Identification of Protein Signatures for AD and MCI in Body Fluids (Blood and CSF)

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

Dedicated to
my beloved Parents and Siblings

Acknowledgement

Praise and gratitude to **Almighty ALLAH**, the Omnipotent, the All-Aware, the most merciful, who showered upon me His blessings throughout the thick and thin of my life and who blessed me with courage, good health, company and support of good teachers and friends to conceptualize, develop and complete my research. Peace and blessings of Allah be upon His dearest **Holy Prophet, Hazrat Muhammad (PBUH)** who exhorted his followers to seek knowledge from cradle to grave.

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Javaria Baseer

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Abstract

Alzheimer's disease (AD) is a debilitating neurodegenerative disease and the most common cause of dementia around the world. The disease is primarily characterized by synaptic degeneration, toxic level of amyloid beta peptides and tau neurofibrillary tangles forming aggregates in the brain. Neuroimaging modalities and body fluids, like blood (serum and plasma) and cerebrospinal fluid (CSF) are the key areas that are being targeted in search of biomarkers for diagnosis, prognosis, testing therapeutic strategies and response to these therapeutic interventions. However, the heterogeneity and multifactorial nature of the disease have rendered many of the reported biomarkers insufficient for clinical use. Since a well-established biomarker with the required level of specificity and sensitivity is lacking, our study focuses on finding a proteomic signature to overcome these issues. We utilized differential proteomic analysis on two datasets of CSF and blood samples (acquired from ADNI and ANM respectively), to uncover a proteomic signature that can diagnose AD and its prodromal stage, MCI. The proteins identified as significantly up-regulated were then validated by ELISA and Mass Spectrometry in the lab. A proteomic signature of four up-regulated proteins was acquired from the two datasets. APOE4 protein was found to be significantly up-regulated in AD and MCI in the CSF samples while Serum Amyloid A-1/A-2 (SAA1/2) was significantly up-regulated in AD and MCI in the blood samples. Lastly, Peptidoglycan Recognition protein (PGLYRP1) and Collagen Alpha-1 (VIII) chain (COL8A1) were significantly up-regulated only in AD of the blood samples while showing no change in MCI samples. COL8A1 protein was validated as a protein with significantly high expression by Mass Spectrometry and Elisa results. The proteins in the acquired signature perform various functions such as lipid metabolism, neuroinflammation and enhancing immunity against cancers and microbial infections. Except, APOE4, which is involved in fat metabolism and is a well-established risk factor for Alzheimer's disease, the other three proteins have not been studied for their role in progression or prevention of AD.

Keywords: Alzheimer's disease, Mild Cognitive Impairment, Proteomics, Dementia.

1. Introduction

There are many different forms of neurodegenerative dementia and the most commonly occurring type is Alzheimer's disease (AD). AD has proven to be a debilitating illness. Globally, 60–80% of dementia cases are Alzheimer's Disease cases (Milà-Alomà, Suárez-Calvet, and Molinuevo 2019). In a report released by the World Health Organization (WHO) in 2012, worldwide, a fresh case of dementia is diagnosed every 4 s, and AD diagnosis happens every 7s (Cornutiu 2015). In the United States, it has been reported that over 6 million people, or 1 out of every 10 individuals aged 65 and above, are suffering from Alzheimer's disease (Mather and Scommegna 2020). In the United Kingdom, in a study in 2019, it was reported that more than 850,000 people, or 1 out of every 14 individuals of 65 years of age or above, were suffering from dementia. The later study estimated that the number could raise to over 1.5 million people with dementia in the United Kingdom if the current rate persists (Wittenberg et al. 2019). As per the Alzheimer's Association fact sheet, the mean life expectancy of the people with AD is estimated to be approximately 7 years after a clinical diagnosis while just 3% of the patients live past 14 years of their initial diagnosis. AD has caused most deaths among the aging population of the United States, preceded in numbers heart disease and cancer only (James et al. 2014). The National Center for Health Statistics reported an alarming rise of 68% in number of deaths caused by AD in the duration of 2000 to 2010. To get a better perspective, the comparison with other fatal diseases is alarming, with deaths caused by human immunodeficiency virus (HIV) infection decreasing by −42%, stroke-related disease by −23%, heart disease by −16%, prostate cancer by −8%, and breast cancer by −2% (Janeiro et al. 2021). Such alarming statistics being reported over the years reinforce the need to discover early diagnosis techniques for AD, with the hopes that early diagnosis would aid in enhancing and developing disease-altering therapeutic strategies.

The most commonly accepted defining feature of AD is accumulation of amyloid plaques and tau neurofibrillary tangles in the brain, which are in turn associated with the synaptic degeneration. As per the definition of the National Institute of Aging and Alzheimer Association (NIA-AA), presence of toxic aggregates of the amyloid-β peptide as plaques and tau neurofibrillary tangles (NFTs) are given the term 'Alzheimer's Disease' (Jack et al. 2018). In the AT (N) classification system, the biomarkers for Alzheimer's disease are grouped into three,

where ‘A’ stands for accumulated amyloid beta (Aβ), ‘T’ stands for tau neurofibrillary tangles, and ‘N’ stands for neurodegeneration (Jack Jr et al. 2016).

In AD, the neurons are degenerated which results in medical symptoms of cognitive decline, most notably dementia. The most common features that characterize AD pathology are; synaptic degeneration, inflammation and cell death in neurons, atrophy of the cortex, and aggregates of Aβ as plaques and tau neurofibrillary tangles in the brain. So far, there is no standardized method for diagnosis of AD and no therapy in place that can treat the disease and/or reverse its adverse effects. There is still a lot of uncertainty on how AD is triggered, however, the relationship between synaptic degeneration and the associated loss in cognition points to a synaptic loss being the main event in AD pathology (Terry et al. 1991), which is then followed by death of neurons, and the formation of tau neurofibrillary tangles together with amyloid beta plaques as the disease progresses. AD diagnostic studies are challenging largely owing to the fact that it is a multifactorial disease. It is a pathologically complex and clinically heterogenic disease which develops over the course of many years due to the contribution of various interrelated factors. Other than that, AD commonly exists with other neurodegenerative diseases present in older people, for instance, taupathy, frontotemporal dementia (FTD), vascular dementia (VaD), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), and Lewy body disease (LBD). Therefore, making an accurate diagnosis of the disease hard to achieve (Janeiro et al. 2021).

1.1 Alzheimer’s Disease Classification

The most agreed upon explanation for the pathogenesis of AD is amyloid cascade hypothesis (Selkoe and Hardy 2016). The primary observation that this hypothesis stands upon is the formation of toxic oligomers of Aβ as the basic building blocks of amyloid plaques. Truncated Amyloid Precursor Proteins (APP) are found in the brain of AD subjects at autopsy as aggregates of Aβ peptides. So far the studies have reported two very specific types of AD dementia: (1) Familial AD or early-onset AD (EOAD), which as the name suggests occurs early in an individual’s life, usually before the age of 65, and is caused by inherited mutations with autosomal dominant family history of AD. This type of AD is very rare and comprises less than 1–5% of all AD cases. (2) late-onset AD (LOAD), which occurs mostly after the age of 65 years in an individual’s life. The primary cause for this type of AD is unclear, with a sporadic and

heterogeneous nature. The most prevalent type of AD is LOAD, comprising more than 95% of all the cases of AD.

The pathogenesis of EOAD or FAD is attributed to inherited mutation in the three genes; presenilin1 (*PSEN1)*, presenilin2 (*PSEN2)* and Amyloid Precursor Protein (*APP*), disturbing normal Aβ processing and forming truncated forms Aβ peptides by using β- and γ-secretase to cleave APP. As per the amyloid cascade hypothesis, increased levels of abnormally cleaved Aβ peptides result in synaptic loss, neurodegeneration, and eventually decrease in cognition (Selkoe and Hardy 2016). These toxic Amyloid beta peptides are the basic units in the protein structure of the amyloid aggregates found in AD patient brain. Of these, the most common Aβ oligomer is Aβ1–40, followed by Aβ1–42. The latter is hydrophobic with an increased level of toxicity and fibrillogenesis, and is also highly pathogenic, providing and running the primary mechanics in the progression of the disease (Zheng et al. 2011).

For the more common, 'sporadic', or late-onset cases, aging and Apolipoprotein E (*APOE-ε4*) allele *ε4* are identified as risk factors. However, the underlying cause for LOAD is explained according to the amyloid cascade hypothesis too. As per this theory, Aβ oligomers are accumulated in the brain to toxic levels, resulting in synaptic dysfunction and neuron loss when a defect in the molecular mechanism fails to clear the toxic levels for Aβ from the brain. A vast amount of research has shown that soluble toxic Aβ peptides impede synaptic activity, adversely affecting learning and memory (Calabrese et al. 2007; Kittelberger et al. 2012; Selkoe and Hardy 2016), and bring about tau hyperphosphorylation (Jin et al. 2011; Oddo et al. 2004). The mutations and abnormalities in these genes, genetic mechanism and signaling pathways that control the clearing process of toxic Aβ in the brain have opened new avenues in search of diagnostic biomarkers and potential therapeutic targets for LOAD.

Increased levels of toxic Aβ peptides may also be encouraged by hypoxia which is very usual in the aging brain, making hypoxia a key factor in triggering of sporadic AD (Zetterberg et al. 2011). However, researches have also shown that at a low physiological concentration, Aβs are neuroprotective and exhibit antimicrobial features against dangerous microbes (Soscia et al. 2010).

1.2 Alzheimer's Disease Risk Factors

Research conducted on epidemiology of AD has have found LOAD to be a multifactorial, sporadic disease with age, genetics, and environmental aspects, for example, nutrition, life style, and physical trauma to the brain as contributing factors for its onset. The most potent risk factor is found to be age. Less than 1% of individuals between the age of 60 to 65 years have AD, and the incidence of diseased individuals rises exponentially to 24–33% at the age of 85 years (Wortmann 2012). According to another study, within AD patients, 4% are younger than 65 years, 6% are between 65 to 74 years of age, 44% are between 75 to 84 years, and 46% are 85 or above (Thies and Bleiler 2012). Studies also suggest that females are more likely to develop AD in comparison to their male counterparts in a ratio of 2 to 1 (2:1) (Viña and Lloret 2010).

Genetics is reported to be another important risk factor for LOAD. However, it is still under investigation exactly how the various genes affect, control or regulate susceptibility to LOAD. So far, the presence of Apolipoprotein E (*APOE-ε4*) allele *ε4* is reported to be the prime genetic factor with an established link of higher risk of LOAD (Green et al., 2009). It has been reported that just one copy of APOE4 allele multiplies the risk of developing LOAD by approximately 3 times, while two copies increases the risk 15 times (Michaelson 2014). However, detection of APOE4 allele in an individual is not enough to predict the development of AD, hinting at the contribution of other factors. Another low-penetrance gene found linked to the onset of LOAD is the sortilin-related receptor (SORL1) gene (Rogaeva et al. 2007). It is imperative to identify more risk genes so that individuals with a genetic potential of developing LOAD can be identified early and can be given therapy for the prevention, or delay the onset of the disease. Since it is possible that a complex network of genes contribute to the onset of a complex disease like LOAD, identification of genetic factors that regulate and influence the pathogenesis of LOAD will also aid in narrowing down genetic biomarkers for the diagnosis and prognosis of the disease.

For FAD or EOAD, genes are the most important risk factors. Inherited mutations in the *PSEN1* (Levy-Lahad et al. 1995), *PSEN2* (Goate et al. 1991) and *APP* (Sherrington et al. 1995) genes have been associated to early-onset FAD. These genes are found on chromosome 14, chromosome 1 and chromosome 21, respectively. There is an increased level of toxic Aβ

peptides in the CNS, especially the brain tissue, of patients carrying these mutations as compared to those without the mutations (Gao et al. 2019).

It has also been observed that monozygotic twins are more susceptible to developing AD as compared to dizygotic twins in case of one of the twins already having AD (Gatz et al. 2006). On the basis of these studies, it can clearly be concluded a genetic factors are dictating the onset of LOAD and more studies are required to identify them.

LOAD has a number of reported environmental risk factors, such as brain injury, cardiovascular diseases, elevated levels of cholesterol, obesity, and high blood sugar levels, together with sedentary lifestyle and improper diet. Studies have shown higher risk of AD in athletes and military populations suffering from traumatic brain injury (TBI) (Lehman et al. 2012; Plassman et al. 2000) and posttraumatic stress disorder (PTSD) (Yaffe et al. 2010) showing that TBI and PTSD also act as strong risk factors for dementia, including LOAD. Alternatively, there is a vast amount of research suggesting that becoming more socially active and connected, engaging in activity that stimulates cognition, teaching and learning, doing exercise and proper diet decreases susceptibility to developing AD.

1.3 What is a Biomarker?

Hulka gave the first definition of biomarkers in 1990 as “quantifiable alterations of cellular, biochemical, or molecular nature in human tissues, cells, or body fluids” (Mueller 1991). According to NIH a biomarker can be described as “A gauge of a biological or pathogenic process, or their response to a therapy, which is measurable and can be quantified and evaluated.” (Biomarkers Definitions Working Group, 2001).

In common terms, a biomarker is a measurable characteristic feature which provides information about the normal and/or altered physiological state, indicating abnormality, disease or a pathology. The quantitative change in a biomarker serves as an indicator of the presence of a disease, its progression and how it is responding to therapeutic intervention. Any measurable chemical and biological entity can serve as a biomarker including proteins and peptides, biological potentials, RNA, microRNA, fats and lipids, as well as morphological and histological cellular characteristics.

A good biomarker exhibits the basic properties of a disease. It helps distinguish a particular disease from other similar diseases, and it can be measured and quantified to predict the development of the disease, diagnose it in its initial phase (serve as an effective early diagnostic tool), as well as the later stages as the disease progresses (can be used as a prognostic tool). An ideal biomarker can be measured accurately, easily, inexpensively and with a sample source that can be collected noninvasively, (blood, urine, saliva, tears, physical movement data etc.). A biomarker is considered well establish only if it can be validated for its results by more studies in various populations, and is found to be sensitive, specific and diagnostically accurate (Jack et al. 2018).

Researchers are conducting studies in search of a clinically useful biomarker for AD that can be used as an early stage diagnostic tool (Thijssen et al. 2021), calculate the susceptibility of developing AD in combination with other risk factors, make an assessment of the effectiveness of therapeutic strategies (Derakhshankhah et al. 2020), follow through as the disease progresses through various prodromal stages (Simrén et al. 2021), differentiate AD from other closely related dementias, and help identify the best therapeutic strategies. So far, a clear-cut diagnosis of AD can be made only after the symptoms of cognitive decline appear in the later stages of the disease, or after the death of the individual when an autopsy can be done to study the aggregation of amyloid plaques and neurofibrillary tangles. As a result, such diagnostic tools are useless for studies that aim to identify individuals susceptible to developing AD or those who have a chance of benefitting from therapeutic intervention. At present, there is not a single well-established AD biomarker that can serve as a test for diagnosing the disease, and are only used as an additional measure to support the clinical judgment (Janeiro et al. 2021).

AD is a neurodegenerative disease with lengthy prodromal stages, the adverse effects of which cannot be reversed as the disease progresses over time. The NIA-AA tasked a working group to design a classification system for AD (2011). This classification system outlined three class for AD: preclinical AD which displays no symptoms of dementia, MCI with dementia, and symptomatic AD with dementia (Albert et al. 2011; Jack et al. 2018; McKhann 2011; Sperling et al. 2011). According to this classification of AD, the working group revisited and revised the previous diagnostic criteria on the basis of specific biomarkers related to the pathophysiological

processes in AD. The criterion set up by Biomarker Definition Working Group assorts the biomarkers for AD into three types: Type 0, Type I and Type II biomarkers.

Natural history of disease is measured by Type 0 biomarkers, which also tracks the progression of the disease through various stages. An example of Type 0 biomarker would be neuroimaging biomarkers of AD, such as a MRI scan to study the atrophy of the brain. Type I biomarkers evaluate the efficacy of therapeutic strategies, such as Cerebrospinal fluid biomarkers. Type II biomarkers are surrogate markers. So far, AD has no biomarkers AD that can be included in Type II.

1.3.1. Biomarker Classification on the basis of their function

Classification of biomarkers is also done on the basis of their function, such as, diagnosis, prognosis, pharmacological role, or a surrogate role. In a similar fashion, the vast number of reported biomarkers are classified into four types: (1) detection biomarkers, used for the detection of specific molecules or chemical entities, especially those related to a certain disease (2) diagnostic biomarkers, used to ascertain if an individual is suffering from a specific disease (3) prognostic biomarkers, used to track the progress disease through its various stages, whether or not therapeutic intervention is done, and (4) predictive biomarker are used for studying the factors that increase susceptibility of a particular illness, and aid in filtering early stage patients who have a greater chance of benefitting from a treatment (Bauer et al. 2006).

1.4 Biomarkers of Alzheimer's Disease

Over the last few decades, studies and researches conducted in the quest for finding adequate diagnostic biomarkers for early detection of AD have made a lot of progress, however, the search for a well-establish, ideal biomarker that may detect AD in its early stages still continues. The National Institute on Aging Working Group has set the criteria for the model biomarker for AD to display sensitivity greater than 80% for detection of AD, and specificity greater than 80% for distinguishing AD from non-AD dementias (Consensus Report of the Working Group on: "Molecular and Biochemical Markers of Alzheimer's Disease" 11The names of the Working Group Members and the names of the Working Group Advisory Committee Members are listed in the Appendix A (section VI). 22The Rea 1998). Few diagnostic biomarkers today are specific

and sensitive enough to meet this set of standards. No biomarker is able to make a definitive diagnosis before the appearance of late-stage symptoms of clinical dementia, or the accumulation of Aβ aggregates and tau neurofibrillary tangles at autopsy. Therefore, none of the currently known biomarkers can be further pushed for clinical trials or used in primary care.

The diagnostic biomarkers presently under investigation for early detection of AD include biomarkers in the CSF, imaging markers such as PET and MRI scans, and biomarkers discovered in peripheral tissues and body fluids like blood, urine, tears and sweat glands. Measuring the levels of Aβ1-42, total tau (t-tau), and phosphorylated tau- 181 (p-tau) is the most studied AD biomarkers in the CSF. However, acquiring samples for conducting studies of CSF biomarkers is a painful and highly invasive procedure requiring a lumbar puncture, and AD being a neurodegenerative disease largely affecting the elderly population, and so it makes CSF biomarkers unsuitable for prognostic studies. A more suitable approach for acquiring diagnostic information is to use the noninvasive neuroimaging technique. At present, Magnetic Resonance imaging (MRI) scans are being used to observe deterioration, Magnetic Resonance Spectroscopy (MRS) measures localized brain region metabolites and PiB PET scans observe sites of amyloid beta deposits. Single-photon emission computed tomography (SPECT) and electroencephalogram (EEG) are also used to detect AD (Scheltens 2009). Although noninvasive, the limitation with imaging modalities is that they required highly skilled operators and specialized facilities for successful operation. Also, neuroimaging modalities are expensive.

Limits and restriction of the currently available and known biomarkers have made the discovery of an easily acquired, least to non-invasive and inexpensive biomarker and signatures an absolute necessity. As such, blood, saliva, tears and urine are primarily being focused upon as avenues in search of model biomarkers and accurate signatures.

Study objectives

Our study will follow through with proteomic analysis of blood and CSF datasets of AD and MCI patients with the following core objectives in mind:

1. To identify and characterize risk factors/signatures in CSF and blood datasets for AD and MCI patients

2. Validation of the identified risk factors/signatures in blood samples of AD and MCI patients.

2. Literature Review

The presence of Aβ and tau aggregates is the primary condition for AD pathology. However, there are a number of other pathologies running parallel to these two, along with pathological processes, such as loss of synaptic function, damage to the blood brain barrier (BBB), immune response and inflammation (Figure 1). As a result, biomarkers are required to study the events of these pathological processes, identify co-pathologies and areas for effective therapeutic intervention. These biomarkers are also required for accurate diagnosis, prognosis, testing therapeutic strategies and the response to these therapeutic interventions. In this review, we will investigate and study CSF and blood biomarkers for AD and MCI that have been reported in the literature till date.

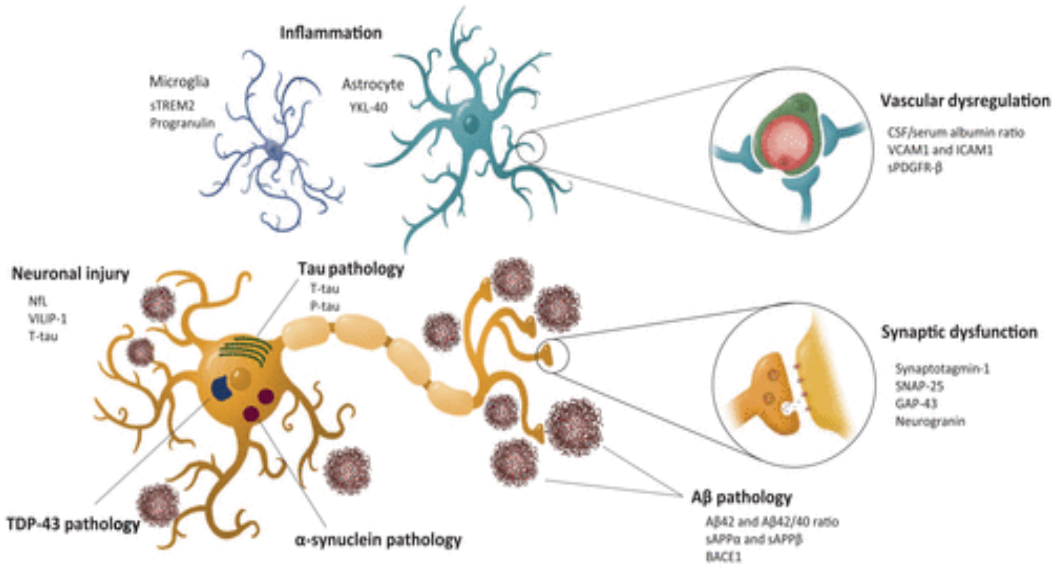


Figure 1: A representation of different pathologies and co-pathologies and the fluid biomarkers related to these pathologies (Milà-Alomà, Suárez-Calvet, and Molinuevo 2019).

2.1 AD biomarkers in CSF

Despite its invasive nature of procurement, the CSF biomarkers are the most widely used and accepted indicators of AD. Studies conducted over the past few decades have discovered and reported quite a few CSF biomarkers for AD. Following are some of the most important ones:

2.1.1 Aβ peptides

Aβ peptides are formed when APP is sequentially truncated by secretases following an amyloidogenic pathway or a non-amyloidogenic pathway. In amyloidogenic pathway, β-secretase enzyme cuts the APP protein at the β-site forming two fragments, the β soluble N-terminal peptide of APP (sAPPβ) and the C-terminal peptide or βCTF. γ-secretase then further slices the βCTF peptide into several smaller peptides with varying lengths. Formed through this amyloidogenic pathway, Aβ-40 consisting of 40 amino acids and Aβ-42 consisting of 42 amino acids are both used as CSF biomarkers for AD. Aβ-40 is largely found in the brain under normal conditions while Aβ-42 is more predominant in amyloid plaques (Haass et al. 2012). Aβ-42 has exhibited clear sensitivity as a biomarker for prognosis from MCI to AD and in diagnosing preclinical stage AD from non-demented controls. It also shows high specificity in distinguishing AD from other non-AD dementias (Ferreira et al. 2014). The γ-secretase cleavage also forms a transcriptional co-factor, the amyloid-β protein precursor intracellular domain (AICD), which is reported to contribute to AD pathology (Flammang et al. 2012).

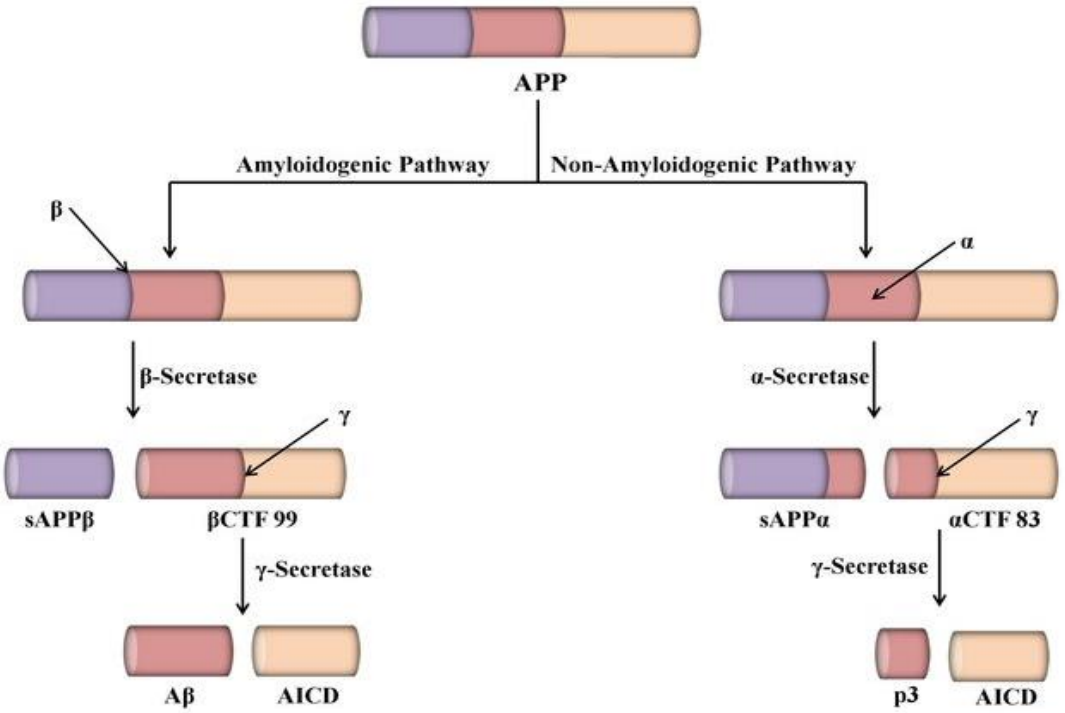


Figure 2: A schematic representation of Amyloidogenic and Non-Amyloidogenic pathway for APP processing (Uddin et al. 2020).

In non-amyloidogenic pathway, the slicing of APP protein is done by the enzyme α-secretase. It forms two fragments; the α soluble N-terminal peptide sAPPα and the APP C-terminal peptide, the αCTF. The latter is again sliced by γ-secretase forming a p3 factor and AICD (Lichtenthaler, Haass, and Steiner 2011).

Another way to predict AD progression more accurately is to use Aβ-40 concentration levels to normalize Aβ-42. Using Aβ-42/Aβ-40 ratio is reported to be a more specific and sensitive biomarker as compared to CSF Aβ-42 only (Lewczuk et al. 2017). CSF Aβ-42/Aβ-38 has exhibited similar results (Janelidze et al. 2016).

2.1.2 Other Aβ pathology biomarkers

sAPPα, sAPPβ peptides and BACE1 activity or protein levels can be measured in CSF but the results they exhibit is not consistent in different studies (Alcolea et al. 2014; Zetterberg et al. 2008).

2.1.3 Tau Proteins

Hyperphosphorylated tau found in neurofibrillary tangles is used as a biomarker for a number of neurodegenerative diseases. These tauopathies vary only in the isoform of total-tau and phosphorylated-tau present in the plaques (V. M.-Y. Lee, Goedert, and Trojanowski 2001).

In AD, p-tau and t-tau is present in elevated levels in the CSF. t-tau levels represent the amount of neuronal loss and degeneration, making it a good biomarker to distinguish AD from aging controls. However, other diseases like Creutzfeldt–Jacob disease (CJD) and Parkinson's disease (PD), acute stroke, frontotemporal lobar degeneration (FTLD) also have elevated levels of t-tau, making it a less specific biomarker for AD (van Harten et al. 2011; Schraen-Maschke et al. 2008)P-tau, on the other hand, is considered a more specific biomarker for AD as it is only slightly overexpressed in other tauopathies. In the ATN classification system, t-tau is used represent neuronal injury and neurodegeneration (N) while p-tau represents tau-pathology (T). Phosphorylations used as a target for p-tau biomarkers are threonine 181 ($p\text{-}tau_{181}$), which is the

most frequently used target, threonine 231, serine 199, and 231 and some C-terminal residues like Serine 396 and 404 (Schöll et al. 2019).

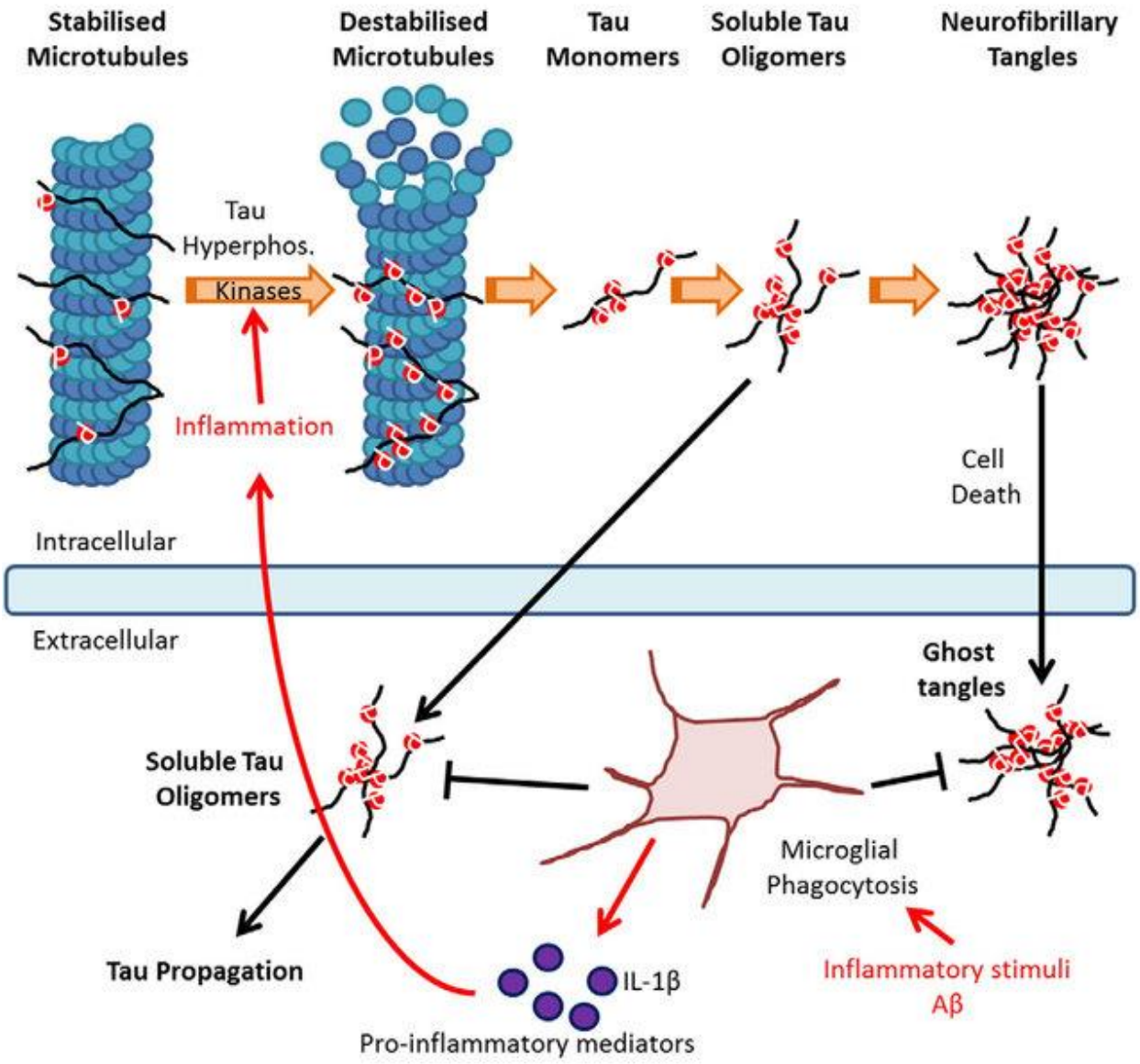


Figure 3: An illustrative representation of the formation of tau neurofibrillary tangles in AD patients (Barron et al. 2017).

The tau proteins in the CSF are better than Aβ as a predictive and prognostic biomarkers, staging and tracking drug response (Aschenbrenner et al. 2018). Deposits of t-tau and p-tau show a better correspondence with cognition and also predict disease progression from nondemented individuals to MCI and MCI to AD dementia more accurately (Ferreira et al. 2014).

2.1.4 Neurofilaments

Axonal and neuronal injuries results in leakage of neurofilaments (NfL) in the CSF, which serve as a nonspecific biomarker for degenerating axons in a number of neurodegenerative disease, including AD (Bos et al. 2019; Skillbäck et al. 2014).

2.1.5 VILIP-1

The neuronal calcium sensor (NCS) protein visinin-like protein 1 is considered a biomarker for damaged neurons (J.-M. Lee et al. 2008). It is highly expressed in AD in comparison to non-demented controls (Kester et al. 2015; J.-M. Lee et al. 2008; Olsson et al. 2016)and can be used as a CSF biomarker to distinguish AD from other dementias. VILIP-1 in CSF is also reported to be good predictive biomarker for MCI patients that might develop AD (Kester et al. 2015; Tarawneh et al. 2015, 2016).

2.1.6 Synaptic Biomarkers

Synaptic degeneration is one of the first events that occur in AD (Arendt 2009) resulting in a number of synaptic biomarkers. Loss of synapse correlates with decline in cognition (DeKosky and Scheff 1990; Terry et al. 1991). There are two types of synaptic biomarkers, axonal or presynaptic biomarkers and dendritic or post synaptic biomarkers.

2.1.6.1 Presynaptic Biomarkers

Presynaptic biomarkers include plasma membrane calcium sensor protein synaptotagmin-1, synaptosomal-associated protein 25 (SNAP-25) present in synaptic vesicles as a factor of SNAP receptor complex (SNARE) and GAP-43 protein which is involved in the development of the neurons and formation of synapses (De la Monte et al. 1989; Neve et al. 1988). Synaptotagmin-1 and SNAP-25 are involved in exocytosis of synaptic vesicles (Südhof and Rizo 1996; Sutton et al. 1998). All three of these presynaptic biomarkers are present in high levels in CSF of AD patients as compared to control (Brinkmalm et al. 2014; Öhrfelt, Brinkmalm, et al. 2016; Remnestål et al. 2016; Sandelius et al. 2019). Interestingly, Synaptotagmin-1 is present in more abundance in MCI as compared to AD (Öhrfelt, Brinkmalm, et al. 2016), whereas GAP-43 has

the potential to be a specific AD biomarker as is present in higher in CSF of AD patients as compared to other neurodegenerative diseases (Remnestål et al. 2016; Sandelius et al. 2019).

2.1.6.2 Postsynaptic Biomarkers

Calmadulin binding protein called Neurogranin is found in high abundance in CSF of AD and MCI. Present in the dendritic spines, neurogranin regulates the calcium signaling and formation of new synapses (Xia and Storm 2005). It exhibits predictive properties for decrease in cognition in cognitively normal individuals. (Bos et al. 2019; Olsson et al. 2016; Tarawneh et al. 2016; Thorsell et al. 2010). A longitudinal study revealed that CSF Neurogranin levels lowers in AD patients as the disease progresses (Sutphen et al. 2018).

2.1.6.2 Other Synaptic Biomarkers

Neural pentraxin 2 (NPTX2), a glutamate receptor binding protein located in excitatory synapse (Xiao et al. 2017), and synaptic vesicle glycoprotein 24 (SVA24), are some other synaptic biomarkers that are reported to decrease in CSF of AD patients. These have an inverse relation with CSF t-tau and p-tau levels (Alzheimer's Association International Conference® 2019 (AAIC®) Scientific Program Committee 2019).

2.1.7 Blood Brain Barrier (BBB) biomarkers

Damage to the cerebrovascular system, such as changes in the blood brain barrier (BBB), is believed to occur even before synaptic degeneration in the pathogenicity of AD (Iturria-Medina et al. 2016; Montagne et al. 2015; Nation et al. 2019; Sweeney et al. 2019). The standard biomarker for measuring alterations to BBB integrity is CSF to serum albumin ratio (Reiber and Peter 2001; Tibbling, Link, and Öhman 1977). In AD studies, CSF/serum albumin ratio had produced contradictory results, hinting to the fact that alteration to the BBB is not feature particular to AD. Several other studies have reported junction protein, proinflammatory proteins and blood clotting factors like fibrinogen and plasminogen as biomarkers for BBB integrity (Sweeney, Sagare, and Zlokovic 2015).

2.1.8 Endothelial markers

Vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) has mostly been reported with conflicting results in several studies, with one study reporting high levels of CSF VCAM-1 and ICAM-1 in MCI and AD patients as compared to unimpaired cognitive controls (Janelidze et al. 2018). This study also reported high levels of IL-15 and Flt-1 in CSF of AD patients.

2.1.9 Inflammatory Biomarkers

Inflammatory immune response is triggered when accumulations of abnormal Aβ peptides and tau neurofibrillary tangles are detected by pattern recognition receptors on microglia and astroglia (Heneka et al. 2015). This immune response can be helpful in clearing the aggregates (Ewers et al. 2019), but it can also be damaging as it can speed up neurodegeneration (Cherry, Olschowka, and O'Banion 2014). As the disease progresses, the effect of inflammatory response continues to switch between beneficial and detrimental response (Fan et al. 2017). A number of neuroinflammatory biomarkers have been studied, however, the results are inconsistent (Brosseron et al. 2014; X.-N. Shen et al. 2019)and these only give a partial explanation of the complicated immune response in AD. More studies are required to identify and establish biomarkers that can provide a more comprehensive picture of the inflammatory response throughout the various stages of AD as it progresses. Some of these biomarkers are discussed here.

2.1.9.1 TREM2 receptors

Triggering receptor expressed on myeloid cells 2 (TREM2) is a plasma membrane receptor of microglial cells. Of the many functions, one of TREM2 role is its involvement in APOE binding and shielding of amyloid aggregates (Atagi et al. 2015; Kleinberger et al. 2014; Zhong et al. 2017). TREM2 mediated microglia response is measured in CSF and blood using its soluble ectodomain sTREM2 which is released into the extracellular space (Kleinberger et al. 2014; Schlepckow et al. 2017). According to several studies, sTREM2 is highly expressed in CSF in AD as compared to controls (Piccio et al. 2016; Rauchmann et al. 2019; Suárez-Calvet et al. 2019; Suárez-Calvet, Araque Caballero, et al. 2016; Suárez-Calvet, Kleinberger, et al. 2016).

Others have contradicted these results (Henjum et al. 2016; Kleinberger et al. 2014). CSF sTREM2 is linked with tau pathology, but shows no relation with Aβ-related neurodegeneration (Suárez-Calvet et al. 2019). Studies have also shown that the increase in CSF sTREM2 levels is not AD-specific and it occurs in other neuroinflammatory diseases whenever neurons are damaged (Alosco et al. 2018; Byrne et al. 2018; Öhrfelt, Axelsson, et al. 2016; Woollacott et al. 2018)

2.1.9.2 Progranulin

Progranulin is a glycoprotein, proteolytic slicing of which results in separate granulins in the extracellular space (Bateman et al. 1990; Bhandari, Palfree, and Bateman 1992). Progranulins have a role in mediating inflammatory response. Animal model studies reveal progranulin having an influence on Aβ and tau pathology (Minami et al. 2014; Takahashi et al. 2017). So far no increase or decrease has been found in the levels of CSF progranulin when AD, MCI and controls were studied comparatively (Körtvélyessy et al. 2015; Morenas-Rodríguez et al. 2016; Nicholson et al. 2014).

2.1.9.3 YKL-40

YKL-40 is a human cartilage glycoprotein present in astroglia in the CNS (Bonneh-Barkay et al. 2012; Querol-Vilaseca et al. 2017). When an inflammatory immune response is triggered, YKL-40 is believed to play a role in remodeling (Bonneh-Barkay et al. 2012). CSF YKL-40 levels are raised in AD patients as compared to control (Craig-Schapiro et al. 2010; Olsson et al. 2016), and it continues to increase as the disease progresses (Lleó et al. 2019; Sutphen et al. 2018)and it has a close correlation with tau pathology. It can be used as a predictive biomarker for cognitively normal individuals to develop MCI (Craig-Schapiro et al. 2010), and individuals with MCI to develop dementia (Kester et al. 2015). However, YKL-40 overexpression in CSF is not AD specific (Alcolea et al. 2014; Illán-Gala et al. 2018)and therefore cannot be term as a biomarker specifically for diagnosing or predicting AD.

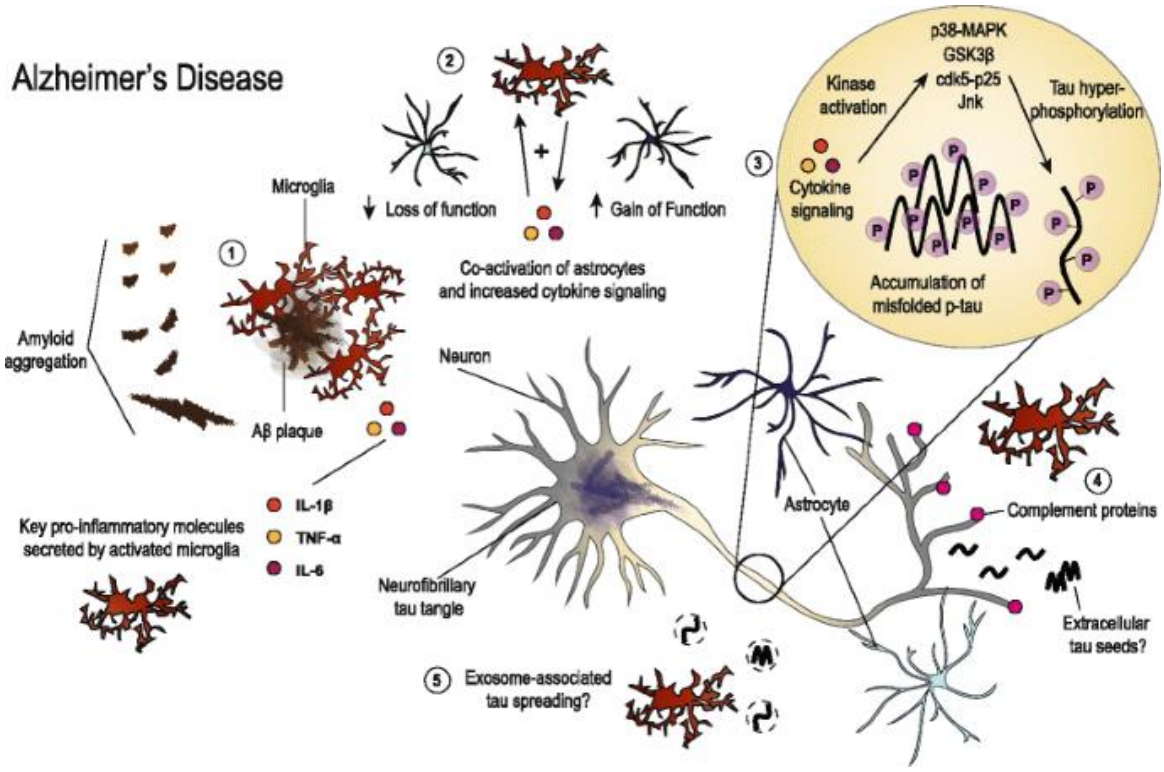


Figure 4: An illustrative description of the hypothesized part played by the inflammatory and gliosis protein in AD (Leyns and Holtzman 2017).

2.1.9.4 Other inflammatory biomarkers

Studies have reported other inflammatory biomarkers such as CSF interferon-inducible protein-10 (IP-10) (Daniela Galimberti et al. 2006), Glial fibrillary acidic protein (GFAP) (Oeckl et al. 2019), Monocyte chemoattractant protein-1 (MCP-1) (Olsson et al. 2016) to be elevated as in MCI and AD patients as compared to non-demented controls, however, the results are inconsistent and contradictory.

2.1.10 TDP-43

TDP-43 is the Transactive response DNA-binding protein of 43 kDa and is reported as aggregates in 20-50% AD individuals (Amador-Ortiz et al. 2007; Josephs et al. 2008; Uryu et al. 2008). It is suggested to be involved in cognitive decline and brain atrophy (Josephs et al. 2008).

2.2 AD biomarkers in Blood

CSF is primarily being used to study AD biomarkers. However, CSF is procured through a painful and invasive lumbar puncture, making it a less desirable mode of sample collection (Engelborghs et al. 2017). For this reason, a lot of focus in being given to identifying blood biomarkers for AD since blood samples are easier to procure, are less invasive and less painful (Hampel et al. 2018).

2.2.1 Aβ peptides

Studies have reported Aβ peptides in blood, however, the results have shown high center to center variability, and do not correspond to the Aβ levels in CSF (Hansson et al. 2010; Olsson et al. 2016). A recent study have shown promising results by quantitating blood Aβ levels using IP-MS, which is immunoprecipitation in tandem with Mass Spectrometry (Kaneko et al. 2014; Nakamura et al. 2018; Schindler et al. 2019). Aβ levels in blood measured in this study show the same levels of sensitivity and specificity as Aβ42/Aβ40 ratio in CSF or amyloid PET scan (Nakamura et al. 2018; Ovod et al. 2017; Schindler et al. 2019). However, further research is still required to replicate and validate the results shown in this study.

2.2.2 Other Aβ pathology biomarkers

sAPPα, sAPPβ peptides and BACE1 activity or protein levels have been reported in plasma but like CSF, the results have not been consistent in different studies (Y. Shen et al. 2018).

2.2.3 Tau proteins

Like blood Aβ peptides, a lot of conflicting results have been reported by several studies regarding blood t-protein levels and a clear consistency is lacking (Olsson et al. 2016). A recent study using IP-MS technique has reported elevated levels of tau in the blood of AD patients compared to non-demented controls (Fossati et al. 2019; Mattsson et al. 2016; Pase et al. 2019). Blood t-tau is a good predictor of declining cognition and developing dementia (Mattsson et al. 2016; Mielke et al. 2017; Pase et al. 2019).

2.2.4 NfL

NfL can accurately be measure in blood and the results correlate to NfL levels in the CSF. In EOAD, blood NfL is observed to increase in AD and MCI patients as compared to normal control years before symptoms appear (Gisslén et al. 2015; Weston et al. 2017). NfL is also useful for staging the disease as the their level rises with progression of the disease (Mattsson et al. 2019; Sánchez-Valle et al. 2018). NfL is also being favored to replace t-tau as a biomarker for neurodegenration (N) in AT (N) classification system for AD (Jack Jr et al. 2016).

2.2.5 VILIP-1

VILIP-1 has been reported to be significantly highly expressed in blood of AD patients (Tarawneh et al. 2011). However, more research is required to replicate and validate these findings.

2.2.6 Endothelial Biomarker

VCAM-1 is present in high levels in blood of AD patients as compared to cognitively normal controls (Ewers, Mielke, and Hampel 2010)

2.2.7 sTREM2

sTREM2 has mostly been studied in the CSF and blood studies are lacking. The few studies conducted used Mass spectrometry and reported no change in plasma or serum sTREM2 levels in AD (Bekris et al. 2018; Kleinberger et al. 2014; Piccio et al. 2016).

2.2.8 YKL-40

Blood YKL-40 studies have reported increased levels of YKL-40 in AD patients in comparison to non-demented controls (Choi, Lee, and Suk 2011; Craig-Schapiro et al. 2010). YKL-40 does not offer much value as a diagnostic biomarker, but it can be used to assess targeted treatment response for neuroinflammation, and disease staging.

2.2.9 Other inflammatory biomarkers

Plasma studies for interferon-inducible protein-10 (IP-10) have given inconsistent results (D Galimberti et al. 2007; Iturria-Medina et al. 2016), Glial fibrillary acidic protein (GFAP) is overexpressed in serum and it correlates to cognitive decline significantly (Oeckl et al. 2019), while serum sirtuin1 levels, which declines with aging, decrease at a faster pace in AD (Julien et al. 2009).

2.2.10 TDP-43

Plasma TDP-43 is reported to have elevated levels in AD and progressive MCI as compared to non-demented controls (Williams et al. 2017).

It is clear from the literature review that biomarkers for AD and MCI are lacking in CSF, and especially in blood. The results for most of the above mentioned biomarkers are inconsistent and contradictory. More research is required to validate the currently reported biomarkers. It is therefore understood that having a protein signature instead of a single biomarker for diagnosis of AD and MCI is a more prudent and feasible approach. In our study, we aimed to analyze a blood sample dataset from ANM, and a CSF sample dataset from ADNI to acquire a proteomic signature for AD and MCI. Differential proteomic analysis would be done to get a protein signature, then the individual proteins in the signature would be validated through Mass Spectrometry and Elisa techniques for their differential expression in AD and MCI.

3. Materials and Methods

3.1 Materials

3.1.1 Datasets

- ANM (AddNeuroMed)
- ADNI (Alzheimer's Disease Neuroimaging Initiative)

Characteristics	ANM	ADNI
Fluid	**Blood plasma**	**CSF**
Cohort	**European**	**American**
Method	**SOMAmer (Slow Off-rate Modified Aptamer) based array**	**Targeted Mass Spectrometry**
Proteins/Peptides measured	**1001 proteins**	**221 proteins, 567 peptides**

Table 1: provides a brief overview of the properties of the two datasets used in this study.

Proteomic datasets were downloaded from ADNI (Alzheimer's Disease Neuroimaging Initiative) database and ANM (AddNeuroMed).

3.1.1.1 ADNI Dataset

ADNI dataset uses CSF samples from an American cohort and collects data using Mass Spectrometry. 1001 proteins were analyzed from AD (n=71), MCI (n=145) and non-demented control (n=87) subjects. More information on the ADNI dataset is available at (http://loni.usc.edu/).

3.1.1.2 ANM Dataset

dataset uses blood plasma samples from a European cohort, comprising of English, Finnish, Greek, Polish, Austrian and Italian subjects and collects proteomics data using SOMAmer (Slow Off-rate Modified Aptamer) based array. 221 proteins and 567 peptides were analyzed from AD

(n= 284), MCI (n=155) and non-demented control (n=193) subjects. More information on the ANM dataset is available at (https://www.synapse.org/).

3.1.2 Softwares used

- Microsoft Excel
- Prism Graphpad

Microsoft excel was used for performing the Differential Proteomic Analysis and graphical representation of the proteomic data. Prism Graphpad was used to help visual the data as graphs and plots.

3.1.3 Laboratory Equipment

- Liquid Chromatography with tandem Mass Spectrometry (LC-MS-MS) from Waters
- SimpleStep ELISA® Sandwich Elisa Kit from Abcam.
- Multichannel pipettes
- Plate washers
- Beakers
- Paper towels
- Spectrophotometer
- Computer

3.1.3 Chemicals

- Ingredients included in human Pro-Collagen I alpha 1 ELISA Kit (ab210966) by SimpleStep ELISA® Sandwich Elisa

3.2 Methods

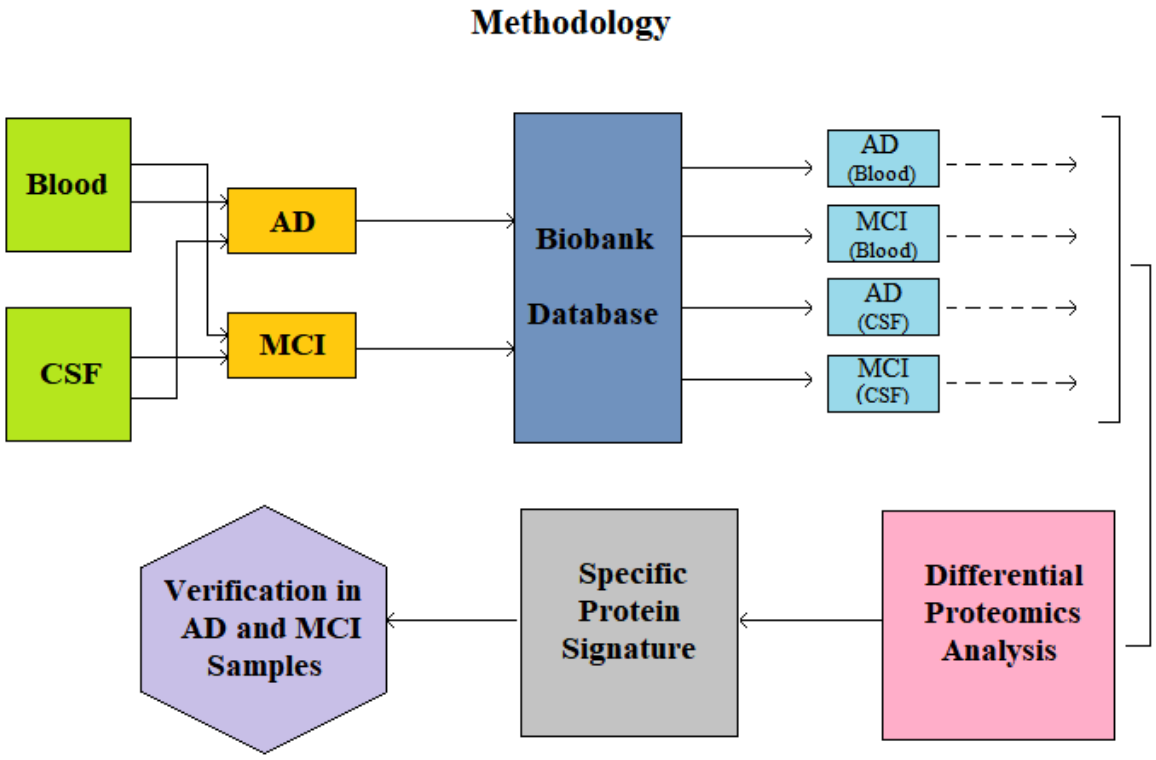


Figure 5: Schematic representation giving a brief overview of the methodology followed in this study.

3.2.1 Differential Proteomic Analysis

- The data was downloaded from ADNI and ANM databases as protein abundances in CSF and blood plasma, respectively, on excel sheets.
- The subjects were sorted and separated as AD vs Control and MCI vs control. Differential Protein Analysis was performed following the steps in (**Figure 6).**
- Ratio of biological replicates/controls was calculated using mean abundances of AD or MCI patient, and non-demented controls for each protein or peptide, then a t-test (p=0.05) was applied to find significance of the changes in protein expressions.
- Sort and filter function was used to find the proteins that were significantly up or down-regulated.

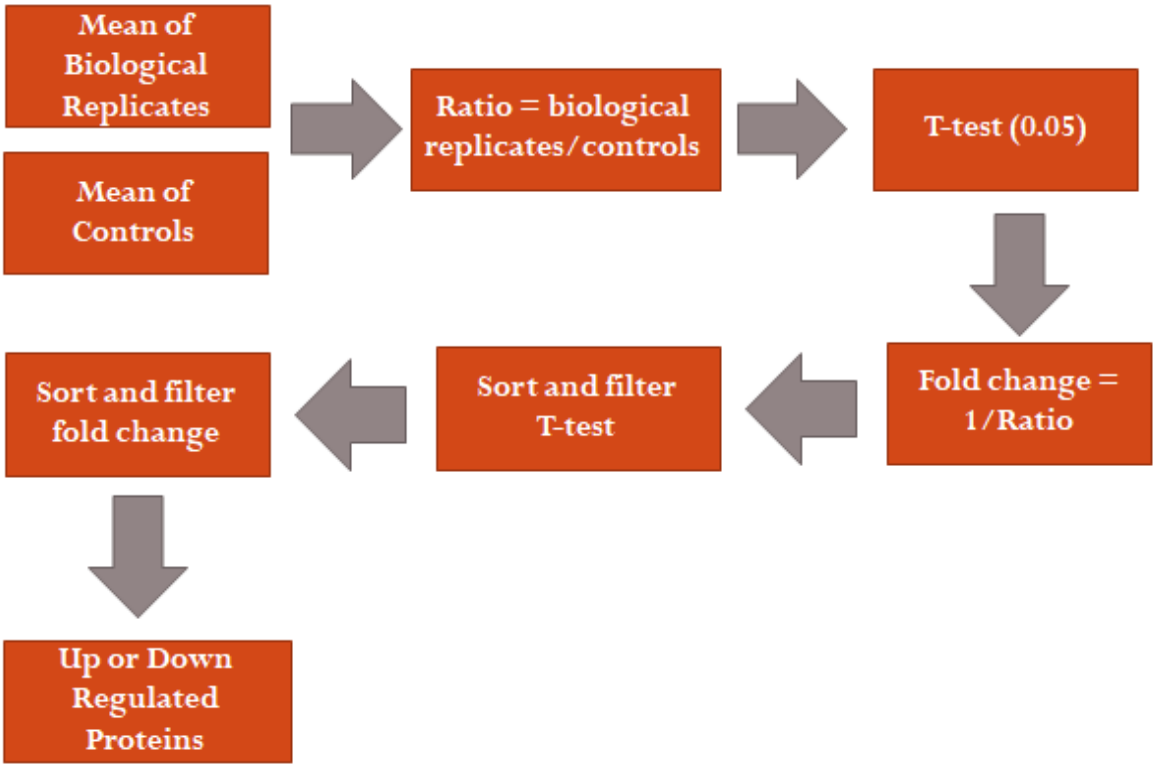


Figure 6: A flowchart representing the steps followed in the Differential Proteomic Analysis to find out the significantly up or down-regulated proteins.

3.2.2 Validation of results

The signature of significantly up or down-regulated proteins concluded from the datasets was validated for results in the laboratory using Mass Spectrometry (LC-MS-MS), and Sandwich Elisa techniques in AD, MCI and non-demented controls.

3.2.2.1 LC-MS-MS

The sensitivity of our results from the Differential Proteomic Analysis was validated using Liquid chromatography with tandem Mass Spectrometry. (LC-MS-MS).

- Extraction of spots on the silver stained 2DEgel for the abnormally expressed proteins is done and then they are followed through the process of Q Exactive hybrid quadrupole mass spectrometry to identify the sequence of these up or down-regulated proteins and peptides.

- After the proteins are identified with the Mass Spectrometer, peptide counts (>2) were accepted so as to eliminate false positives in the proteomic data. The peptide threshold was kept at 95%
- False Discovery Rate (FDR) maintained at 0.01% at minimum as a qualification basis for proteins that were identified.

3.2.2.2 ELISA

The protein validated through the Mass Spectrometry performed in the laboratory was followed in an Elisa experiment to confirm its specificity to Alzheimer's Disease. 16 plasma samples (n=16) were investigated for presence of significantly up-regulated Collagen alpha-1 (VIII) chain (Table 2).

Diagnosis	Number of Samples
Alzheimer's disease	**4**
Creuzfeld Jacob Disease (CJD)	**4**
Dementia with Lewy bodies (DLB)	**4**
non-demented controls	**4**

Table 2: The specificity validation cohort comprised equal number of samples of Alzheimer's disease (n=4), Creuzfeld Jacob Disease (CJD) (n=4), Dementia with Lewy bodies (DLB) (n=4) and non-demented controls (n=4).

Human Pro-Collagen I alpha 1 ELISA Kit (ab210966) by SimpleStep ELISA® Sandwich Elisa, Abcam was used to carry out the investigation.

- The platform of the Elisa kit is a 96 well microtiter plate with 8x12 well strips. The Assay is set up and samples and reagents are prepared. The base of each well is pre-coated with an anti-tag monoclonal antibody which is bound to an affinity tag. This affinity tag will bind the anti-tag antibody to the capture antibody.

- The sample, which contains the target analyte, tagged capture antibodies and conjugated detection antibodies are mixed in a buffer to form an antibody cocktail and added into the wells. This mixture is allowed to incubate for one hour. During this incubation time, the capture antibody binds to the target analyte at one end and to the anti-tag antibody via the affinity tag at the other end, binding the target proteins to the microtiter plate. On the other hand, the target protein also binds to the detection antibody, which is conjugated to Horseradish peroxidase (HRP). In this way, the target protein is sandwiched between the two types of antibodies, and therefore the name, Sandwich Elisa.

- After incubation the well is washed to remove the residual matrix protein or unbound antibodies.

- Detection Reagent 3,3’5,5’-Tetramethylebenzidine (TMB) is added to the wells and incubated for 10 minutes. The TMB solution reacts with HRP.

- After incubation the well is washed to remove the residual matrix protein or unbound antibodies.

- Stop solution is added and colors changes from blue-green to yellow in proportion to the bound sandwich complex.

- The absorbance is then read on a standard spectrophotometer at 450nm

4. Results

4.1 Phenotypic Results

The phenotypic data was divided into CSF cohort (ADNI) and Blood cohort (ANM), on the basis of the type of sample used for quantification of the proteins. The phenotypic data was analyzed separately. It comprised of factors such as:

- Age
- Gender
- Marital status
- MMSE scores

4.1.1 Age

The age distribution of the subjects in the CSF dataset varied from 56 to 89.6 years of age. The data was divided in seven age groups with a gap of five years each. Following bar graph displays the age distribution of the CSF cohort.

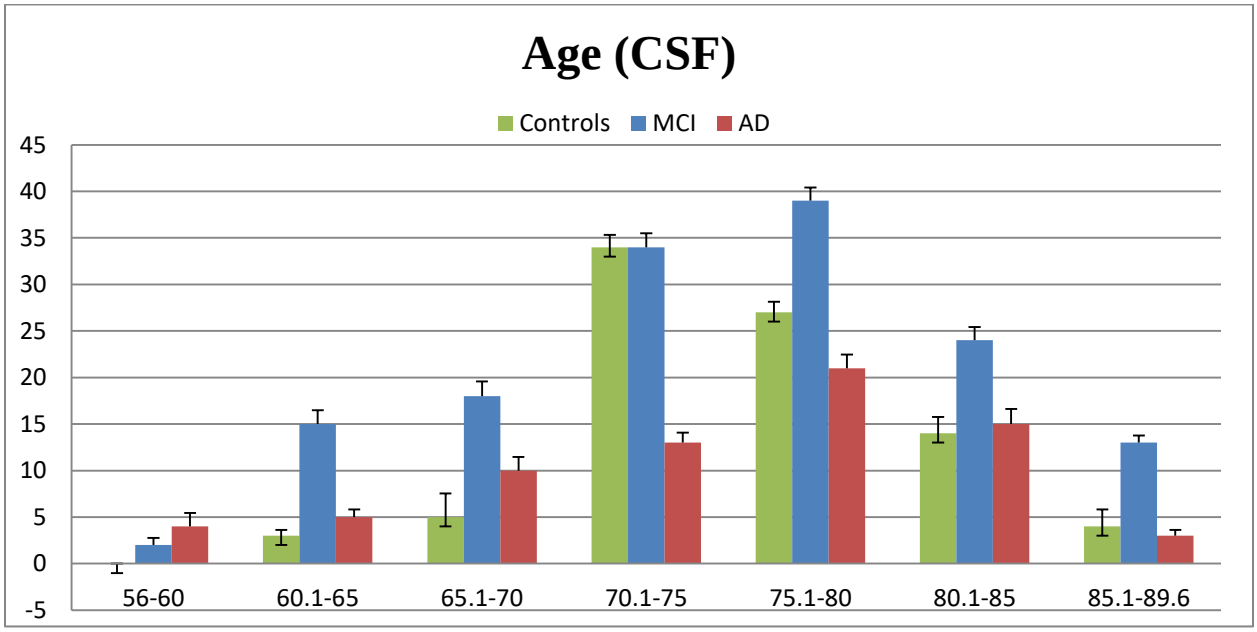


Figure 7: A graphic representation of the age distribution of Non-demented controls (in green), MCI (in blue) and AD (in red) for the CSF dataset.

Age	Controls	MCI	AD
56-60	**0**	**2**	**4**
60.1-65	**3**	**15**	**5**
65.1-70	**5**	**18**	**10**
70.1-75	**34**	**34**	**13**
75.1-80	**27**	**39**	**21**
80.1-85	**14**	**24**	**15**
85.1-89.6	**4**	**13**	**3**

Table 3: Age distribution of Controls, MCI and AD patients is shown in this table as seven groups, each with a five years age gap.

For blood dataset, the ages vary from 55 years to 90 years. The data was divided in seven age groups with a gap of five years each. Following bar graph displays the age distribution of the CSF cohort.

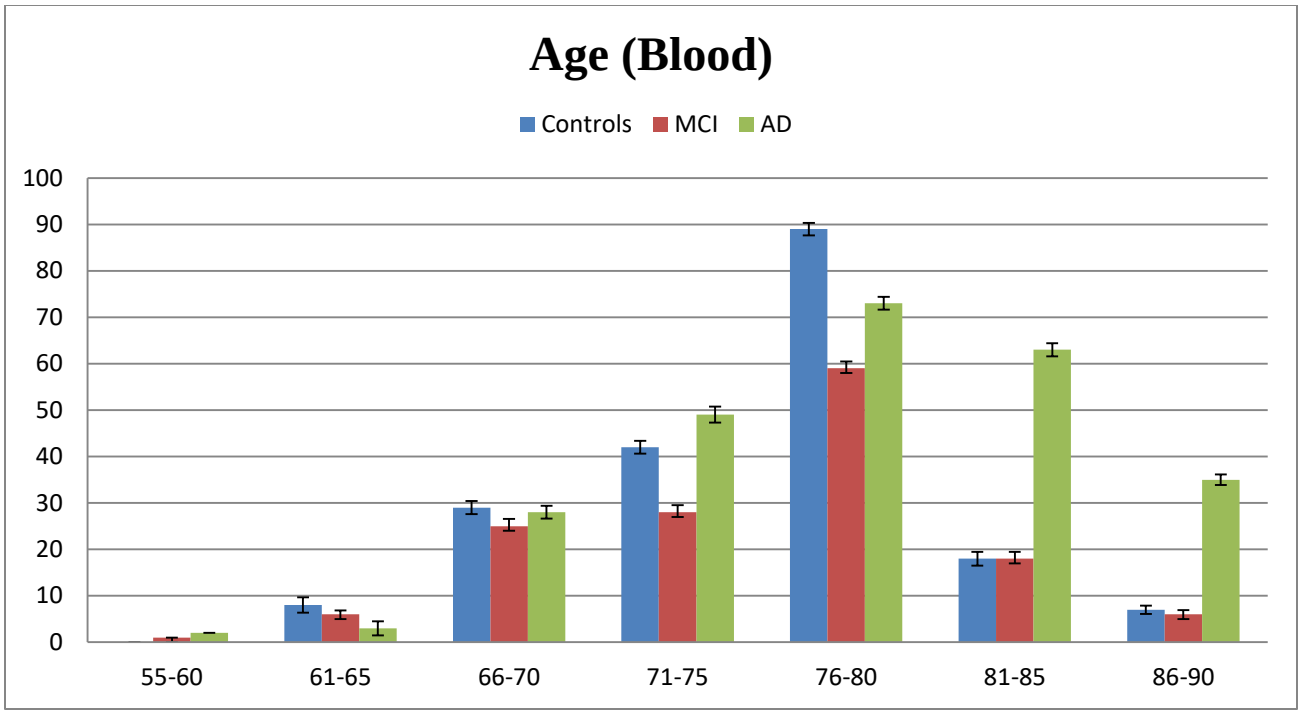


Figure 8: A graphic representation of the age distribution of non-demented controls (in green), MCI (in blue) and AD (in red) for the blood sample dataset

Age	Controls	MCI	AD
55-60	0	1	2
61-65	8	6	3
66-70	29	25	28
71-75	42	28	49
76-80	89	59	73
81-85	18	18	63
86-90	7	6	35

Table 4: Age distribution of blood data for controls, MCI and AD patients is shown in this table as seven groups, each with a five years age gap.

4.1.4 Gender

Figure 9 shows gender distribution for cognitively normal controls (n=44 males and n=43 females), MCI subjects (n=99 males and n=46 females), and AD subjects (n=39 males and n=32 females) for the CSF cohort.

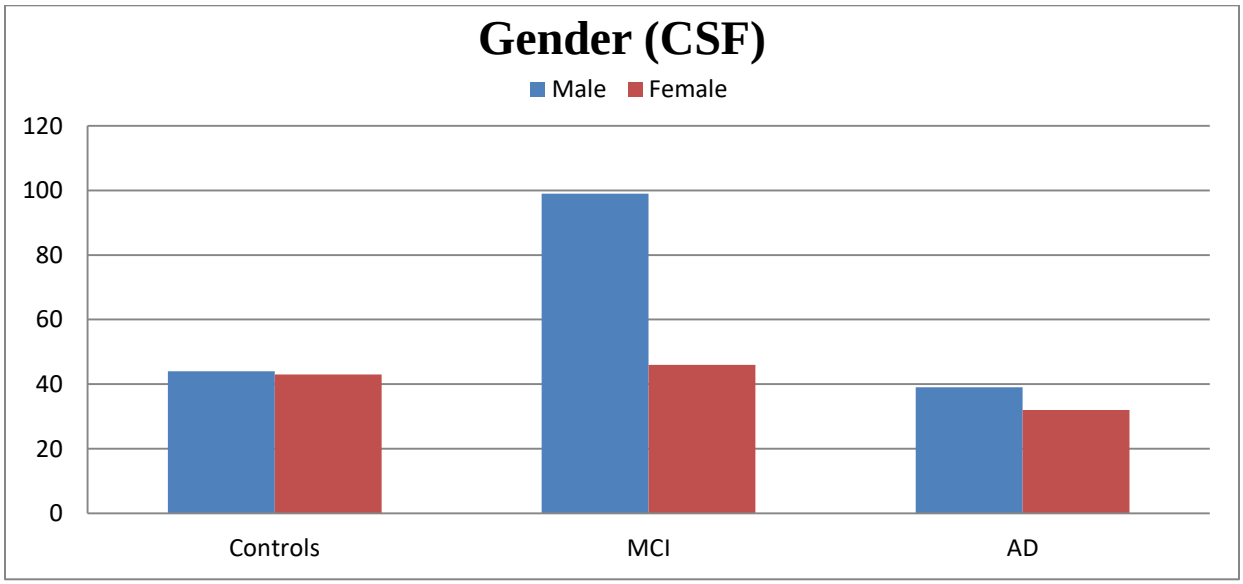


Figure 9 shows the gender distribution of the CSF cohort.

Diagnosis	Male	Female
Controls	**44**	**43**
MCI	**99**	**46**
AD	**39**	**32**

Table 5 shows the gender distribution for CSF cohort in tabulate form.

Figure 10 shows gender distribution for cognitively normal controls (n=102 males and n=91 females), MCI subjects (n=90 males and n=65 females), and AD subjects (n=191 males and n=93 females) for the blood cohort.

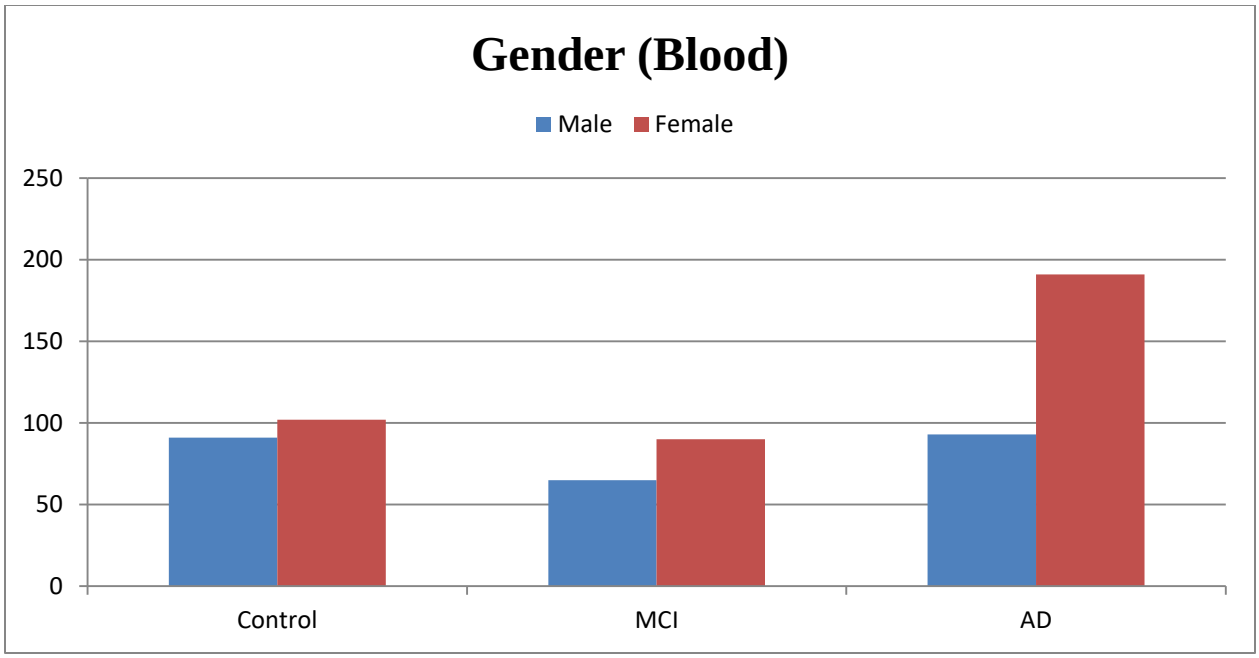


Figure 10 shows the gender distribution of the blood cohort.

Diagnosis	Male	Female
Controls	**91**	**102**
MCI	**65**	**90**
AD	**93**	**191**

Table 6 shows the gender distribution for CSF cohort in tabulate form.

4.1.2 MMSE

MMSE (Mini Mental State Exam) is also known as the Folstein test. It is a questionnaire with 30 points to evaluate skills related to language, alertness, spatial orientation, memory and Visio-spatial aspects. MMSE has traditionally been used to measure cognitive decline, ascertain dementia and estimate its progression for clinical and research purposes. The test scores are divided into the following categories: normal cognition (25-30 score), mild dementia (21-24), moderate dementia (10-20) and severe dementia (90 or lower). The following bar graph displays the MMSE test scores of the subjects in both CSF and Blood-based cohorts.

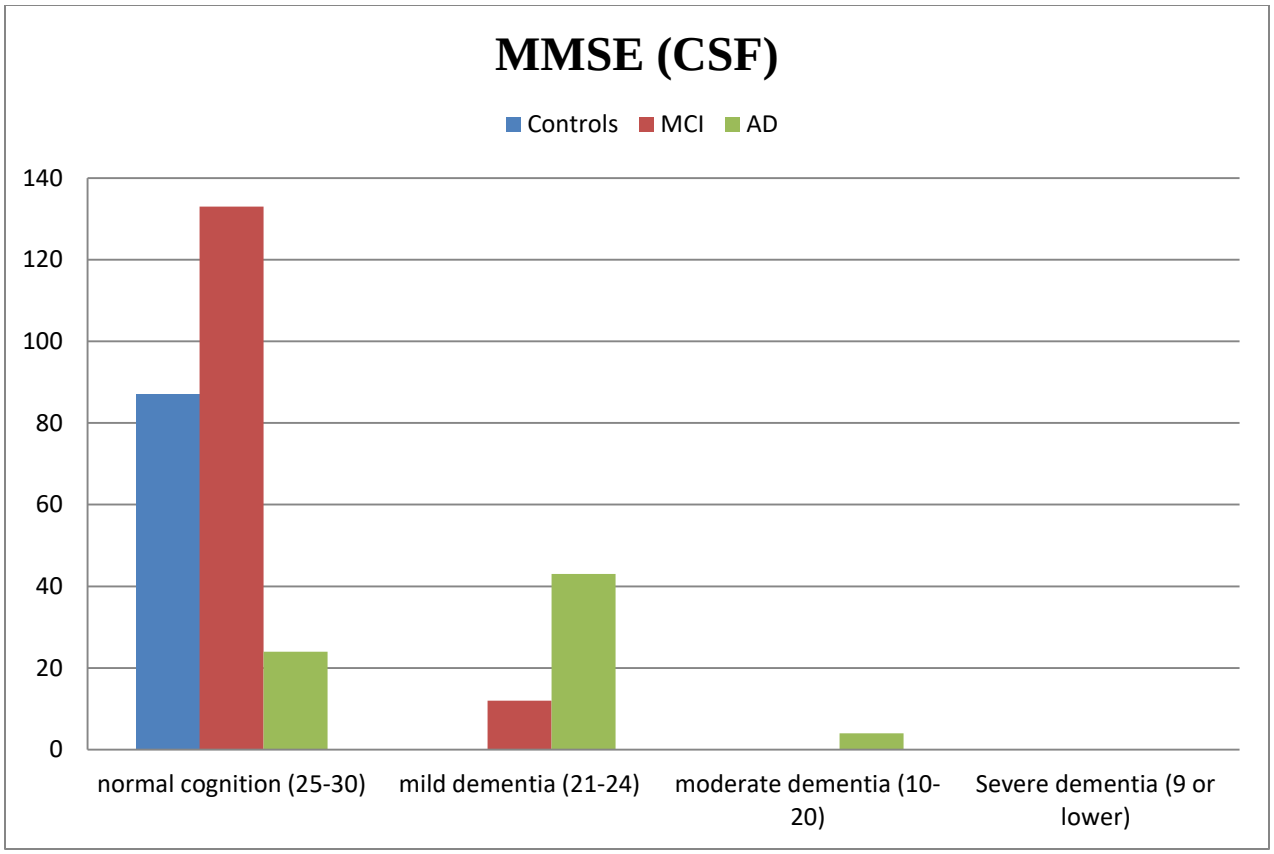


Figure 11: Graphical representation of MMSE test scores showing normal cognition (25-30), mild dementia (21-24), moderate dementia (10-20) and severe dementia (90 or lower), for control (in blue), MCI (in red) and AD (in green) among the CSF sample subjects.

MMSE	Controls	MCI	AD
normal cognition (25-30)	**87**	**133**	**24**
mild dementia (21-24)	**0**	**12**	**43**
moderate dementia (10-20)	**0**	**0**	**4**
Severe dementia (9 or lower)	**0**	**0**	**0**

Table 7: Table showing the number of subjects in the CSF cohort among controls, MCI and AD falling against MMSE test score categories of normal cognition (25-30), mild dementia (21-24), moderate dementia (10-20) and severe dementia (90 or lower).

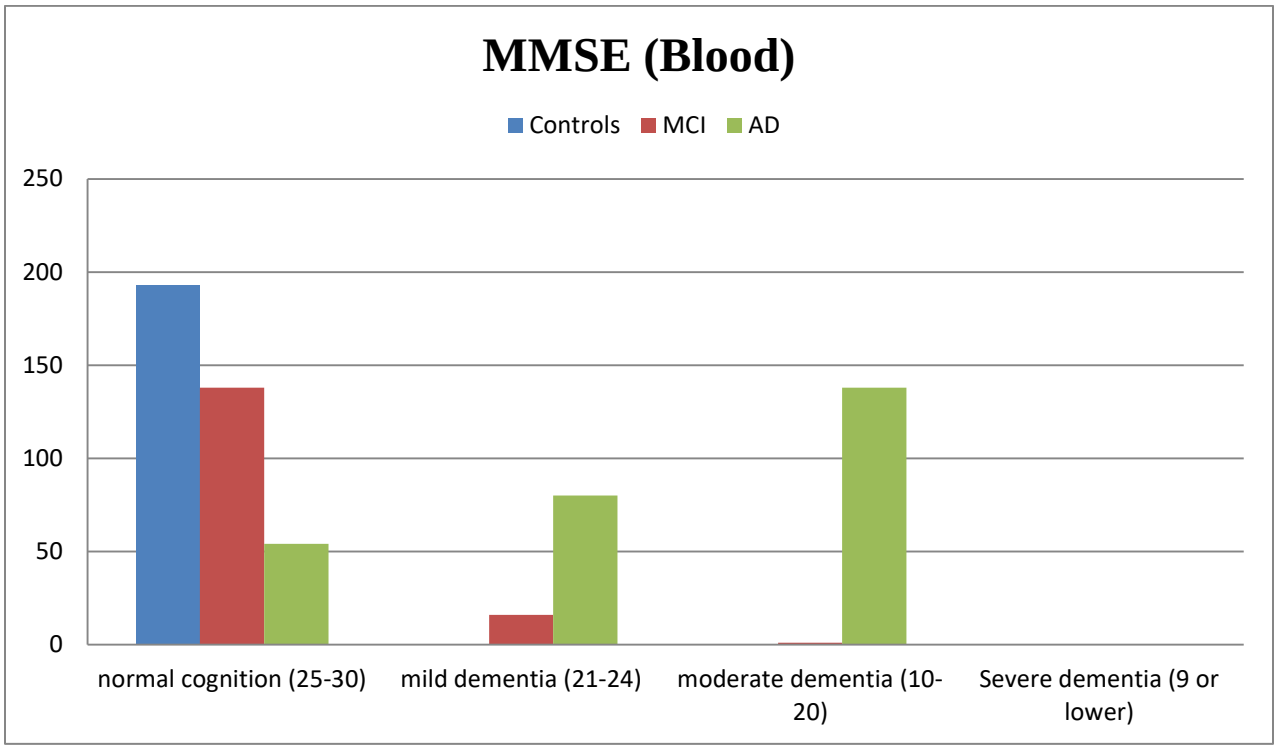


Figure 12: Graphical representation of MMSE test scores showing normal cognition (25-30), mild dementia (21-24), moderate dementia (10-20) and severe dementia (90 or lower), for control (in blue), MCI (in red) and AD (in green) among the blood sample subjects.

MMSE	Controls	MCI	AD
normal cognition (25-30)	**193**	**138**	**54**
mild dementia (21-24)	**0**	**16**	**80**
moderate dementia (10-20)	**0**	**1**	**138**
Severe dementia (9 or lower)	**0**	**0**	**0**

Table 8: Table showing the number of subjects in the blood cohort among controls, MCI and AD falling against MMSE test score categories of normal cognition (25-30), mild dementia (21-24), moderate dementia (10-20) and severe dementia (90 or lower).

4.1.3 Marital Status

Marital Status data was only available for CSF cohort. The subjects were divided into four categories based on their marital status: married, divorced, widowed and never married. The following bar graph displays the number of subjects against each category.

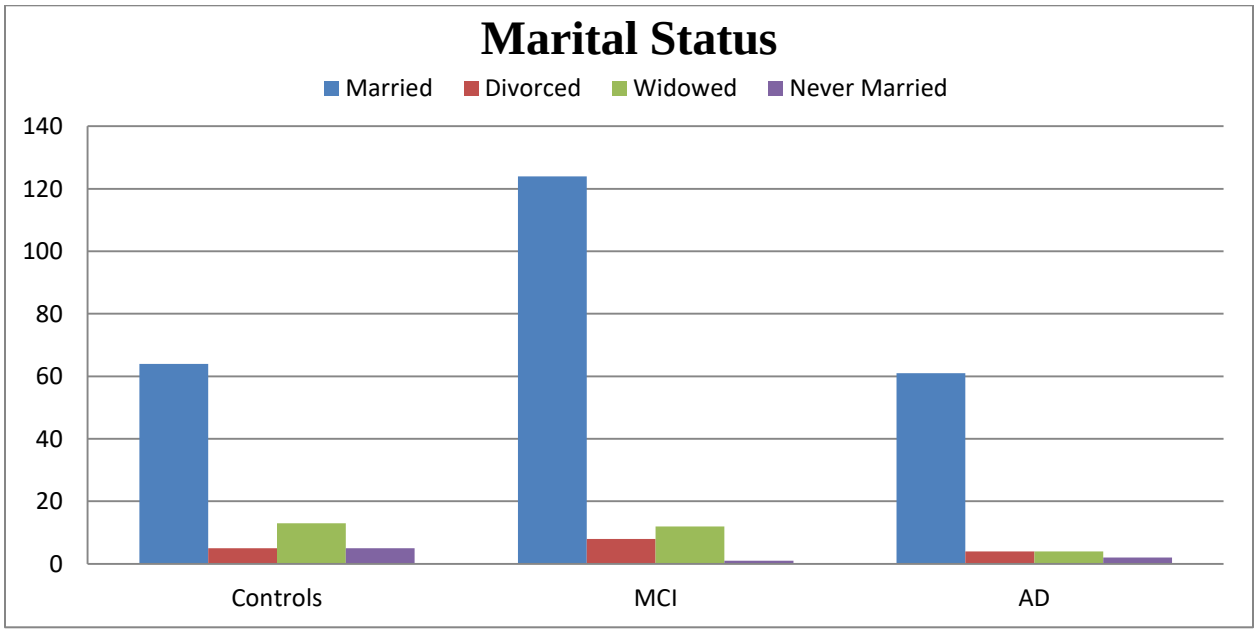


Figure 13 displays the number of control, MCI and AD subjects that were married, divorced, widowed or never married for the CSF cohort.

Diagnosis	Married	Divorced	Widowed	Never Married
Controls	**64**	**5**	**13**	**5**
MCI	**124**	**8**	**12**	**1**
AD	**61**	**4**	**4**	**2**

Table 9: The number of subjects is shown against their marital status as married, divorced, widowed or never married in the CSF cohort.

4.2 Genotypic Results

A number of genes are known to play a role in triggering Alzheimer's disease but one of the most important and established genetic risk factor is *APOE* allele 4. Apolipoprotein E (APOE) protein involved in fats metabolism and is the principle cholesterol carrier in brain. The APOE alleles (E2, E3 and E4) of the control, MCI and AD subjects of both, CSF and blood, cohorts are displayed as the following graphs.

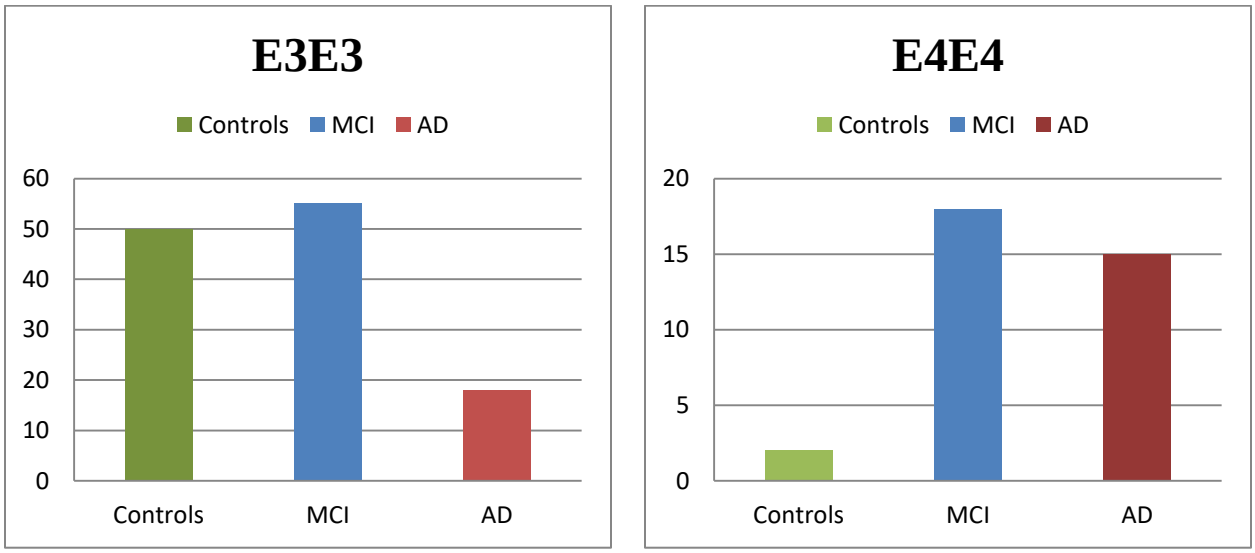


Figure 14: Graphical display of the number of controls (in green), MCI (in blue) and AD (in red) subjects with *E3E3* and *E4E4* allelic combinations of *APOE* gene for CSF cohort.

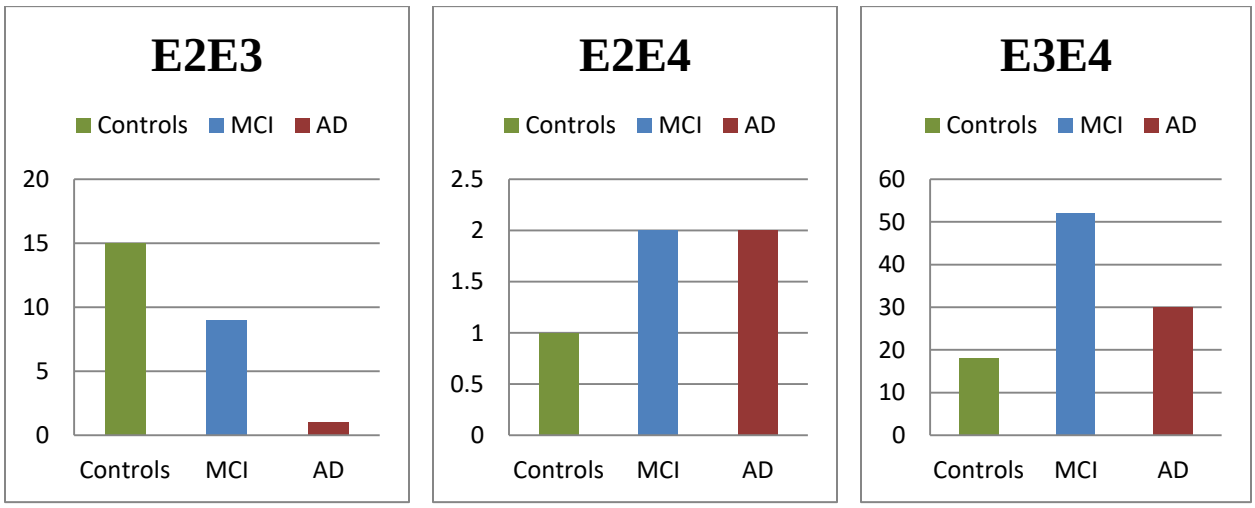


Figure 15: Graphical display of the number of controls (in green), MCI (in blue) and AD (in red) subjects with *E2E3*, *E3E4* and *E2E4* allelic combinations of *APOE* gene for CSF cohort.

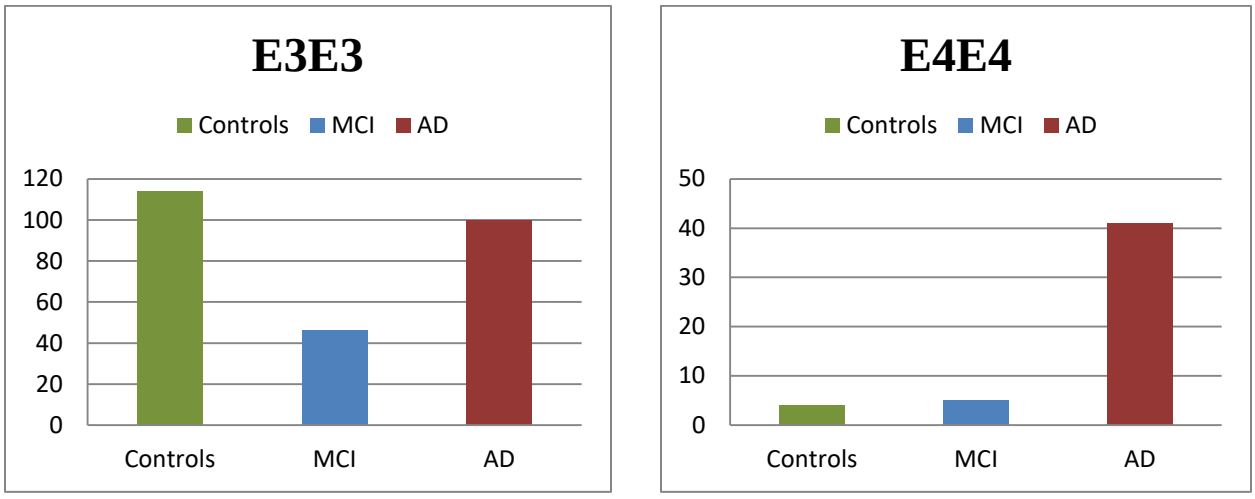


Figure 16: Graphical display of the number of controls (in green), MCI (in blue) and AD (in red) subjects with *E3E3* and *E4E4* allelic combinations of *APOE* gene for blood cohort.

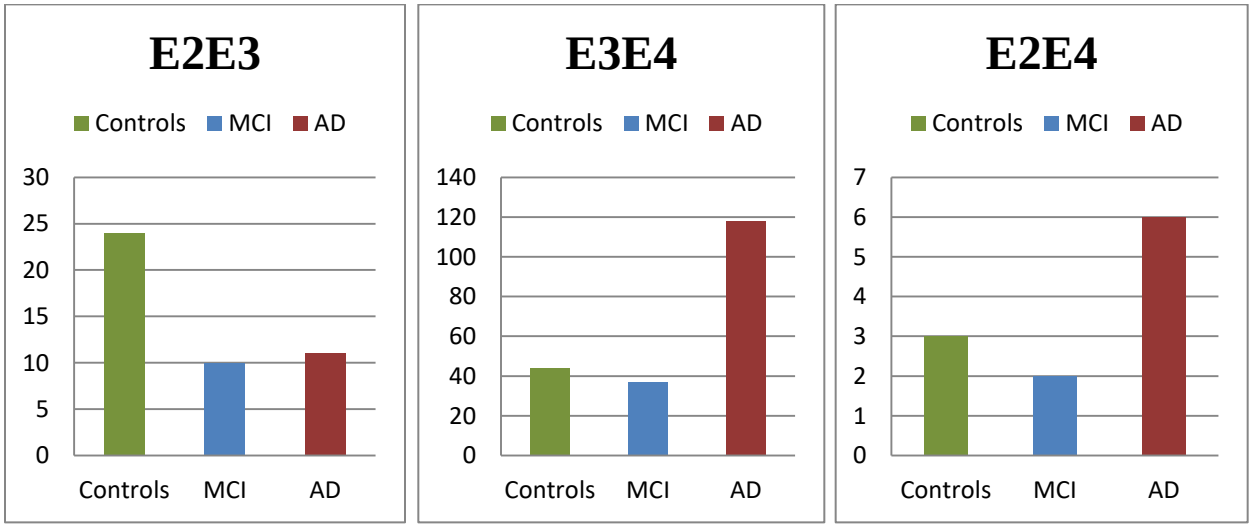


Figure 17: Graphical display of the number of controls (in green), MCI (in blue) and AD (in red) subjects with *E2E3*, *E3E4* and *E2E4* allelic combinations of *APOE* gene for blood cohort.

4.3 Proteomic Results

Proteome is protein analogue of the genome, and the term itself is the amalgamation of the two words. It is the detailed study of the complete protein expression of an individual. Proteomic study vary from genomic studies in that the proteome is dynamic and keeps changing over time and conditions, whereas the genomic composition mostly remains. Identifying an unusual pattern

of protein expression is called proteomic signature. In our study, we used differential proteomic analysis to identify a signature shown in **Figure 18**

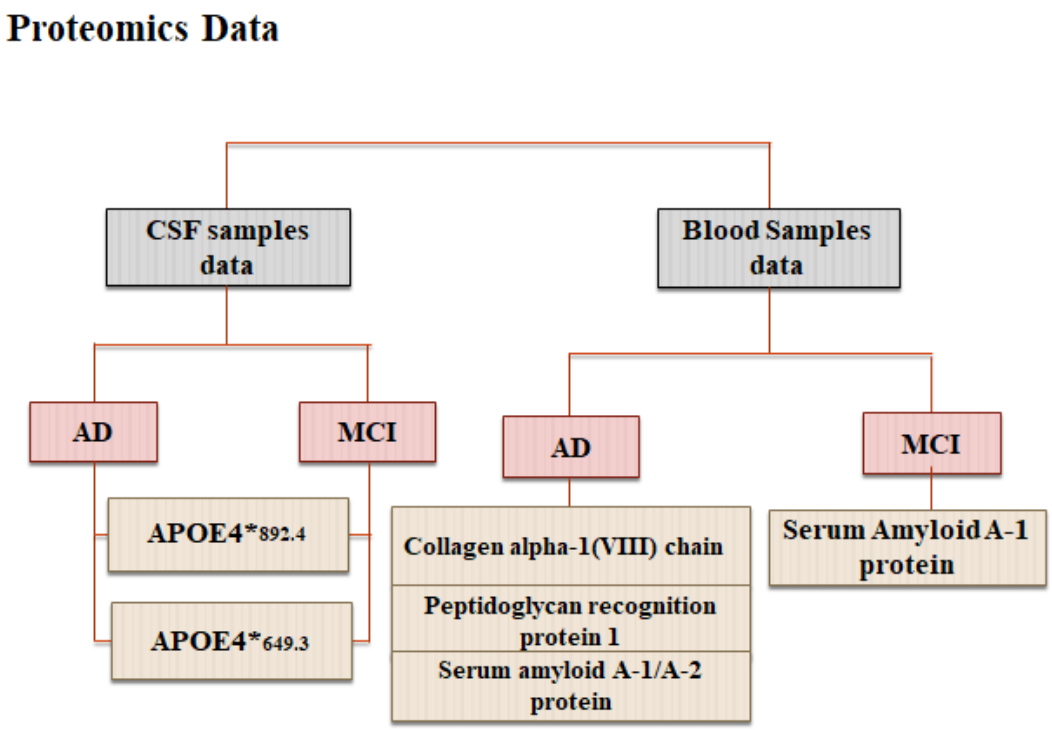


Figure 18: A flow chart explaining the obtained proteomic signature from CSF and blood samples data. AD and MCI CSF samples had significantly up-regulated APOE4 proteins, while MCI blood had highly expressed Serum Amyloid A-1 protein and AD blood samples had highly expressed collagen alpha-1 (VIII) chain, peptidoglycan recognition protein 1, and Serum Amyloid A-1/A-2 protein.

In CSF samples, APOE4 protein was found to be highly up-regulated in MCI and AD subjects as compared to controls. Two peptides for APOE4 were obtained, one APOE4$^{*892.4}$ slightly heavier than APOE4$^{*649.3}$ due to differential slicing and post translational modification of the peptide chain during Mass Spectrometry as shown in **figure 19**.

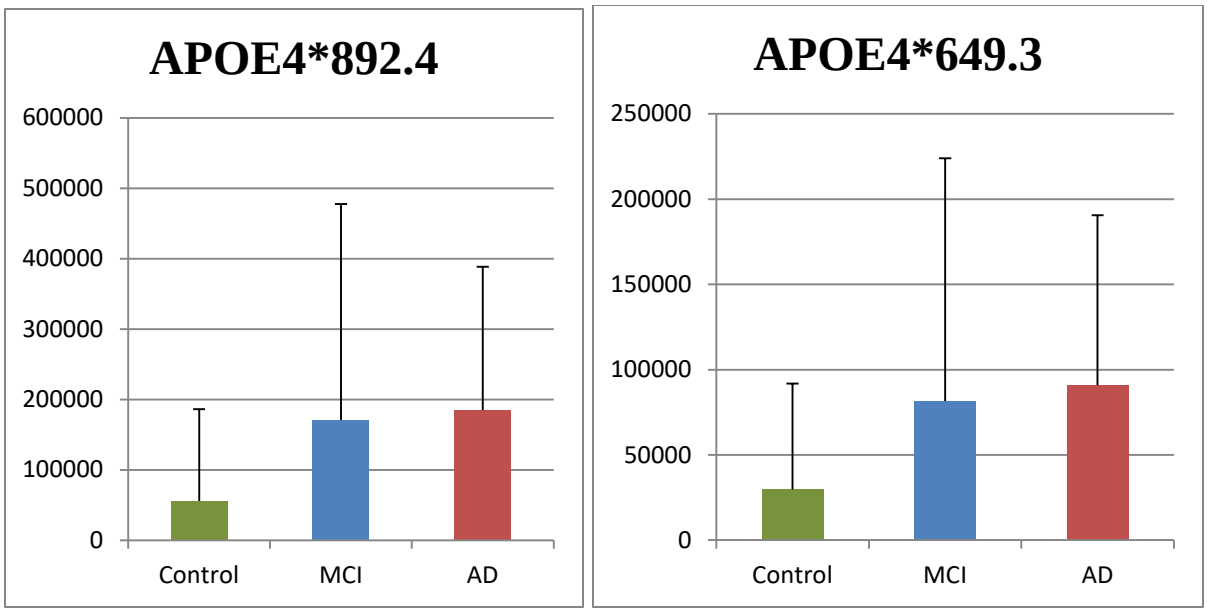


Figure 19: The graphs represent significantly high abundance of APOE4 (In two forms APOE4$^{*892.4}$ and APOE4$^{*649.3}$) in MCI (in blue) and AD (in red) subjects as compared to non-demented controls (in green).

In blood samples, MCI subjects had significantly up-regulated Serum Amyloid A-1 protein in their plasma as compared to non-demented controls.

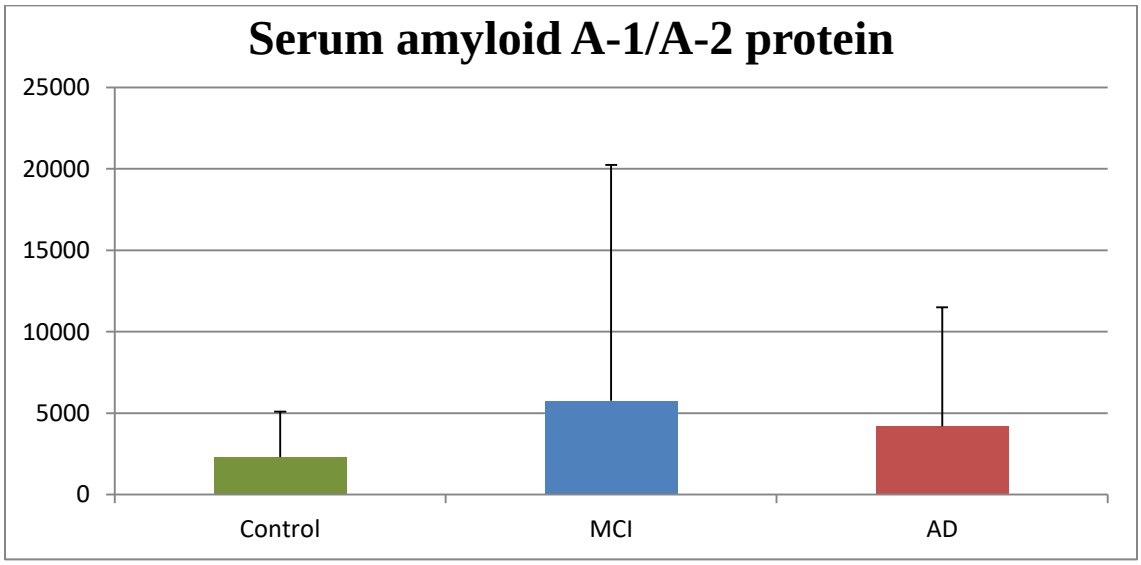


Figure 20: Graphical representation of significantly high abundance of Serum Amyloid A-1/A-2 in MCI (in blue) and AD (in red) subjects as compared to non-demented controls (in green) in plasma of the blood cohort.

On the other hand, AD blood samples had three protein that were significantly up-regulated as compared to non-demented controls, namely, collagen alpha-1 (VIII) chain, peptidoglycan recognition protein 1, and Serum Amyloid A-1/A-2 protein shown in **figure 20, 21** and **22** respectively.

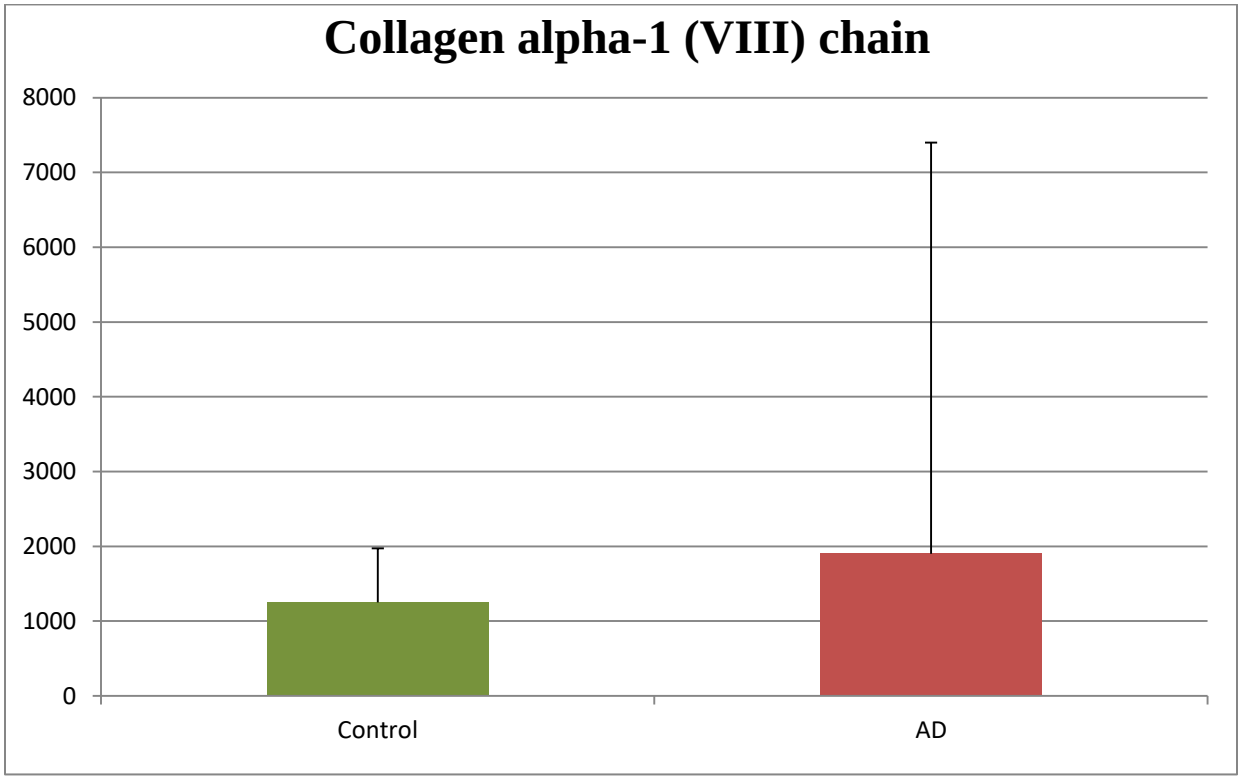


Figure 21: Graphical representation of significantly high abundance of collagen alpha-1 (VIII) chain in MCI (in blue) and AD (in red) subjects as compared to non-demented controls (in green) in plasma of the blood cohort.

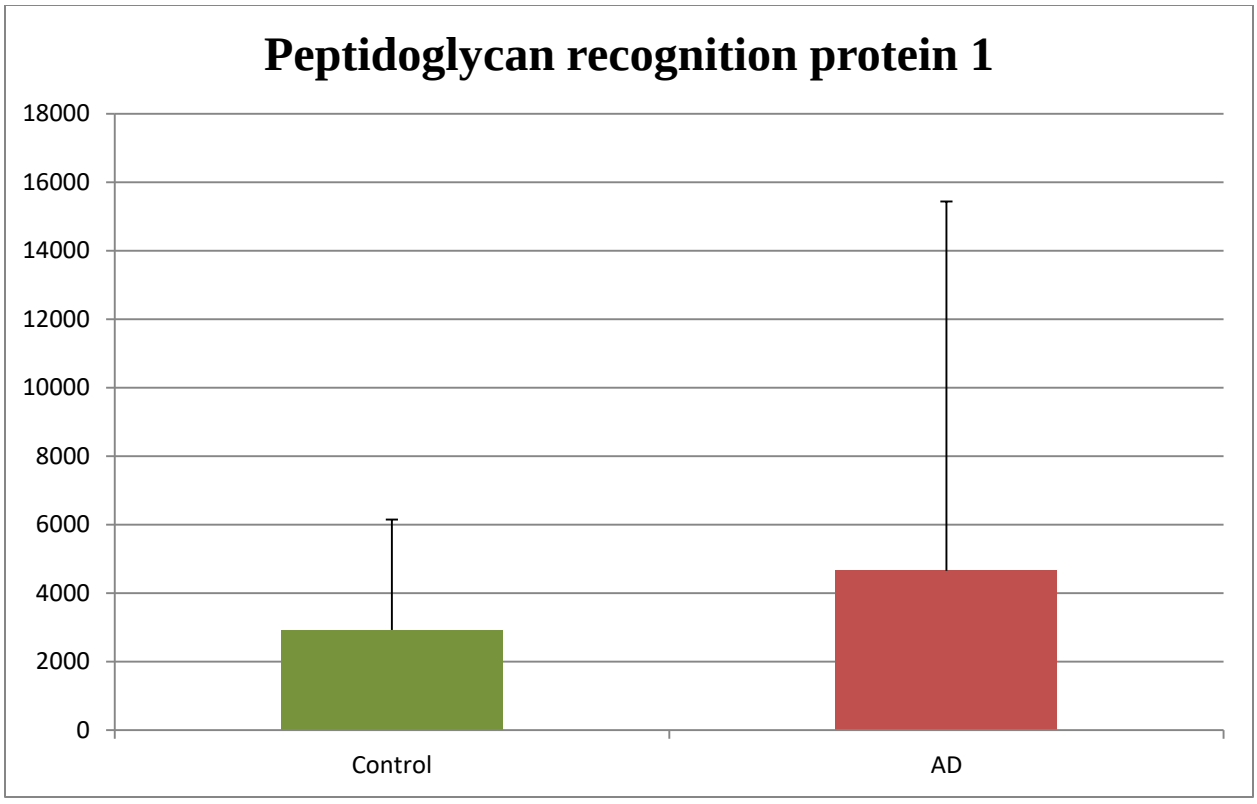


Figure 22: Graphical representation of significantly high abundance of peptidoglycan recognition protein 1 in MCI (in blue) and AD (in red) subjects as compared to non-demented controls (in green) in plasma of the blood cohort.

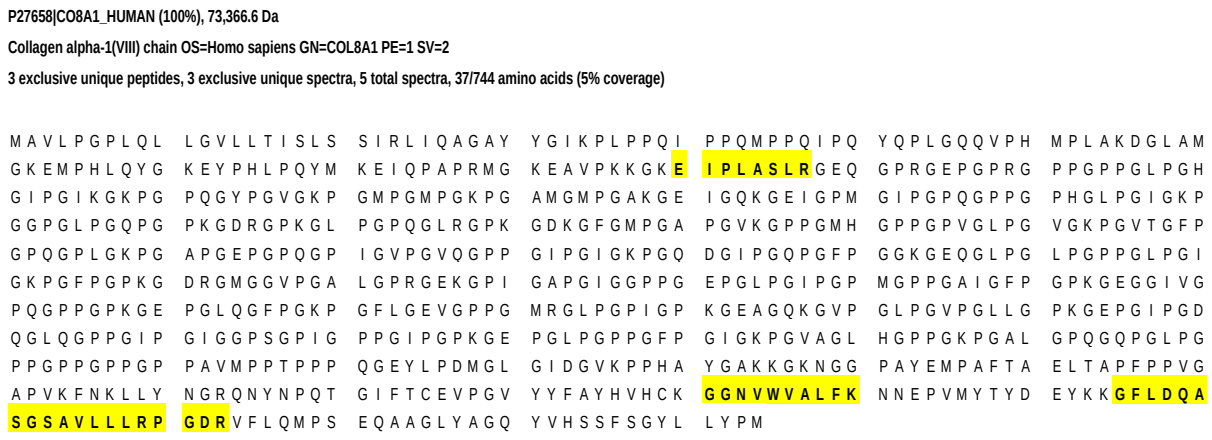
The numerical values for abundances of each protein in the proteomic signature are given in the **table 10**.

	AD	MCI	Controls
APOE4*649.3	90879.76	81449.84	29784.68
APOE4*892.4	185070.1	170684.9	56121.27
Serum Amyloid A-1 Protein	4193.032	5752.28	2296.087
Collagen alpha-1 (VIII) chain	1901.613	1370.12	1251.485
Peptidoglycan recognition protein 1	4657.526	3170.446	2923.17

Table 10: The abundances of each protein is given in tabulate form of AD, MCI and controls.

4.4 Validation Results

The above mentioned protein signature obtained in this study was validated for sensitivity and specificity to Alzheimer's disease in the laboratory. APOE4 of CSF cohort is extensively validated through literature. So, each protein of the blood cohort was validated through Mass Spectrometry. Our results confirmed the significant over-expression of Collagen alpha-1 (VIII) chain protein.


P27658|CO8A1_HUMAN (100%), 73,366.6 Da

Collagen alpha-1(VIII) chain OS=Homo sapiens GN=COL8A1 PE=1 SV=2

3 exclusive unique peptides, 3 exclusive unique spectra, 5 total spectra, 37/744 amino acids (5% coverage)

MAVLPGPLQL LGVLLTISLS SIRLIQAGAY YGIKPLPPQI PPQMPPQIPQ YQPLGQQVPH MPLAKDGLAM
GKEMPHLQYG KEYPHLPQYM KEIQPAPRMG KEAVPKKGKE IPLASLRGEQ GPRGEPGPRG PPGPPGLPGH
GIPGIKGKPG PQGYPGVGKP GMPGMPGKPG AMGMPGAKGE IGQKGEIGPM GIPGPQGPPG PHGLPGIGKP
GGPGLPGQPG PKGDRGPKGL PGPQGLRGPK GDKGFGMPGA PGVKGPPGMH GPPGPVGLPG VGKPGVTGFP
GPQGPLGKPG APGEPGPQGP IGVPGVQGPP GIPGIGKPGQ DGIPGQPGFP GGKGEQGLPG LPGPPGLPGI
GKPGFPGPKG DRGMGGVPGA LGPRGEKGPI GAPGIGGPPG EPGLPGIPGP MGPPGAIGFP GPKGEGGIVG
PQGPPGPKGE PGLQGFPGKP GFLGEVGPPG MRGLPGPIGP KGEAGQKGVP GLPGVPGLLG PKGEPGIPGD
QGLQGPPGIP GIGGPSGPIG PPGIPGPKGE PGLPGPPGFP GIGKPGVAGL HGPPGKPGAL GPQGQPGLPG
PPGPPGPPGP PAVMPPTPPP QGEYLPDMGL GIDGVKPPHA YGAKKGKNGG PAYEMPAFTA ELTAPFPPVG
APVKFNKLLY NGRQNYNPQT GIFTCEVPGV YYFAYHVHCK GGNVWVALFK NNEPVMYTYD EYKKGFLDQA
SGSAVLLLRP GDRVFLQMPS EQAAGLYAGQ YVHSSFSGYL LYPM

Figure 23: Raw MS/MS data for Collagen alpha-1 (VIII) chain from scaffold database is shown as a peptide sequence with 3 exclusive peptide fragments, that were detected, highlighted (in yellow). The Collagen alpha-1 (VIII) chain protein from *Homo sapiens* has a weight of 73,366.6 Da. The three exclusive unique peptides include EIPLASLR, GGNVWVALFK and GFLDQASGSAVLLLRPGDR, from which 3 exclusive unique spectra were identified with 37/744 amino acids of the sequence and 5% coverage.

Valid	Weight	Sequence	Prob	Mascot Ion score	Masc...	Masc...	NTT	Modifications	Observed	Actual Mass	Charge	Start	Delta Da	Stop	Delta PPM	# Oc...	Rete...	Other Prot...	Intensity	Spectrum ID	TIC
☑	1.0	(K)EIPLASLR(G)	99%	23.6	25.0	23.6	2		449.77	897.53	2	110	-0.0013	117	-0.14	0				Elution from: 27.75 to 29.23 period: N_Youness_2111...	545100
☑	1.0	(K)EIPLASLR(G)	98%	20.6	25.0	20.6	2		449.77	897.53	2	110	-0.0013	117	-0.14	0				Elution from: 27.75 to 29.23 period: N_Youness_2111...	1617000
☑	1.0	(K)GGNVWVALFK(N)	100%	47.0	25.0	40.6	2		545.81	1,089.60	2	671	-0.0089	680	-0.82	0				Elution from: 38.13 to 38.38 period: N_Youness_2111...	387600
☑	1.0	(K)GFLDQASGSAVLLLRPGDR(Y)	100%	54.9	25.3	54.3	2		658.02	1,971.05	3	695	-0.0011	713	-0.55	0				Elution from: 34.88 to 35.30 period: N_Youness_2111...	975600
☑	1.0	(K)GFLDQASGSAVLLLRPGDR(Y)	99%	23.7	25.3	23.7	2		658.02	1,971.05	3	695	-0.0011	713	-0.55	0				Elution from: 34.88 to 35.30 period: N_Youness_2111...	445300

Figure 24: Tabulate form of the peptide sequence with highlighted detected peptide shown in figure 23. This table shows the probability and the amino acid sequence and the sequence coverage of Collagen alpha-1 (VIII) chain protein.

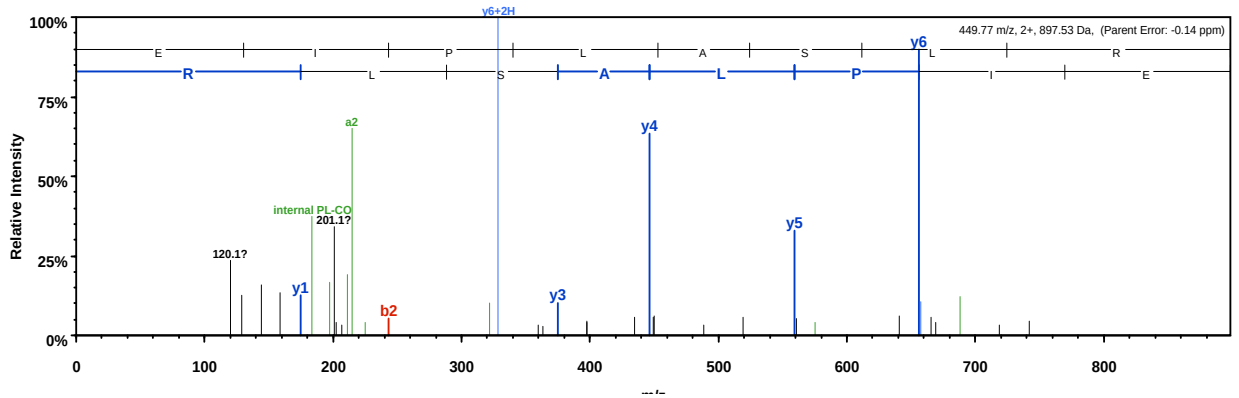


Figure 25: A sample of the spectrum from the Mass Spectrometry results for Collagen alpha-1 (VIII) chain is shown as a graph. The mass to charge ratio (m/e) is displayed on the x-axis while corresponding relative intensities of each m/e is shown on y-axis. The graph also numbers the b-ions from left to right on the N-terminus, while y-ions are numbered right to left on the C-terminus.

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	130.0			112.0	E	898.5	449.8	881.5	880.5	8
2	243.1			225.1	I	769.5	385.3	752.5	751.5	7
3	340.2			322.2	P	656.4	328.7	639.4	638.4	6
4	453.3			435.3	L	559.4		542.3	541.3	5
5	524.3			506.3	A	446.3		429.2	428.3	4
6	611.3	306.2		593.3	S	375.2		358.2	357.2	3
7	724.4	362.7		706.4	L	288.2		271.2		2
8	898.5	449.8	881.5	880.5	R	175.1		158.1		1

Figure 26: This is a spreadsheet version of the graph in figure 25 for Collagen alpha-1 (VIII) chain, called the fragmentation table. The ions corresponding to the peaks of the sample spectrum in figure 25 are colored coded as red for red for b-ions, blue for y-ions and green for ions that are neutral or lost.

Collagen alpha-1 (VIII) chain protein was then validated using Sandwich Eliza kit (Human Pro-Collagen I alpha 1 ELISA Kit (ab210966) by SimpleStep ELISA®). It was found that the target protein was highly expressed in Alzheimer's while in non-demented controls, the protein expression remains normal (**Figure 27**).

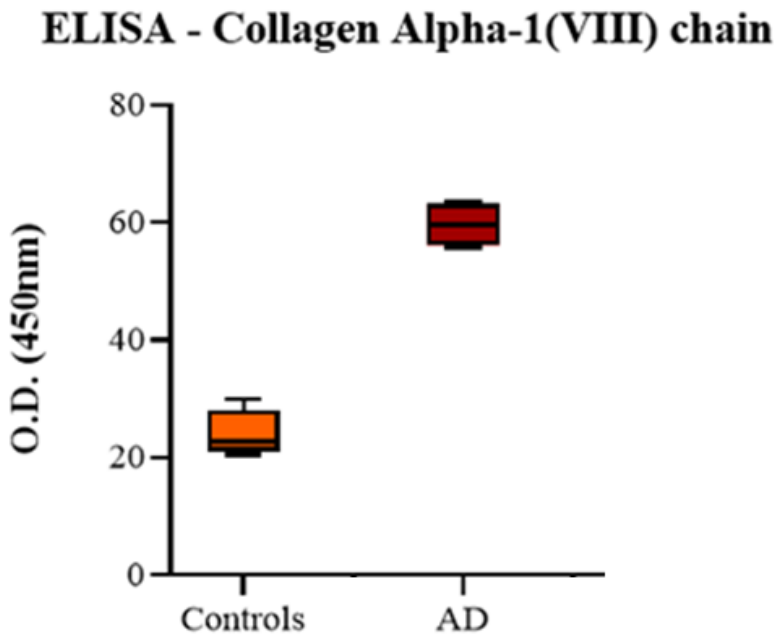


Figure 27: The box and whiskers plot represents the abundances of Collagen alpha-1 (VIII) chain against non-demented controls. The observance for AD shows significantly higher expressions as compared to MCI, DLB and controls.

To conclude our results, in the phenotypic data analysis, age remains to be a strong risk factor. For AD and MCI, genotypic analysis of APOE gene conformed to previously reported results of E4 allele being the more common allelic form of APOE as compare E2 and E3. In our proteomic analysis, APOE4 protein was significantly up-regulated in the CSF samples for both AD and MCI from ADNI cohort. In blood samples from ANM cohort, Serum Amyloid A-1/2 protein was significantly over-expressed in both AD and MCI cases. In AD blood samples, Peptidoglycan recognition protein and Collagen alpha-1 (VIII) chain protein were also significantly up-regulated. Lastly, Collagen alpha-1 (VIII) chain shows significantly high expression after validation in blood samples.

5. Discussion

5.1 Phenotypic Data

In our study, age remains to be a proven risk factor (L E Hebert et al. 2010), and the number of AD and MCI cases increase with each increasing age bracket. The earliest reported case for both AD and MCI is >55 years of age in both datasets. However, AD and MCI cases appear in increasing numbers after the age of 65, with the highest number of AD and MCI patients falling in the age brackets of >70 to 85 years for both, ADNI and ANM datasets. This increasing trend of cases as the age increases is validated by previous studies (Liesi E Hebert et al. 2013).

Women are reported to develop AD more than men (Liesi E Hebert et al. 2013). In contrast in our study, the ADNI dataset reports 2:1 male to female ratio for MCI cases. AD numbers are also higher in males than females as compared to controls, in which case the male to female ratio is almost equal. In ANM dataset however, MCI male to female ratio is 3:2, while AD male to female ratio is 1:2. The higher number of female AD cases in ANM cohort is in accordance with a previous study (2020 Alzheimer's disease facts and figures 2020). This higher incidence in women is partly credited to woman living longer than men, and age is known to be a more influential risk factor for developing AD.

Most of the subjects in both datasets had MMSE score of 25-30 showing normal cognition. In ADNI cohort, 8.27% of the MCI and 60.6% of the AD patients exhibit mild dementia (21-24 mmse score), while 5.6% of the AD patients exhibit moderate dementia (10-20 mmse score). In ANM cohort, 10.3% of the MCI patients and 29.4% AD patients had mild dementia, while 0.6% of the MCI patients and 50.74% of the AD patients exhibited moderate dementia. This higher number of a more severe dementia cases in AD as compared in MCI in ANM cohort can be explained by increasing neuro-degeneration by the formation of amyloid beta plaques and tau neurofibrillary tangles as the disease progresses.

In ADNI cohort, marital status is also investigated. Married people are reported to have half the risk of developing AD compared to people who never married, while divorced or widowed subjects who stay such are at three times risk of developing AD as compared to married subjects (Håkansson et al. 2009). In our study it is found that the number of married MCI cases is almost

twice that of married control and married AD cases. 85% of MCI cases and 85.9% of AD cases were married, showing marriage to be a risk factor for MCI and AD which is in contrast to the results reported in previous studies. However, the deviation from previously reported studies can be explained by the fact that in the ADNI dataset, the major proportion of the cohort consists of subjects that were married (82%) compared to divorced, widowed and never married which comprised a combined percentage of 18%.

From the analysis done so far on the two datasets, between age, gender, and marital status, only age seems to play a role in developing AD. Gender and Marital status does not seem to have much influence on the development on the disease.

5.2 Genotypic Data

A number of genes are reported to increase the risk of AD, however, the only gene to have an established risk associated with the development of the AD is Alipoprotein-e4 (APOE-e4) gene. APOE protein is involved in fats metabolism and is the principle cholesterol carrier in brain. It has three allele e2, e3 and e4. e4 allele is reported to increase the risk of AD compared to e3 allele. In the ADNI dataset, there are 14.6% of e3e3 and 4% of e2e3 genotype are AD cases, compared to 30% of e3e4, 40% of e2e3 and 42.8% of e4e4. In the ANM dataset, similarly, presence of e4 allele raises the AD cases from 38% of e3e3 and 24% of e2e3 to 54.5% of e2e4, 59% of e3e4 and 82% of e4e4. Increased number of AD in subjects with e4 allele is due to accumulation of beta amyloid in their brains (Jansen et al. 2015). They also develop dementia at a younger age compared to subjects with e2 and e3 allele (Spinney 2014).

5.3 Proteomic Data

Analysis of the two datasets produced a signature of four proteins. APOE4 protein was found to be significantly up-regulated in cohort for both AD and MCI. In the blood cohort, Serum Amyloid A-1 protein was significantly up-regulated in both AD and MCI. However, AD from blood cohort also had a significantly high expression of peptidoglycan recognition protein -1 (PGLYRP1) and Collagen alpha-1 (VIII) chain, as well.

APOE4

APOE4 was significantly up-regulated in CSF samples for both AD and MCI. The over-expression of this protein is vastly reported and validated in studies on Alzheimer's disease. As mentioned before, APOE4 expression has been associated with increased risk of developing the disease and its early onset (Hunsberger et al. 2019). APOE protein is involved in fats metabolism being the principle cholesterol carrier in brain. The exact mechanism for how APOE4 increases the risk of AD is not well understood. However, researches have attempted to investigate the reason and it is believed the problem may be of the inability or abnormal fat processing in the brain cells' which plays a role in Alzheimer's disease. It is still unclear if APOE4 loses a neuroprotective role and/or attains a more toxic one (Kim, Basak, and Holtzman 2009). A recent study suggests APOE4 disturb lipid homeostasis which results in accumulation of unsaturated fats and lipid droplets in iPSC-derived astrocytes (Sienski et al. 2021).

Serum Amyloid A-1/A-2

Serum Amyloid A-1/A-2 proteins (SAA1/2) exhibited significantly high expression in blood for both AD and MCI in our study. Serum amyloid proteins are involved in amyloid fibril formation, which is a normal process. However, diseases such as Alzheimer's disease occur when there is more amyloid formation compared to clearance.

Studies suggest that CNS glia are triggered to produce acute phase protein and mast cells chemo-attractants like SAA proteins as a result of inflammation in the AD brain to attract mast cells to sites of amyloid aggregation (Jones, Nair, and Gupta 2019). SAA1 intensifies amyloid accumulation and also leads cognitive decline. It also exacerbates the decline in memory by intensifying neuronal inflammation in conditions with abundant amyloid aggregates (Jang et al. 2019).

Peptidoglycan recognition protein (PGLYRP1)

Peptidoglycan recognition protein is an innate immunity protein Tag7 with many roles to strengthen the body defense mechanisms against tumors and microbial attacks. PGLYRP1 primary function is bactericidal by binding to its receptor, murein peptidoglycans, in Gram-positive bacteria (Kang et al. 1998).

It also has a role in triggering programmed cell death by forming a complex with HSPA1A, which is also called heat shock protein 70 (HSP70), by activating TNFR1 receptor in the membranes of tumor cells (Yashin et al. 2015). Alzheimer's disease commonly exhibits protein misfoldings which are prone to producing toxic accumulates. HSP70 is a known for playing important functions against protein misfolding and preventing aggregations. HSP70 is considered a therapeutic target for arresting AD progression and treating it (Lu et al. 2014). However, once it forms a Tag7-HSP70 complex with PGLYRP1, it interacts with TNFR1 receptor and activates calpins, which makes lysosomal membranes porous, initiating necroptosis in tumor cell (Yashin et al. 2016). High expression of PGLYRP1 may point to the formation of a similar complex with HSP70 with a necroptosis function.

PGLYRP1can also induce lymphocyte migration, especially the NK cells towards areas of infection to kill the virus (Dukhanina et al. 2015)or the cells infected by it, or tumor cells (Sharapova, Ivanova, Prasolov, et al. 2017; Sharapova, Ivanova, Soshnikova, et al. 2017). These lymphocytes and NK cells may help clear the amyloid aggregation through phagocytic activity.

Monocyte cell surface receptor TREM-1, an immunoglobulin receptor, interacts with PGLYRP1 to induce cytotoxicity (Read et al. 2015; Sharapova, Ivanova, Soshnikova, et al. 2017). TREM-1 receptor is also expressed on microglia and its variant rs6910730^{G} is known to play a part in AD pathology. The high expression of PGLYRP1 receptor can be explained on the basis of this cytotoxic activity with TREM1. It has been reported that TREM1 regulates amyloid beta clearance by phagocytosis through monocytes in the CNS. On the other hand, its variant rs6910730^{G} encourages AD pathogenesis by inhibiting this phagocytic activity of TREM1 (Saadipour 2017).

Collagen Alpha-1 (VIII) chain

Collagen Alpha-1 (VIII) chain protein (COL8A1) is significantly up-regulated in the blood of AD cases. It is the only protein in the signature acquired in this study from blood proteins that was validated for sensitivity and specificity in the laboratory. COL8A1 is the core constituent of the basement membrane of the corneal endothelial cells (Xu et al. 2001). It may be involved in AD pathology like Collagen XXV alpha 1 (COL25A1), which is expressed in neurons and bind to amyloid aggregates (Tong et al. 2010). COL8A1 is also expressed during repair process in

mast cells and astrocytes which are involved in Alzheimer's disease inflammatory response (González-Reyes et al. 2017; Rüger et al. 1994; Shaik-Dasthagirisaheb and Conti 2016)

Conclusion

To conclude, our study has analyzed datasets from ADNI and ANM to demonstrate a protein signature for AD and MCI in different cohorts. In the phenotypic data analysis, age remains to be a strong risk factor. For AD and MCI, genotypic analysis of APOE gene conformed to previously reported results of E4 allele being the most common allelic form as compare E2 and E3. Furthermore APOE4 protein was significantly up-regulated in the CSF samples for both AD and MCI from ADNI cohort. The exact mechanism of its risk to AD is unknown and more studies are required to understand the reason behind its increased risk and early onset property. In blood samples from ANM cohort, Serum Amyloid A-1/2 protein was significantly over-expressed in both AD and MCI cases. Serum amyloid proteins have a role in amyloid fibril formation and neuroinflammation in areas of high amyloid deposition. In AD blood samples, Peptidoglycan recognition protein and Collagen alpha-1 (VIII) chain were also significantly up-regulated. The primary function of Peptidoglycan recognition protein is to strengthen body defenses against microbial attack and tumors. It is known to interact with several enzymes and proteins such as HSP70 that are involved in neuroprotection in Alzheimer's disease. Lastly, Collagen alpha-1 (VIII) chain shows significantly high expression after validation in blood samples.

The protein signature obtained in this study requires validation and further studies to gain more useful insights into their underlying mechanism and role in development of AD. This signature may be potentially used as a diagnostic tool for AD and MCI, specifically the three proteins from ANM data can be useful in developing a less invasive blood test for diagnosis of AD.

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