediate protein variants as described [48] (Fig 1.3). A phage display library essentially consists of a repertoire of phages, each 'displaying' a protein (scFv) in the minor or major coat protein. These phage libraries typically have $10^8$ variability and increase the odds of selecting a specific scFv against an antigen (intermediate protein variants). Sample of the phage library before and after enrichment is incubated with minute quantities of the antigen on mica and scanned using an AFM probe. AFM is based on the principle of Hooke's law - deflective forces between the probe tip and surface of mica is recorded to obtain topography of the surface scanned. It allows observation of the entire panning process before and after every selection step. The selected antibody fragments are characterized using ELISAs, IHC and western blots. These scFvs can serve as a potential diagnostic tool in differentiating between AD and FTD. Both AD and FTD are the leading causes of early onset dementia. Both have overlapping symptoms and pathologies which often leads to misdiagnosis.

### 1.6 Alzheimer's Disease

Alzheimer's disease is the leading cause of dementia and affects the ageing population. Despite advances in imaging techniques and progress in assessing an individual's mental state, there is a lack of accurate diagnostics to detect early stages of AD. Depending on age of onset of symptoms, AD can be classified as early or late onset.

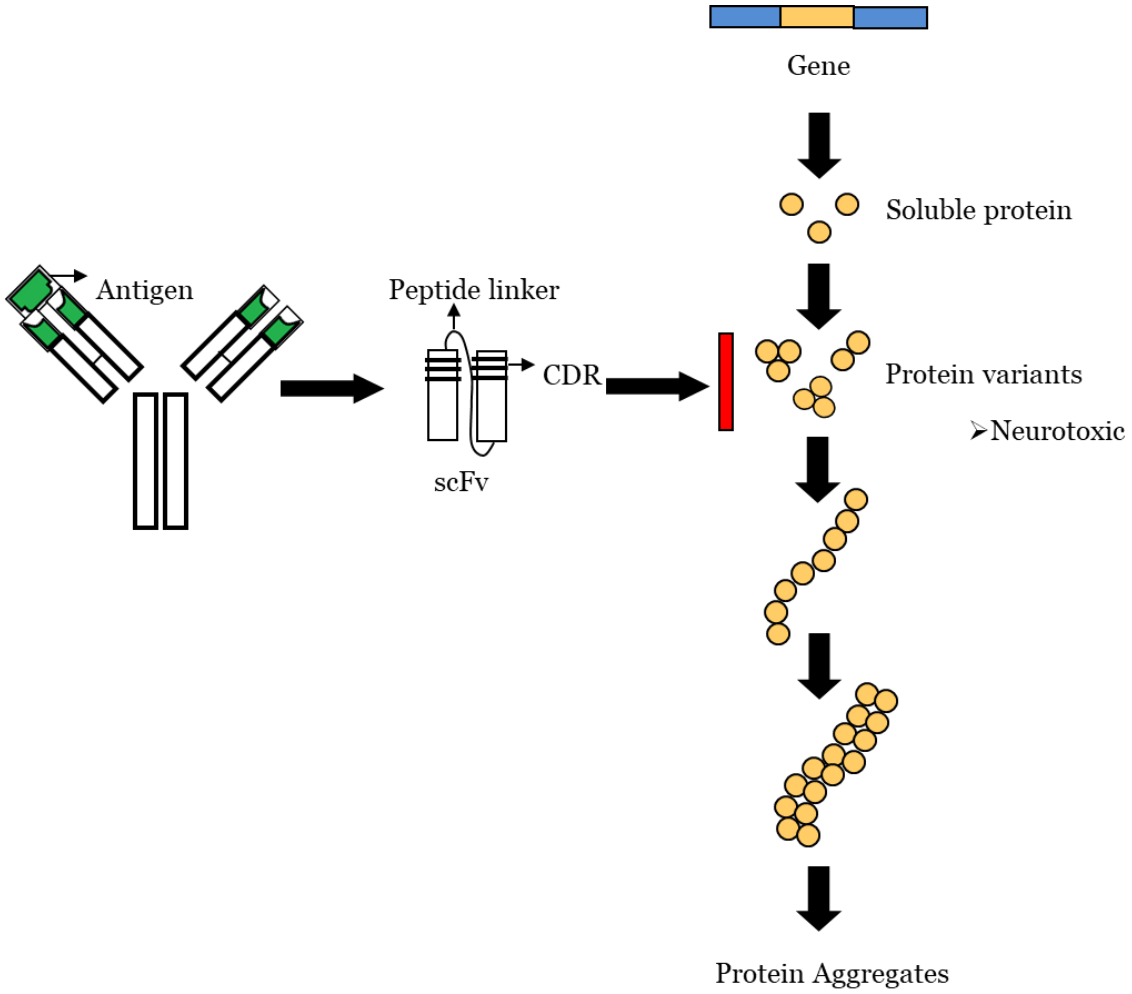



Figure 1.2 Diagnostic and Therapeutic Intervention using scFvs against Intermediate Protein Variants

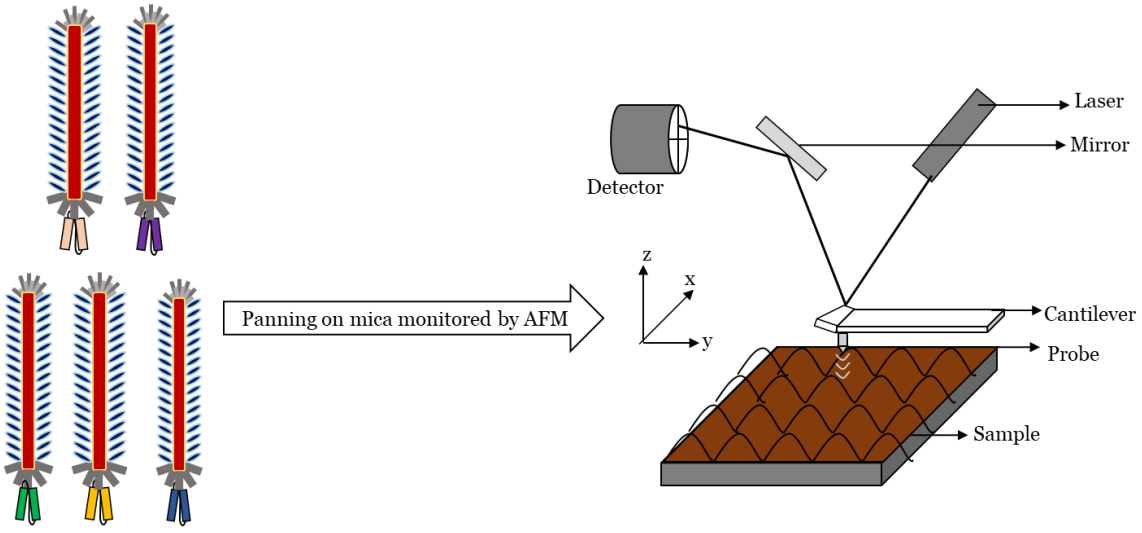



Figure 1.3 AFM based panning process using phage display library.

While early onset is rare and typically has a genetic component (family history), late onset has a sporadic onset and constitutes a large fraction of the AD cases. This chapter deals with understanding etiology of AD and characterizing protein variants that could serve as biomarkers of disease.

### *1.6.1 Neuropathology of AD*

Alzheimer's disease has a unique and distinct neuropathology. AD has plaques mainly composed of Aβ and tangles composed of tau. There is severe atrophy of the brain regions accompanied by extensive neuronal loss.

Amyloid plaques are visualized either using Congo Red or Thioflavin-S staining. Based on staining patterns, the plaques can be classified as dense-core or diffuse plaques. Dense core plaques usually consist of fibrillar amyloid surrounded by neuritic plaques, astrocytes and microglia. Dense plaques are associated with memory loss and with AD. Diffuse plaques, on the other hand, consist of amorphous amyloid and does not stain with Congo red and Thioflavin-S [50,7]. Diffuse plaques are not surrounded by glial cells [51]. They are a part of normal aging and are found widespread in elderly individuals. The amyloid plaques can be visualized by PET and Single Photon Emission Computer Tomography (SPECT).

Neurofibrillary tangles are primarily composed of hyperphosphorylated tau. Due to hyperphosphorylation and truncation of tau, they form Paired Helical Filaments (PHF) which leads to tangles. The core of PHF mainly consists of a repeat domain which is responsible for the rich β-pleated structure. The N-terminal and C-terminal surround the core forming the outer structure of PHF. Tau tangles and PHFs are visualized by PET scans and can also be observed via atomic force microscopy (AFM). NFTs can also exist as diffuse or pre-NFTs which are healthy neurons (intact axon, dendrite and nucleus) that have mild tau staining within the neuron. NFTs can further be classified as intraneuronal NFTs

(iNFT) or extraneuronal NFTs (eNFTs) depending on the location of tau staining. In iNFT, there is intense tau staining within the neuron and the nucleus is not centered due to the presence of intraneuronal fibrillar tau aggregates. In eNFT, there is extraneuronal tau staining in dying neurons, that do not stain for the nucleus or the cytoplasm [52]. Neuropil threads are also frequently found along with NFTs in AD. They occur due to the breakdown of axons and dendrites containing hyperphosphorylated tau.

*1.6.2 Genetics*

ApoE gene located on chromosome 19 codes for apolipoprotein E and is indicated as a major risk factor in late onset AD. Apolipoprotein regulates the metabolism and transport of lipoproteins in the blood. Every individual has two copies of the ApoE gene with three possible alleles ε2, ε3, ε4 resulting in a total of six possible combinations - ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4. Several studies have indicated that ε4 allele increases the risk of AD compared to other alleles [53] while ε2 confers a protective effect [54]. In individuals carrying one or two copies of the ε4 allele, age of onset of dementia dramatically decreases from 84 to 68 years and the rate of cognitive decline is also rapid compared to other allele combinations [53,55]. There is also increased amyloid burden [56,57] and decreased olfactory function (identification of odors) in ε4 homozygous AD individuals [58]. There is also a decrease in Aβ42 levels accompanied by an increase in tau levels in Cerebrospinal Fluid (CSF) of AD individuals carrying the ApoE ε4 allele [59,60].

Genetic factors associated with early onset AD includes mutations in presenilin-1, presenilin-2, and amyloid precursor protein (APP) [61,62,63]. APP is a protein that is responsible for formation of synapse, transport and neural plasticity. Proteolytic processing of APP by either α-secretase or β-secretase followed γ-secretase leads to the formation of APP Intracellular Domain (AICD) and p3 (non-amyloidogenic pathway) or

AICD and Aβ peptide (amyloidogenic pathway) respectively. sAPPα has higher neuroprotective effect against Aβ induced toxicity compared to sAPPβ [64].

Presenilin genes are involved in making PS1 and PS2 proteins respectively. PS1 protein is a subunit of γ-secretase enzyme which is responsible for the proteolytic cleavage of APP. PS2 acts along with other enzymes in the processing of APP and is also involved in transmitting signals from the transmembrane to the nucleus to activate genes.

More than 50 different mutations in APP, 100 mutations in PS1 and 11 mutations in PS2 have been identified which relates to early onset AD [65]. These mutations lead to altered APP conformations or aberrantly folded γ-secretase. This leads to defective proteolytic processing of APP leading to the formation of sticky Aβ peptide. These Aβ peptides have a high tendency to aggregate leading to plaques in AD.

*1.6.3 Inflammation in AD*

Inflammatory responses in AD typically involve glial cells which surround plaques and tangles in the brain. Glial cells surround neurons and provide insulation while also supplying nutrients, maintaining synaptic junction, recycling neurotransmitters and serving as scavengers in removal of dead cells. In central nervous system, inflammatory responses involve microglia and macroglia like astrocytes, oligodendrocytes, radial cells and ependymal cells are involved. There are several studies that have probed into the role of astrocytes and microglia in AD. While microglia serve as scavengers and are involved in the removal of dead neurons plaques etc., astrocytes are involved in maintaining ion balance and regulating the blood brain barrier. In AD, there is an increase in astrocytes and microglia during the progression of the disease and they positively correlate with NFTs compared to amyloid plaques [51,66]. Synaptic dysfunction, neuronal loss and cognitive

decline present in AD also correlates with microglia and astrocyte activity. These indicate the importance of NFTs and inflammation in AD.

*1.6.4 Aβ and tau*

Amyloid beta is a short peptide produced because of proteolytic cleavage of amyloid precursor protein with β-secretase followed by γ-secretase. γ-secretase does not have a precise cleavage site resulting in peptides of varying lengths. Two of the prominent peptides found in AD are Aβ40 and Aβ42 which are present in plasma, CSF, interstitial fluid and brain tissue of AD. The longer, hydrophobic Aβ42 peptide is extremely prone to aggregation compared to Aβ40. Several studies have indicated that there is an increase in Aβ42/Aβ40 ratio in AD which is a reliable measure compared to Aβ42 measurement alone [67,68]. Soluble Aβ is present at a six-fold higher concentration in individuals with AD compared to controls [68]. These soluble Aβ oligomers increase with age and occurs prior to plaque formation. However, studies have indicated that compared to Aβ oligomers, tau variants and NFTs correlate better with cognitive decline, and severity of dementia associated with AD [69,70].

The primary component of NFTs and PHFs is tau, a microtubule associated protein. It is a natively unfolded protein encoded by the MAPT gene located on chromosome 17. MAPT consists of a total of 16 exons and alternative splicing of messenger Ribonucleic Acid (mRNA) transcripts coding exon 2, 3 and 10 results in six tau isoforms of varying lengths (Fig 1.4). The isoforms have either 0, 1 or 2 inserts close to the N-terminal region and they contain either a 3 repeat or 4 repeat domains near the carboxy terminal. The isoforms vary in length between 352 and 441 amino acids. 3R and 4R tau isoforms are both developmentally regulated. While 3R tau is present primarily during fetal development, both 3R and 4R are present in equal proportion in adults [71]. In AD and

other neurodegenerative diseases, the equilibrium between 3R and 4R tau is disrupted. Studies have also indicated that there are disease and brain region specific variations with respect to these isoforms [72]. These variations can lead to increased vulnerability of different brain regions to the occurrence of tau. Common post translational modifications that occur in AD brain includes hyperphosphorylation, truncation, ubiquitination, acetylation, nitration, oxidation of tau etc. These modifications lead to abnormal tau that aggregates leading to dissociation of tau from microtubules thus affecting microtubule dynamics and axonal transport in AD.

### *1.6.5 Spread of Aβ and tau*

Aβ deposits occur in AD brain regions in a hierarchical manner. Aβ deposits begin in the neocortex and spread into the allocortex. Further regions that are affected as the disease progresses includes diencephalic nuclei, striatum, and the basal forebrain. In later stages of AD, Aβ deposits further spread into the brainstem and cerebellum [73].

Tau, a component of NFTs, also has a characteristic distribution in AD brains. In the initial stage, tau deposits are found in the trans-entorhinal and entorhinal cortex regions. This is followed by further tau spread affecting the hippocampus. As the disease progresses, there is a steady increase in the occurrence of NFTs throughout the cortex [74].

Tau is essential for Aβ induced neurotoxicity and removal of endogenous tau improves deficits produced by Aβ [75,76].

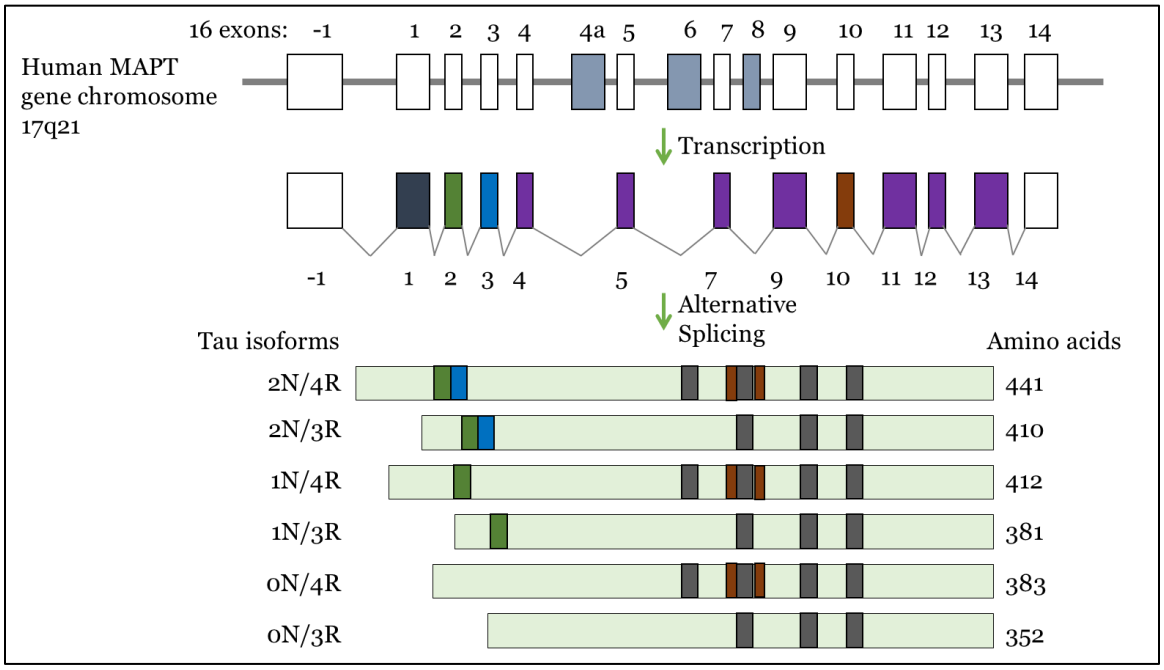



Figure 1.4 Alternative splicing of MAPT gene leading to the formation of six different tau isoforms ranging between 352 amino acids and 441 amino acids.

Due to the distribution patterns of tau, prion like propagation mechanism of tau from one neuron to another have been studied. Seeding experiments in vivo and in vitro have shown the ability of aggregated tau variants to recruit healthy tau to form seeds further extending the aggregation process. These aggregated tau species can spread via i) receptor mediated endocytosis, ii) exosomes, iii) diffusion and iv) nanotubes [77,44].

## 1.7 Frontotemporal Dementia

Frontotemporal dementia is the second leading cause of early onset dementia following Alzheimer's disease [78]. It affects individuals in the age group of 30 – 50 years. Based on pathology and symptoms, there are three major subtypes of FTD – i) Behavioral FTD, ii) Progressive Non-Fluent Aphasia (PNFA), iii) semantic dementia (SD). Progressive Supranuclear Palsy (PSP) and Corticobasal Degeneration (CBD) also fall under the FTD umbrella and have motor involvement. Individuals affected by FTD have issues with behavior, mood swings, motor deficits, and progressive language and memory issues.

### *1.7.1 Neuropathology of FTD*

FTD has a distinct pathology where different regions of the brain involving the frontal and temporal lobes are affected [79]. In bvFTD, there is atrophy of the frontal and anterior temporal regions; in PNFA both frontal and temporal regions are affected; and in SD the temporal region is primarily affected. In CBD there is atrophy in frontal, temporal and parietal lobes while in PSP there is involvement of basal ganglia, brain stem and cerebellum. In certain cases, subcortical regions and substantia nigra are also affected. The pathology found in FTD cases based on the protein aggregates is classified into three subtypes: i) FTD-Tau, ii) FTD-TDP and iii) FTD- Fused in Sarcoma (FUS) [80]. FTD-tau includes intraneuronal tau positive inclusions; FTD-TDP includes TDP-43 positive, tau negative and α-synuclein negative inclusions; and FTD-FUS includes FUS positive

inclusions. While FUS pathology is observed in only ~10% of FTD cases, TDP-43 and tau are the most commonly observed pathologies in FTD cases (Fig 1.5 a). Studies have shown that these subtypes have overlapping molecular pathology, making diagnosis difficult [2,81,82] despite progress in imaging techniques and CSF biomarkers.

There are currently several imaging techniques like diffusion tensor imaging, functional-MRI (voxel based changes) and PET scan that have shown promise in FTD diagnosis [83,84,85]. These techniques have been demonstrated on a small scale and are focused on measuring anatomical similarities within FTD and assessing differences between FTD and other dementias. Relying only on imaging for diagnosis has limited potential since FTD falls under a spectrum with a wide range of anatomical representations.

*1.7.2 Genetics*

Unlike AD and other dementia types, FTD has a strong genetic component and family history associated with it. Mutations in several genes have been identified including MAPT, TARDBP, C9orf72, GRN, FUS, VCP, and CHMP2B [65,86,87,25]. Some of these mutations are inherited in an autosomal dominant manner and are responsible for familial FTD while others are responsible for sporadic cases of FTD.

Approximately 40 mutations in MAPT gene located on chromosome 17 have been recognized that is associated with FTD. These mutations lead to build up of either 3R or 4R tau leading to pathological tau positive aggregates in CBD, PSP and certain FTD cases. Approximately 68 mutations in GRN gene, which codes for progranulin have been identified. These mutations alter the function of granulin which regulates lysosomal activity and survival of neurons in the brain. 30 mutations have been identified in Valosin-Containing Protein (VCP) which is responsible for clearing aggregated proteins in the ubiquitin protease system. These mutations lead to altered enzyme which impairs its

ability to clear protein aggregates. Mutations in C9orf72 have been associated with ALS and FTD. This leads to hexanucleotide repeat expansion which affects RNA and protein homeostasis. Mutations have also been identified in TARDBP gene which codes for Transactive response DNA binding protein. All these mutations lead to protein aggregates (TDP pathology) in FTD [88].

A risk factor recently identified in a genome wide association study is the link between TMEM mutation with the development of FTD-TDP. TMEM106B is involved in lysosomal transport and regulation of dendrite morphology. TMEM106B variant increases expression of TMEM106B which increases the risk for FTD-TDP. The occurrence of TMEM106B variant in individuals having mutations in the GRN gene also increases the risk of FTD-TDP [89]. A polymorphism occurring in MAPT gene called H1 haplotype is common among individuals with PSP. This polymorphism increases the risk of PSP and the occurrence of tau pathology.

Most of the mutations identified in FTD is primarily involved in clearing aggregated protein (ubiquitin proteasome pathway or autophagy) which results in disruption of RNA and protein homeostasis. This disruption drives a feed forward loop which results in TDP aggregation in FTD. More than 90% of the FTD cases develop either tau negative, TDP positive pathology or tau pathology further underlining the importance of TDP and tau in the etiology of FTD.

*1.7.3 TDP-43 and tau*

As discussed in Chapter 2, MAPT gene codes for tau which is a microtubule associated protein. Mutations in the MAPT gene lead to abnormal accumulation of tau protein leading to gradual neuronal loss and cognitive decline associated with FTD.

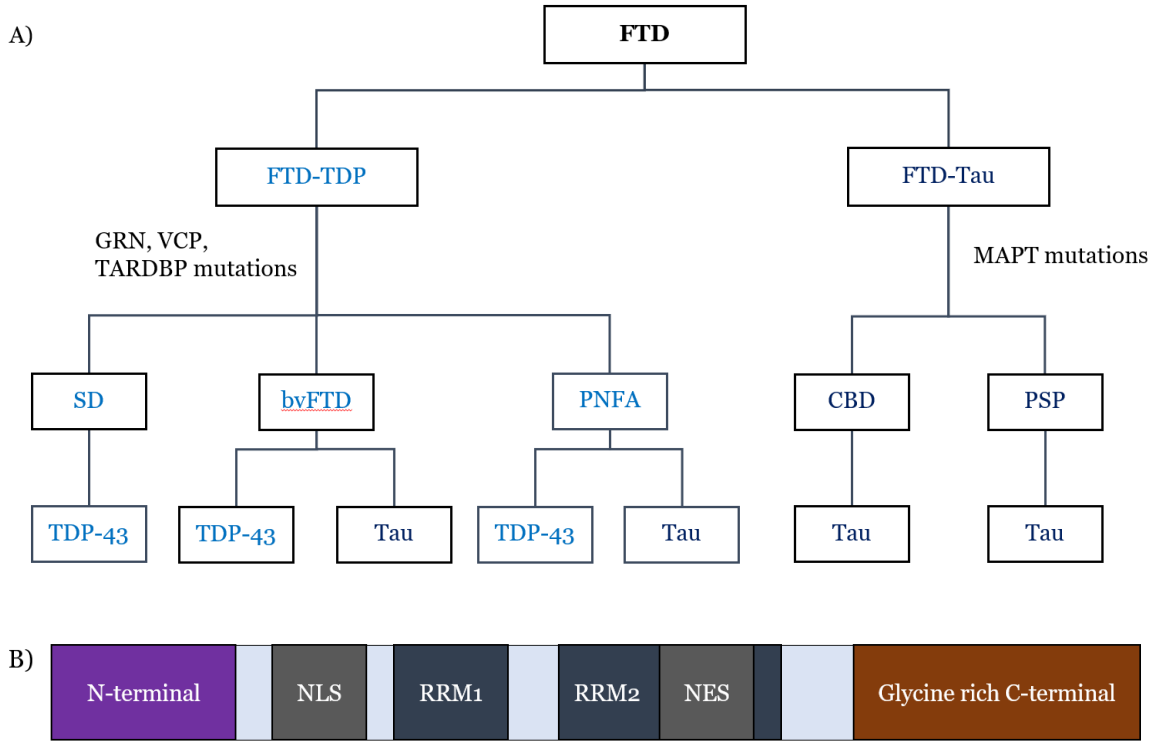



Figure 1.5 A) Adapted from 90. Pathological (FTD-TDP and FTD-Tau), genetic and clinical phenotypes (Semantic Dementia, SD; Progressive Non-Fluent Aphasia, PNFA; Behavioral FTD, bvFTD; Corticobasal Degeneration, CBD; Progressive Supranuclear Palsy (PSP) in Frontotemporal Dementia. It shows the overlap of TDP and tau pathology in the FTD subtypes. Since FUS pathology represents less than 10% of FTD cases, it has not been included in the schema, B) Structure of TDP-43 protein coded by the TARDBP gene. TDP-43 consists of five functional domains: Nuclear localization signal (NLS), two RNA binding domains, Nuclear Export Signal (NES) and a highly conserved Glycine rich C terminal.

FTD individuals with a tau pathology develop speech and motor deficits (parkinsonism) over time such as tremors, gait changes etc. and include FTD with parkinsonism, CBD, PSP etc. TDP-43 is a TAR DNA binding nuclear protein, 414 amino acids in length coded by the TARDBP gene (Fig 1.5 b). TDP-43 has multiple functional domains which include a glycine rich C-terminal similar in structure to the C-terminal of prion protein, which is extremely prone to aggregation. It also consists of two highly conserved RNA binding domains (RRM1 and RRM2) and a Nuclear Localization Sequence (NLS) close to the N-terminal domain, responsible for nuclear import of TDP.

TDP-43 is a common molecular pathology in the FTD-ALS spectrum and is observed in more than 50% of the FTD cases [88]. It plays a key role in transcription and translation processes and is involved in alternate splicing, mRNA transport, micro RNA (miRNA) biogenesis, formation of stress granules, and serves as a shuttle between the nucleus and cytoplasm [91]. TDP-43 levels are autoregulated by TDP-43 dependent splicing of 3'UTR region of its own mRNA. Mutations in FTD and age-related changes affect TDP-43 levels which disrupt several aspects of protein and RNA homeostasis (Figure 1.6). This includes genes controlled by TDP-43 which disrupt protein clearing pathways such as ubiquitin proteasome, and autophagasome. This further disrupts autoregulation of TDP-43 driving its aggregation in FTD. TDP-43 is thus translocated to the cytoplasm [92] and location and type of aggregates [93] differ in the clinical subtypes of FTD [81].

*1.7.4 Spread of TDP-43*

Type A TDP aggregates are present in bvFTD and PNFA and consists of short Dystrophic Neurites (DN) and oval Neuronal Cytoplasmic Inclusions (NCI). Type B has moderate NCI and few DN throughout the cortex and is present in bvFTD and FTD-Motor Neuron Disease (FTD-MND).

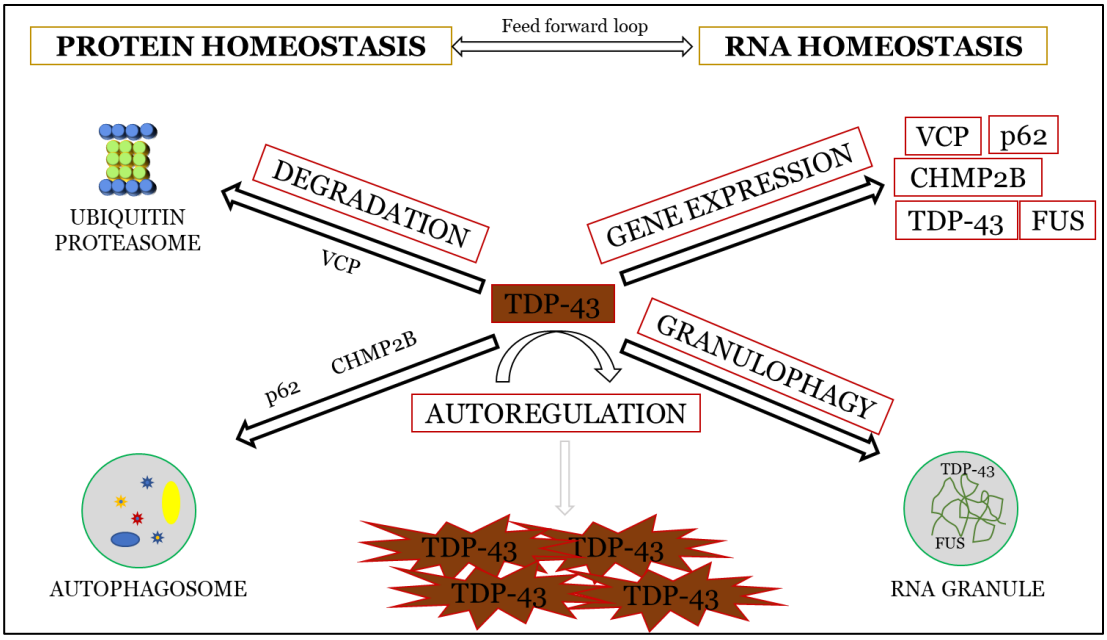



Figure 1.6 Disruption of RNA and protein homeostasis in Frontotemporal Dementia

Type C is present in individuals with SD and bvFTD and predominantly consists of elongated DN in upper cortical layers with few NCI. Type D occurs in individuals with VCP mutations and consists of abundant DN and lentiform neuronal intranuclear inclusions (NII).

Although the pathogenic mechanisms is not known, several studies indicate that TDP-43 can spread in a prion like fashion from neuron to neuron through the axonal pathway [94,95,96,97,98]. TDP-43 is also implicated in ALS, where different strains of TDP-43 have been shown to spread at different rates in *in vitro* models, indicating presence of multiple toxic TDP variants [95,99].

Different TDP-43 conformations with different levels of toxicity resulting in different pathologies (TDP type A-D) and disease phenotype have been identified [100]. These TDP-43 variants exist due to post-translational modifications such as hyperphosphorylation, polyubiquitination and truncation leading to C-terminal fragments that are toxic [92,101,102,103,104].

Studies have indicated that in bvFTD, phosphorylated TDP-43 progresses in a sequential manner along the axonal pathway as the disease progresses. pTDP-43 lesions are initially observed in orbital cortex, and amygdala. It further spreads to mid-frontal and anterior cingulate cortex, temporal lobe, striatum, and thalamus. In advanced cases, pTDP-43 is present in motor cortex, spinal cord anterior horn with increased presence in the visual cortex in the final stages.

Despite progress in imaging biomarkers, differentiation of FTD subtypes and AD based on atrophy regions are not conclusive due to the involvement of multiple brain regions in behavior and language, which is affected. Elevated levels of TDP 43 and tau are found in circulating CSF of FTD, ALS and AD patients compared to healthy controls [92,105,106]. TDP-

43 has also been detected in the plasma of individuals with FTD and AD [107]. However, there is extensive overlap in the TDP-43 and tau species detected between the FTD, ALS and AD cases. There is a lack of accurate blood-based biomarker that is capable of distinguishing FTD from AD and healthy controls.

We propose that FTD specific TDP-43 variants and AD specific tau variants can be used as unique biomarkers in early antemortem diagnosis distinguishing FTD from AD and other neurodegenerative diseases. We have identified a unique panel of scFvs capable of recognizing TDP variants that are present in human FTD patients and tau variants in human AD patients but not in age-matched cognitively normal controls. AD and FTD specific variants are described in detail in the following chapters.

Chapter 2

Alzheimer's Disease

## 2.1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease that affects memory and behavior. AD along with many other neurodegenerative diseases is associated with altered folding of key neuronal proteins. For AD, aggregation of amyloid-beta and tau, primary components of the hallmark extracellular plaques and intracellular neurofibrillary tangles, respectively, are key targets. While the plaques and tangles are comprised of fibrillar aggregates of these proteins, many recent studies indicate that small soluble oligomeric aggregates of amyloid beta and tau play major roles in the pathogenesis and spread of disease.

Tau is a natively unfolded microtubule associated protein due to its very low hydrophobic content. The protein contains a projection domain, a basic proline-rich region, and an assembly domain that contains either three or four repeats (3R or 4R) of a conserved tubulin-binding motif as a result of alternative splicing of exon 10 [71,108,109]. Tau 4R isoforms have better microtubule binding and stabilizing capabilities compared to the 3R isoforms, and while 3R tau is expressed at the fetal stage, 3R and 4R are present in equal proportions in the adult human brain. Mutations that alter splicing of tau transcript and the ratio of 3R to 4R tau isoforms can lead to neurodegenerative disease [108,109]. In AD, tau undergoes several post-translational modifications which include aggregation, phosphorylation, glycosylation, glycation, ubiquitination, cleavage or truncation, (reviewed in:[110]). Tau can aberrantly fold into various aggregate morphologies which include ß-sheet rich fibrillar forms that result in the formation of paired helical filaments and neurofibrillary tangles [111,112]. Hyperphosphorylation of tau decreases the affinity of tau to the microtubules which

in turn affects axonal [113,114]. Therefore, tau in human brain tissue can exist in a variety of different lengths and morphologies and with multiple post-translational modifications.

Accumulation of tau is necessary for the development of cognitive deficits in AD models caused by over-expression of Aβ [76,115]. While neurofibrillary tangles (NFTs) have been implicated in mediating neurodegeneration in AD and tauopathies [116–118], animal models of tauopathy have shown that memory impairment and neuron loss do not associate well with accumulation of NFT [119]. Animal studies showed that a reduction in neuronal loss and improvement in memory could occur even with accumulation of NFTs [120], and that neuronal loss and NFT pathology did not always colocalize [121–123]. Oligomeric tau aggregates correlate well with AD progression [52,124,125] and high levels of tau oligomers were detected in the frontal lobe cortex [126125] and in CSF during early stages of AD [127,128]. Various oligomeric tau aggregates have been shown to propagate in AD brains through prion-like and receptor mediated mechanisms [129]. For example, tau pathology spreads throughout the brain from early to late stage [50], and seeding with tau aggregates can propagate altered tau folding from the outside to the inside of a cell [96]. Studies have also shown that brain extract from a transgenic mouse with aggregated mutant human tau can transmit tau pathology throughout the brain in mice expressing normal human tau [130] and inducing aggregation of human tau induces formation of tau aggregates and tangles composed of both human and normal murine tau (co-aggregation) [131]. Therefore, many diverse studies indicate oligomeric tau aggregates are involved in neurodegeneration and therefore selective AD related tau variants may be excellent early diagnostic and therapeutic targets for AD.

Because of the diversity of tau forms in healthy and AD brain, it would be very beneficial to develop reagents that selectively bind to tau variants that are uniquely present in AD brain. Here we develop antibody-based reagents that specifically bind tau variants that are

present in human AD brain samples, but not cognitively normal age matched control samples and show that the reagents have potential diagnostic and therapeutic value for AD.

## 2.2 Materials and methods

### *2.2.1 Brain Tissue and sera samples*

Human brain tissue, sections and sera samples were obtained from Dr. Thomas Beach, director of the Brain and Body Donation Program at Banner Sun Health Research Institute (BBDP; http://www.brainandbodydonationprogram.org) [132,133]. 7 different pathologically confirmed AD and 2 cognitively normal brain tissue samples were obtained from two different brain regions - the superior frontal gyrus and middle temporal gyrus. Brain sections for IHC were obtained from the superior frontal cortex. Sera samples were obtained from 10 pathologically confirmed AD (Braak stage V and VI) and 10 control cases.

### *2.2.2 Brain tissue homogenization*

Frozen brain tissue samples were briefly homogenized as described previously [46]. Briefly tissue was sonicated in cold lysis buffer: 25 mM HEPES NaOH (pH 7.9), 150 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5% Triton-X-100, 1 mM dithiothreitol, protease inhibitor cocktail. The homogenized sample was centrifuged, and the supernatant was frozen in -80°C. The presence of fibrillar tau was verified by immunohistochemistry by staining AD and healthy control brain tissue slices with 1:1000 dilution of commercial AT8 antibody (Thermo Fisher Scientific, Catalog # MN1020) against phosphorylated tau.

### *2.2.3 Tau protein immunoprecipitation*

Two polyclonal tau antibodies, PA1 against amino-acids 240-450 of tau and PA5 against amino-acid 1-286 (Thermo Fisher Scientific) were used to immunoprecipitate tau.

Antibody conjugates were captured using the Pierce Crosslink IP Kit A following the manufacturers' protocols. The antibody is first bound to the protein A/G agarose which is chemically crosslinked to the resin to prevent the antibody from eluting off the column. AD brain tissue homogenates were pooled as per the Braak stage classification into Braak stage III and Braak stage V samples. Tau immunoprecipitation was performed on the pooled AD Braak stage III, Braak stage V and healthy control samples. The integrity of the eluted tau was preserved by neutralizing the low pH elution buffer with 1M Tris, pH 9.5 as recommended by the manufacturer. The immunoprecipitated tau samples were probed with 1:1000 dilution of commercially available AT8 antibody (Thermo Fisher Scientific, Catalog # MN1020) to verify the presence of tau.

*2.2.4 Phage library preparation*

Tomlinson I, J and Sheets phage libraries each having a diversity of $10^8$ were grown as described [134] and equal phage titers from each library of $10^{13}$ were pooled for biopanning studies.

*2.2.5 Biopanning – AD tau specific morphologies*

A series of negative and panning steps followed by positive panning were performed as described [48] to obtain AD-tau specific reagents (Fig 2.1). Negative or subtractive selection steps were performed to remove phage that bound to non-target sticky protein samples using bovine serum album and aggregated protein morphologies using α-synuclein aggregates. Additional negative panning steps against monomeric tau, healthy tissue samples and healthy tau samples were performed to remove all antibody fragments that bound to those forms of tau found in healthy individuals. Atomic force microscopy (AFM) imaging was performed after every negative panning step to ensure removal of all antibody fragments against the off-target antigens. Phage that did not bind to any of the off- target

antigens were used for the final positive selection round performed against tau immunoprecipitated from pooled AD Braak stage III and V brain tissue samples. For the positive panning step, the tau preparation was deposited on mica since only nanogram quantities of the antigen are needed and the process can be monitored via AFM imaging. Phage were eluted using trypsin and TEA and grown on LB – Amp plates overnight at 37°C.

*2.2.6 Phage and scFv purification*

DNA sequences were obtained from recovered phage to ensure that the phage expressed full length antibody fragments. After sequence validation, phage were amplified as described [134]. Phage titers were performed to verify the concentration of phage (~$10^9$ pfu/mL). Soluble scFv protein separate from the phage was also produced for each phage by transforming the plasmid to HB2151 strain of E coli. The scFvs were grown and purified using a protein A Sepharose affinity column (GE Healthcare) as described [45,48]. Molecular size of the scFvs were checked by western blot using anti c-myc (9E10) monoclonal antibody (1:1000 dilution) which recognizes the c-myc region in the scFv followed by goat anti-mouse HRP (1:2000 dilution). The DNA sequences of the scFvs were also validated using MAFFT, a multiple sequence alignment software.

*2.2.7 Tau monomer phage biotinylation*

To obtain a detection phage for sandwich ELISA we required a phage particle displaying an scFv that bound all forms of tau including monomeric and oligomeric. To generate this scFv, we used the phage preparation eluted after negative panning with BSA and aggregated α-synuclein, and used this in a single positive panning step using commercially available tau monomer deposited on mica as described [48]. The detection phage binds tau

variants present in both AD and healthy control samples and does not compete for the same binding sites as the capture scFv in sandwich ELISA.

*2.2.8 ELISA assay*

A phage ELISA was initially performed to assess the specificity of each recovered phage for tau variants present in AD but not control brain tissue as described [135]. Pooled AD and control human brain tissue homogenates were used to coat the plates followed by detection with each of the phage clones. This assay was used to evaluate binding specificity of all the phage for AD over healthy control samples.

Soluble antibody fragments (scFv) (ADT-1, ADT-2, ADT-3, ADT-4, ADT-5 and ADT-6) were produced for the six phage samples that had the highest reactivity with the AD samples. The soluble scFvs were then used as capture antibodies in a sandwich ELISA to test reactivity with AD (n=10) and cognitively normal control sera (n=10) samples as described [132,133].

*2.2.9 Longitudinal sera analysis*

Sera samples were obtained over multiple timepoints from 25 individuals that started cognitively normal and converted to AD and 25 individuals that remained cognitively normal throughout the timeline (~10 years). The samples were taken from Mayo Clinic collections obtained by Dr. Steven Younkin and provided with the assistance of Dr. Terrone Rosenberry at the Mayo Clinic College of Medicine in Jacksonville, Florida. Four to five different timepoints were obtained for every individual. Based on clinical diagnoses samples were designated as pre-mild cognitive impairment (pre-MCI), MCI and AD. Case have not been pathologically validated. Data obtained with ADT-2, ADT-4 and ADT-6 scFvs were analyzed with respect to gender, genotype, MMSE scores.

*2.2.10 Immunohistochemistry*

Five anti-tau scFvs (ADT-1, ADT-2, ADT-3, ADT-4 and ADT-5) converted to IgG format were produced and purified by MIGS LLC (New Hampshire, USA). for evaluation in immunohistochemistry and toxicity assays. Concentration of the purified IgGs were assessed using a BCA kit.

Brain sections from the superior frontal cortex were treated with either a commercially available antibody against phosphorylated tau (AT8, Covance, 1:2000) or the five anti-tau IgGs (1:100). The sections were further treated with fluorescently tagged goat anti-mouse IgG (red) and goat anti-human IgG (green) (1:1000) respectively and the non-specific background was blocked with 0.03% Sudan black for 5 minutes. The sections were observed and imaged with Leica SP5 [47]. Commercial AT8 antibody is visualized in red, anti-tau IgG in green and DAPI, which stains the nucleus, in blue.

*2.2.11 Toxicity Assay*

The ability of the different anti-tau IgGs to block toxicity of tau variants contained in human AD tissue were assessed using cultured neuronal cells. Tau immunoprecipitated from human AD brain was used to induce toxicity in the human neuroblastoma cell line, SH-SY5Y. Once the SH-SY5Y neuronal cells reached confluence they were introduced into a 6-well plate. 100 μg/mL of immunoprecipitated tau from AD or control post mortem brain tissue was introduced into the wells. The cells were then incubated with either a commercial polyclonal tau antibody PA5 (Thermo Fisher), or one of the anti-tau IgGs – ADT-1, ADT-2, ADT-3, ADT-4 or ADT-5. After 12 hours of incubation, toxicity and damage to the cells were measured using a lactate dehydrogenase assay kit [136].

*2.2.12 Statistical Analysis*

Measurements on the ELISAs are represented as ratios of luminescence measurement for each sample with respect to the background (no antigen) control. Reactivity of each test sample was obtained relative to the average signal of the control group. Any sample with a ratio greater than 1 is considered a positive signal. Statistical significance was assessed using SPSS software (version 24) and one-way ANOVA was performed with LSD post-hoc analyses with significance at $p<0.05$. To determine the accuracy of these anti-tau scFvs in detecting AD over healthy controls, Receiver Operating Curves (ROC) were plotted based on the reactivity of the anti-tau scFvs with postmortem verified AD and healthy control sera. Sensitivity and specificity of the anti-tau scFvs were obtained by setting the cutoff as the average value of the healthy controls. Area Under the Curve (AUC) was calculated as described [137]. AUC greater than 0.8 is considered a good diagnostic test while 0.5 (straight line) indicates the test cannot significantly differentiate between AD and healthy controls.

## 2.3 Results

*2.3.1. Tau protein immunoprecipitation*

Tau was immunoprecipitated from pooled AD samples with confirmed tangle pathology and control brain tissue homogenates. When probed with commercial AT8 antibody, AD samples contained high molecular weight phosphorylated tau which was not present in the control samples (Fig 2.2). The immunoprecipitated AD and control tau samples were used for biopanning studies.

*2.3.2. Biopanning*

A series of negative or subtractive panning steps were utilized to remove all phage from the initial pooled phage library that bound to several off-target antigens including BSA,

aggregated α-synuclein, healthy control tissue and any forms of tau immunoprecipitated from healthy control tissue. Essentially complete removal of all phage binding these off-target antigens was verified by AFM imaging (Fig 2.3). After completing the subtractive panning process, a single round of positive panning using the immunoprecipitated tau from AD brain tissue was performed.

Thirty-five phages were recovered from the positive selection step and based on ELISA, 11 showed selective binding to the AD brain tissue samples compared to the control brain tissue samples (Fig 2.4). The six phages (ADT-1, ADT-2, ADT-3, ADT-4, ADT-5 and ADT-6) with the highest reactivity and specificity for pooled AD over control human brain tissue homogenates were selected for further analyses. We expressed soluble scFv from each of the six clones and evaluated reactivity of each scFv with individual AD (n=7) and control (n=2) brain tissue homogenates by sandwich ELISA. All six scFvs showed reactivity with tau variants present in each of the human AD tissue samples, but not with tau variants in the cognitively normal control brain tissue samples (Fig 2.5).

*2.3.3 Sera sample analysis*

We next tested whether the 6 selected scFvs (ADT-1, ADT-2, ADT-3, ADT-4, ADT-5 and ADT-6) also selectively bind tau variants present in sera samples of human AD (n=10) but not age-matched controls (n=10). All 6 scFvs preferentially bound tau variants present in all 10 AD sera samples compared to cognitively normal controls (Fig 2.6). Receiver Operator Curves (ROC) were plotted for the six anti-tau scFvs to determine their ability to select tau variants in sera samples from AD compared to healthy control cases (Fig 2.7). Five of the six anti-tau scFvs have high sensitivity and specificity to tau variants in AD.

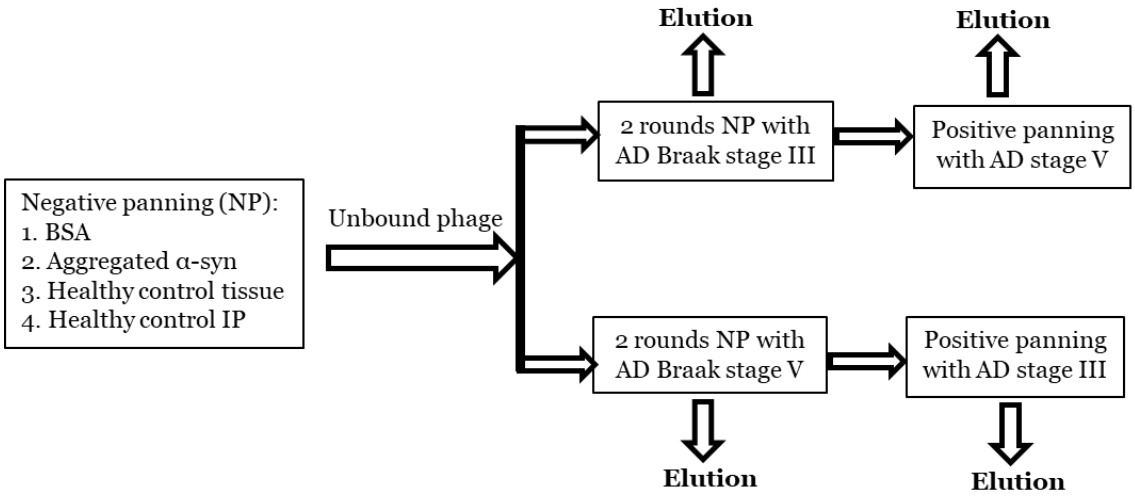



Figure 2.1 Flow diagram outlining steps in the panning protocol. Negative selection to remove non-specific clones was followed by positive selection with tau immunoprecipitated from AD Braak stage III and Braak stage V.

The Area Under Curve (AUC) values for ADT-2, ADT-4 and ADT-6 (0.91, 0.96 and 0.94) suggest these reagents are excellent candidates for detecting AD specific tau variants present in human sera samples. When combined, ADT-2, ADT-4 and ADT-6 scFvs recognized tau variants with 90% sensitivity and 90% specificity with 0.96 AUC. These three anti-tau scFvs were therefore used in further studies to analyze the presence of the respective tau variants in longitudinal AD and control sera samples.

*2.3.4 Longitudinal sera analysis*

*Tau variants in sera:* Sera samples from 25 individuals that converted to AD over a 10-year timeline (N=119) based on clinical examination and 25 individuals that remained as controls (n=121) over the course of the study were analyzed for the presence of tau variants recognized by the ADT-2, ADT-4 and ADT-6. Sera samples from individuals that converted to AD showed higher average reactivity toward each of the tau variants tested (blue bars Fig 2.8a) compared to samples from individuals that did not convert to AD (gray bars Fig 2.8a). Based on one-way ANOVA analysis, all three anti-tau scFvs were able to distinguish the converters from the non-converters. The AD converter and control groups were broken into groups depending on APOE genotype. Both ApoE3,3 and ApoE3,4 genotypes had significantly higher tau levels in the AD converters compared to the controls (Fig 2.8b).

*Gender analysis:*

We also analyzed the samples to determine if there were any gender differences. ADT-4 recognized tau variants preferentially present in females (n=51) while ADT-6 recognized tau variants preferentially present in males (n=68) (Fig 2.9a). The gender selectivity was maintained when the samples were further grouped according to their ApoE genotype (Fig 2.9b). The ADT-2 scFv did not demonstrate any gender selectivity.

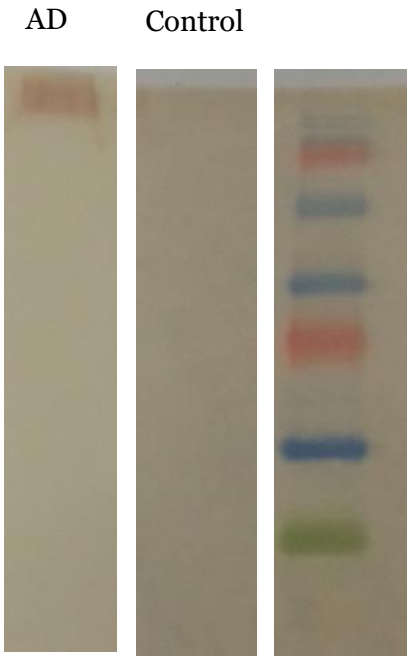
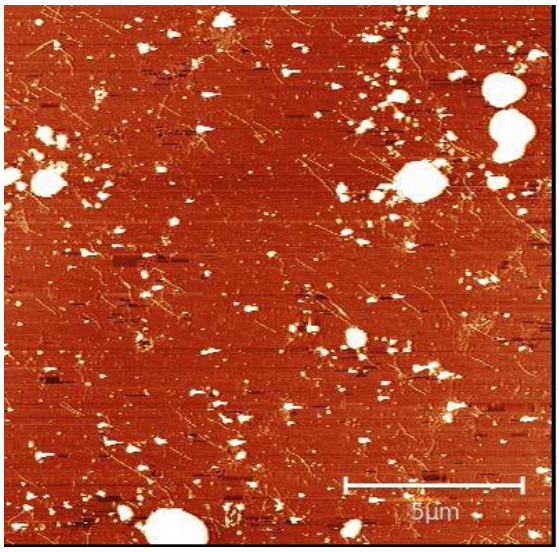
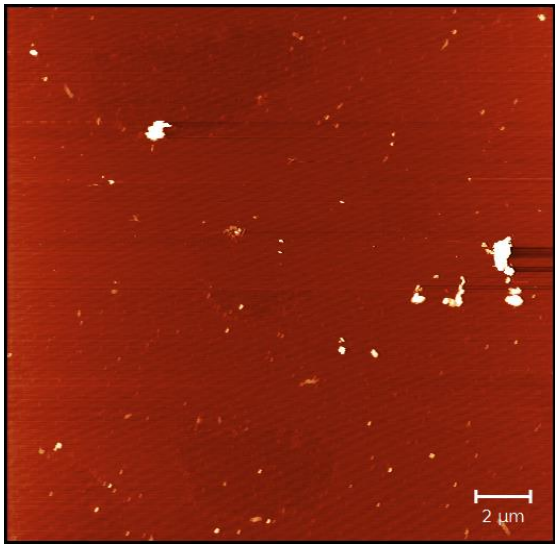



Figure 2.2 Western blot staining with anti-phosphorylated tau antibody AT8. Blot shows presence of high molecular weight phosphorylated tau species in AD sample with its absence in the control sample.




Binding to BSA (round 1) at 20µm




Binding to healthy control tau IP (round 8) at 20µm

Figure 2.3 AFM images before and after the panning process. Image above shows phage binding to BSA before negative panning. The image below has no phage binding to control tau IP indicating successful negative panning.

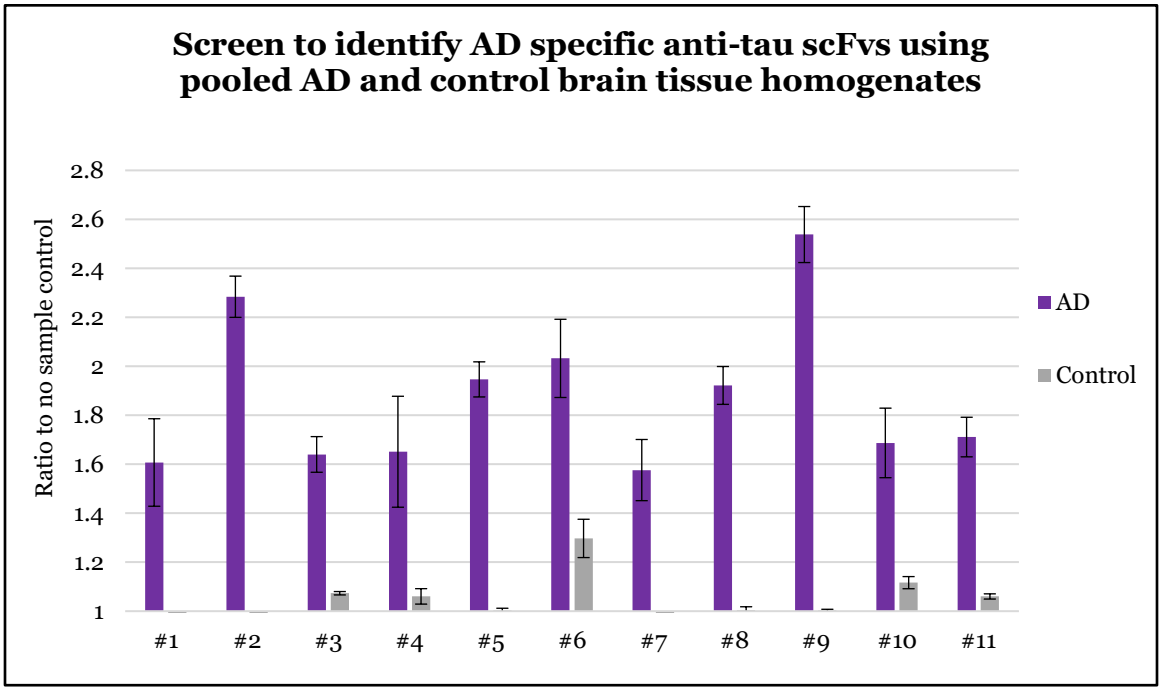



Figure 2.4 Indirect ELISA assay to identify AD specific anti-tau clones using pooled and control brain tissue homogenates. X axis represents the various clones and Y axis represents luminescence signal ratio to no sample control. All the clones have high levels of binding to AD compared to controls.

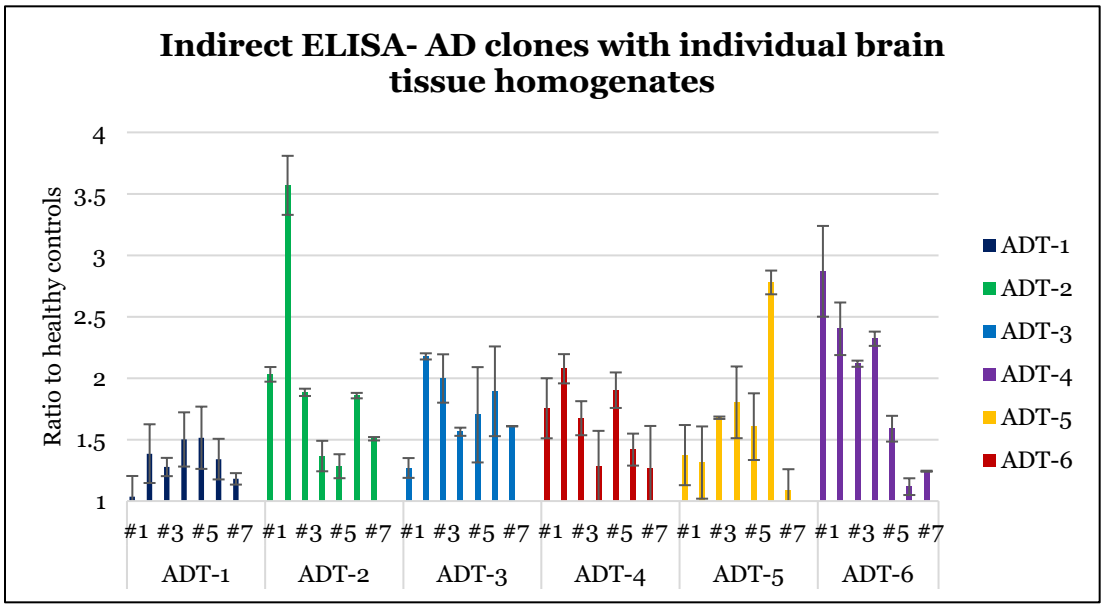



Figure 2.5 Indirect ELISA screening reactivity of six anti-tau phage candidates with individual AD brain tissue homogenates. X axis represents individual brain tissue homogenates and Y axis represents luminescence signal ratio to healthy controls. All the candidates have high levels of binding to individual AD brain tissue homogenates compared to controls.

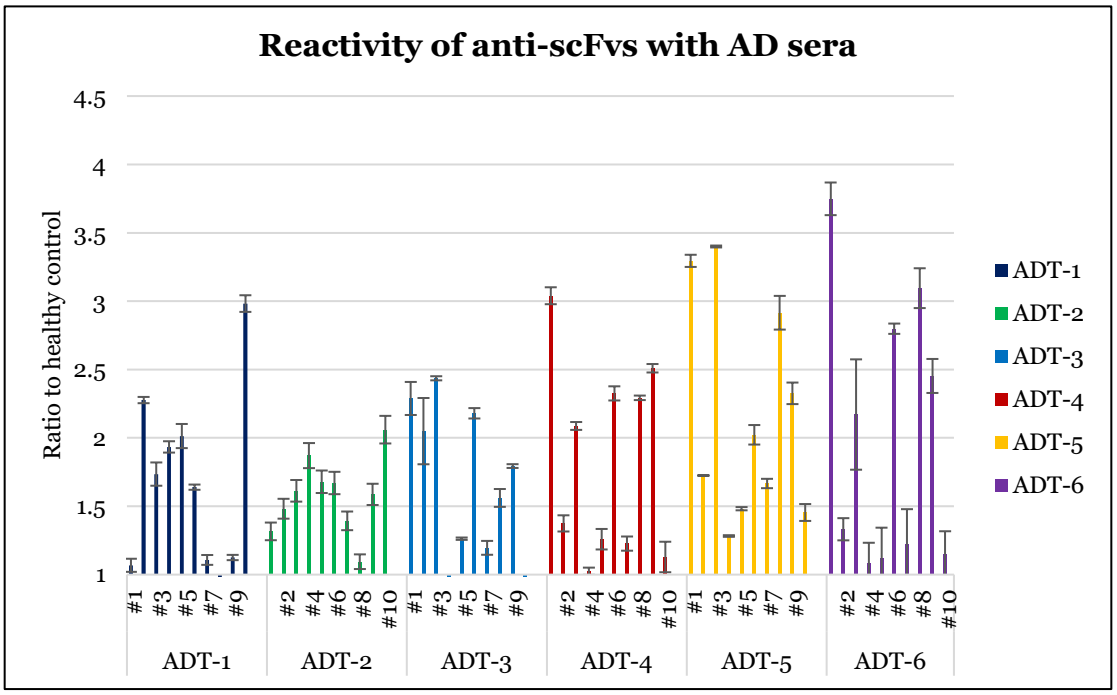



Figure 2.6 Sandwich ELISA screening reactivity of six anti-tau scFvs with individual AD sera. X axis represents individual AD sera samples and Y axis represents luminescence signal ratio to healthy control. All the 6 AD-tau scFvs have high levels of binding with all the 10 AD sera.

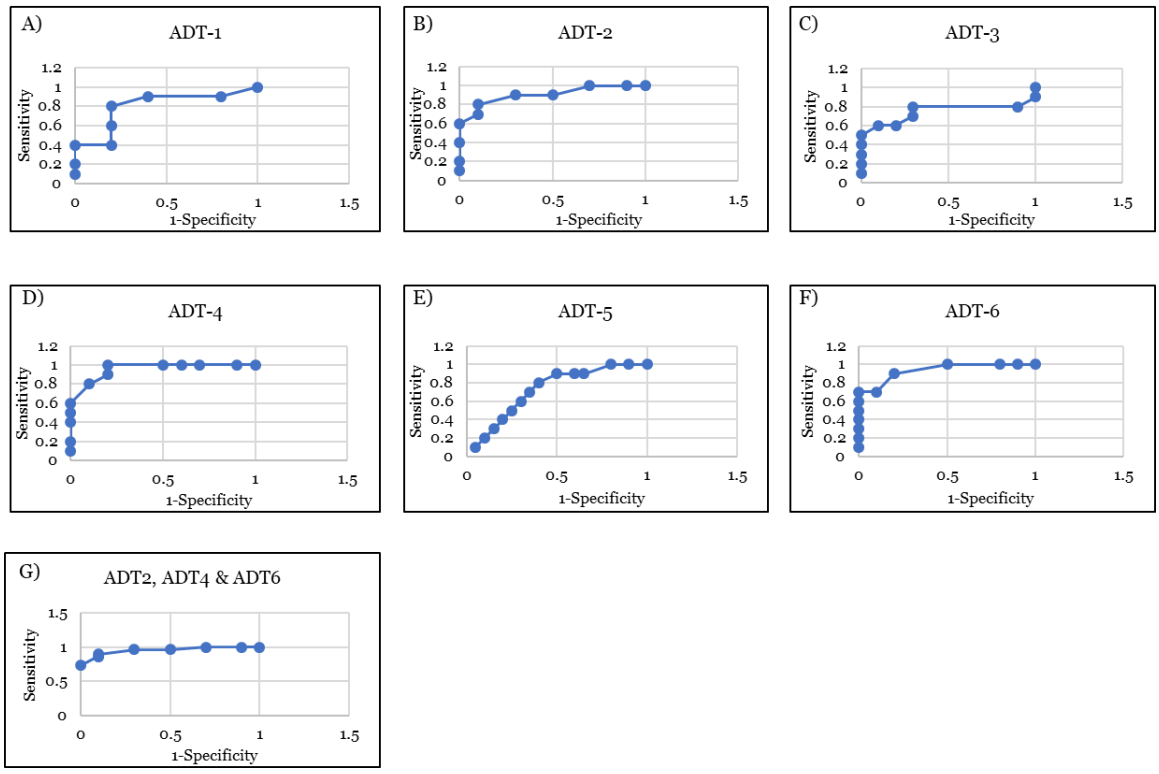



Figure 2.7 ROC curves of the six anti-tau scFvs based on reactivity with AD and control sera. Y-Axis represents sensitivity of the anti-tau scFvs to identify tau variants in AD and X-Axis represents false positive rate. Five of the six anti-tau scFvs (A-D, F) have high sensitivity and specificity to AD tau variants compared to cognitively normal controls. G) ADT-2, ADT-4 and ADT-6, together, have 90% sensitivity and 90% specificity.

*AD Progression:*

The sera samples were grouped according to classification based on neurological exam reports. Samples were classified as either pre-MCI, MCI, or AD depending on their neurological exam and performance on mini mental state examination (MMSE). ADT-4 scFv has significantly higher reactivity with samples obtained from patients during the MCI stage compared to samples taken during pre-MCI and AD stages (Fig 2.10a) suggesting that ADT-4 recognizes a tau variant preferentially generated during the MCI phase. The preference of ADT-4 for tau variants generated during the MCI stage is present in both females and males (Fig 2.10b).

### *2.3.5 Immunohistochemistry*

The location of the different tau variants in human AD brain tissue were analyzed by immunohistochemistry. Human post mortem AD and control brain sections from the superior frontal cortex were probed using the IgG version of five of the anti-tau scFvs (ADT-1, ADT-2, ADT-3, ADT-4 and ADT-5) and the commercial AT8 anti-phosphorylated tau antibody. As expected, none of the antibodies showed significant reactivity with control brain sections, however widespread positive staining was observed in the AD brain sections (Fig 2.11). There is extensive extracellular colocalization of commercial AT8 and four of the IgGs (ADT-1, ADT-3, ADT-4 and ADT-5). Interestingly, ADT-2 does not colocalize with AT8 and based on morphology may recognizes tau along the axons.

### *2.3.6 Therapeutic potential of anti-tau scFvs*

Since all five IgG versions of the scFvs tested (ADT-1, ADT-2, ADT-3, ADT-4 and ADT-5) preferentially bound human AD tissue and sera samples compared to age-matched cognitively normal samples, we next determined which of the IgGs could neutralize toxicity of the tau variants present in human AD brain tissue. We added

immunoprecipitated tau from human AD brain tissue to human neuronal SH-SY5Y cells and determined toxicity when co-incubated with different concentrations of either a control IgG, a commercial anti-tau IgG or the IgG version of each of the five anti-tau scFvs. Significant reduction in toxicity was observed at concentrations of 0.1μg/mL in ADT-1, ADT-2, ADT-4 and ADT-5 compared to commercial antibody which was effective only at a dose of 10μg/mL (Fig 2.12). ADT-3 was not as effective as the other IgGs in reducing toxicity.

## 2.4 Discussion

AD is a neurodegenerative disease for which there are currently no effective therapeutics. Both Aβ and tau have been shown to play a critical role in AD pathogenesis (as reviewed in [138]). Several intermediate variants of tau that are generated prior to the formation of intracellular tau tangles which have been suggested to play a major role in disease pathogenesis. It has also been shown that these tau variants progressively spread from one cell to another in a neurotoxic manner [50,96,130] and are synaptotoxic and responsible for spread of the disease [115,119,123,126]. Reagents that can selectively recognize the different tau variants would provide tools to facilitate diagnosis of AD and represent potential novel therapeutics.

Here, we generated a pool of antibody-based reagents that selectively bind different tau variants that are present in human AD but not age-matched control samples. These morphology specific reagents recognize tau variants present in both brain tissue homogenate and circulating sera from AD patients but not age-matched controls. We showed that several of the reagents are excellent biomarkers of AD, and that they also represent promising therapeutics that can selectively bind toxic tau variants.

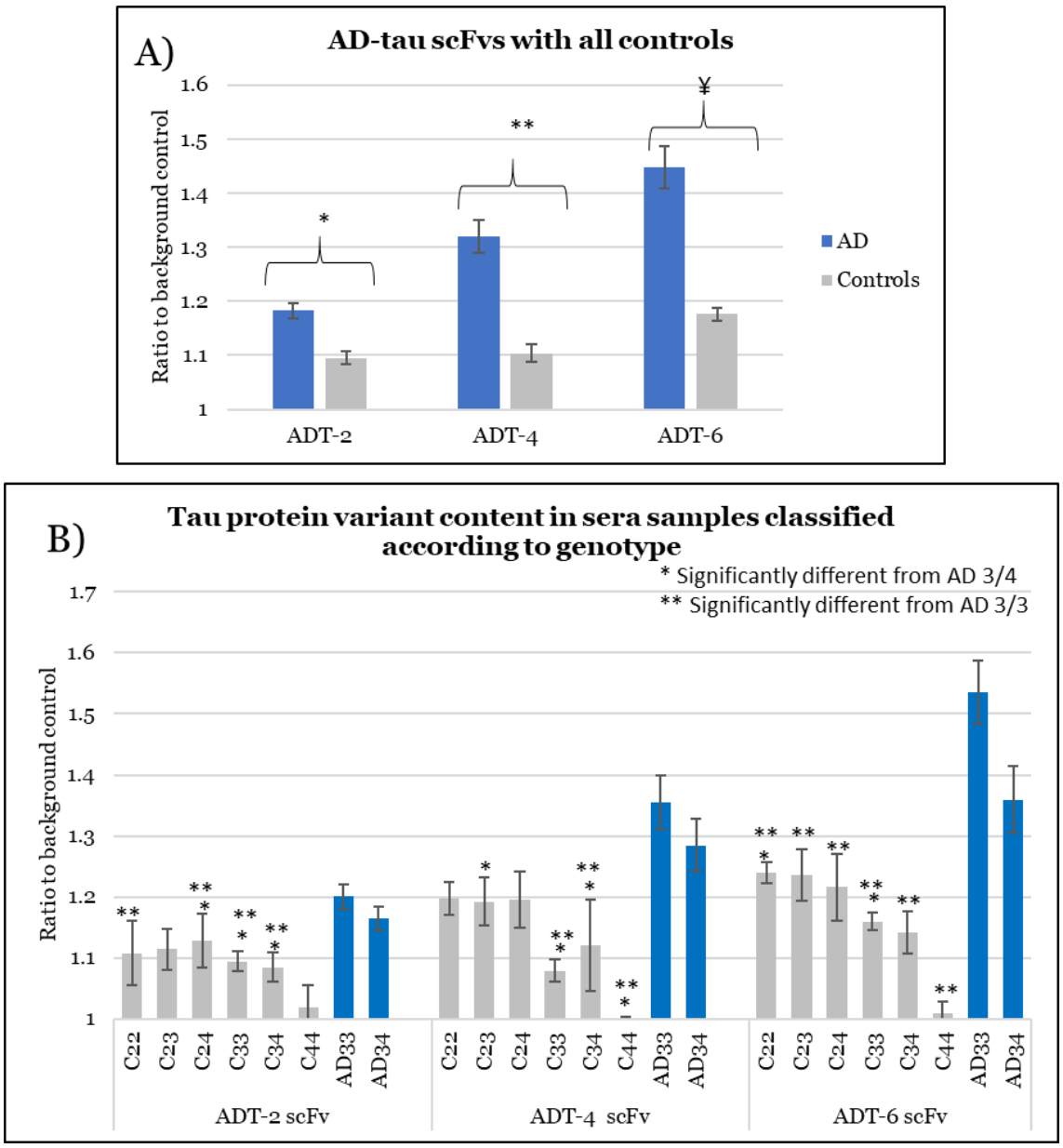



Figure 2.8 Reactivity of anti tau scFvs ADT-2, ADT-4 and ADT-6 with longitudinal sera samples. X-axis represents sera samples classified as AD and controls and Y-axis represents ratio to background control. A) represents ratios of AD (n=25) and control (n=25) samples, B) represent Reactivity of ADT-2, ADT-4 and ADT-6 respectively with AD and control samples classified according to their genotype (AD 3/3, 3/4, Control 2/2, 2/3, 2/4, 3/3, 3/4, 4/4).

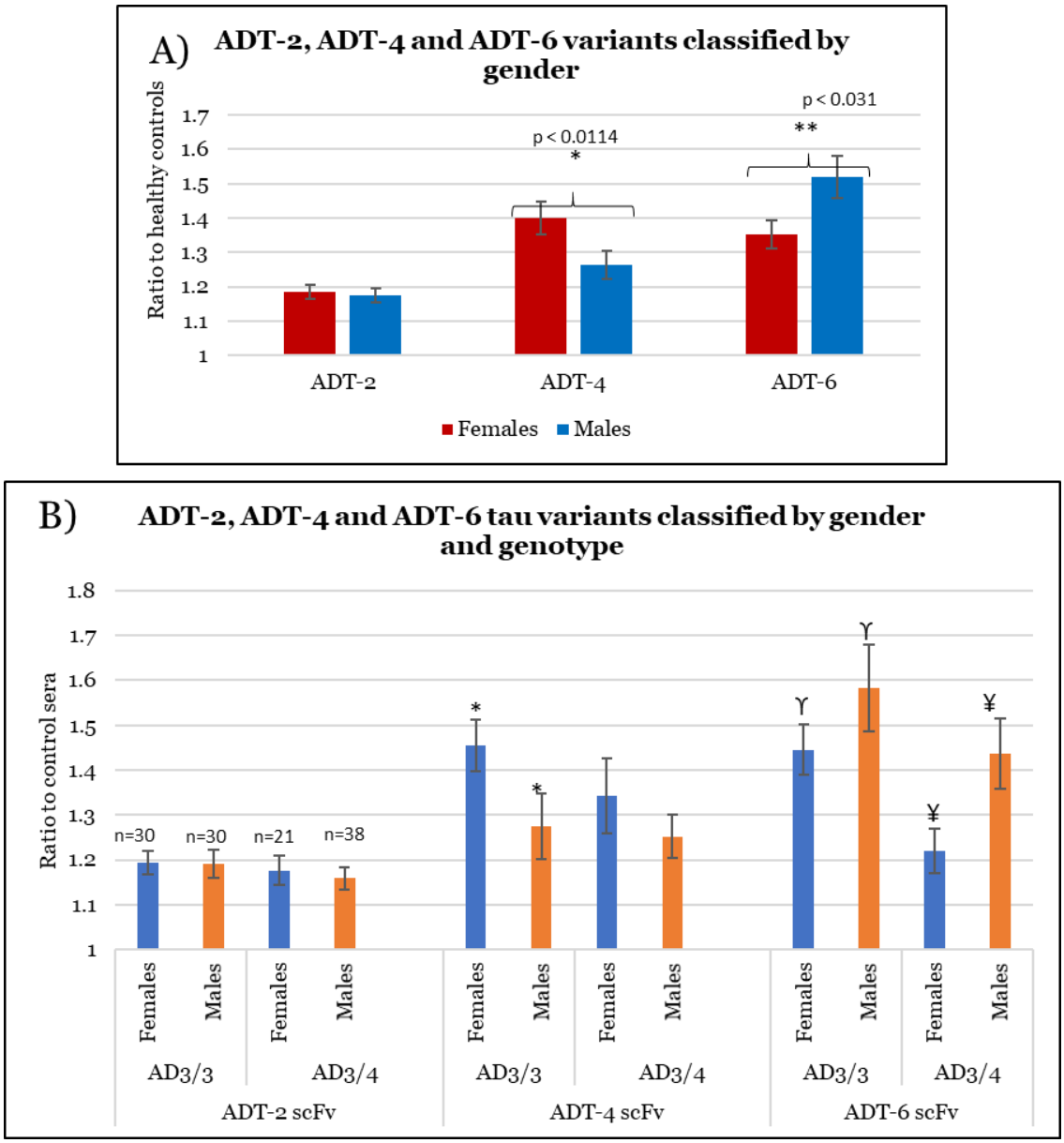
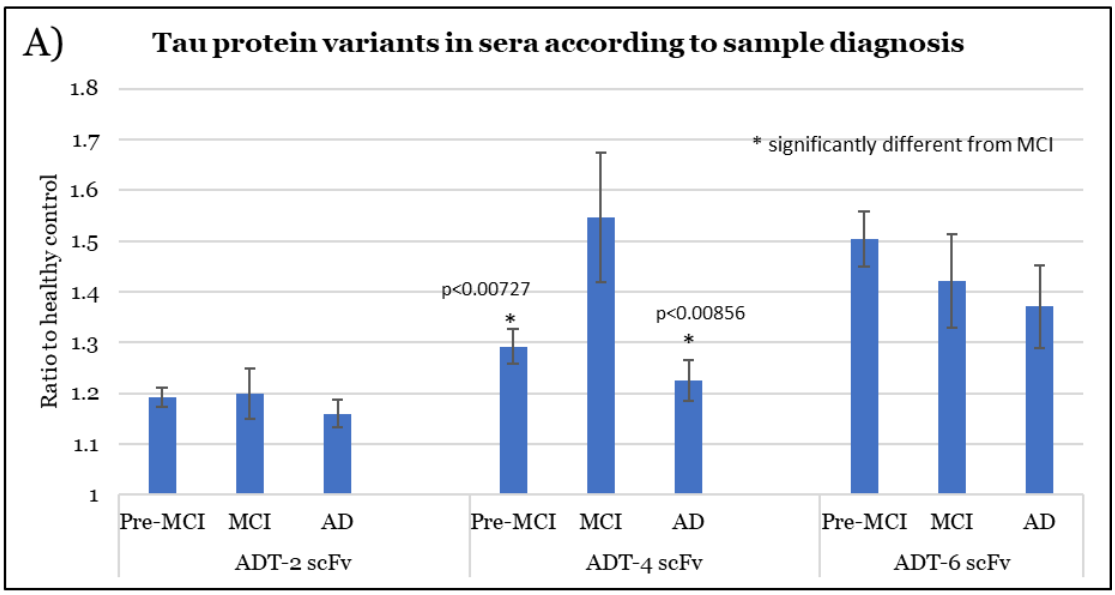



Figure 2.9 Reactivity of anti tau scFvs ADT-2, ADT-4 and ADT-6 with AD sera (n=119) A) classified according to their gender (X-Axis) and reactive protein variant content with respect to healthy controls (Y-Axis). ADT-4 ($p<0.0114$) and ADT-6 ($p<0.031$) have significant gender differences, B) represents reactivity classified according to their genotype (AD 3/3, 3/4) and gender. ADT-4 and ADT-6 retained their gender selectivity at 90% CI in AD 3/3 genotype (ADT-4: $p<0.019$, ADT-6: $p<0.084$) and AD 3/4 genotype (ADT-6: $p<0.060$).



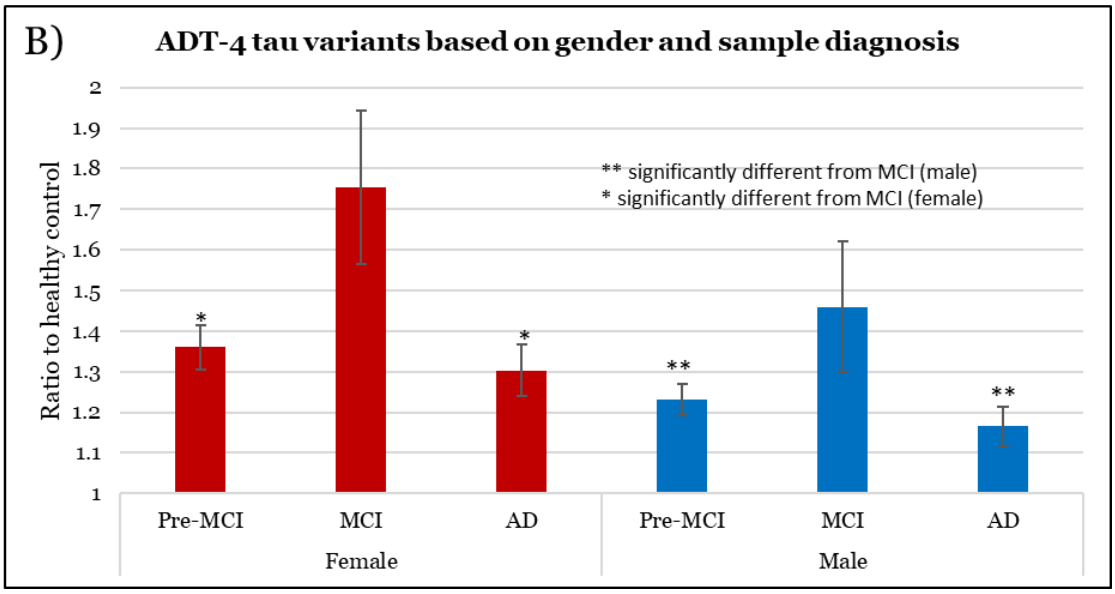
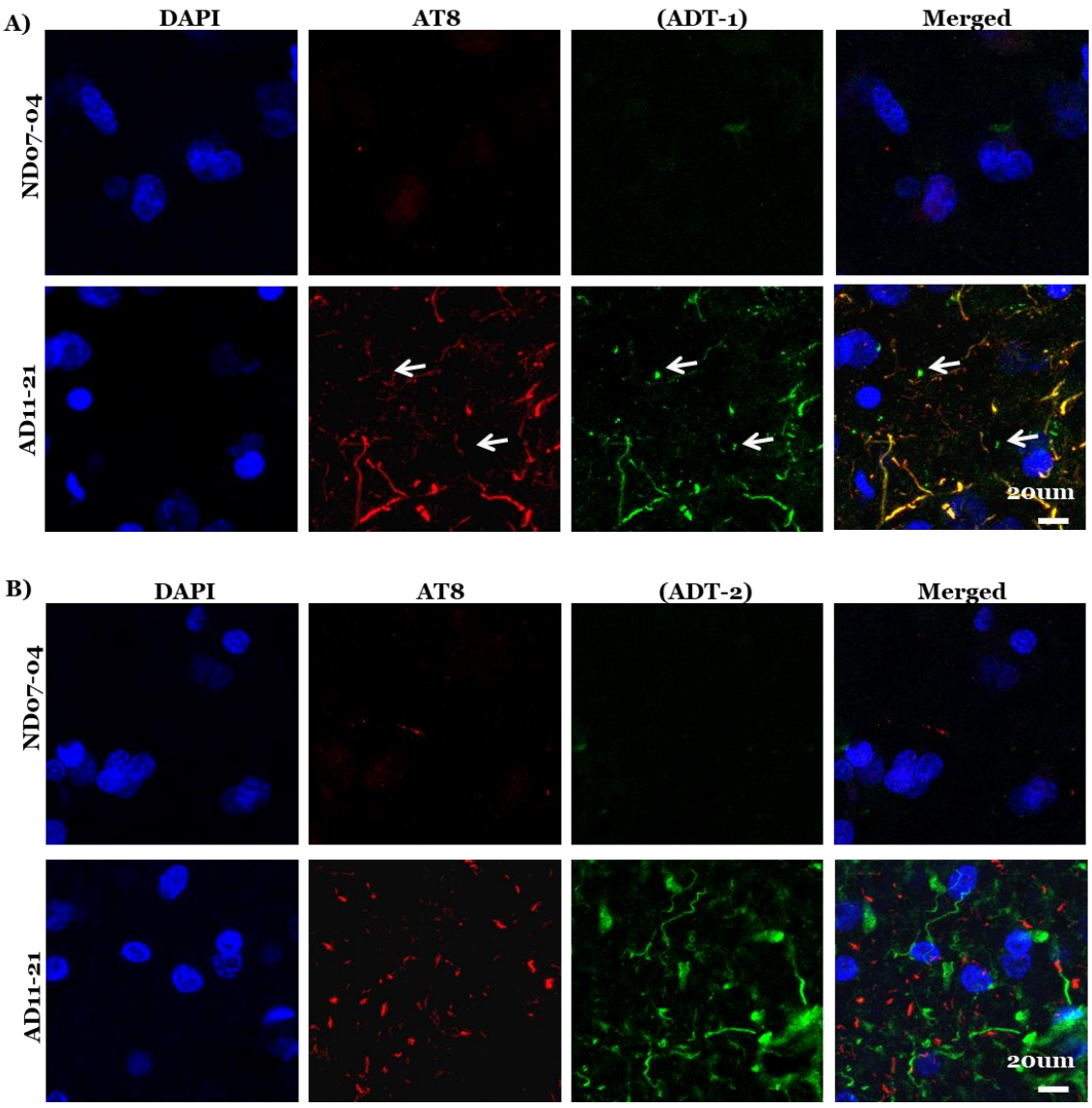
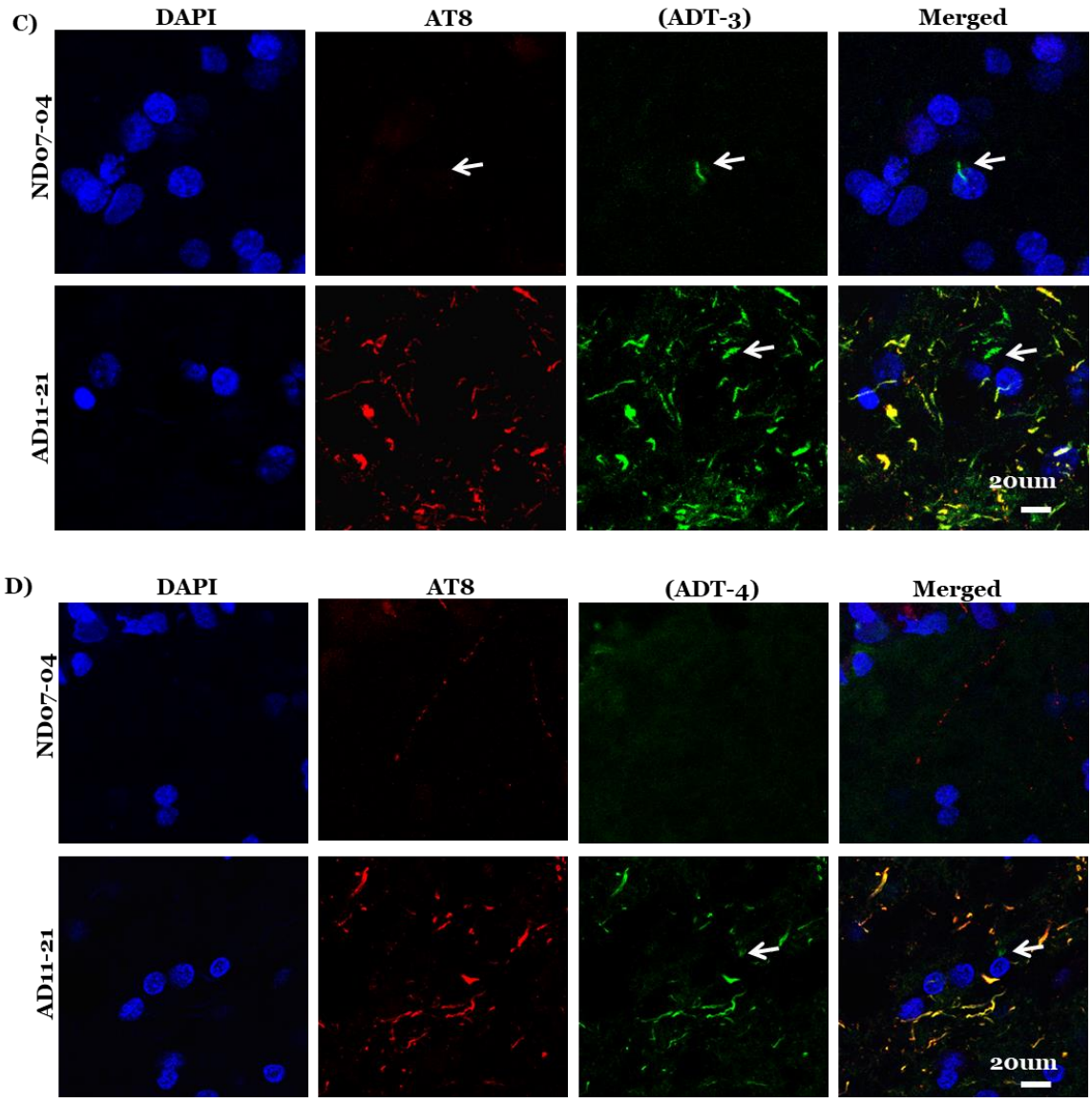




Figure 2.10 Reactivity of anti tau scFvs ADT-2, ADT-4 and ADT-6 with AD sera samples (n=119) classified based on A) sample diagnosis (Pre-MCI, MCI and AD), B) gender and sample diagnosis. Results indicate that ADT-4 tau variants were significantly high in samples classified as MCI compared to pre-MCI and AD groups in both females and males.


A)
DAPI
AT8
(ADT-1)
Merged
ND07-04
AD11-21
20um
B)
DAPI
AT8
(ADT-2)
Merged
ND07-04
AD11-21
20um


C)
DAPI
AT8
(ADT-3)
Merged
ND07-04
AD11-21
20um
D)
DAPI
AT8
(ADT-4)
Merged
ND07-04
AD11-21
20um

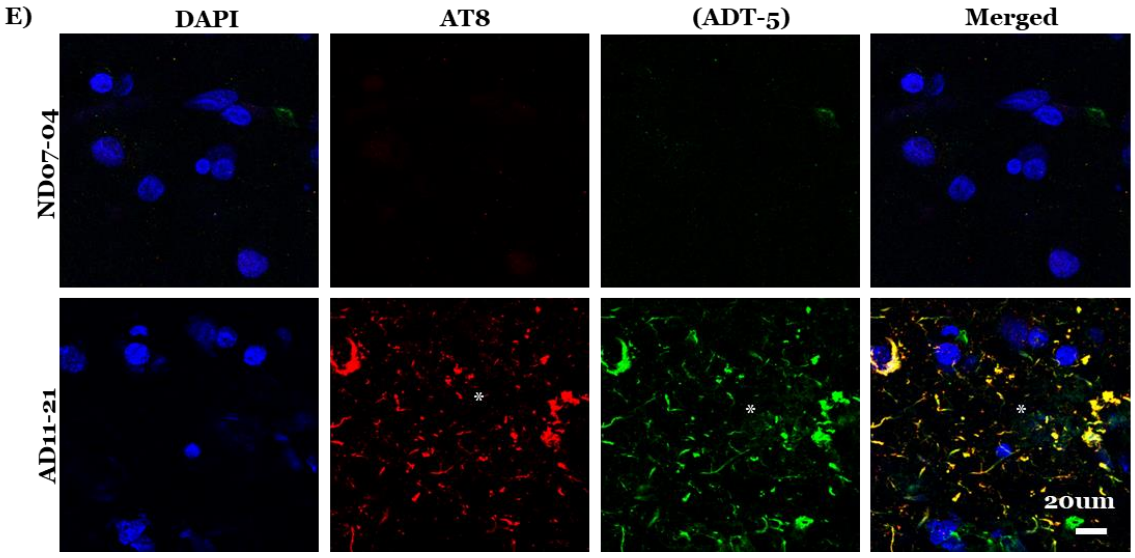



Figure 2.11 Reactivity of anti-tau IgGs with tau variants in AD and control brain tissue slice using IHC. Primary antibodies were applied with mouse against phosphorylated tau (AT8, Covance, 1:2000) and human against tau variants A) ADT-1, B) ADT-2, C) ADT-3, D) ADT-4, E) ADT-5 respectively, 1:100) on a shaking stage overnight, 4C. Goat anti-mouse IgG and goat anti-human IgG with fluorescence at the concentration of 1:1000 were used respectively as secondary antibodies for 1 hour at room temperature. The sections were washed with PBS 3 times and the non-specific background was blocked with 0.03% Sudan black for 5 minutes. The sections were observed and imaged with Leica SP5.

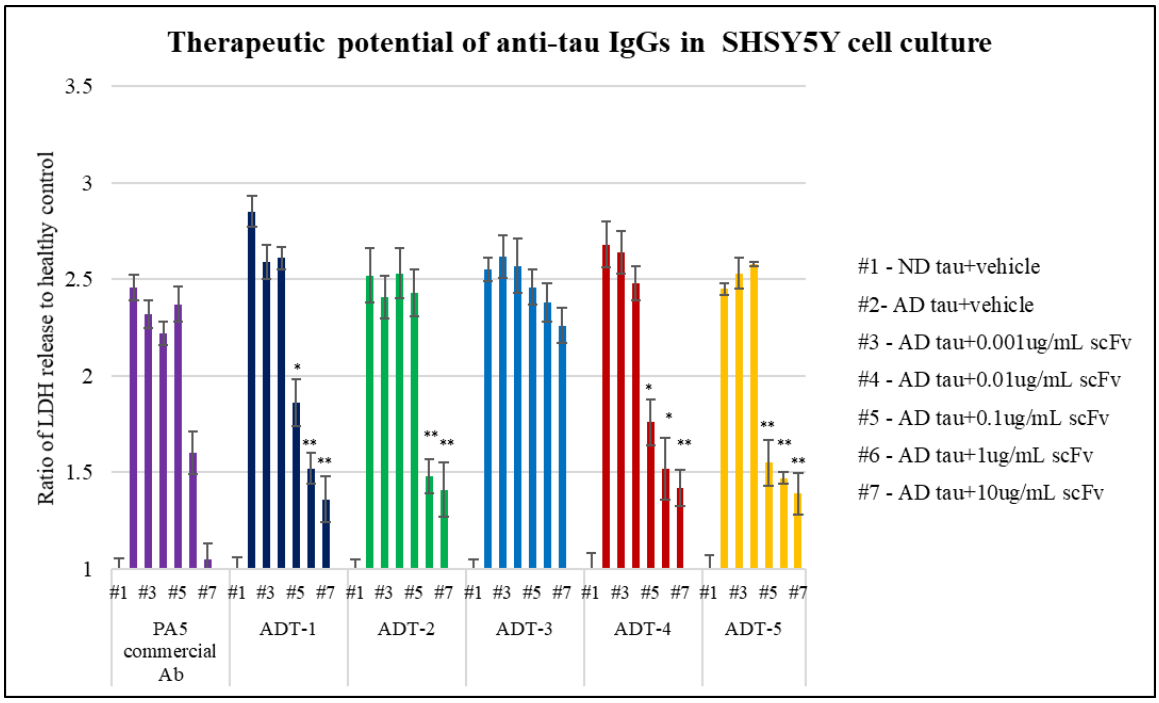



Figure 2.12 Therapeutic potential of anti-tau IgGs in SH-SY5Y neuroblastoma cell line. Cells were treated with AD brain derived tau IP and with different concentrations of either polyclonal antibody PA5 or IgGs ADT-1, ADT-2, ADT-3, ADT-4 and ADT-5 for 12 hours. The cell damage and toxicity were tested by measuring lactate dehydrogenase (LDH). *p < 0.5, **p < 0.01. ADT-3 did not inhibit toxicity of AD brain derived tau IP, while the other 4 IgGs inhibit toxicity more effectively than a polyclonal anti-tau preparation.

Since tau pathology is not unique to Alzheimer's Disease and is also observed in other tauopathies like Frontotemporal Dementia [139], these reagents will be further screened with a larger sample set to eliminate reagents that have any reactivity with other neurodegenerative diseases. As a result, we could obtain reagents that are morphology and disease specific which can help in differentiating neurodegenerative diseases and serve as unique biomarkers of disease.

Chapter 3

Frontotemporal Dementia

## 3.1 Introduction

Frontotemporal dementia is the second leading cause of early onset dementia following Alzheimer's disease [78]. FTD is diverse and involves atrophy of the frontal and temporal regions of the brain affecting language, memory and personality [79]. Based on prominent pathological protein inclusions -TDP, tau or FUS, FTD is classified as either FTD-TDP, FTD-tau or FTD-FUS [80]. Studies have shown that these subtypes have overlapping molecular pathology, making diagnosis difficult [2,81,82], despite progress in imaging techniques and CSF biomarkers.

There are currently several imaging techniques like diffusion tensor imaging, functional-MRI (voxel based changes) and PET scan that have shown promise in FTD diagnosis [83–85]. These techniques have been demonstrated on a small scale and are focused on measuring anatomical similarities within FTD and assessing differences between FTD and other dementias. Relying only on imaging for diagnosis has limited potential since FTD falls under a spectrum with a wide range of anatomical representations.

Apart from imaging, current CSF based biomarkers for FTD focus on measuring p-tau, tau and Aβ42 which is downregulated in certain FTD subtypes compared to AD [140–143]. While a fraction of FTD cases demonstrate AD pathology [40,144–146], over 50% of Alzheimer's cases present with TDP pathology [147–150] rendering AD based biomarkers (p-tau and Aβ) unreliable. Therefore, there is a need for biomarkers that can differentiate FTD from other diseases.

Although there is a familial component to FTD with mutations identified in MAPT, C9orf72 and GRN, extensive TDP-43 pathology has been observed in both familial and sporadic cases of FTD [25,86,87]. TDP-43 is a TAR DNA binding nuclear protein, 414 amino acids in length coded by the TARDBP gene. TDP-43 is a common molecular pathology in the FTD-ALS spectrum and is observed in more than 50% of the FTD cases [88]. It plays a key role in transcription and translation processes and is involved in alternate splicing, mRNA transport and serves as a shuttle between the nucleus and cytoplasm [91]. In FTD, TDP-43 is translocated to the cytoplasm [92] and location and type of aggregates [93] differ in clinical subtypes of FTD [81]. Elevated levels of TDP 43 are found in circulating CSF of FTD and ALS patients [92,105]. Although the pathogenic mechanisms is not known, several studies indicate that TDP-43 can spread in a prion like fashion from neuron to neuron through the axonal [94–98]. TDP-43 is also implicated in ALS, where different strains of TDP-43 have been shown to spread at different rates in *in vitro* models, indicating presence of multiple toxic TDP variants [95,99]. Different TDP-43 conformations with different levels of toxicity resulting in different pathologies (TDP type A-D) and disease phenotype have been identified [100]. These TDP-43 variants exist due to post-translational modifications such as hyperphosphorylation, polyubiquitination and truncation leading to C-terminal fragments that are toxic [92,101–104]. Currently, there is a lack of accurate blood-based biomarkers for FTD irrespective of familial or sporadic origin. We propose that FTD specific TDP-43 variants can be used as unique biomarkers in early antemortem diagnosis distinguishing FTD from other neurodegenerative diseases. We have identified a unique panel of scFvs capable of recognizing TDP variants that are present in human FTD patients but not in age-matched cognitively normal controls.

## 3.2 Materials and methods

### *3.2.1 Human specimens*

Human brain tissue homogenates from motor cortex of FTD (n=3), ALS (n=3) and healthy controls (n=2) and immunoprecipitated TDP-43 from these cases were provided from Georgetown Brain Bank (Georgetown University Medical Center). These samples were used in the initial AFM based screening. Human postmortem brain tissue sections from the superior frontal cortex and sera samples from FTD and control were provided by Dr. Thomas Beach, director of the Brain and Body Donation Program at Banner Sun Health Research Institute (BBDP; http://www.brainandbodydonationprogram.org) [132,133]. The brain sections were used for immunohistochemistry studies and sera samples (FTD-TDP (n=12), FTD-Tau (n=12), AD (n=11)) used in ELISA characterization studies.

### *3.2.2 Panning using immunoprecipitated TDP-43*

Frozen brain tissue samples were briefly homogenized as described previously [46]. Briefly tissue was sonicated in cold lysis buffer: 25 mM HEPES NaOH (pH 7.9), 150 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5% Triton-X-100, 1 mM dithiothreitol, protease inhibitor cocktail. The homogenized sample was centrifuged, and the supernatant was frozen in -80°C.

TDP-43 protein was immunoprecipitated from brain tissue homogenates which were pooled (3 FTD samples and 2 healthy controls) using a commercial polyclonal antibody against TDP-43 protein (ProteinTech Inc, Chicago, IL; Catalog # 10782-2-AP) as validated in [151]. The immunoprecipitated samples were probed with 1:1000 dilution of commercially available anti-TDP antibody (ProteinTech Inc, Chicago, IL; Catalog # 10782-2-AP) to verify the presence of TDP-43.

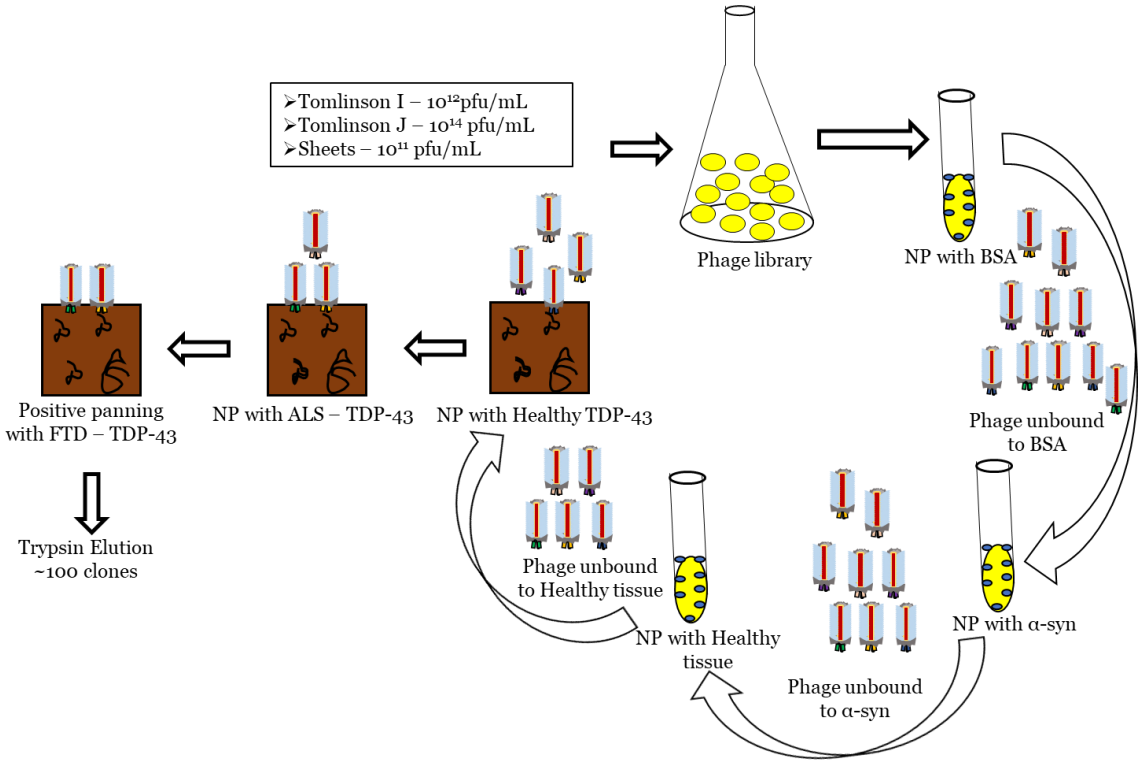



Figure 3.1 Schematic of panning protocol. Negative panning was performed against BSA, aggregated α-synuclein, healthy tissue and TDP-43 immunoprecipitated from healthy tissue and human postmortem ALS brain tissue. Positive selection was against TDP-43 immunoprecipitated from human postmortem FTD brain tissue.

A combination of commercially available phage display libraries - Sheets, Tomlinson I and Tomlinson J with a variability of $10^8$ and concentration of $10^{12}$ pfu/m were used for the panning process (Fig 3.1) [134]. We utilized an AFM based selection process that uses exhaustive subtractive panning steps to remove non-specific phage binding clones as well as clones binding to off-target antigens including antibody fragments that bound to TDP-43 forms from healthy individuals and from ALS patients as described previously [48]. Atomic force microscopy (AFM) imaging was performed after every negative panning step to ensure removal of all antibody fragments binding these off-target antigens. Phage that did not bind to any of the off-target antigens was used for the final positive selection round performed against TDP immunoprecipitated from pooled FTD brain tissue samples. For this positive panning step, the TDP IP preparation was deposited on mica since only nanogram quantities of the antigen are needed and the process can be monitored via AFM imaging. Phage were eluted using trypsin and TEA and grown on LB – Amp plates overnight at 37°C.

*3.2.3 Phage and scFv purification*

Phage obtained after the positive selection were sequenced to ensure that they encoded complete scFv sequences. After sequence validation, phage were amplified as described [134]. Phage titers were performed to verify the concentration of phage (~$10^9$ pfu/mL). Soluble scFv were also prepared by transforming the plasmids from each phage into *E. Coli* strain HB2151. An overnight culture was used for growing scFv in 2xYT media at 37°C for 3-4 hours. The scFvs were grown and purified using a protein A Sepharose affinity column (GE Healthcare) as described [48][45]. Molecular size of the scFvs were checked in both the supernatant and lysate fraction via western blot with anti - c-myc 9e10 primary antibody (1:2000 dilution) followed by secondary antibody goat anti-mouse HRP (1:2000

dilution) [48]. The DNA sequences of the scFvs were also validated using MAFFT, a multiple sequence alignment software.

### 3.2.4 TDP phage biotinylation

An aliquot of the remaining phage pool that was recovered after exhaustive negative panning with BSA, and aggregated α-synuclein and TDP-43 immunoprecipitated from healthy control tissue was used to select a detection phage for sandwich ELISA [48]. A phage expressing an scFv that binds to all forms of TDP-43 contained in both FTD and ALS samples was selected to increase signal to noise ratio in ELISA. This phage was biotinylated using the EZ-Link Pentylamine-Biotinylation kit (Thermo Scientific, USA) as described [135]. The detection phage binds TDP variants present in both FTD and ALS samples and does not compete for the same binding sites as the capture scFv in sandwich ELISA.

### *3.2.5 FTD tissue and sera analysis*

Brain tissue homogenates from FTD (n=3), ALS (n=3) and healthy individuals (n=2) were pooled together and used for the initial screening assay as described previously [135]. The pooled brain tissue homogenate was used to coat the plates and tested for reactivity with each of the phages. This assay was used to evaluate binding specificity of all the phage clones for FTD over ALS and cognitively normal control samples.

Soluble antibody fragments (scFv) (FTD-TDP1, FTD-TDP2, FTD-TDP3, FTD-TDP4 and FTD-TDP-5) were produced for each of the phages that had a high signal with the FTD brain tissue homogenates in the indirect ELISA. The scFvs were used as the capture antibody in a sandwich ELISA to test reactivity with sera samples (12 FTD-TDP, 12 FTD-tau and 10 healthy controls) as described in [132,133]. The bound species was detected by biotinylated TDP phage to amplify the signal to noise ratio. Signal ratios were determined

by comparing the signal obtained for each scFv with the FTD sera to healthy controls and plotted as described [135].

*3.2.6 Western blot analysis*

A 15% non-denaturing PAGE gel was used to analyze the molecular weight of TDP species recognized by the FTD-TDP2 scFv. The resolving and stacking gels were prepared without SDS. 5X-Running buffer (15g Tris+72g Glycine in 1L) and 2X-loading buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 1% Bromophenol Blue) were also prepared without SDS detergent.

Protein samples were diluted in loading buffer and this mixture was loaded directly onto the gels without heat denaturation. Samples including TDP-43 immunoprecipitated from three different FTD individuals and two healthy controls were analyzed. The gel apparatus was set at 70V for 30 minutes followed by 100V for approximately 3 hours until the marker was well separated. A nitrocellulose membrane was used to transfer the separated bands from the gel using standard western protocol [152]. The blot was incubated at RT with 2% milk powder in 1X PBS followed by incubation with FTD-TDP2 scFv supernatant overnight at 4°C. The blot was then washed with 1X PBS thrice followed by incubation with anti- c-myc (9e10) primary antibody (1:2000 dilution) for 2 hours at RT. The blot was further washed with 1X PBS followed by incubation with goat anti-mouse HRP (1:1000 dilution) at RT for 45 minutes. After a final wash with 1X PBS, a colorimetric DAB substrate was added, and the blot was developed as per manufacturer's protocol [152]. A duplicate gel was stained using Coomassie brilliant blue stain for 1 hour at RT followed by destaining at 4°C overnight.

*3.2.7 Competition ELISA*

To determine if the five FTD-TDP scFvs were binding to similar or different epitopes, a competition ELISA was performed as described [135,153]. Each of the five FTD-TDP scFvs were pre-incubated with FTD sera at 37°C for 1 hour. During the addition of antigen, 1:100 dilution of FTD sera or FTD sera pre-incubated with FTD-TDP scFvs were used.

*3.2.8 Immunohistochemistry*

Human postmortem tissue sections from superior frontal cortex were incubated with FTD-TDP2 and FTD-TDP3 scFvs respectively (1:100) on a shaking stage overnight at 4°C. Primary antibodies against c-myc region of scFv (Sigma, 1:1000, rabbit) and MAP2 (Covance, 1:400, mouse) were applied to the tissue sections for 3 hours at room temperature. Goat anti-rabbit IgG (green) and goat anti-mouse IgG (red) with fluorescence at the concentration of 1:1000 were used respectively as secondary antibodies for 1 hour at room temperature. The sections were washed with PBS 3 times and the non-specific background was blocked with 0.03% Sudan black for 5 minutes. The sections were observed and imaged with Leica SP5. Commercial MAP2 antibody is visualized in red, anti-TDP scFv in green and DAPI, which stains the nucleus, in blue.

*3.2.9 Toxicity Assay*

TDP-43 for the toxicity assay was immunoprecipitated from human postmortem FTD and control brain tissue using four commercial antibodies - A16583 (cell signaling), ab190963 (Abcam), 10782-2-AP and 12892-1-AP (ProteinTech). The human neuroblastoma cell line, SH-SY5Y was used for toxicity studies. Cells were grown in serum free media on 6 well plate and once they reached confluence, toxicity was induced by incubating the cells with 1 µg/mL of TDP-43 IP from FTD or control. The cells were then incubated with commercial anti-TDP antibody ab190963 (Abcam, 1µg/mL), or one of the anti-TDP scFvs – FTD-

TDP1, FTD-TDP2, FTD-TDP3, FTD-TDP4 and FTD-TDP5. After 12 hours of incubation, toxicity was measured using a lactate dehydrogenase assay kit [136].

*3.2.10 Statistical Analysis*

Luminescence signals obtained on the ELISAs were plotted as a ratio with respect to either background or healthy controls. Statistical significance was assessed using SPSS software (version 24) with $p<0.05$. Receiver operating curves (ROC) and area under the curve (AUC) were computed based on the reactivity of the five FTD-TDP scFvs with FTD-TDP, FTD-Tau and healthy control sera. Sensitivity and specificity of the FTD-TDP scFvs were also obtained by setting the cutoff as the average value of the healthy controls.

## 3.3 Results

*3.3.1 Phage and scFv purification*

Serial rounds of subtractive panning were performed to remove phage that bound off target antigens including BSA, homogenate from healthy human brain tissue and TDP-43 immunoprecipitated from pooled ALS brain tissue homogenates. A single round of positive selection was then performed using immunoprecipitated TDP-43 from pooled FTD brain tissue (Fig 3.2). Eighty phage clones were recovered from the positive panning step, 17 of which contained complete scFv sequences without deletions, insertions or stop codons. These 17 phages were screened to ensure they selectively bound human FTD brain tissue, but not ALS or healthy brain tissue homogenates using pooled FTD and pooled ALS tissue homogenates. All 17 phage preparations showed high reactivity to pooled FTD tissue and little to no reactivity to pooled ALS tissue homogenates (Fig 3.3).

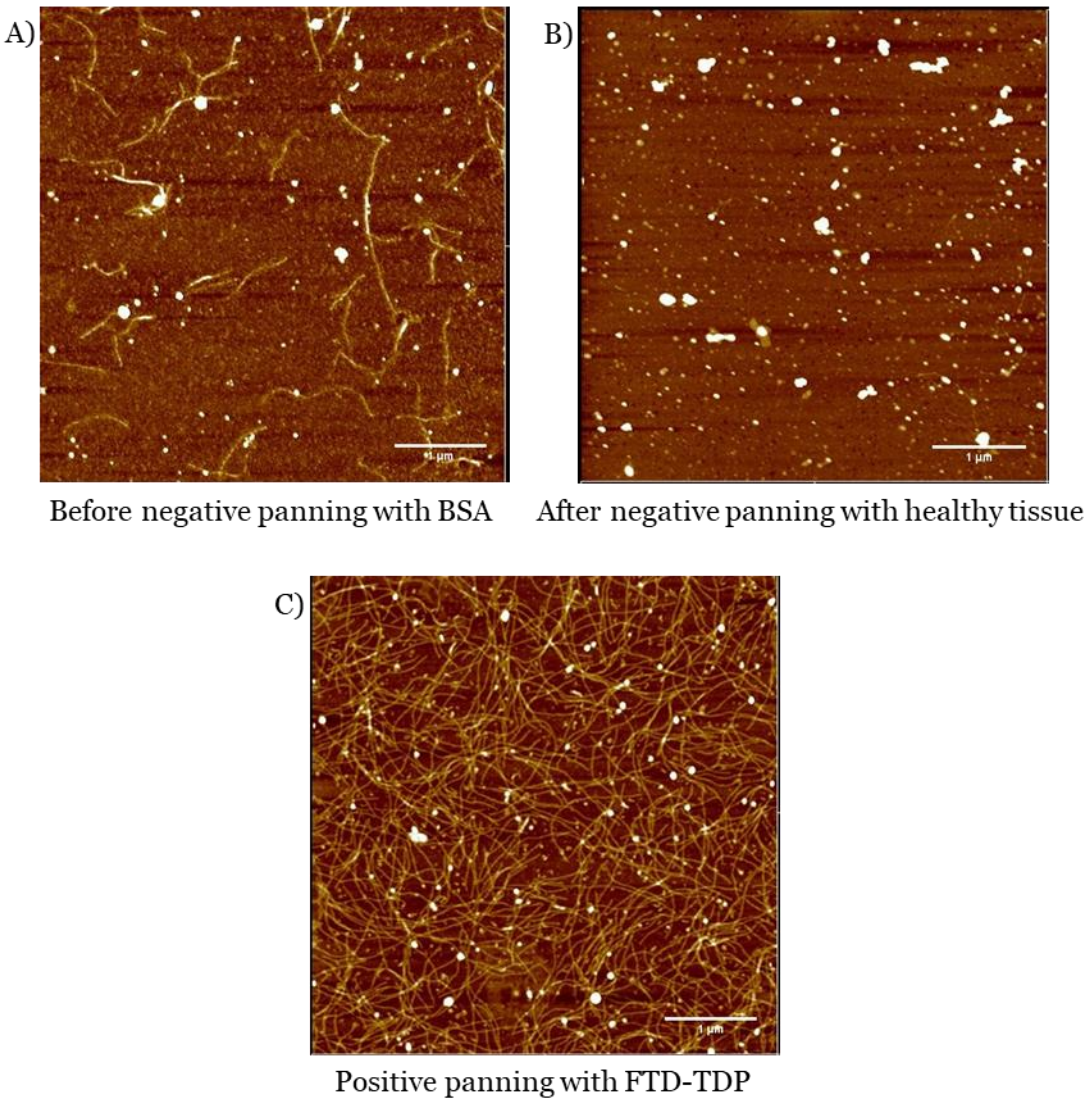



Figure 3.2 AFM images before and after negative panning process. A) phage binding to BSA is observed prior to negative panning, (B) no phage binding is observed after multiple rounds of negative panning with healthy control tissue[48], (C) phage binding with FTD-TDP IP after positive selection

Based on the initial ELISA screening, we selected the 8 phages with the highest reactivity with pooled FTD but no reactivity with pooled ALS and pooled healthy control tissue homogenates for further testing with individual FTD (n=6) and age-matched cognitively normal (n=2) brain homogenates (Fig 3.4). The 8 phage samples reacted with each of the FTD brain tissue homogenates, however each phage had a different binding pattern among the FTD patients suggesting that they bind different TDP variants.

The five phage samples with the strongest reactivity toward the individual FTD tissue samples without reactivity towards the control samples were expressed as scFvs and used to determine if the TDP variants could also be detected in sera. The five scFvs (FTD-TDP1, FTD-TDP2, FTD-TDP3, FTD-TDP4 and FTD-TDP5) were used to assay sera samples from FTD-TDP (n=12), FTD-tau (n=12), AD sera (n=11) and controls (n=10) (Fig 3.5). Four of the scFvs (FTD-TDP1, FTD-TDP2, FTD-TDP3 and FTD-TDP4) have significantly higher reactivity to FTD-TDP and FTD-tau sera samples compared to AD sera samples, while the fifth scFv (FTD-TDP5) had high reactivity with all the FTD and AD samples.

None of the scFvs studied here discriminated between FTD-TDP and FTD-tau sera samples, though four of them did discriminate between FTD and AD samples suggesting that some TDP variants are unique to FTD, while others are involved in both FTD and AD. The sensitivity and specificity of each of the five anti-TDP scFvs for FTD-TDP and FTD-tau are shown (Table 3.1). All the scFvs have area under curve (AUC) >0.84 implying high sensitivity and specificity of the scFvs in selecting FTD sera over healthy controls.

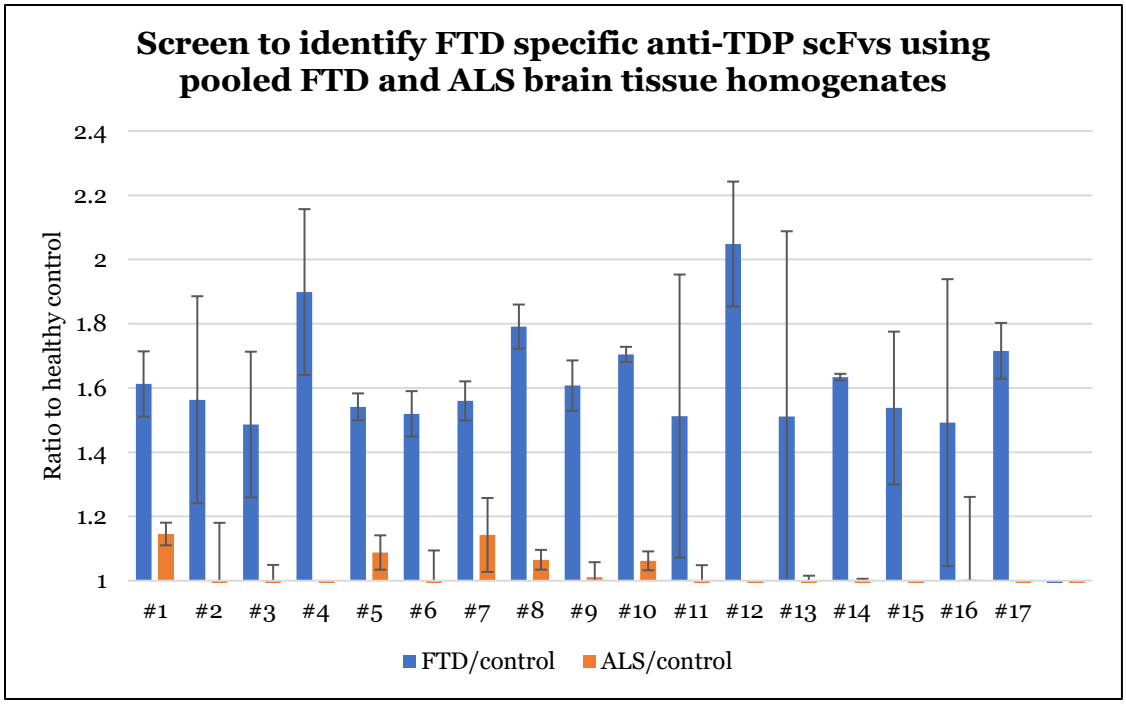



Figure 3.3 Indirect ELISA screening for FTD specific anti-TDP scFvs using pooled human FTD brain tissue samples (n=3), pooled ALS brain tissue samples (n=3) and healthy controls (n=2).

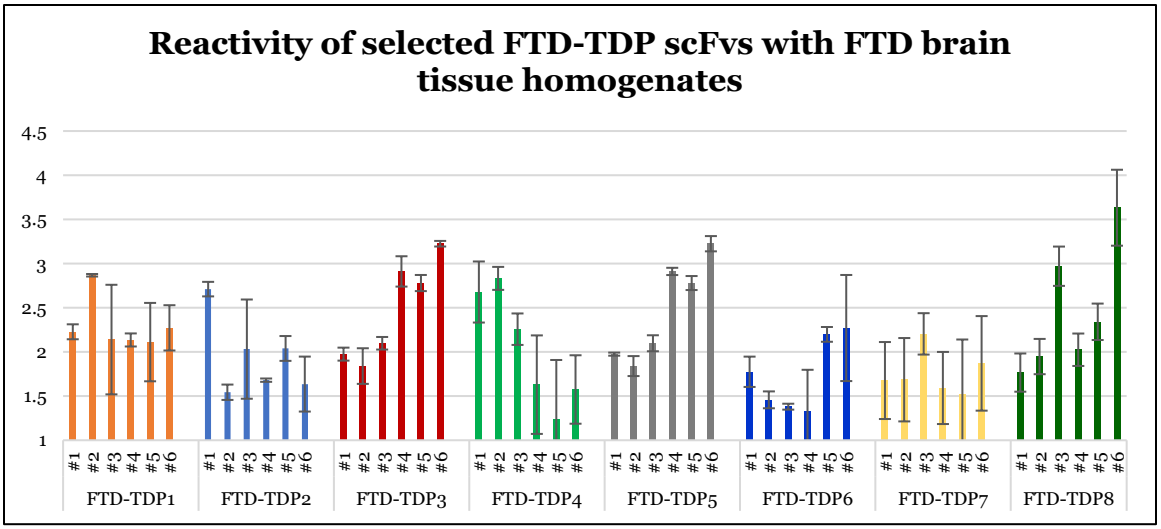



Figure 3.4 Indirect ELISA Indirect ELISA screening reactivity of eight anti-TDP scFv candidates with individual FTD brain tissue homogenates (n=6). X-Axis represents anti-TDP scFvs and Y-Axis represents ratio to cognitively normal healthy controls.

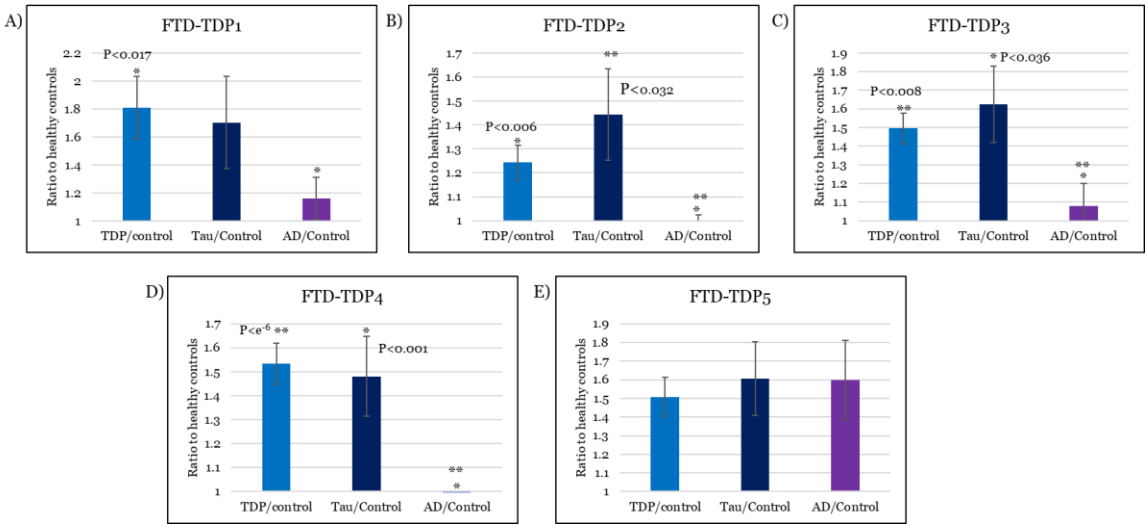



Figure 3.5 Sandwich ELISA characterizing reactivity of five anti-TDP scFvs with FTD and AD sera. 4 of the 5 anti-TDP scFvs selectively bind to both FTD-TDP (n=12) and FTD-tau (n=12) sera and has relatively little to no binding to AD sera (n=11). FTDP-TDP5 was the only scFv that had reactivity with FTD-TDP, FTD-Tau and AD sera over cognitively normal healthy controls.

### *3.3.2 Western blot analysis*

We assume that the FTD selective scFvs bind conformational epitopes of TDP-43 that are involved in FTD since the scFvs did not bind TDP variants present in healthy age-matched control tissue. To verify that the scFvs were binding a conformational epitope, we analyzed PAGE gels under denaturing and native conditions by probing with a commercial anti-TDP antibody and the FTD-TDP2 scFv (Fig 3.6). Under denaturing conditions, the commercial antibody but not FTD-TDP2 recognized TDP variants in control and FTD brain tissue IPs (~70kDa), while under native conditions, FTD-TDP2 recognizes a disease variant of TDP present in FTD (~70kDa) but not controls.

### *3.3.3 Competition ELISA*

To determine if the five different scFvs against the FTD related TDP variants were binding different epitopes, we performed a competition ELISA where each scFv was tested with FTD sera (no competition) or FTD sera preincubated with one of the other 4 scFvs (competition) (Fig 3.7). If any two scFvs recognize the same epitope, we expect a significant reduction in ELISA signal. One-way ANOVA analysis indicated there was not any difference between the control samples and those with added scFv indicating that the scFvs bind unique epitopes.

### *3.3.4 Immunohistochemistry*

Two anti-TDP scFvs were further studied using IHC analysis of human postmortem FTD and control brain tissue sections. The FTD-TDP2 and FTD-TDP3 scFvs were utilized for the IHC analyses since they had high expression levels and high reactivity with FTD over cognitively normal controls in tissue and sera analyses. Fluorescence tagged secondary antibodies were used to visualize the microtubule associated protein (MAP2) (Red) and the bound scFvs (green) (Fig 3.8).

| | FTD-TDP | | | FTD-Tau | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|
| scFv | Sensitivity | Specificity | AUC | Sensitivity | Specificity | AUC | Sensitivity | Specificity | AUC |
| FTD-TDP1 | 91.66% | 100% | 0.99 | 75% | 75% | 0.85 | 75% | 87.5% | 0.92 |
| FTD-TDP2 | 75% | 100% | 0.87 | 75% | 100% | 0.82 | 75% | 100% | 0.84 |
| FTD-TDP3 | 100% | 87.5% | 0.99 | 91.66% | 87.5% | 0.96 | 95.83% | 100% | 0.99 |
| FTD-TDP4 | 100% | 83.3% | 0.97 | 75% | 75% | 0.79 | 75% | 87.5% | 0.88 |
| FTD-TDP5 | 100% | 100% | 1 | 100% | 100% | 1 | 100% | 100% | 1 |

Table 3.1: Sensitivity and Specificity of Five Anti-TDP FTD scFvs based on reactivity with FTD-TDP (n=12), FTD-tau (n=12) and control sera (n=8). Sensitivity and specificity were calculated for FTD-TDP, FTD-tau and both FTD subtypes – AUC values greater than 0.95 are highlighted.

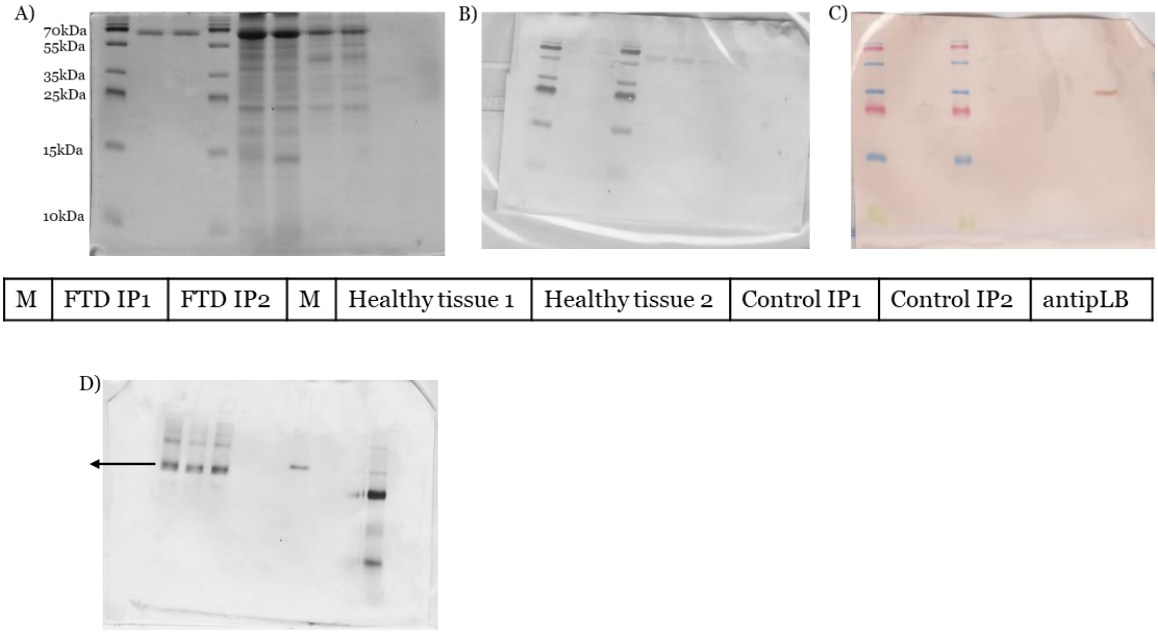



Figure 3.6 Reactivity of anti-TDP scFv FTD-TDP2 with TDP IP from FTD and healthy control under denaturing (A-C) and non-denaturing conditions (D). A) Stained gel under reducing and denaturing conditions, B) Commercial TDP antibody identifying TDP species in FTD and healthy controls, C) FTD-TDP2 scFv does not recognize TDP species under denaturing conditions, D) Native PAGE was run under non-reducing and non-denaturing conditions and FTD-TDP2 scFv recognizes disease variant of TDP present in FTD and not healthy controls.

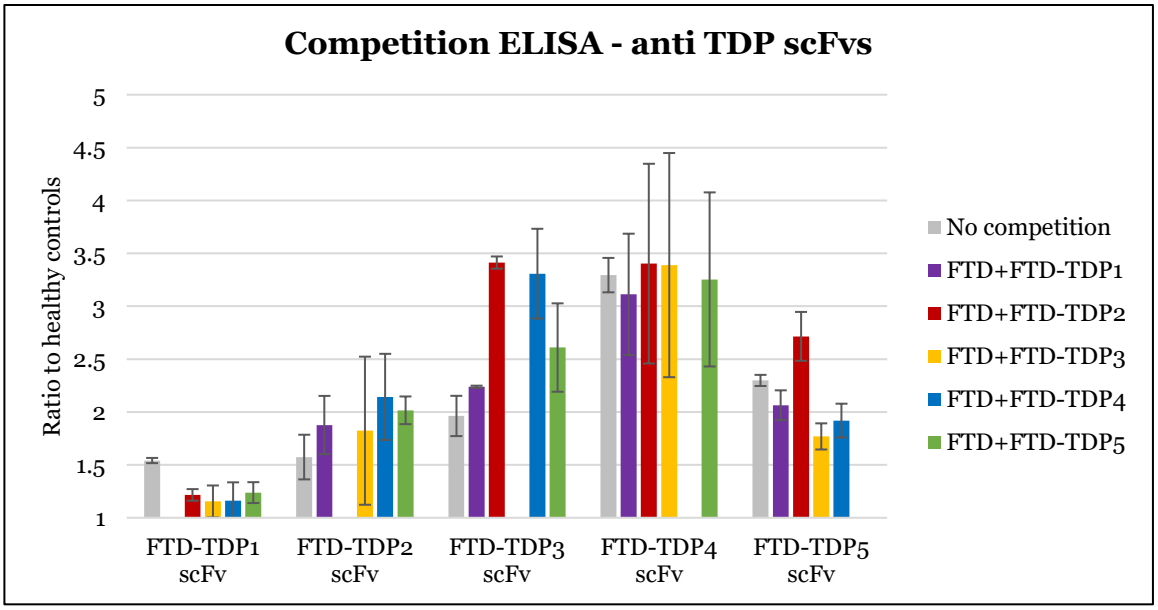



Figure 3.7 Competition ELISA of 5 anti-TDP scFvs. X-axis represents each scFv and Y-Axis resents ratio to age matched controls. Each scFv was tested with FTD sera (1 FTD-TDP +1 FTD-tau) (no competition) or FTD sera pre-incubated with each of the other four scFvs (competition). One-way ANOVA analysis indicate no significant difference between the no competition and competition.

Although MAP2 staining is present in both FTD and control case, there is no anti-TDP scFv staining with the control case. In the FTD case, there is extensive anti-TDP scFv staining indicating that the FTD-TDP scFvs recognize disease specific TDP variants. Both the anti-TDP scFvs have similar staining surrounding the nucleus (blue) in FTD tissue indicating presence of intraneuronal TDP variants in the cytoplasm.

### *3.3.5 Toxicity assay*

When incubated with neuronal cells, the TDP-43 sample immunoprecipitated from human post-mortem FTD brain tissue induced significantly increased toxicity toward cultured SH-SY5Y cells compared to TDP-43 immunoprecipitated from cognitively normal human brain tissue (Fig 3.9). Five anti-TDP scFvs with high selectivity for FTD sera over the controls (FTD-TDP1, FTD-TDP2, FTD-TDP3, FTD-TDP4 and FTD-TDP5) and a commercial antibody against TDP-43 were separately co-incubated with the cells at a concentration of 1μg/mL to block TDP variants from interacting with the cells. Four of the scFvs, FTD-TDP1, FTD-TDP2, FTD-TDP3 and FTD-TDP5 significantly reduced toxicity of the TPD-43 while the commercial anti-TDP-43 antibody did not reduce toxicity and the FTD-TDP4 scFv only slightly decreased toxicity at the concentration studied. These results indicate that FTD related TDP variants are toxic to neuronal cells, and that selectively targeting the TDP variants may be an effective therapeutic for treating FTD and potentially other related neurodegenerative disease.

## 3.4 Discussion

TDP-43 pathology is commonly observed in a vast number of FTD cases and TDP-43 variants are observed in CSF and sera making it an ideal candidate for antemortem FTD diagnosis [105,140,154–156]. We generated a panel of scFvs that selectively bind FTD specific TDP-43 variants using an AFM-based biopanning protocol [48].

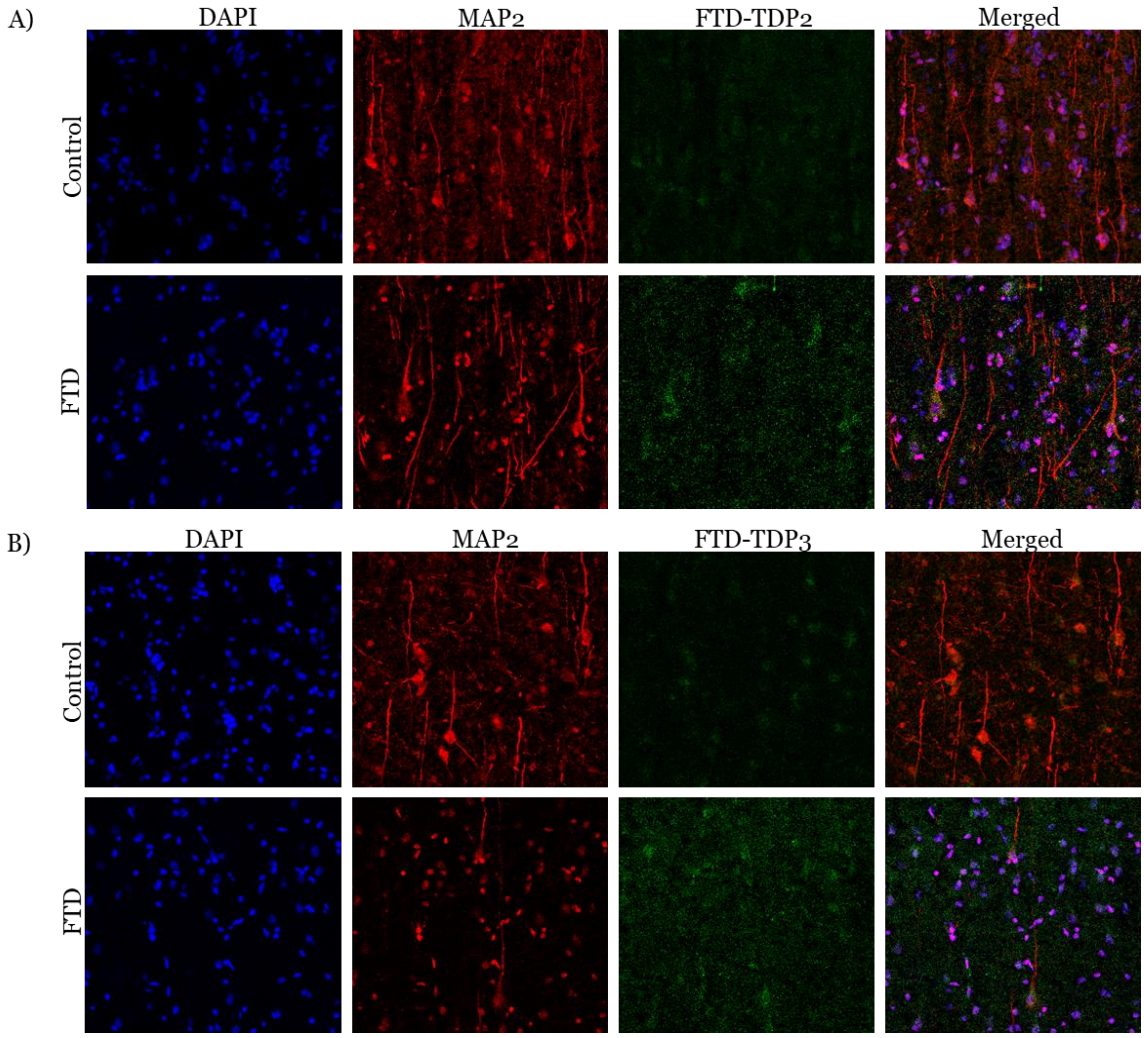



Figure 3.8 Reactivity of anti-TDP scFvs with TDP variants in FTD and control brain tissue slice using IHC. Tissue sections were incubated with A) FTD-TDP2 and B) FTD-TDP3 respectively (1:100) on a shaking stage overnight at 4C. Primary antibodies against c-myc region of scFv (Sigma, 1:1000, rabbit) and MAP2 (Covance, 1:400, mouse) were applied followed by goat anti-rabbit IgG (green) and goat anti-mouse IgG (red) with fluorescence. The FTD sections show positive staining with anti-TDP scFvs compared to control.

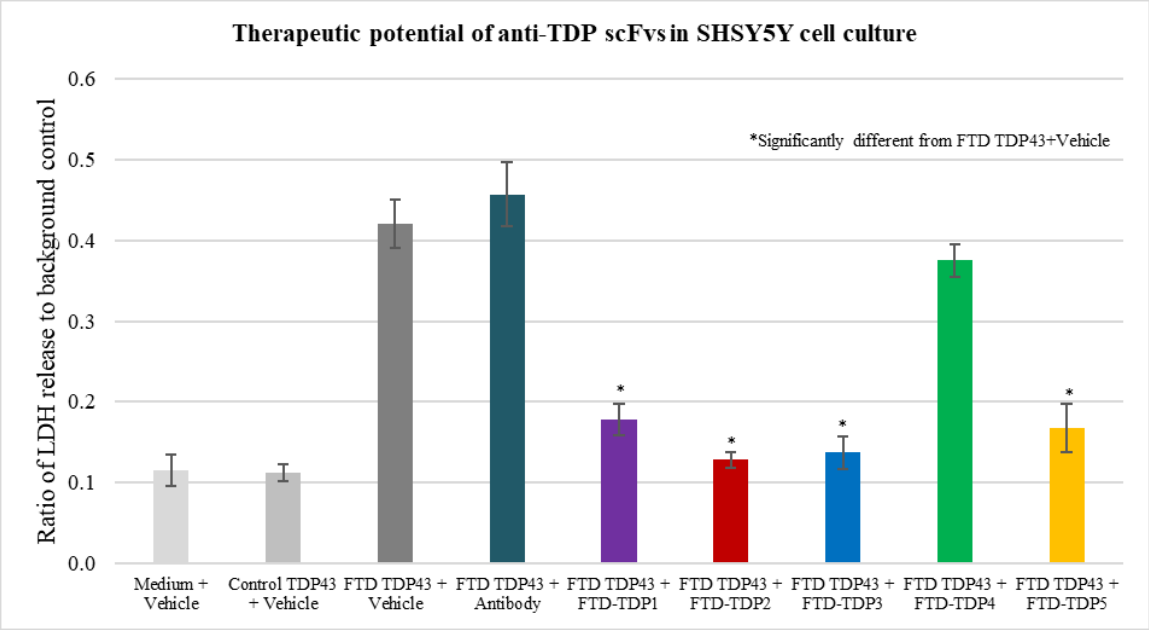



Figure 3.9 Therapeutic potential of anti-TDP scFvs in SH-SY5Y neuroblastoma cell line. Cells were treated with TDP-IP derived from human FTD and control brain tissue. The cells were further treated with a commercial anti-TDP antibody (ab190963, Abcam, 1µg/mL) or anti TDP scFvs (FTD-TDP1, FTD-TDP2, FTD-TDP3, FTD-TDP4 and FTD-TDP5) for 12 hours. The cell damage and toxicity were tested by measuring lactate dehydrogenase (LDH). FTD-TDP4 did not inhibit toxicity of FTD brain derived TDP IP, while FTD-TDP1, FTD-TDP2, FTD-TDP5 inhibit toxicity more effectively than commercial TDP antibody.

Five scFvs that had high reactivity with individual FTD brain tissue over control tissue (FTD-TDP1, FTD-TDP2, FTD-TDP4 and FTD-TDP5) were further analyzed using sera samples from FTD-TDP, FTD-tau, AD and control cases. Four of the five scFvs tested showed high reactivity with FTD sera but not AD or cognitively normal controls, while one scFv showed high reactivity with the FTD and AD sera cases. Even though around 50% of AD cases have prominent TDP pathology [147,148], it is apparent that TDP pathology in FTD cases has some distinct differences from that in AD cases. Although FTD sera has been classified as FTD-TDP and FTD-tau based on postmortem pathology reports, studies have shown that there is an overlap of tau and TDP-43 pathology in FTD cases [30]. Here we also observed that TDP pathology between the FTD-TDP and FTD-tau cases are quite similar.

TDP-43 undergoes several post-translational modifications and occurs as C-terminal fragments of varying lengths [104,157]. Previous studies have indicated that a 70kDa TDP species is present in FTD brain tissue studies [158]. Here, we show that FTD-TDP2 scFv recognizes a conformation specific 70kDa species present in FTD and not in cognitively normal control tissue samples and that this variant is localized in the cytoplasm of neurons in FTD brain tissue but not healthy controls. Other neurodegenerative diseases like motor neuron disease, AD, dementia with Lewy bodies and Huntington's disease also exhibit TDP pathology [159–161]. Investigating overlap in TDP pathology in these different diseases will be the subject of future studies. We also showed that the TDP variants present in FTD brain are toxic to neuronal cells, and that selectively targeting the toxic variants may be an effective therapeutic option for treating FTD and other TDP related diseases.

Chapter 4

Biomarkers for Alzheimer's Disease and Frontotemporal Dementia

## 4.1 Introduction

AD and FTD are the leading causes of early onset dementia and have a very similar underlying pathology. While amyloid plaques and NFTs are the hallmark features of AD, TDP-43 and tau aggregates are key to FTD. Clinical diagnosis is based on neurological and neuropsychological exam to assess various aspects of the individual such as memory, behavior, speech and motor skills. There is overlap of symptoms between FTD and AD, especially in the initial stages making diagnosis difficult. The pathology involved is complex making diagnosis challenging, even at autopsy. Studies have shown that FTD cases are misdiagnosed as AD [4,42]. Despite advances in neuroimaging, and in CSF and blood-based biomarkers [83,162–164], there is lack of disease specific biomarkers capable of differentiating FTD from AD and healthy controls.

### *4.1.1 Imaging biomarkers*

Several imaging techniques exist that can detect structural changes in the grey and white matter regions of the brain involved in dementia. Grey matter changes are assessed by structural MRI. Diffusion tensor imaging, on the other hand, measures structural changes in the white matter. In this method the rate and direction of diffusion of water is measured which is sensitive to the cellular environment thereby detecting early pathological changes occurring in the brain. Apart from structural changes, functional changes in the brain that accompany dementia are measured using specific techniques. These include FDG-PET which uses fluorodeoxyglucose as a tracer [165]. This method helps measure reduced glucose metabolism in different brain regions associated with dementia. Arterial spin labeling, and

functional MRI are also used to measure some of the early structural changes that accompany dementia.

Other methods involve using specific tracers for Aβ and tau such as Pittsburgh B (PiB) compound which recognizes amyloid changes in the brain but PiB is not specific to AD and recognizes amyloid changes that occur in certain subtypes of FTD [165,166]. Tracers for tau include Flortaucipir, THK5117 and PBB3. However, due to the complexity of tauopathies and diverse brain regions involved, there is considerable overlap in the uptake of these tracers in different neurodegenerative diseases [39]. Although there is considerable progress in PET and MRI imaging, there is a significant overlap between the dementia subtypes [4,41,43,167]. Tracers for Aβ and tau need to be further characterized to assess its potential as biomarker tool in differentiating between dementia subtypes. Although imaging provides ability to identify structural and functional changes in the brain, a more precise biomarker is required to distinguish neurodegenerative diseases.

*4.1.2 CSF biomarkers*

Since cerebrospinal fluid is closely linked and surrounds the brain, pathological changes occurring in the brain due to dementia can be observed by biochemical analysis of the CSF. Aβ and tau which are involved in the pathology of AD and TDP-43 and tau which are involved in the pathology of FTD are analyzed. In CSF of AD cases, there is decrease in Aβ and increase in p-tau and total tau compared to healthy controls [168]. Although Aβ pathology occurs in AD and Aβ to p-tau or total tau ratios are core biomarkers used in the diagnosis of AD [127,142,143,169], several studies focus on similar measurements in FTD. There is a decrease in Aβ levels and increase in p-tau and total tau in FTD compared to AD [162]. TDP-43 and total tau measurements in CSF of ALS and FTD cases are also elevated compared to healthy controls [170]. These measurements are however, not unique to FTD

due to the overlap in pathology and occurrence of elevated CSF tau and CSF TDP-43 in AD and ALS respectively [43,106,171].

*4.1.3 Blood based biomarkers*

Apart from CSF, several studies have focused on measuring plasma levels of Aβ, tau and TDP-43 in AD and FTD [48,107,153,154,156,169,172,173]. Plasma measurements have advantages such as being precise, and easily accessible compared to CSF. It provides a means of monitoring disease progression and measuring effectiveness of therapeutic interventions with the possibility of multiple measurements over time. Review of plasma measurements of Aβ40, Aβ42 and ratio of Aβ42 to Aβ40 yielded 86% sensitivity with only 50% and 70% specificity in AD[173]. Tau plasma levels, on the other hand, were elevated in different dementias compared to healthy controls. However, there is considerable overlap in plasma tau profiles between FTD, AD and other dementia subtypes [172]. Plasma levels of phosphorylated TDP-43 are elevated and progranulin levels are decreased in FTD individuals carrying mutations in GRN or C9orf72 compared to remaining FTD (without mutation) and healthy controls [154,174]. These mutation specific changes identify specific genetic form of FTD (a small fraction of the FTD population) from remaining FTD and healthy controls. Hence, they are not ideal candidates to help distinguish both sporadic and familial type of FTD from other dementias. Other studies indicate that there is an overlap between FTD, AD and healthy controls in the detection of plasma TDP-43 [107]. These studies underline the importance of identifying FTD and AD specific biomarkers.

Although tau and TDP-43 have been implied in the pathogenesis of Alzheimer's and FTD, the occurrence and progression of tau and TDP conformational protein variants associated with each disease is unique [99,130]. We hypothesize that there is a spectrum of protein variants that play a role in these neurodegenerative diseases. Some of these variants may

be unique to either AD or FTD as they are understood today. However, some will likely be common to both AD and FTD, and perhaps to other dementias. Hence there is a need for biomarkers capable of identifying protein variants specific to AD and FTD. Chapters 2 and 3 focus on isolating scFvs against these specific protein variants –tau and TDP-43 in AD and FTD respectively [48].

The goal is to use a panel of scFvs to identify unique TDP-43 and tau protein variants present in FTD and AD respectively. Detection of these protein variants will be able to facilitate early diagnosis of FTD from AD and healthy controls and will also provide a means for disease staging and monitoring therapeutic strategies.

## 4.2 Materials and methods

### *4.2.1 Brain Tissue and sera samples*

Human postmortem FTD, AD and control brain tissue; and sera samples from FTD-TDP (n=12), FTD-Tau (n=12), AD (n=11) and controls (n=10) were obtained from Dr. Thomas Beach, director of the Brain and Body Donation Program at Banner Sun Health Research Institute (BBDP; http://www.brainandbodydonationprogram.org) [132,133].

### *4.2.2 ELISA assay*

Sandwich ELISAs were performed to detect anti-TDP and anti-tau protein variants in FTD, AD and healthy control sera as discussed [135]. Anti-tau and anti-TDP scFvs were coated on the plate followed by incubation with the sera samples. Tau and TDP species in sera are detected via biotinylated tau phage (against monomeric tau) and biotinylated TDP phage (capable of identifying TDP species in ALS, FTD and healthy controls) respectively. A chemiluminescent substrate was used, and the luminescence signal was measured using Wallac Victor$^2$ microplate reader.

## 4.3 Results

### *4.3.1 scFv selection and characterization*

TDP and tau IP were obtained from FTD and AD brain as described [48]. scFvs were identified against the IP and further characterized on ELISAs. Based on the initial screening and expression of the scFvs, a total of five scFvs against TDP variants in FTD and five scFvs against tau variants in AD were selected for further characterization with sera. The sera were classified into different subtypes based on pathology reports and analyzed with the anti-TDP and anti-tau scFvs against FTD and AD respectively.

### *4.3.2 Sera sample analysis*

Three of the anti-TDP scFvs and two of the anti-tau scFvs that had interesting trends on the initial screen were further analyzed with sera (Fig 4.1). All luminescence values were plotted as ratio to healthy controls. While X-Axis represented individual sera samples, Y-Axis represented ratio to healthy controls. FTD-TDP3 and FTD-TDP4 had significantly higher binding to FTD-TDP and FTD-tau sera but not to the AD sera. FTD-TDP5, on the other hand, identified TDP variants present in both subtypes of FTD and AD sera.

Anti-tau scFvs ADT-2 and ADT-6 were of the utmost interest on the ELISA analysis. ADT-2 identified tau species that were unique to AD and FTD-tau subtype and these values were significantly higher than FTD-TDP group. ADT-2 had little to no reactivity with the FTD-TDP sera samples. This indicates that ADT-2 is possibly identifying a tau variant that overlaps between FTD-tau subgroup and AD. ADT-6, however identified unique tau species present only in AD sera but not in FTD-TDP or FTD-Tau.

ROC curves for the five different scFvs were plotted with the AD sera and FTD sera cases and sensitivity, specificity and Area Under Curve (AUC) were recorded (Table 4.1). FTD-

TDP3 and FTD-TDP4 has high sensitivity and specificity to FTD sera with AUC values of 0.99 and 0.88. They however have poor ROC curves with AD sera. Hence, they are good biomarker candidates for TDP variants in FTD. FTD-TDP5 has 100% sensitivity and specificity with FTD sera and has AUC of 1 and 0.81 with FTD and AD sera. Hence FTD-TDP5 is an excellent biomarker in recognizing TDP variants in both FTD and AD sera. The AD-tau scFvs (ADT-2, ADT-6) has high sensitivity and specificity with AD sera with AUC of 0.91 and 0.94. These scFvs also have poor sensitivity and specificity with AD sera making them excellent biomarkers for AD. Together, the panel of these 5 scFvs are capable of recognizing FTD and AD and differentiating between them.

### 4.4 Discussion

Mutations in several genes such as APP, PS1, PS2, MAPT, TARDBP, VCP, C9orf72, GRN have been linked with AD and FTD [25,26,28,30,86,175]. Each of these genes code for specific proteins such as Aβ, tau and TDP-43 which assume an altered folding state leading to pathological aggregates in AD, FTD and other neurodegenerative diseases [46,103,125]. While Aβ and tau pathology are hallmark features of AD, this pathology is also observed in the FTD-ALS spectrum [62,71]. Similarly, although TDP-43 pathology is implicated mainly in the FTD-ALS spectrum, it is also observed in AD (Fig 4.2) [43].

There are intermediate protein variants that correlate better with cognitive decline and different clinicopathological phenotypes compared to the pathology observed at autopsy [48,49]. These intermediate protein variants occur prior to plaques, tangles and TDP aggregates and determine the clinicopathological phenotype. Hence it is important to identify these biomarkers since they will give a better idea of the protein variants underlying the different clinicopathological phenotype.

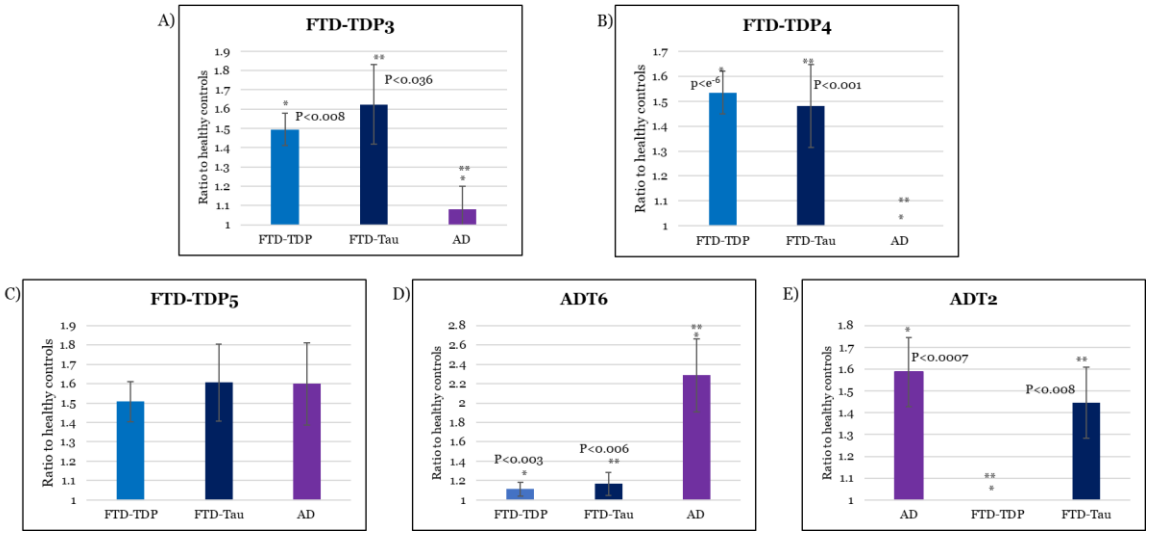



Figure 4.1 Reactivity of anti-tau scFvs and anti-TDP scFvs against AD (n=10), FTD-TDP (n=12) and FTD-tau sera (n=12) relative to cognitively normal age matched controls (n=10). FTD-TDP2 and FTD-TDP4 selected TDP variants in FTD over AD sera, FTD-TDP5 selected TDP variants in FTD and AD sera. ADT-2 selected tau variants in AD and FTD-tau sera and ADT-6 selected tau variants in AD but not FTD.

| | FTD sera | | | AD sera | | |
|---|---|---|---|---|---|---|
| scFv | Sensitivity | Specificity | AUC | Sensitivity | Specificity | AUC |
| FTD-TDP3 | 0.95 | 1 | 0.99 | 0.46 | 0.62 | 0.53 |
| FTD-TDP4 | 0.75 | 0.88 | 0.88 | 0.27 | 0.38 | 0.24 |
| FTD-TDP5 | 1 | 1 | 1 | 0.64 | 1 | 0.81 |
| ADT-2 | 0.42 | 0.5 | 0.44 | 0.8 | 0.9 | 0.91 |
| ADT-6 | 0.42 | 0.2 | 0.28 | 0.9 | 0.8 | 0.94 |

Table 4.1: Sensitivity, Specificity and Area Under Curve (AUC) Values of the Five Different scFvs with FTD and AD Sera.

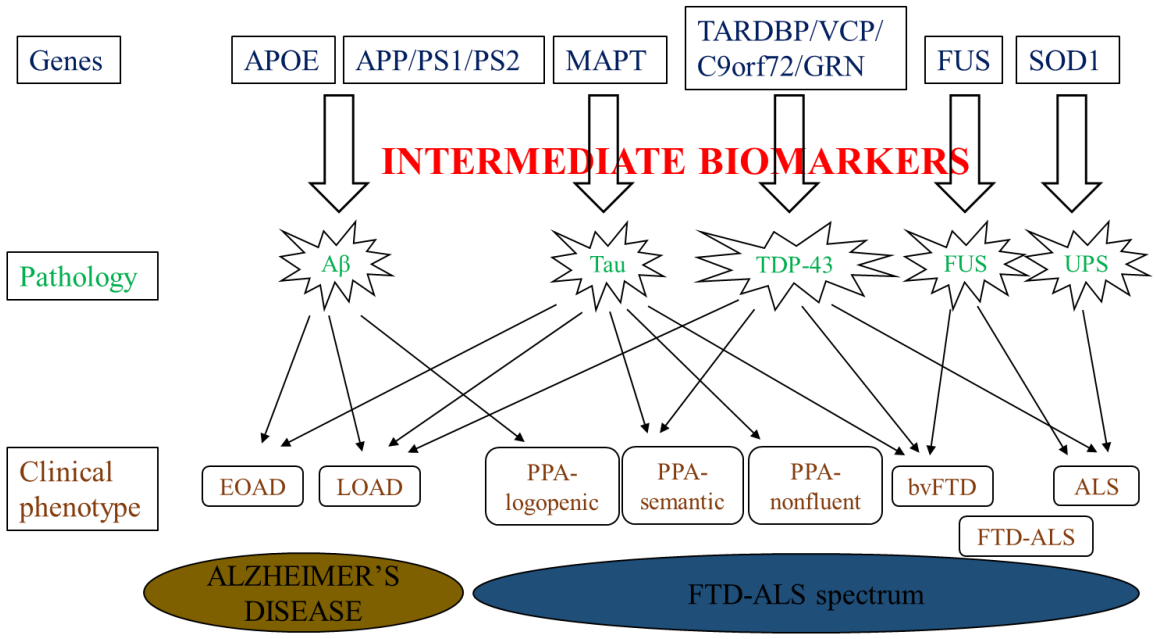



Figure 4.2 Genetic, pathological and clinical phenotypes associated with Frontotemporal Dementia and Alzheimer's Disease. Intermediate protein variants help distinguish between the different clinicopathological subtypes.

Classifying a disease based on the pathology observed at autopsy is not useful for diagnosing dementia especially prior to onset of symptoms. Simplifying a cluster of symptoms and complex brain pathology observed at autopsy results in generic treatment options which are often not successful leading to failed clinical trials. Identification of intermediate protein variants will help in generating protein variant profiles for every individual that will aid in personalized diagnostics and therapeutics. We have successfully used a scFv panels to measure tau and TDP protein variant levels, thereby identifying changes occurring at the molecular level.

Despite these scFvs being selected against immunoprecipitated TDP and tau from FTD and AD brains, they were capable of recognizing protein variants in brain tissue, CSF and sera samples of diseased individuals over controls. This indicates that the protein variants recognized by these scFvs in the brain are also present in the circulating sera, making them viable biomarker candidates. The panel of scFvs consist of an anti-tau scFv that can identify unique tau variants in AD but not FTD; another anti-tau scFv against tau variants present in AD and FTD-tau but not FTD-TDP; an anti-TDP scFv against variants in FTD but not AD; and another anti-TDP scFv against TDP variants in FTD and AD.

# Chapter 5

## Conclusion and Future Direction

Dementia, including AD and FTD is the among the top leading causes of death in the aged population. AD and FTD are characterized by distinct pathologies resulting in cognitive decline and certain motor deficits [6,30,176]. There are currently no ways to slow down progression, prevent or cure AD and FTD. Severe brain trauma and repetitive head injuries also leads to similar symptoms and pathologies observed in AD, and FTD [177]. Despite advances made in imaging and neuropsychological exam, reaching a definitive diagnosis is challenging especially with overlap of symptoms and complex brain pathology [43,107,147]. The need for biomarkers capable of differentiating these neurodegenerative diseases from healthy individuals, especially in the early stages, is important. Although there is an overlap of TDP-43, tau and Aβ pathology between the FTD-ALS spectrum and AD, we hypothesize that unique protein variants are present in each disease compared to healthy controls. These protein variants can serve as biomarkers of disease and can be used to monitor disease progression and effectiveness of therapeutic intervention.

### 5.1 Isolation and characterization of scFv against tau variants in AD

Aβ and tau are hallmark features of AD pathology. While Aβ does not correlate well with cognitive decline, tau variants present prior to tangle formation correlate with memory loss and cognitive decline associated with AD [52,124,125,178]. scFvs capable of identifying these unique conformations or variants of tau can serve as biomarker tools in AD. We have successfully identified anti-tau scFvs against human postmortem AD brain derived tau compared to healthy controls. These anti-tau scFvs were characterized using ELISA assays and six different scFvs (ADT-1 – ADT-6) have shown considerable promise. These anti-tau scFvs can recognize unique tau variants present in human AD brain tissue and sera

over cognitively normal healthy controls. Longitudinal sera analysis with three of the anti-tau scFvs (ADT-2, ADT-4 and ADT-6) have consistently selected AD over controls. Therapeutic potential of five of these scFvs showed that four of the five anti-tau scFvs significantly reduced toxicity levels at much lower concentration (0.1µg/mL) than commercial antibody which was effective only at 1µg/mL concentration. We thus have a panel of six anti-tau scFvs that can recognize unique tau variants present in AD.

### 5.2 Isolation and characterization of scFv against TDP variants in FTD

TDP-43 pathology is common in FTD, ALS, AD and other neurodegenerative diseases [43,179–181]. In FTD, TDP which shuttles between the nucleus and cytoplasm begins to aggregate in the cytoplasm. Depending on the region of the brain and any underlying genetic mutations, distinct TDP aggregates exist. Different conformations of TDP exist which correlate with the clinicopathological phenotype and cognitive decline in FTD. scFvs that can identify unique TDP variants present in FTD but not in healthy controls can serve as biomarkers. We have identified five FTD-TDP scFvs (FTD-TDP1 – FTD-TDP5) that identify different TDP variants present in FTD but not in cognitively normal healthy controls. These anti-TDP scFvs identify TDP variants present in human postmortem FTD brain tissue and sera compared to controls in ELISA assay. Therapeutic potential of these scFvs have also been tested in neuroblastoma SHSY5Y cell line. The anti-TDP scFvs significantly reduced toxicity caused by the TDP IP from FTD brains compared to commercial antibody.

### 5.3 Characterization of FTD and AD sera using scFv panel

A panel of anti-tau and anti-TDP scFvs were used to characterize AD and FTD sera. Of the five anti-TDP and six anti-tau scFvs, we have identified three anti-TDP scFvs and two anti-tau scFvs capable of distinguishing FTD from AD. Together these scFvs can identify

unique TDP variants present only in FTD; TDP variants present in FTD and AD; tau variants present only in AD; and tau variants present in AD and FTD-tau sera. AD and FTD are currently defined by the hallmark pathological features and the symptoms observed in these patients. However, for diagnosis, and staging of disease progression, the pathology-based classification is not ideal since there is an overlap of symptoms and overlap of pathology between the dementia subtypes (AD and FTD). The current classification also does not provide adequate insight into the molecular profile of individual cases prior to the onset of symptoms and brain pathology.

Measurement of intermediate protein variants which correlate with cognitive decline can provide molecular profile for dementia subtypes and serve as biomarker tools. The panel of five scFvs recognized unique tau and TDP protein variants in what is referred to as AD and FTD today. Some of the scFvs identified protein variants that overlap between AD and FTD. This indicates that several neurodegenerative disease cases do not have a pure pathology and do not conform to a dementia subtype. This makes mixed pathology a common occurrence in dementia subtypes, making diagnosis difficult. scFvs that can identify protein variants which overlap between neurodegenerative diseases can aid in better diagnosis of dementia subtypes. As a panel of scFvs, these biomarkers can help identify protein variants which change as the disease progresses. Identification of protein variants in individual sample can aid in generating protein variant profile for each case. Personalized diagnostics and therapeutics can be designed based on the individual's unique protein variant profile which can help in monitoring as well as treating the underlying condition.

## 5.4 Isolation and characterization of scFv against tau variants in TBI

Traumatic Brain Injury often leads to the individual developing a neurodegenerative disease like AD and Chronic Traumatic Encephalopathy (CTE) [177]. Depending on the severity and the number of injuries to the brain, they develop a wide array of symptoms and pathology like tau tangles which overlap with dementia subtypes. We have identified scFvs using a combination of phage display libraries and AFM based screening against tau immunoprecipitated from TBI CSF. Initial ELISA assays identified several scFvs that recognized tau variants in pooled and individual TBI CSF over healthy controls. Further screening shows that TBI-T2 and ADT1 recognize unique tau variants present in TBI that is not present in AD or healthy control sera.

Anti-tau scFvs obtained against tau IP from AD however recognize tau variants in AD and TBI sera but not healthy controls. These results indicate that while the TBI tau scFvs recognize tau variants unique to TBI, the TBI sera also possess tau variants which overlap with AD. This indicates that these TBI individuals have elevated levels of tau variants, 20-30 years post injury, which overlap with AD. Thus, these anti-tau scFvs identify individuals at risk of developing neurodegenerative diseases like AD.

## 5.5 Future Work

We have successfully identified a panel of scFvs that identify tau and TDP variants in AD, TBI and FTD. We hypothesize that every individual suffering from dementia (AD, FTD etc.) has different levels of diverse protein variants present in their system. Identification of these intermediate protein variants will help generate a protein variant profile. These protein variant profiles change as the disease progress, as shown in longitudinal AD sera analysis (Section 2.2.9). These scFvs identify AD over controls while generating a profile that also varies from one individual to another. Thus, the protein variant profile can help

identify toxic variants unique to an individual at any given time. Future work involves analysis of sera samples with scFvs against Aβ, α-synuclein, TDP-43, tau and other protein variants to generate a heat map or profile indicating levels of different protein variants and the type of therapeutic intervention required.

Mass spectrometry analysis and X-Ray crystallography can identify conformations of the protein variants recognized by the anti-tau and anti-TDP scFvs. Toxic protein variants identified by this analysis can be compared between neurodegenerative diseases to identify any overlap. This analysis will also help identify specific post-translational modifications that increase the toxicity of the variants causing neuronal loss and cognitive decline associated with neurodegenerative diseases. These protein variant profiles generated using ELISAs along with mass spectrometry analysis can be used to provide personalized therapeutic intervention based on the presence of specific toxic protein variants.

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# APPENDIX A

## TRAUMATIC BRAIN INJURY

## A.1 Introduction

Traumatic brain injury occurs frequently in individuals who participate in contact sports, war veterans/military personnel and individuals who experience an external trauma to the brain causing edema and inflammation in the brain. Pathological examination has revealed that these individuals have Aβ plaques, phosphorylated tau tangles and intraneuronal and glial TDP-43 aggregates [182] that are also found in several neurodegenerative diseases.

TBI is classified as mild, moderate or severe TBI based on severity of injury. In mild TBI, the injury may be accompanied by loss of consciousness typically less than 30 minutes. In moderate/severe TBI, the injury is accompanied by loss of consciousness greater than 30 minutes and less than 24 hours, sometimes even leading to coma. Irrespective of the severity, TBI individuals experience, confusion, mood swings, and cognitive deficits. Depending on the severity, they also experience motor deficits, often requiring physiotherapy to regain motor function. Within the TBI cases, some have faced a single traumatic event while others have faced multiple injuries in succession preventing any recovery. ApoE ε4 carriers and exposure to concussions (contact sports, war veterans etc.) is a risk for increased damage after TBI [183].

Severe brain trauma has led to development of neuropathology that overlaps with neurodegenerative diseases like Chronic Traumatic Encephalopathy such as plaques, tau tangles, TDP-43 aggregates and in some cases Lewy bodies [177,184]. TBI is accompanied by cognitive and motor deficits which overlap with dementia subgroups like Alzheimer's, Parkinson's, and Frontotemporal dementia. Both, number of TBI events, and severity (with occurrence of loss of consciousness) increases the risk and accelerates the age of onset of dementia [185]. Risk of AD increases and age of onset is accelerated in individuals

who carry the ApoE ε4 allele or have a history of TBI. However, age of onset is the earliest and the rate of cognitive decline is rapid in individuals who are both ApoE ε4 carriers and have a history of TBI [186,187]. Loss of consciousness accompanying TBI accelerates age of onset of cognitive decline associated with FTD [186] and increases risk of early onset of AD [188]. Several factors like repetitive head injury and genetic factors increase the risk of TBI individuals to develop neurodegenerative diseases like AD, PD, ALS and FTD several years after the injury[9],[10]. It is important to identify TBI individuals at risk of developing neurodegenerative diseases like AD. Hence there is an imminent need for a biomarker capable of identifying at risk TBI individuals.

A majority of the TBI cases that die shortly after injury are reported to have hyperphosphorylated tau accumulation in the form of neurofibrillary tangles especially in the superficial layers of the cortex[191]. Despite occurrence of other pathologies in TBI, there is an initial elevation in plasma and CSF tau levels after a TBI event which correlate well with severity of the injury. High levels of tau during the initial period after an injury are indicative of a longer recovery period [192–196]. Therefore, measurement of tau levels is important in identifying at risk individuals who can potentially develop other neurodegenerative diseases several years after injury.

A.2 Materials and methods

Cerebrospinal fluid samples were obtained from four individuals with head injury and four healthy controls. The samples were obtained from Dr. Thomas Beach, director of the Brain and Body Donation Program at Banner Sun Health Research Institute (BBDP; http://www.brainandbodydonationprogram.org) [132,133]. TBI (n=18), AD (n=5) and control sera (n=3) samples were provided by Dr. Kristine Yaffe. Sera were obtained from TBI individuals who had suffered at least one TBI occurrence that was severe enough to require

medical attention during some point in their life. The time since the last TBI was in most cases 25-50 years previous. The controls were self-reported and did not recall having TBI.

Tau was immunoprecipitated using a commercial crosslink IP kit (Pierce) from pooled TBI and pooled control CSF samples as previously described [48]. Two commercial anti-tau antibodies – PA1 against amino acid 240-450 of tau and PA5 against amino-acid 1-286 (Thermo Fisher Scientific) was used to cover all tau isoforms. Eluted tau IP was neutralized with 1M Tris, pH 9.5 as per the manufacturer's protocol to preserve the integrity of the tau IP.

Dotblot with the immunoprecipitated tau samples from pooled TBI and pooled control CSF was performed as described [48]. Commercial anti-tau antibody PA1 and PA5 (Thermo Fisher Scientific) (1:1000) was used as primary and goat anti rabbit-HRP (1:2000) was used as a secondary detection antibody. Both the elute and flow through from the IP protocol was tested for reactivity.

To isolate antibody fragments against tau protein variants from TBI CSF, a combination of three different phage display libraries – Tomlinson I, J and Sheets were used at a concentration of $10^{13}$ pfu/mL. The library were grown as described previously [48]. Several subtractive panning steps to remove phage binding to off target antigens were performed as described [48] including subtractive panning rounds against healthy control CSF and tau IP from control CSF. The subtractive panning steps ensure removal of non-specific phage binders and any phage binding to generic forms of tau present in healthy control CSF. A final round of positive selection was performed on mica using tau IP from TBI CSF essentially as described [48].

Trypsin was used to elute the bound phage and the eluted phage clones were streaked on a LB Ampicillin plate and grown overnight at 37°C. Individual clones were further grown

overnight at 37°C in 2xYT media. Phage was purified as described [45] and used for initial characterization studies. Selected phage clones were transformed into HB2151 strain of E coli to express scFv which was purified using protein A column.

Phage that binds to tau monomer was used as a secondary detection reagent in sandwich ELISA. This phage was selected by probing commercially available tau monomer protein (rPeptide) with enriched phage library (after negative selection with BSA and α-synuclein). The phage was biotinylated using a commercial biotinylation kit (Pierce) to enhance the signal to noise ratio in ELISA assays.

Indirect and sandwich ELISA were performed as described to characterize the anti-tau scFvs obtained after the exhaustive panning process [48,135]. Pooled TBI and control CSF samples was used to coat the plates and milk was used subsequently to block the wells. Each of the phage clones were tested with pooled TBI and pooled control CSF samples. Secondary anti-M13 and chemiluminescent substrate was used for detection. The luminescence was measured using a spectrophotometer and represented as a ratio with respect to no sample control. Further screening was performed with TBI, AD and control sera in a sandwich ELISA as described previously [135].

A.3 Results

Tau was successfully immunoprecipitated from TBI and control CSF (Fig 5.1). Exhaustive negative panning was performed against BSA and aggregated α-synuclein to remove non-specific binders as described [48]. The panning process was verified by AFM imaging (Fig 5.2). The phage obtained at this point was used for selecting a detection reagent for sandwich ELISAs. Negative panning was also performed against healthy control CSF and tau IP from pooled control CSF. The phage pool remaining after subtractive panning was then used for positive selection against tau immunoprecipitated from pooled TBI CSF.

TM1D phage that binds to monomeric tau was tested for reactivity with Alzheimer's human brain tissue homogenates, TBI and healthy control CSF (Fig 5.3). Due to its strong reactivity to tau variants in healthy controls and diseased samples, the TM1D phage was further biotinylated to be used in subsequent sandwich ELISAs. Approximately twenty clones were recovered from the panning against tau IP from pooled TBI CSF samples and the integrity of the scFvs were validated by DNA sequencing. Of these twenty clones, seven of them had complete sequences and expressed well, which were tested in the initial indirect ELISA screening.

The seven complete scFvs recovered from panning against tau variants present in TBI CSF but not control CSF were tested with pooled TBI and control CSF to verify binding specificity using the indirect ELISA (Fig 5.4). Those scFvs that had high binding ratios to the TBI CSF sample were expressed as soluble scFvs (TBI-T1, TBI-T2, TBI-T3, TBI-T4 and TBI-T5) and assayed for reactivity with individual TBI and control CSF using a sandwich ELISA (Fig 5.5) Each of the five TBI scFvs had significantly higher reactivity with all four of the TBI CSF samples compared to the control CSF samples (Fig 5.5). Each scFv showed a different binding pattern to the individual TBI samples suggesting that the scFvs bind different tau variants. Of all the five TBI-tau scFvs, TBI-T2 was sensitive and had high binding to all four TBI samples. Hence TBI-T2 was selected for analysis with TBI sera.

TBI-T2 and our AD-tau selective scFvs (ADT-1, ADT-2, ADT-3, ADT-4 and ADT-5) were further tested with human TBI, control and AD sera samples obtained from veterans (Table 5.1).

We wanted to assess the risk to the TBI individuals of developing neurodegenerative diseases like Alzheimer's Disease and if there was any overlap in the tau variants recognized by the AD-tau scFvs and TBI-T2 scFv. Both the AD-tau selective and TBI-T2

scFv specifically bound to tau morphologies in TBI sera compared to the healthy controls. Binding ratios of TBI-T2 and five AD selective scFvs toward 18 TBI and 5 AD samples are shown (Fig 5.6). TBI-T2 and ADT-1 were able to distinguish mild to severe TBI cases compared to healthy controls with strong signals in mild and severe TBI cases with slight signal in 2 of the 5 AD cases compared to the controls. These results indicate that unique tau variants are still generated by patients that received TBI even 30 to 50 years earlier.

The AD-tau scFvs on the other hand selected both TBI and AD cases. The four AD-tau scFvs (ADT-2, ADT-3, ADT-4 and ADT-5) selected all 5 of the AD cases. They have strong signals with mild TBI and 2 of the 5 severe TBI cases. Since the anti-tau scFvs obtained after panning with AD brain derived tau also identified the same TBI patients, these scFvs have potential value in differentiating TBI and healthy individuals and identifying patients at increased risk of incurring neurodegenerative diseases.

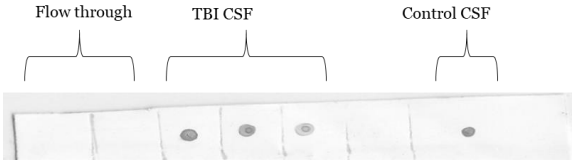


Figure A.1 Dotblot assay confirming the presence of immunoprecipitated tau from TBI and control CSF samples in the IP elutes following immunoprecipitation protocol.

| Sample # | Classification | Age | MMSE |
|---|---|---|---|
| 1 | TBI | 87 | 29 |
| 2 | TBI | 60 | 26 |
| 3 | TBI | 78 | 21 |
| 4 | TBI | 83 | 30 |
| 5 | TBI | 96 | 26 |
| 6 | TBI | 73 | 27 |
| 7 | TBI | 74 | 27 |
| 8 | TBI | 84 | 30 |
| 9 | TBI | 62 | 30 |
| 10 | TBI | 86 | 28 |
| 11 | TBI | 86 | 29 |
| 12 | TBI | 52 | 28 |
| 13 | TBI | 62 | 27 |
| 14 | TBI | 86 | 29 |
| 15 | TBI | 65 | 28 |
| 16 | TBI | 81 | 28 |
| 17 | TBI | 66 | 30 |
| 18 | TBI | 95 | 29 |
| 19 | Control | 90 | 30 |
| 20 | Control | 72 | 30 |
| 21 | Control | 71 | 30 |

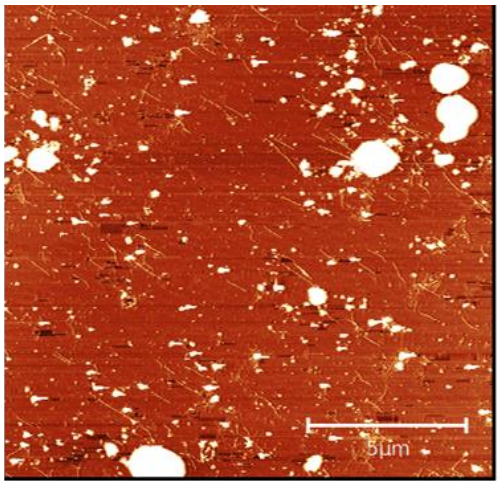
Table A.1 Age and MMSE Scores of the TBI and Control Cases.

Most of the anti-tau clones tested exhibited a strong signal with two of the TBI samples (Fig 5.6, sample 1 in severe TBI and sample 10 in mild TBI) potentially indicating that this patient has the highest risk of incurring a tau based neurodegenerative disease such as Alzheimer's or Frontotemporal dementia.

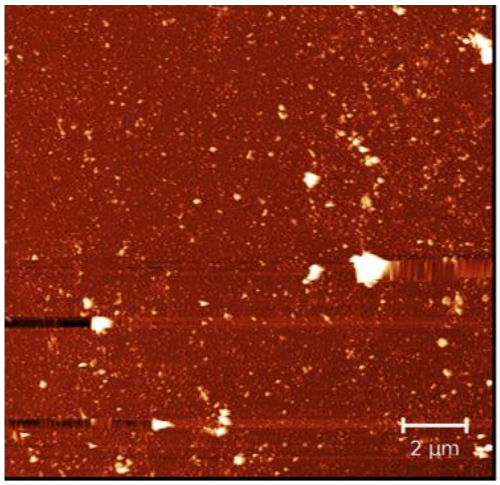
The different tau clones tested all exhibited slightly different patterns with the 18 TBI patients' samples suggesting that the tau scFvs may be binding different tau variants. These results indicate that the anti-tau scFvs have potential value as tools to identify TBI related tau variants in blood samples.

A.4 Discussion

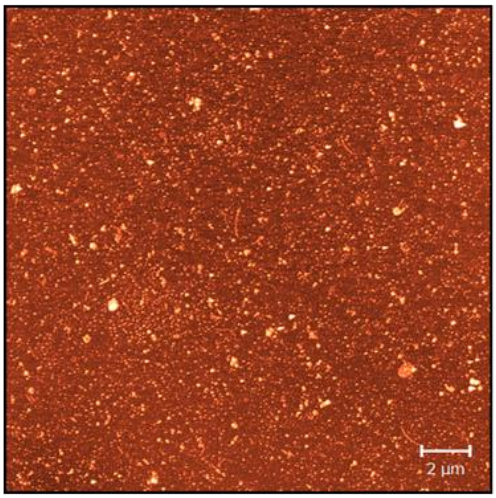
Traumatic brain injury occurs in individuals who are at risk of exposure to concussion[196]. War veterans who have encountered explosions, individuals engaged in contact sports like soccer, hockey and boxing and any activity that causes rapid acceleration or deceleration of head experience such injuries [177,191,193,194,196]. Depending on the location and severity of the TBI, individuals develop a multitude of symptoms and pathology. Tau plays a prominent role in TBI and acute levels of plasma and CSF tau are observed immediately after an injury[177,192]. We have identified anti-tau scFvs that identify TBI related tau variants which overlap with tau variants present in AD sera. Our panel of scFvs also include AD specific anti-tau scFvs which recognize tau variants in AD but not TBI. This indicates that the TBI individuals have circulating tau variants 20-30 years after injury that correspond to tau variants present in AD. Thus, our panel of scFvs can identify TBI individuals at risk of developing neurodegenerative diseases like AD over healthy controls making them ideal biomarker candidates. These anti-tau scFvs need to be tested with a larger sample set to ensure it retains its selectivity of TBI over controls.



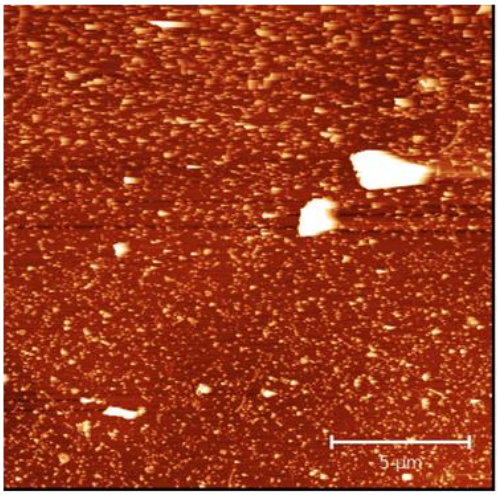

Binding to BSA (round 1) at 20µm




Binding to BSA (round 34) at 20µm




Binding to aggregated α-syn (round 1) at 20µm




Binding to aggregated α-syn (round 12) at 20µm

Figure A.2 AFM images before negative panning with BSA and after negative panning with α-synuclein. Abundant phage binding is observed before panning with BSA. At the end of several rounds of negative selection, the phage no longer binds to BSA or aggregated α-synuclein.

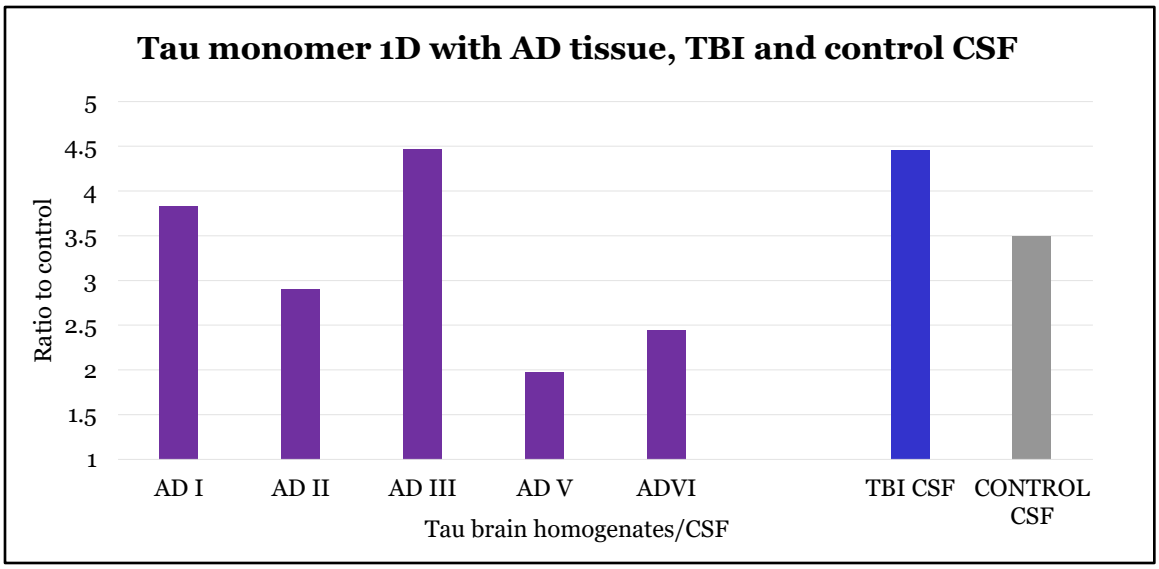



Figure A.3 Indirect ELISA of tau monomer 1D (TM1D) binding to different Braak stages of AD brain tissue, TBI and control CSF samples. This clone can potentially be used for detection in sandwich ELISA.

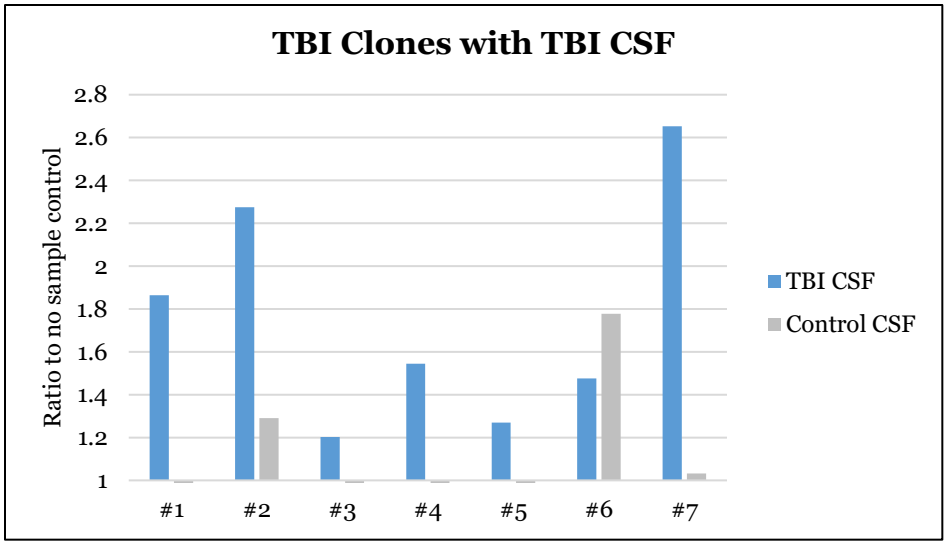



Figure A.4 Initial indirect ELISA screening with 7 different TBI clones with pooled TBI and pooled control CSF. X-Axis represents different clones and Y-Axis represents ratio to no sample background.

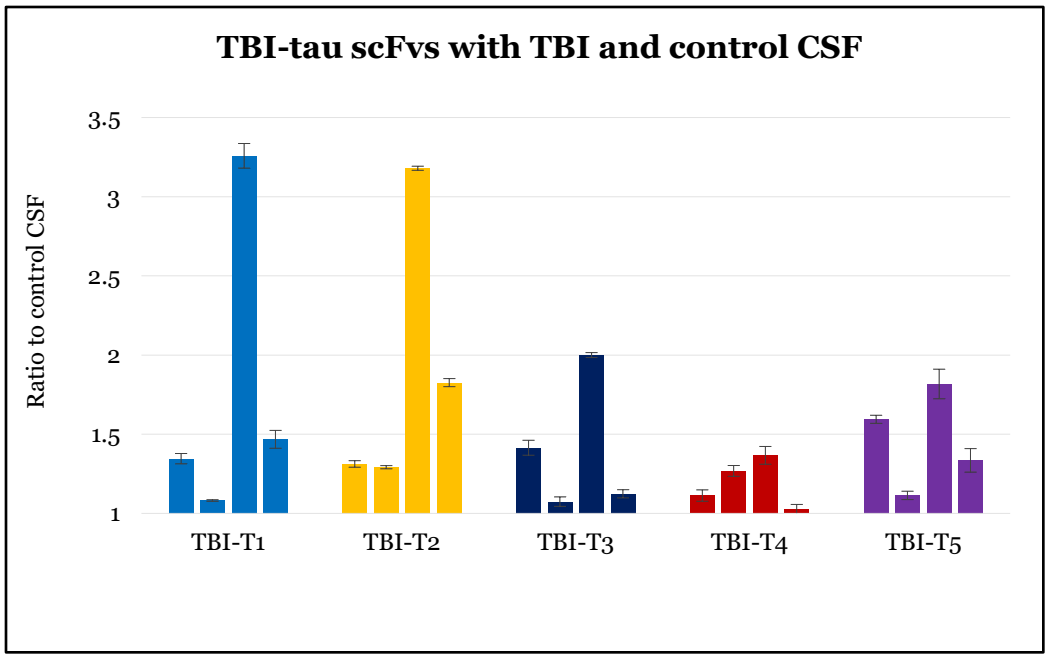



Figure A.5 Screening of 5 TBI clones with individual TBI and control CSF. X-Axis represents different clones and Y-Axis represents ratio to healthy control. All five TBI clones picked out all the TBI cases and had different binding patterns.

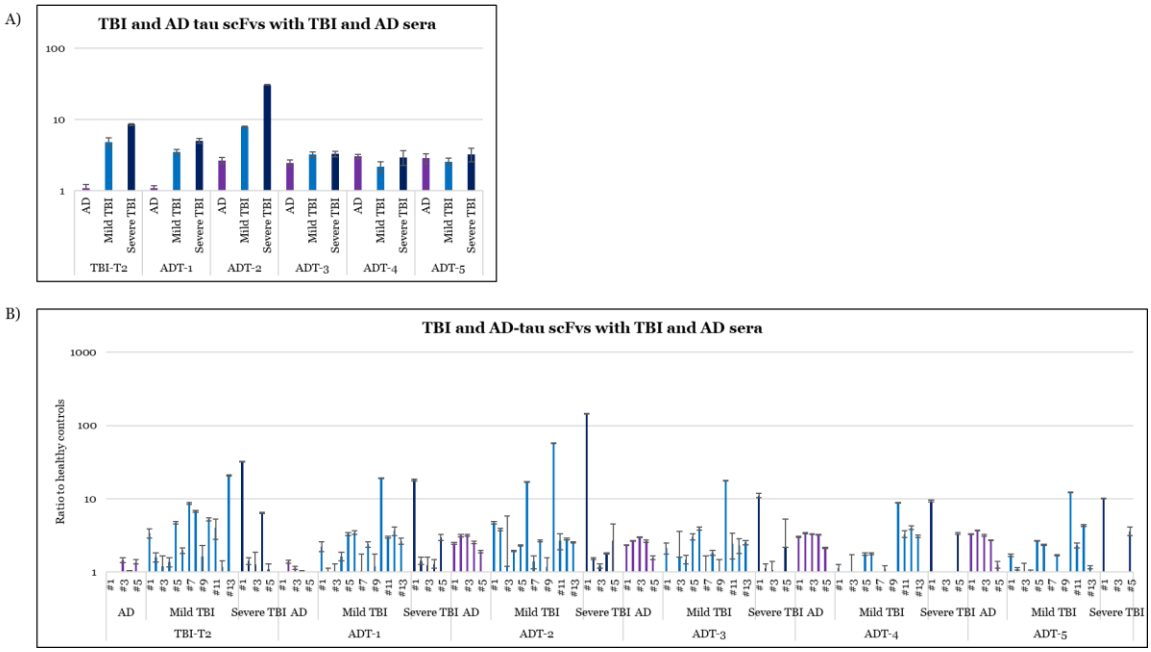



Figure A.6 Sandwich ELISA of TBI scFvs TBI-T2 and ADT-1, and AD tau scFvs ADT-2, ADT-3, ADT-4, ADT-5 with TBI and AD sera. X axis represents sera samples grouped as AD, mild TBI, moderate/severe TBI and Y axis represents ratio to healthy control. Binding ratios to AD are very low in the TBI scFvs with increasing binding ratio from mild to severe TBI. AD-tau scFvs have binding to AD with higher binding to mild and moderate/severe TBI.