### Isolation and Characterization of Distinct Populations of Amyloid β

## Aggregates Using Size Exclusion Chromatography and Structure-Specific

## Antibody

by

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#### Abstract

One of the most significant challenges in the study of Alzheimer's disease (AD) is the elucidation of causative agents. AD belongs to a family of diseases characterized by proteinopathy-misfolded amyloidogenic proteins forming aggregates. Pathogenic amyloid aggregates are capable of prion-like propagation, serving to seed template misfolding and propagate aggregation. Literature strongly implicates amyloid- $\beta$  aggregates (A $\beta$ ) as causative agent in AD, impairing synaptic transmission, causing cell death, and serving to propagate cytotoxic insults. Canonical protein aggregation proceeds in three distinct stages: with misfolding of initial monomers, followed by spontaneous formation of oligomers, and ending with deposition of insoluble fibrils as AD plaques in the brain. A $\beta$  oligomers (A $\beta$ O) have been shown to exert the most potent toxicity and propagation of aggregation both *in vitro* and *in vivo*, with monomers and fibrils being orders of magnitude weaker. As literature suggests the structure of amyloidogenic proteins confer toxicity; great efforts are being made to understand their structural and functional biology, including Aβ. However, Aβ aggregation is complex and nonlinear, and the transience of oligomeric populations in solution hamper efforts to identify specific disease-causing isoforms. Literature also shows significant differences in toxicity and aggregation kinetics between synthetic and biologically-derived A $\beta$ Os. As A $\beta$ O causes AD, great efforts are being made to neutralize aggregate activity, with therapeutic antibodies at the forefront. However, all antibodies that underwent clinical trials up to date have failed to meet their efficacy endpoints, despite showing plaque clearance. These antibodies targeted regions of A $\beta$  sequence, which led to broad spectrum reactivity to A $\beta$  monomers and aggregates. This observation emphasizes the importance of elucidating ABO-specific epitopes for targeted therapy. It is thus imperative to identify and isolate individual populations of ABOs from both

synthetic preparations and biological sources for target determination. In my project, size exclusion chromatography (SEC) was used to separate and collect A $\beta$  aggregates by size, and the seeding ability of various A $\beta$ Os was examined using Thioflavin-T assay. Finally, surface plasmon resonance (SPR), a state-of-the-art label-free technology that monitors protein-protein interactions in real-time, was employed to determine the specificity of antibodies developed inhouse targeting structural features unique to A $\beta$ O.

### Lay Summary

Alzheimer's disease (AD) is the most common form of progressive senile dementia. Evidence strongly implicates amyloid-beta (A $\beta$ ), a protein fragment naturally produced in the brain, as the cause of AD. A $\beta$  is an amyloidogenic protein, which, when misfolded, can aggregate into oligomers, causing damage and seeding further aggregation. Studies on A $\beta$ aggregate structure are complicated by multiple species existing simultaneously in equilibrium, each with a different level of toxicity and seeding activity. Thus, it is imperative to pinpoint one or several species for study. In this study, A $\beta$  oligomers will be separated by size exclusion chromatography, and seeding ability will be analyzed by the thioflavin-T assay. Using an antibody—protein that targets a specific protein structure, I will examine oligomers by filter trap assay. Finally, I will utilize surface plasmon resonance, a state-of-the-art label-free method, to analyze aggregate-antibody binding interaction in real-time, advancing knowledge in AD therapeutic development.

### Preface

The entirety of this thesis was conducted at Dr. Neil Cashman's laboratory in the Department of Medicine at the University of British Columbia Point Grey campus.

Protocol for generating  $A\beta$  oligomers from recombinant peptide used in this thesis was provided to me by Dr. Ebrima Gibbs. Brain homogenates used in this thesis were provided to me by Dr. Andrei Roman. I generated the  $A\beta$  oligomers and designed, as well as carried out, the experiments, data collection, and data analysis in Chapter 3.

Formulas for Thioflavin-T assay data analysis were provided by Dr. Luke McAlary. Mouse PMN310 antibody was provided by Dr. Ebrima Gibbs. I designed and carried out the experiments, data collection, and data analysis in Chapter 4.

Chapter 5 of this thesis was conducted in collaboration with Dr. Ebrima Gibbs. I was responsible for the generation of A $\beta$  oligomers and preparation for experimentation. The experimental design was done in collaboration with Dr. Ebrima Gibbs, who also carried out the testing, data collection, and analysis. I am truly grateful for the contribution and help from Dr. Ebrima Gibbs in supporting the completion of my thesis.

This thesis aimed to examine the binding of PMN310, an antibody targeting for conformation-specific to A $\beta$  oligomers, to different oligomer species, with a preference for smaller, low molecular weight species. Chapter 3 characterized the heterogeneous oligomer population generated from recombinant A $\beta$  at different incubation lengths, as well as from human brain homogenates. The second hypothesis was that different A $\beta$  oligomer species exhibit different pathogenic activity, which was tested in Chapter 4, along with examining PMN310 binding to these species. Chapter 5 sought to test the hypothesis using an alternative methodology to characterize the samples examined in chapter 3 and 4.

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## List of Abbreviations

ABC: ATP-binding cassette transporter AChE: acetylcholinesterase AChEI: acetylcholinesterase inhibitor AD: Alzheimer's disease ADAM: a disintegrin and metalloproteinase ADDL: Aβ-derived diffusible ligand Akt: Ak strain transforming ALS: amyotrophic lateral sclerosis APH-1: anterior pharynx-defective 1 ApoA1: apolipoprotein A1 ApoE: apolipoprotein E APP: Aβ precursor protein ATP: adenosine triphosphate Aβ: amyloid-beta AβO: amyloid-beta oligomer BACE1: β-amyloid cleaving enzyme 1 BBB: blood-brain barrier

BCA: bicinchoninic acid

BDNF: brain-derived neurotrophic factor

BuChE: butyrylcholinesterase

CAA: cerebral amyloid angiopathy

CBF: cerebral blood flow

CNS: central nervous system

CR: caloric restriction

CSF: cerebrospinal fluid

CV: column volume

Da: dalton

DHA: docosahexaenoic acid

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

ECL: enhanced chemiluminescence

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

EOAD: early-onset AD

FA: fatty acid

Fc-receptor: fragment crystallizable region

FDA: United States Food and Drug Administration

FDG: 18F-fluorodeoxyglucose

FTD: frontotemporal dementia

FTIR: Fourier-transform infrared spectroscopy

GABA: gamma-aminobutyric acid

GLUT1: glucose transporter 1

GLUT3: glucose transporter 3

GnHCl: guanidine hydrochloride

HCl: hydrochloride

HFIP: hexafluoroisopropanol

hIgG1: human IgG1

HMW: high molecular weight

HPLC: high-performance liquid chromatography

HRP: horseradish peroxidase

IgG1: immunoglobulin G1

IWG: International Working Group

kDa: kilodalton

LMW: low molecular weight

LTD: long-term depression

LTP: long-term potentiation

mAb: monoclonal antibody

MCI: mild cognitive impairment

MDa: megadalton

min: minute

ml: milliliter

MRI: magnetic resonance imaging

MS: mass spectrometry

mTOR: mammalian target of rapamycin

NaOH: sodium hydroxide

NFL: neurofilament light chain

NIA-AA: National Institute of Aging and Alzheimer's Association

NINCDS-ADRDA: Neurological and Communicative Disorders and Stroke and the Alzheimer's

Disease and Related Disorders Association

nm: nanometer

NMDAR: N-methyl-D-aspartate receptor

NMR: nuclear magnetic resonance

NVC: neurovascular coupling

OSA: obstructive sleep apnea

PA: physical activity

PBS: phosphate-buffered saline

PD: Parkinson's disease

PEN-2: presenilin enhancer 2

PET: positron emission tomography

PI3K: phosphoinositide 3-kinases

PiB: Pittsburg compound B

PICUP: photo-induced cross-linking of unmodified protein

PSEN1: presenilin 1

PSEN2: presenilin 2

pTau: hyperphosphorylated tau

PUFA: polyunsaturated fatty acid

REM: rapid eye movement

RU: response unit

SASP: senescence-associated secretory phenotype

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEC: size exclusion chromatography

Simoa: single-molecule array

SNAP25: synaptosomal-associated protein, 25kDa

SNARE: soluble NSF attachment proteins receptor

SNP: single nucleotide polymorphism

SPR: surface plasmon resonance

SRBD: sleep-related breathing disorder

SREBP2: sterol regulating element-binding protein 2

STY1: synaptotagmin-1

SV-AUC: sedimentation velocity analytical ultracentrifugation

TBI: traumatic brain injury

TBS: tris-buffered saline

TBS-T: tris-buffered saline-tween-20

ThT: thioflavin-T

tTau: total tau

UV: ultraviolet

μl: microliter

µm: micrometer

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# Dedication

I dedicate this work to my parents, who supported me materially and spiritually, and to God my Lord and Savior, for I did all things through Your power.

### **Chapter 1: Introduction**

#### **1.1 Alzheimer's Disease**

Alzheimer's Disease (AD) is a disease characterized by incurable and progressive neurodegeneration, memory loss, and cognitive decline. AD is the most common form of senile dementia, accounting for 60-70% of dementia and afflicting 47.5 million people worldwide in 2015 (Goure *et al.*, 2014 and Santos *et al.*, 2017). This number is projected to reach over 115 million patients by 2050, with 13.5 million patients projected to be in the US alone and an estimated economic loss of a trillion dollars (Um and Strittmatter, 2013; Cummings *et al.*, 2020; Bateman *et al.*, 2012). A delay of AD onset by five years through therapeutic intervention can reduce the number of AD dementia cases by 57% and nearly half the amount of Medicare spending in the US, from \$627 billion to \$344 billion (Sperling *et al.*, 2011).

Two pathognomonic markers of AD are extracellular neuritic plaques, composed mainly of aggregated amyloid- $\beta$  (A $\beta$ ), and intracellular neurofibrillary tangles, composed of hyperphosphorylated tau (pTau) (Blennow *et al.*, 2006). Evidence strongly suggests that the formation of A $\beta$  aggregates precedes cellular disturbances, which lead to downstream pathologies, such as synaptic dysfunction, excitotoxicity, metabolic disturbances through mitochondrial dysfunction, proteasomal dysfunction, and cell death (Mattson, 2004). The presence of and changes in the level of A $\beta$  aggregates in the brain and CSF are indicative of AD progression and are routinely examined for diagnosis (Trojanowski *et al.*, 2010). Tau aggregates, formed by hyperphosphorylation of tau, exert pathological effects intracellularly, overwhelming proteasome and causing cell death (Jack *et al.*, 2010). However, the presence of hyperphosphorylated tau is not indicative of AD, as pTau is also observed in other neurodegenerative diseases and by itself is sufficient for causing dementia, forming a family of diseases characterized by tauopathies, and evidence has also shown pTau aggregation happens downstream of  $A\beta$  aggregation.

Disease progression proceeds in three distinct stages: preclinical, prodromal, and dementia (Dubois et al., 2010). Patients in preclinical AD-which may begin a decade before diagnosis—are asymptomatic but possess synaptic and cellular disruptions with associated disease biomarkers (Hardy and Selkoe, 2016). These patients progress to the prodromal stage, in which the earliest cognitive deficits became detectable on diagnostic tests (Dubois et al., 2010). The diagnosis of mild cognitive impairment (MCI) in patients potentially with prodromal AD deserves a special mention. MCI is not indicative of AD, serving as a general label of the cognitive deficit without clear disease etiology, and it is mostly used to exclude probable cases of AD that do not sufficiently fulfill the criteria for AD. Patients at the prodromal stage of AD will start experiencing a disrupted quality of life due to memory and attention deficits, along with changes in mood and the development of depression (Breijyeh and Karaman, 2020). Further progress to moderate AD is signified by pronounced memory deficits and aberrant behaviors that are detectable by family members and close associates as the pathology spreads to the cerebral cortex. At this stage, patients will start forgetting the names of friends and families and having difficulties reading, writing, and speaking. In severe AD, degeneration progresses through the rest of the cortex, causing cognitive and functional impairments. Patients will be unable to recognize any former associate and be bedridden, requiring assistance for normal day-to-day functions. Death often occurs as a result of complications from the inability to communicate and difficulties with several activities, such as swallowing, coughing, and urination.

#### **1.2 AD Etiology**

#### 1.2.1 Genetic Factors

Despite the majority of AD diagnoses being sporadic cases, there is a strong genetic linkage to AD pathology. In 1906, Dr. Alois Alzheimer identified an unknown form of pre-senile progressive dementia in a patient in her early 50's, noting the two particular abnormalities plaques and fibrillary tangles—that are now known to be the pathognomonic hallmarks of AD (Thomas and Fenech, 2007). Glenner and Wong observed similar early progressive dementia in Down's Syndrome patients, presenting plaques in their brains, and isolated the substance, identifying it as a 4.2kDa peptide—later termed amyloid-β due to AD plaques displaying similar iodine staining as starch (O'Brien and Wong, 2011). Subsequent isolation of plaque from AD patient brain concluded the substance to be one and the same. Genetic analysis linked this 4.2kDa peptide—which is produced from amyloid precursor protein (APP)—to chromosome 21, which explains AD-like disorders in Down's Syndrome patients with trisomy 21 (Hardy and Selkoe, 2002). Later discovery showed that Down's Syndrome patients with trisomy 21 lacking the APP coding region do not develop AD-like disorders, but individuals with duplication of APP gene result in clinical symptoms. Further studies revealed that mutations in the APP gene could lead to more aggregation-prone cleavage products, and with the discovery of proteases responsible for cleavage of APP (Di Paolo and Kim, 2011), mutations leading to enhanced activity and production of  $A\beta$  have been found in highly-penetrant forms of familial AD. Twin studies in AD lineages illustrated a clear genetic linkage in disease occurrence and onset to mutations within APP and associated proteins, with monozygotic twins having a significantly higher rate compared to heterozygotic twins, both of which are significantly higher rates than unrelated controls (Ertekin-Taner, 2007). Sequencing of the genome of sporadic AD patients also

showed the same mutations present in familial AD playing a role in pathogenesis. A populationwide twin study further demonstrated that sporadic AD has an 80% heritability (Dubois *et al.* 2006). The connection between sporadic and heritable, familial AD, as well as early AD-like symptoms observed in Down's syndrome patients, centered around A $\beta$ , provided strong evidence for the pathogenic roles of A $\beta$ , particularly mutations that lead to increased production or accelerated aggregation.

Aside from mutations in APP and its proteolytic processing, a number of genes have been implicated in AD pathogenesis, with apolipoprotein E (ApoE) emerging as the most significant and substantiated AD-relevant gene (Hardy and Selkoe, 2016). Apo-E is a member of the lowdensity lipoprotein family and acts as a cholesterol transporter. In the CNS, ApoE gene is expressed by astrocytes and comes in three polymorphic alleles—  $\varepsilon_2$ ,  $\varepsilon_3$ ,  $\varepsilon_4$ —with  $\varepsilon_3$  isoform being the most prevalent among the global population, followed by  $\varepsilon 4$  and  $\varepsilon 2$  (Liu *et al.* 2013). However, ApoE ɛ4 allele (ApoE4) is specifically enriched within AD patient populations and is correlated with earlier age of onset and plaque deposition. Carriers of ApoE4 also showed poorer and more rapid cognitive decline and increased progression from MCI to AD. In contrast, ApoE ε2 has a protective effect against AD, having a lower occurrence than ε4 and ε3 (Serrano-Pozo et al., 2021). The effect of ApoE polymorphism is dose-dependent, with  $\varepsilon 4/\varepsilon 4$  having the worst prognosis and  $\varepsilon 2/\varepsilon 2$  having the greatest protection (Bu *et al.* 2013). The mechanism of pathogenesis for ApoE lies in its A $\beta$  clearance activity, capturing the peptide for transport to degradation, with ApoE  $\varepsilon$ 4 binding A $\beta$  least avidly out of the three polymorphs (Arbor *et al.*, 2016). Furthermore, ApoE ɛ4 performs less efficiently as a cholesterol transporter, affecting membrane fluidity, which enhances APP protease activity for A $\beta$  production.

Recent studies also implicated the role of the ATP-binding cassette (ABC) family of transporter proteins in causing a number of neurodegenerative and amyloidogenic diseases, including AD (Hardy and Selkoe, 2016). These proteins are expressed ubiquitously within the body, where a majority of them function in an ATP-dependent manner for the removal and uptake of materials, especially in the liver and kidneys (Behl et al., 2020). ABCs are mainly found on astrocytes, microglia, and parenchymal cells in the CNS, functioning to regulate the movement of materials across the blood-brain barrier (BBB), as well as maintaining lipid and sterol homeostasis. They are responsible for lipidating lipoprotein ApoA1 and ApoE, and dysregulation of ABCs links closely with the lipoprotein-dependent increase in A $\beta$  deposition. Clearance of A $\beta$  from brain parenchyma is mediated by ABC in a transcellular manner, and studies in both mice and post-mortem human brain slices have found that downregulation of ABC transporters correlates with increased A $\beta$  deposition seen in AD, with up to 80% of which also exhibiting cerebral amyloid angiopathy (CAA). Other ABCs work as anion transporters and serve a neuroprotective role in relieving oxidative stress through toxin removal and antioxidant transport. Within the ABC family, ABCA7 has been strongly linked to AD prevalence, with multiple single nucleotide polymorphisms (SNP) shown to increase the risk for AD by influencing APP processing (Bungau et al., 2020). Reduced ABCA7 expression due to SNP accelerates AD symptoms and plaque formation. Studies show that downregulation of ABCA7 leads to increased APP processing through increased cell surface internalization and retention of APP in mitochondria-associated endoplasmic reticulum membrane, where  $\beta$  and  $\gamma$ -secretases are located. Furthermore, ABCA7 mediates  $\beta$ -secretase expression through regulating sterol regulating element-binding protein (SREBP2), preventing APP processing. Expression of ABCA7 in macrophages and microglia is also found to regulate phagocytosis, with an

upregulation observed in AD patient brains that may be explained as a compensatory response to  $A\beta$  burden.

#### 1.2.2 Non-Genetic Factors

#### 1.2.2.1 Effect of Aging

The three major mechanisms by which AD arises: insufficient clearance of  $A\beta$ , increased Aß aggregation and increased Aß production strongly suggest a host of non-genetic risk factors in disease causation. Aging represents the most significant risk factor in neurodegeneration, including AD, as brain clearance and blood-brain barrier integrity decrease with age (Thelen and Brown-Borg, 2020 and Farral and Wardlaw, 2007)). The nine hallmarks of aging are: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, mitochondrial dysfunction, cellular senescence, dysregulated nutrient sensing, stem cell exhaustion, and altered intercellular communication and immune function (Thelen and Brown-Borg, 2020). Ample evidence from the literature has shown many of the mutations observed in familial AD leading to altered A $\beta$  production or aggregation can be present in sporadic cases, thus deterioration of genetic integrity—whether through a buildup of DNA damage or altered DNA repair mechanisms—over time certainly serves as a potential instigator of AD. Furthermore, the degeneration and breakdown of brain vasculature due to cell death and dysfunction of the proteasome, all of which lead to loss of proteostasis, result in impaired clearance and accumulation of waste, including A $\beta$  (Kayed and Lasagna-Reeves, 2013). The buildup of A $\beta$ increases the likelihood for aggregation, leading to downstream cell death, inflammation, and oxidative stress. The link between senescence and AD is complicated and multifaceted. Senescence occurs in nearly all cell types in the human body in response to accumulated, irreparable damages occurring as a result of chronic or severe stress that does not reach the level

of activating programmed cell death (Saez-Atienzar and Masliah, 2020). The resulting senescence-associated secretory phenotype (SASP) is characterized by the expression of proinflammatory compounds (Saez-Atienzar and Masliah, 2020). Aside from being a stress response, senescence also participates in tissue homeostasis and remodeling during development, wound healing, and tumor suppression. Studies have shown an intimate link between the process of aging to senescence, as exemplified by the discovery of senescent cells in individuals with accelerated aging diseases and experiments in mouse models (Saez-Atienzar and Masliah, 2020). Evidence supporting senescence as a cause for AD can be seen by the upregulation of senescent markers in cell types associated with AD pathogenesis. Studies suggest that Aβ aggregates can trigger senescence in astrocytes and microglia and subsequent increase in hyperphosphorylation of tau, which mediate a plethora of downstream damages resulting in AD symptoms. Dysfunction of astrocytes and microglia also leads to deterioration of A $\beta$  clearance, promoting the formation of toxic aggregates, and the senescence of these cells creates a chronic inflammatory environment, thus exacerbating neurodegeneration (Saez-Atienzar and Masliah, 2020). However, all of the above-described phenomena leading to glial cell senescence could occur as a consequence, not cause, of AD pathology and associated damages. The phenomenon of aging causes and leads to all three major pathways for AD pathogenesis, hence the predominance of sporadic AD as late-onset cases.

One particular risk factor implicated in the development of AD that is pertinent to aging is sleep disturbance. Changes in sleep patterns can occur naturally in the aging process, barring any psychiatric disorders, as a result of alteration in neurotransmitter production and degeneration of CNS structures and pathways. Studies show a natural increase in nighttime awakenings due to poor maintenance of sleep, poor quality of sleep, and daytime sleepiness with

age (Peter-Derex et al., 2015). Older individuals may also experience difficulty in entering sleep, which is a consequence of the decline in melatonin production with age, leading to disturbances in the natural circadian cycle. Some studies suggest melatonin possesses antioxidant, cytoprotective, and even anti-amyloid functions. Sleep is essential to the majority of higher cognitive functions in humans, with different stages of sleep responsible for different categories of memory consolidation and maintenance of physical and psychological health of the brain, in addition to the rest of the body (Peter-Derex et al., 2015). Evidence also shows a decline in some sleep-dependent memory consolidation processes with age, while some remain unaffected or even improved with age. Processes linked to neurodegenerative diseases also fluctuate along with the sleep cycle. Levels of cerebrospinal fluid (CSF) AB and pTau change throughout the natural circadian cycle, with the lowest level recorded during the third to fourth hour of sleep, and disturbances of deep sleep with audio stimuli disrupt this phenomenon to levels observed during waking hours (Peter-Derex et al., 2015). Thus, interruption of normal sleep patterns contributes significantly to increased risk of AD and other neurodegenerative diseases. Sleep abnormalities manifest as co-morbidities in up to 45% of AD cases, and the symptoms reported parallel natural disruptions due to aging but at greater severity (Peter-Derex et al., 2015). Many of the perturbations of normal sleeping behavior-regular circadian cycle, depth, and length of sleep-precede the development of AD, occurring early in pathogenesis and often leading to MCI before AD diagnosis, and studies show the severity of symptoms vary according to AD progression, correlating positively with the severity of dementia. One major variance in sleep abnormality that arose as a result of aging and AD is disruption of the rapid eye movement (REM) sleep (Peter-Derex et al., 2015). While REM sleep remains relatively consistent throughout the early aging process, and significant alteration only occurs much later, AD patients present defective REM sleep compared to age-matched controls. REM sleep is tied to cholinergic neurotransmission, a process affected by degeneration of the forebrain and brainstem structures, as observed in AD. Several sleep-related risk factors reviewed by the literature are insomnia, duration of sleep, poor sleep quality, circadian cycle abnormalities, and obstructive sleep apnea (OSA). According to Bubu et al. in 2017, individuals presenting these factors are 1.55 times more likely to develop AD, 1.65 times for cognitive impairment, and 3.78 times for preclinical AD. Sleep disturbances are found to have a more significant impact on younger individuals as they age and predisposes them to cognitive impairment and AD compared to older individuals. Duration of sleep as a risk factor encompasses both shorter and longer than usual—7 to 8 hours for healthy adults-length, with studies showing a higher risk for long periods of sleep. Shorter sleep duration, poor sleep quality, and insomnia may arise as a result of lifestyle choices, sleeping environment, or psychological conditions, such as depression or anxiety, both of which are observed in pre-AD and AD patients. Amongst these risk factors, obstructive sleep apnea (OSA) presents the greatest risk potential for developing AD at 2.37 times more likely than healthy control, compared to 1.86 and 1.62 for sleep duration and poor sleep quality, respectively. OSA is representative of the family of sleep-related breathing disorder (SRBD), and studies have shown it as a risk factor of cognitive decline (Peter-Derex et al., 2015). The link between OSA and AD is complicated, as while evidence implicates OSA as a risk factor for AD, up to 40-70% of AD patients develop OSA, with increasing severity as dementia progresses. In addition to disruption of sleep quality and duration, OSA also leads to hypoxia of the brain, which activates downstream pathways that lead to neural damage, cell death, and a pro-amyloid state (Bubu et al., 2020). However, dysfunction of breathing that culminates in OSA may be comorbid to degeneration or lesion of the neural pathways controlling breathing in the progression

of AD (Peter-Derex *et al.*, 2015). A recurring theme evident amongst the host of non-genetic risk factors in AD is the cyclic nature of the occurrence of symptoms associated with said risk factors presenting itself as co-morbidities, which exacerbate the disease pathology.

#### 1.2.2.2 Effect of Diet

The case of Apo-E lipoprotein being a major genetic risk factor in AD alludes to the role of lipid and cholesterol metabolism in AD pathogenesis (Saez-Orellana et al., 2020). The varying effect of Apo-E isoforms on the CNS stems from differences in lipidation and resulting stability, which impact their ability to serve as carriers of A $\beta$  (Hyman *et al.*, 2021). Lipid homeostasis is also heavily implicated in the pathogenesis of AD, and a large body of literature is starting to explore the effect of diet on neurodegeneration. A precursor protein (APP) possesses a cholesterol-binding domain that can direct its subcellular localization, affecting its hydrolysis by  $\beta$  and  $\gamma$ -secretases, which are membrane-associated and intramembrane-cleaving proteases, respectively (Saez-Orellana et al., 2020). Aß aggregates have been shown to insert into and perforate lipid membranes, and this behavior takes place preferentially at cholesterol-rich regions (Arbor *et al.*, 2016). And many of the proteins shown to interact with or be affected by  $A\beta$ , including  $\beta$  and  $\gamma$ -secretases, are localized within lipid rafts—regions of membrane enriched with cholesterols and sphingolipids and exhibiting increased rigidity (Saez-Orellana et al., 2020). Lipid rafts are also essential in synaptic transmission and plasticity and processes shown to be vulnerable in the earliest stages of AD. Caveolae-membrane invaginations similar to lipid raft in composition and rigidity found on the cell membrane and membrane-bound organelles -also promote APP processing to  $A\beta$ , and studies show an upregulation of caveolae level in the hippocampus and frontal cortex in AD patients.

The role of lipids in the pathology of AD reaches beyond its effect on APP processing. As precursors in the signaling pathways for activation of microglia, lipids play a major role in the damaging inflammatory response to Aβ aggregates (Baranowski et al., 2020). Literature delving into detriments of prolonged dietary intake of saturated fats and cholesterol, as often seen in the so-called Western diet, has been shown to negatively impact cognition through not only exacerbation of inflammation in the CNS but also upregulation of the Aß generating pathways. Neuroinflammation also causes disruption of the cerebral vasculature and contributes to the microhemorrhages observed in AD (Thelen and Brown-Borg, 2020). On the other hand, studies show suppression of the expression or function of proteins involved in cholesterol and fatty acid pathways such as statin treatments in the CNS, result in alleviation of cognitive decline, neurodegeneration, and amyloid burden in animal models of AD (Saez-Orellana et al., 2020). Neuroinflammation as a response to toxic A $\beta$  aggregate insult feedback upon itself due to cell death, leading to further dysregulation in the clearance and generation of A $\beta$ . Lipids also play an essential role in neurogenesis, synaptic transmission, and plasticity, all processes commonly disrupted in early or prodromal stages of neurodegenerative diseases (Saez-Orellana et al., 2020). A diet high in saturated long-chain fatty acids (FA) and low in polyunsaturated fatty acids (PUFA) is associated with neurodegenerative diseases, along with neurological dysfunction and psychiatric disorders. As the human body cannot generate PUFA through de novo synthesis, dietary intake of PUFA has significant consequences on cognitive development. And within the family of PUFAs, docosahexaenoic acid (DHA) level is found to decrease in plasma and brain of AD patients (Saez-Orellana et al., 2020). While increased intake of DHA does not alleviate cognitive deficits, prolonged supplement is associated with a lower risk of developing neurodegenerative diseases.

Lipid homeostasis and metabolism represent just one facet of the complex effect diet present as a risk factor for developing AD. Prolonged dietary intake of substances that generate radical oxygen species has also been shown to negatively impact cognition and lead to neurodegeneration, as the resulting oxidative stress overburdens the endogenous system and cause cell death and DNA damages that may culminate in neurodegenerative diseases (Thelen and Brown-Borg, 2020). Increased caveolae expression found in AD patients-structures that lead to increased APP processing—also promotes oxidative stress (Saez-Orellana et al., 2020). Similar to the neuroprotective effect seen in suppressing cholesterol and fatty acid pathways or reducing dietary intake, a diet rich in antioxidants has been shown to benefit cognition and reduce decline observed with aging. Studies on the Mediterranean diet enriched in PUFAs and antioxidants reveal potential benefits in lowering the risk of developing neurodegeneration, along with a reduced chance of cardiovascular diseases, which have also been tied to AD as potential risk factors (Baranowski et al., 2020). An expanding field of research on diet and brain energetics also reveals the potential benefit of caloric restriction (CR) and fasting, which are common strategies in alleviating the effect of aging, as prevention for AD and similar neurodegenerative diseases (Thelen and Brown-Borg, 2020). Studies in rodents show a significant increase in lifespan when administered a calorie-restricted diet or placed on intermittent fasting, and the underlying mechanism is implied to involve mTOR and related metabolic signaling pathways that respond to stress. In the context of the CNS, neurovascular coupling (NVC) is a metabolic response to active regions of the brain by increasing delivery of oxygen and glucose, and this process is disrupted as a consequence of aging, through damages caused by oxidative stress and inflammation (Thelen and Brown-Borg, 2020). CR and intermittent fasting activate pro-survival pathways, such as the generation of antioxidants and

anti-inflammatory agents, and autophagy, and prevent these damages from occurring. An additional facet to controlled diet is its protective effect on insulin signaling, and a large body of research has explored insulin resistance as a potential contributor to AD pathogenesis. Prolonged intake of high concentrations of sugar causes insulin resistance not only in the bodily circulation, leading to diabetes mellitus but also resistance in the CNS. AD patient brains have shown a decreased level of glucose transporter and sensor GLUT1 and GLUT3, which allows glucose to be moved into the brain (Thelen and Brown-Borg, 2020). Insensitivity to glucose can lead to starvation and subsequent degeneration of brain vasculature and neuropathology. Inflammation can also be upregulated as a result of insulin resistance, and studies show insulin signaling to be essential to regulating Aβ and tau processing (Thelen and Brown-Borg, 2020). Similar to CR and fasting, ketogenic diet-foods that lead to the production of ketone bodies from FA by the liver as an alternative fuel for the brain-can improve insulin sensitivity, and studies in AD models show a reduction of amyloid load, inflammation, oxidative stress, and improve cognition (Saez-Orellana et al., 2020). While the field of study on the effect of diet in regard to neurodegeneration requires further exploration, the findings so far suggest an unignorable correlation between the two.

#### 1.2.2.3 Effect of Exercise

A large and growing body of investigation is exploring the effect of physical activity (PA) and the lack thereof on cognition and development of neurodegeneration. While the field presents no unanimity, studies looking at AD models and human patients point to neuroprotective benefits of exercise for not only healthy individuals but also patients with mild cognitive impairment (MCI) and preclinical AD (Valenzuela *et al.*, 2020). Physically active individuals are 35-38% less likely to develop cognitive decline when compared to age-matched

individuals with a sedentary lifestyle (Valenzuela et al., 2020). A consistent regimen of exercise in adherence to the minimum recommended 150 minutes a week of moderate to vigorous PA leads to a 40% reduction in risk for AD. Furthermore, many of the implicated modifiable, nongenetic risk factors of AD, such as cardiovascular pathologies, diabetes, and obesity, can be prevented or alleviated by a consistent regimen of physical activities. The mechanism underlying the benefits of exercise is the increase in the secretion of neurotrophic factor, the most important of which is the brain-derived neurotrophic factor (BDNF), which promote neuronal survival and synaptic plasticity (Valenzuela et al., 2020). A decrease in BDNF is found in the blood and brain of AD patients in the early stage of the disease. Studies show that even a single event of exercise can lead to the production of BDNF in both healthy individuals and AD patients, with a positive effect on cognition. Additionally, neural imaging in AD patients who engage in aerobic exercise over a period of six months shows an increase in hippocampal volume. Further research into the BDNF-induced cognitive improvement revealed the rescue is not simply due to neurogenesis, as pharmacologically inducing hippocampal neurogenesis in AD mouse model fails to improve cognition (Valenzuela et al., 2020). Rather, a combination of neurotrophic factors, such as BDNF, and neurogenesis is necessary to rescue cognition. The exact mechanism behind how exercise promotes BDNF production is unclear, but a number of cytokines produced in muscles, termed myokines, and metabolic products from muscle contractions are being implicated. Cathepsin B, one such myokine, is found upregulated in the plasma of human and animal models in response to exercise, and this peptide can cross the BBB to increase BDNF expression in the hippocampus (Valenzuela et al., 2020). In a mouse model, knockout of the cathepsin B gene eliminates the neurogenesis and improved memory resulting from exercise. Another myokine, Irisin, produces a similar effect by crossing the BBB and stimulating hippocampal neurogenesis
through increasing BDNF expression (Valenzuela *et al.*, 2020). This finding is further corroborated by studies showing a decreased level of irisin in AD patient hippocampus, and expression of irisin correlate positively to exercise-induced memory improvement in AD mouse model. Lactate, a by-product of glycolysis that increases during prolonged PA, also stimulates BDNF expression by crossing the BBB through mono-carboxylate transporter (MCT), in addition to being essential for long-term memory formation (Valenzuela *et al.*, 2020). Inhibition of lactate transporter or astrocytic metabolic process can result in impaired memory consolidation, as well as decrease BDNF production, and these deficits can be rescued by exogenous administration of lactate. Prolonged PA, along with fasting and CR, can induce the production of ketone bodies, which also stimulate BDNF production.

A major risk factor of AD is cardiovascular disease and its co-morbidities, such as hypertension and diabetes, and these elements accelerate cognitive decline in individuals with and without AD. One potential mechanism for increased risk of AD lies in disrupted cerebral blood flow (CBF) due to vascular aging, potentially impairing clearance of A $\beta$  (Valenzuela *et al.*, 2020). Studies show consistent PA can resist vascular aging and promote cerebral angiogenesis with a concomitant decrease in amyloid burden (Valenzuela *et al.*, 2020). The effect of exercise on A $\beta$ deposition can be seen in carriers of ApoE4 allele, and when imaged with Pittsburgh compound B positron emission tomography (PiB PET), the amount of exercise the individual regularly engages in negatively correlated with the amyloid load. Through modifying metabolic processes, PA also exerts protective effects against inflammation and oxidative stress in individuals of all age groups and disease status (Valenzuela *et al.*, 2020). The neuroprotective effect of PA does not last for the lifetime of the individual, and an individual previously active may suffer from cognitive decline due to deterioration in health and mobility as a consequence of aging or extraneous circumstances (Valenzuela *et al.*, 2020). A longitudinal study looking at more than 4000 older adults reported a reduced risk for dementia among individuals with more PA four years from the initial record, but no difference was observed in a second follow-up 14 years from baseline. The selection and intensity of PA also alter the physiological effects, with highly intense exercises over a prolonged period of time shown to cause an increase in pro-inflammatory cytokine production and oxidative stress.

### 1.2.2.4 Effect of Trauma

Trauma to the brain can occur as a result of events leading to deformation of the brain, whether through direct impact of the head or from rapid change in acceleration, such as whiplashes seen in contact sports or car accidents. A growing field of study looking at the effect of traumatic brain injury (TBI) on cognitive health depicts a strong, causative link to various neurodegenerative diseases, including AD, and the effect can manifest decades after the initial, acute damage and symptoms (Abrahamson and Ikonomovic, 2020). The mechanism underlying the connection between TBI and AD is multifaceted, but disruption of brain vasculature and neuroinflammation represent the major contributors after the initial damage caused by mechanical compression and stress on the brain. Studies show that different regions of the brain display varying degree of vulnerability to damages caused by TBI, with the cortex and limbic system being most vulnerable—structures most affected during early AD pathogenesis (Abrahamson and Ikonomovic, 2020). Damages to the brain vasculature led to impaired efflux of material, including amyloidogenic peptides responsible for neurodegenerative diseases, from the brain, as revealed by post-mortem autopsy of individuals who suffered from TBI displaying vascular deposits of AB and pTau reminiscent of AD pathology. A rapid rise in extracellular AB level is also reported in acute TBI cases, suggesting a buildup of A $\beta$  due to reduced removal and

release of material from lysed cells, in addition to possible upregulation of amyloidogenic APP processing (Abrahamson and Ikonomovic, 2020). Cells associated with the vasculature, such as pericytes and astrocytes, are also responsible for capturing molecules for removal, and these pathways are also impaired during TBI (Abrahamson and Ikonomovic, 2020). BBB integrity has been shown to break down after TBI, shown by magnetic resonance imaging (MRI) study of young athletes in contact sport after concussive injuries displaying elevated plasma biomarkers in the CSF, and this increase in BBB permeability lasts up to two months post-incidence (Abrahamson and Ikonomovic, 2020). Impaired removal of amyloidogenic peptides caused by TBI can further exacerbate the initial damage, as deposition of peptides into plaques stiffens the vessels, leading to greater dysfunction and cell death-a cycle identical to the progression of AD. Evidence also suggests that the same non-genetic risk factors for AD-cardiovascular disease, obesity, and diabetes-determine the severity and subsequent damages of a TBI episode by predisposing vasculature to injury (Abrahamson and Ikonomovic, 2020). One of the genetic risk factors of AD, namely ApoE isoforms, has also been shown to mediate TBI severity through facilitating clearance of AB and other amyloidogenic peptides, and studies have shown a slower recovery from vascular <sup>18</sup>F-fluorodeoxyglucose dysfunction and BBB breakdown. The disruption of the vasculature and BBB, along with direct injury of the brain, results in hypoxia, exacerbating cell death and promoting neuroinflammation as a survival response (Green et al., 2020). Studies show an infiltration and accumulation of immune cells through the BBB to the site of trauma in TBI, and the concomitant release of pro-inflammatory cytokines also contribute to increased permeability of the BBB (Abrahamson and Ikonomovic, 2020). Inflammation causes disruption in the normal scavenging activity by glial cells and pericytes of the vascular and upregulates the production of  $A\beta$ , all phenomena observed in AD. Injuries to neurons can

also cause axonal white matter pathology, where myelin sheaths break down and become phagocytosed by microglia, a process that occurs naturally in aging and contributes to microglial dysfunction in AD. Prolonged inflammation also leads to increased oxidative stress through mitochondrial disruption, a process also associated with AD. The effect of inflammation in TBI reaches beyond the breakdown of Aβ clearance and increased Aβ production, as studies show that chronic pathological inflammation interferes with pathways regulating circadian cycle and sleep (Green *et al.*, 2020). Patients who suffered TBI reported an increase in daytime sleepiness, as well as disruption in the onset of sleep, hypersomnia, insomnia, and fatigue, illustrating sleep disturbance as an additional link between TBI and AD pathogenesis. While many studies have explored the consequences of severe, acute TBI, increasing effort is made to examine the deleterious effects of chronic, mild TBI on cognition and dementia later in life (Wu *et al.*, 2020). Studies looking at animal models and human patients suggest that individuals suffering from mild TBI present similar pathologies to acute, severe TBI and increased risk for developing neurodegenerative diseases.

## 1.2.2.5 Effect of Cognitive Reserve

The concept of cognitive reserve hypothesizes that differences in cognitive performance among individuals with similar physical profiles and neuropathology are attributable to factors extraneous to those affecting physiology (Soldan *et al.*, 2017). A similar and related concept is the idea of brain maintenance, which postulates the preservation of brain structure and wellbeing can resist cognitive decline (Nilsson and Lövdén, 2018). The difference between cognitive reserve and brain maintenance lies in the processes in question: whereas cognitive reserve seeks to explain the varying susceptibility to cognitive decline among the population, brain maintenance examines changes over time and the means to prevent or resist these changes. Many

criticisms directed toward the study of cognitive reserve targeted its heterogeneous and ambiguous definition—with some relating it to socioeconomic status and others simply as factors unexaminable by standard tests—and the fact that many mechanistic experiments monitor physiological changes as the output, which contradicts its conception as non-physiological factors affecting cognition (Nilsson and Lövdén, 2018). The nature of epidemiological studies also predisposes cognitive reserve research to errors and biases. Nevertheless, a large and growing body of evidence supports the overarching theme of cognitive reserve and neurodegeneration in both animal models and humans, that there exists a positive correlation between activities enhancing cognitive reserve and decreased risk for dementia. A number of factors are frequently examined in cognitive reserve studies, with education level, occupation, and early life stressors being the most common. The length and level of education can significantly affect lifetime risk for AD, with individuals receiving less than 8 years of formal education being 2.2 times more likely to develop AD than those with more than 8 years of education (Lesuis et al., 2018). Language skills and training early in life protect against AD, and studies show that individuals raised in bilingual environment are less likely to develop dementia. Epidemiological studies also reveal that study participants with low socioeconomic status are at 2.25 times greater risk for AD than participants with higher educational and occupational level (Lesuis *et al.*, 2018). These effects last for the lifetime of the individuals, unlike PA, even in the absence of cognitively stimulating tasks and responsibilities later in life. However, such stimulations may still be beneficial for the elderly, as shown by a 38% decrease in risk for dementia among older individuals who regularly engage in leisure activities (Lesuis et al., 2018). Furthermore, the benefit of engaging in cognitive reserve enhancement extends even to individuals with genetic risk factors, such as carriers of the ApoE4 allele (Wang et al., 2017).

Evidence in support of occupation in cognitive reserve enhancement are more mixed, but several aspects of work, such as physical exertion, problem-solving, and learning, are all beneficial for cognitive health and tied to the same processes in contributing to cognitive reserve as education (Wang et al., 2017). In contrast, stressors occurring during early life can also negatively impact the individual throughout life, such as the death of parents or negligence and abuse (Lesuis et al., 2018). Studies looking at people who have lost one or both parents between 0 to 18 years old found an increased occurrence of AD amongst this group. Synaptic plasticity represents the main mechanism potentially underlying the neuroprotective impact of cognitive reserveenhancing activities, with increased connectivity and strength of the synaptic network likely posing greater resilience against cytotoxic insult and perturbation by AB and other amyloidogenic peptides (Lesuis et al., 2018). Studies showing the significance of early life stimulations—with education and cognitive challenges having the greatest effect during the "critical period" when the brain is most plastic—supports this hypothesis. Similarly, acute or chronic stress during childhood negatively affect synaptic connectivity, and studies using animal models show that a lack of postnatal maternal care results in a decrease in dendritic connections in the hippocampus (Lesuis et al., 2018). Reduced neural complexity and strength of connection may then predispose the brain to damage by A $\beta$ . Stress also activates pathways leading to neuroinflammation and upregulating A $\beta$  production that increases the risk of cognitive decline and AD. An interesting aspect of cognitive reserve is the phenomenon where the onset of cognitive decline and dementia is delayed, but the deterioration is much more rapid, suggesting that while cognitive reserve enhancement may build resilience against pathogenesis, the advent of symptoms occurs once a threshold of pathogenic agent is surpassed with severity corresponding to the level of accumulated pathology (Lesuis et al., 2018).

#### 1.3 Diagnosis of AD

#### 1.3.1 Past and Current Diagnostic Tools and Criteria

The earliest diagnostic criteria for AD are founded on the categorization of AD as "dementia," with clinical symptoms of cognitive dysfunctions that debilitate normal social and quality-of-life activities (Blennow and Zetterburg, 2018). Primarily determined by cognitive and behavioral changes, the Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) added further, mainly exclusionary standards for the diagnosis of AD for patients with dementia in 1984, emphasizing the need for examination of pathognomonic hallmarks to ascertain the diagnosis. As no fluid biomarkers or detection technique aside from autopsy were available at the time, patients were diagnosed as "probably AD" until post-mortem examination can be carried out. Major leaps in technologies, along with discovery and novel revelations on AD pathogenesis and disease progression, led to a major paradigm shift in 2007 when the International Working Group (IWG) proposed new criteria for AD diagnosis and research, which was followed by the National Institute of Aging and Alzheimer's Association (NIA-AA) working groups in 2011 (Blennow and Zetterburg, 2018). During this time, the presence of MCI and the long preclinical stage of AD were acknowledged, and diagnosis of AD shifted to become more biomarker-based, focusing on changes in tau,  $A\beta$ , brain glucose metabolism, and hippocampal volume (Scheltens et al., 2016). A revision occurred in 2014, where changes in brain volume and glucose metabolism became secondary for diagnosis and serve only as signs for disease progression (Blennow, 2017). Since then, the examination of core AD biomarkers is used to diagnose up to 70% in the clinical setting, with great importance in the administration of therapeutics.

Current diagnostic criteria consist of fluid biomarker detection and neuroimaging utilizing MRI and PET. AB42, total tau (tTau), and pTau CSF levels represent the core of the AD fluid biomarkers, which are examined using ELISA (Blennow, 2017). Pathological changes of brain vasculature and structure atrophy are carried out using MRI (Rathore et al., 2017). PET is employed in conjunction with <sup>18</sup>F-fluorodeoxyglucose (FDG) to detect changes in metabolic activity of the brain as a measure for synaptic dysfunction. A more recent technique employed is PET imaging of fluorescent agents that bind to  $A\beta$  plaques with high sensitivity, with three FDA-approved ligands that are offshoots from Pittsburgh compound B currently in use: florbetapir, florbetaben, and flutemetamol. A decrease in CSF Aβ42 level, along with an increase in tTau and pTau, occurs in patients with MCI, and longitudinal monitoring of these biomarkers can have up to 85-90% sensitivity and specificity in predicting progression to AD (Weller and Budson, 2018). The recent refinement to diagnostic evaluation now focuses on  $A\beta 42/A\beta 40$ rather than A $\beta$ 42 level alone, where A $\beta$ 40 serves as a control for A $\beta$  expression that helps to better pinpoint patients most likely to show AD progression (Blennow and Zetterburg, 2018). CSF tau levels are reflective of neurodegeneration, while CSF A $\beta$ 42 is indicative of AD. Increase of tTau in CSF is not specific to AD, as this phenomenon occurs in all neurodegenerative diseases-with the highest observed in Creutzfeldt-Jacob disease-while pTau level increases only in diseases where deposits of neurofibrillary tangles occur and is specific to AD when taken together with a concurrent change in A $\beta$  level (Blennow and Zetterburg, 2018). Several caveats prevent these core AD CSF biomarkers from achieving higher diagnostic accuracy: the first being the biomarker difference between AD patients and healthy individuals represent a continuum instead of clear distinctions, as the peptides examined are normally produced in people at various levels, and the second being the concurrent pathologies

by amyloidogenic peptides, such as TDP43 and  $\alpha$ -synuclein, in AD patients. Additionally, variability in result between test centers, with A $\beta$ 42 test especially affected, frequently occur due to differences in techniques, equipment, and reagents (Blennow and Zetterburg, 2018). Nevertheless, CSF biomarkers remain a major diagnostic tool due to their correlation with disease progression. The development of fully automated assay systems, along with validated and standardized reference measurement procedures and reference materials, will minimize and possibly eliminate errors in inter-laboratory analysis. The combination of all three biomarkers also serves as a stringent cut-off, as healthy levels of A $\beta$ 42, tTau, and pTau can almost exclude AD as a diagnosis in patients with MCI (Scheltens *et al.*, 2016).

Reduction in CSF A $\beta$ 42 level in AD patients was reported as early as 1995, but the cause for this phenomenon was unknown (Blennow and Zetterburg, 2018). The correlation between the drop in CSF A $\beta$ 42 level and amyloid deposition was not made until an autopsy study in 2003, and in 2006, in vivo imaging using PET showed amyloid deposits in individuals with low CSF A $\beta$ 42 level, regardless of cognitive impairment. Since then, amyloid PET is a standard diagnostic tool for AD, with a concordance rate of 90% with CSF A $\beta$ 42 (Blennow and Zetterburg, 2018). Current understanding illustrates reduced A $\beta$ 42 level as a result of deposition in the brain, and studies support this hypothesis, as individuals with low CSF A $\beta$ 42 but negative amyloid PET is three times more likely to show positive amyloid PET in a follow-up than individuals with normal CSF A $\beta$ 42 level (Blennow and Zetterburg, 2018). This result suggests a change in CSF A $\beta$ 42 level as an earlier biomarker than amyloid PET status. The high concordance rate between CSF A $\beta$ 42 level and A $\beta$  PET state also suggests interchangeability for clinical diagnosis. Recent development in ligand for tau now allows for PET scan of tau tangles, but the correlation between CSF tau biomarkers and tau PET is weak (Blennow and Zetterburg, 2018). When

examined closer, tau PET result holds a moderate correlation with CSF pTau, but no correlation with CSF tTau. Interestingly, while an increase in CSF tTau and pTau levels correlate strongly with each other and with the progression of MCI to AD, tau PET status remains normal in preclinical AD cases that later progress to confirmed AD. Increased tau PET correlated positively with the severity and site of atrophy, as well as defective glucose uptake and metabolism, suggesting that while CSF tau level reflects a state of neurodegeneration, tau PET indicates the stage and progress of neurodegeneration.

### 1.3.2 Future of AD Diagnostic Tools

While the core AD CSF biomarkers serve well as diagnostic for the main pathogenic agents, there is an ongoing search for additional markers of other pathologies in AD. Synaptic dysfunction and damage represent major pathophysiology in AD, with ample evidence showing that both A $\beta$  aggregates mediate a variety of AD symptoms through disrupting both the synapses and dendrites. Neurogranin is of special interest as a potential AD biomarker (Blennow, 2017). As a protein expressed on dendrites of excitatory neurons in the hippocampus and cortex, neurogranin plays an important role in long-term potentiation (LTP). Neurogranin, enriched in the associative cortical region, level drops in AD as a result of dendritic loss. A high CSF level of neurogranin has been found in confirmed AD cases, and many reports show elevated levels in prodromal AD. An increase in CSF neurogranin also positively correlates with future hippocampal atrophy and reduction in glucose metabolism. Interestingly, neurogranin level remains stable in other neurodegenerative diseases, such as Parkinson's disease and frontotemporal dementia, and elevation of neurogranin in the CSF may be specific to AD. Components of the SNARE complex-presynaptic proteins responsible for the release of synaptic vesicles—are also implicated as potential AD biomarkers (Blennow and Zetterburg,

2018). In particular, synaptosomal-associated protein 25 (SNAP25), a protein found on synaptic vesicles, and synaptotagmin-1 (STY1), a membrane protein responsible for vesicle release, levels are decreased in AD brain, similar to neurogranin, with a concomitant increase in CSF in both prodromal AD and AD dementia.

The "holy grail" for the future of AD diagnosis is the discovery and utilization of blood biomarkers (Blennow, 2017). Current techniques, while adequate and well-validated, suffer from two main challenges: difficulty of procedure and infrastructure. With advances in technology and medical technique, lumbar puncture—a necessity for collecting CSF samples—is a relatively safe operation, though the associated risk and potentially debilitating consequence, along with the side effect of the process, cannot be completely eliminated (Blennow, 2017). Furthermore, the nature of the procedure requires its execution to be in a highly controlled environment, limiting application. MRI and PET also require highly specialized medical infrastructure and technicians to maintain their operation. The prohibitive cost of operating these machines, in addition to auxiliary materials such as fluorescent ligands used for amyloid PET, prevents widespread and affordable screening of AD and other neurodegenerative diseases. With studies showing that AD pathogenesis begins up to two decades before the first symptom, a cheap and scalable diagnostic tool could revolutionize AD treatment. However, the development of technology to detect blood AD biomarkers needs to overcome two major challenges: the first being the minuscule amounts of brain-derived molecules that are able to pass through the BBB into the systemic circulation, which is overwhelmed by the abundant mixture of unrelated proteins, and the second being breakdown and clearance of brain biomarkers by bloodborne proteases, liver, and kidneys, respectively (Blennow, 2017). Up until very recently, standard immunochemical assays cannot achieve the level of sensitivity necessary for detecting brain-

derived biomarkers in blood. However, with the development of ultrasensitive techniques such as immunomagnetic reduction (IMR) and single-molecule array (Simoa)—which captures biomolecules using magnetic beads in femtoliters of sample for detection with enzymeconjugated antibody and digital quantification—analysis of proteins at sub-picogram per milliliter concentration is now possible (Blennow, 2017).

Efforts to validate AD CSF biomarkers, namely Aβ and tau, in blood utilizing novel technologies and techniques are raising optimism. While weak, there is a significant correlation between plasma level—namely decrease in Aβ level and increase in tau level—with CSF level and concurrent physiological biomarker changes (Blennow and Zetterburg, 2018). A large overlap between AD patients and healthy control results is present, precluding the effectiveness of plasma AB and tau ad biomarkers. Systemic production of AB serves to inflate and confound detection of brain-derived A $\beta$ , and the hydrophobic nature of A $\beta$  leads to binding with proteins in blood, interfering with assay. Tau can exist in various truncated forms, even in CSF, with this heterogeneity may be translated into blood. Further refinements in methodology and reagents are needed to resolve these complications and raise the accuracy of the assays. One interesting blood biomarker currently in the study is the axonal neurofilament light chain (NFL), which showed a strong correlation between plasma and CSF level (Blennow and Zetterburg, 2018). A significant increase in blood NFL level is observed in AD cases, with accuracy comparable to core AD CSF biomarkers. While less significant, changes in NFL level were also present in individuals with MCI, and the highest increase occurs in MCI cases with positive amyloid PET imaging. This change predicts accelerated cognitive decline, hippocampal atrophy, and glucose metabolic dysregulation. Studies of individuals with familial AD mutations show increased blood NFL level not only in symptomatic cases but also presymptomatic cases, with NFL level predictive of year of onset and subsequent decline. Of note, NFL is not specific to AD, but the ease of testing will allow it to serve as a rapid, cheap, and non-invasive examination in patients with cognitive deficit to determine further action.

### 1.4 Amyloid Beta

### 1.4.1 Amyloid $\beta$ Description

APP processing is carried out by three proteases— $\alpha$ ,  $\beta$ , and  $\gamma$ -secretase—in two distinct routes, amyloidogenic and non-amyloidogenic pathways. Both pathways occur constitutively in healthy individuals, with non-amyloidogenesis being the dominant pathway. The nonamyloidogenic pathway involves  $\alpha$ -secretase, consisting of at least two members of the ADAM family of metalloproteases, and y-secretase, an intramembranous protease complex consisting of presenilin, nicastrin, APH-1, and PEN-2 (Blennow et al., 2006). In the amyloidogenic pathway,  $\beta$ -secretase, an integral membrane aspartyl protease  $\beta$ -site APP cleaving enzyme 1 (BACE1), takes the place of  $\alpha$ -secretase. Cleavage of APP by  $\alpha$ -secretase occurs on the extracellular Nterminal tail within the A $\beta$  site—whereas  $\beta$ -secretase cleaves right before, and the subsequent intramembrane cleavage by  $\gamma$ -secretase releases a small peptide fragment p3, consisting of residue 17-40 to 42 of AB. Under physiological conditions, AB peptide exists in a vast number of isoforms with various N-terminal and C-terminal cleavage states due to the non-specific endocarboxypeptidase activity of  $\gamma$ -secretase (Selkoe and Hardy, 2016). In addition, A $\beta$  peptides can also receive a number of amino-acid modifications subsequent to cleavage—all of which add up to create the complex biology of Aβ peptide *in vivo* (Wildburger *et al.*, 2017). The different cleavage states confer a varying degree to the aggregation propensity of A<sup>β</sup> peptide, with additional C-terminal amino acids adding to hydrophobicity and vice versa for N-terminal amino acids. A $\beta$ 1-40 and A $\beta$ 1-42 are the most abundant isoforms of the peptides and are frequently

employed and analyzed in experimentations, with A $\beta$ 1-40 being the major isoform. While A $\beta$ 1-40 is the most abundant cleavage state, other N-terminal cleavage states can also exist in A $\beta$ x-40, with x ranging from 1-11 (Wildburger *et al.*, 2017). C-terminal variations are in even greater diversity, ranging from 20 to 43 (Dunys *et al.*, 2018, Wildburger *et al.*, 2017). These variations often occur as a result of secondary cleavages or by processes outside of the amyloidogenic pathway.

As a constitutively expressed and produced peptide,  $A\beta$  may serve important physiological functions within the brain. The bioflocculant hypothesis represents the earliest theory of A $\beta$  function, which postulates that A $\beta$  may harbor the role as a sequester of metal ions, pathogens, and harmful proteins inadvertently released to be gathered by microglia (Brothers et al., 2018). This idea is further expanded and specified A $\beta$  as a possible antimicrobial peptide, a type of innate immune peptide with general microbicide activity, due to the ability for aggregated A $\beta$  to perforate lipid membranes and form pores resembling that of the canonical antimicrobial peptide (Arbor et al., 2016). Studies also found an interesting correlation between AD and lowered risk for cancer (Arbor et al., 2016). This correlation is exemplified in the naked mole rat—well known for being cancer-resistant, which harbors Aß buildup at the same level as AD model mice without memory deficit. Application of AB onto cultured cancer cells inhibits growth, and high A $\beta$  level inhibits capillary development, an important process in cancer growth. A  $\beta$  may also act as a form of vascular seal in the event of leaks or injuries to the BBB (Arbor et al., 2016). This line of thought originates from observations that various components of the coagulation cascade colocalize with A $\beta$  plaques on blood vessels, and the removal and clearance of these plaques result in edema and microhemorrhage. Animal studies modeling injuries resulting in BBB damage and bleeding found an increase in A $\beta$  plaque counts, and this

phenomenon is also present in mice modeling chronic hypertension, which leads to vascular damage and is a major risk factor for AD. In line with this theory,  $A\beta$  may possess a general neuroprotective effect against brain injuries (Arbor et al., 2016). Studies cited in support of this hypothesis show an upregulation of A $\beta$  in TBI patients that accumulate at sites of injury but do not correlate with cognitive impairments. Animal models show that this Aß accumulation resolves over time, and mice with BACE1 knockout displayed worse outcomes compared to normal mice. A similar trend is present in mice models of stroke, with APP knockout mice more likely to decrease following vascular occlusion. Finally, A $\beta$  is implied to play a role in mediating synaptic transmission (Arbor *et al.*, 2016). Evidence reveals that during neuronal activity, APP is transported to postsynaptic terminal and cleaved to produce  $A\beta$ , which binds to presynaptic neuron to promote further transmitter release. Mice with APP knockout displayed impaired LTP, which can be rescued with exogenous human A $\beta$ 1-42. A $\beta$  may mediate LTP through the cholinergic pathways, as well as excitatory neurons with NMDA receptors (NMDAR). Cognitive impairment due to pharmaceutical agents that reduce A $\beta$  production seen in clinical trials was also cited as support. All lines of research looking at physiological functions of A<sup>β</sup> will require further examination to clarify mechanisms of action, contradictions with current understanding on Aβ biology in AD pathogenesis, and omission of other participants, such as pTau, in actions.

### 1.4.2 Aggregation of Amyloid $\beta$

A large body of literature has shown A $\beta$ 1-42 to be the major neurotoxic species in the pathogenesis of AD. As one of the two most common proteoforms along with A $\beta$ 1-40, the change in the A $\beta$ 40/A $\beta$ 42 is indicative of AD pathogenesis. A $\beta$  is an intrinsically disordered peptide that exists as an extended random coil (Roychaudhuri *et al.*, 2013). However, in solution, A $\beta$  rapidly adopts a  $\beta$ -hairpin structure, and this change in conformation instigates subsequent

aggregation into higher-order forms toward eventual deposition as plaque composed of insoluble A $\beta$  fibrils. Both A $\beta$ 40 and A $\beta$ 42 undergo this structural change, but A $\beta$ 42 displays faster aggregation kinetics and increased stability with its additional C-terminal hydrophobic amino acids. Manipulation of the C-terminus chemical profile by changing amino acids can alter aggregation characteristics. Increasing hydrophobicity at the C-terminal tail of A $\beta$ 40 by amino acid substitution will render it more aggregation-prone and toxic than wildtype A $\beta$ 42 and mutating several key amino acids in A $\beta$ 42 C-terminus abrogates its aggregation. The contribution of various other truncated A $\beta$  species to aggregation is complex and subject of ongoing investigation, but a similar trend is observed for N-terminally truncated A $\beta$  proteoforms being enriched in insoluble material in the AD brain (Wildburger *et al.*, 2017).

Aggregates of  $A\beta$  peptides can exist in a diverse spectrum of conformations in solution. Canonically, aggregation begins with the association of  $A\beta$  monomers in aggregative,  $\beta$ -hairpin state, leading to the formation of  $A\betaO$ , which then proceed through monomer additions and eventually result in insoluble fibrils that are clinically observed as plaques (Roychaudhuri *et al.*, 2009). These three stages of aggregation, resulting in fibrillogenesis, similarly proceed in three phases: a lag phase, where conversion and aggregation of  $A\beta$  monomers occur to form oligomers, followed by an exponential elongation phase, and finally a plateau phase when all  $A\beta$ monomers in solution are consumed.  $A\beta$  fibrils are composed of pairs of tightly interdigitated  $A\beta$ monomers, termed steric zipper, in cross- $\beta$  motifs stacked in the direction of fibril axis (Riek and Eisenburg, 2016). A review by Chiti and Dobson in 2006 shows a great heterogeneity within amyloid fibril structure, with  $\beta$ -sheets being parallel and in register or antiparallel along the stacking, and that fibril formation and arrangement is a common product shared among all amyloidogenic, even potentially misfolded normal, proteins. Similarly, A $\beta$ Os consist of a great continuum of aggregate species, and increasing evidence suggests that the canonical model of aggregation fails to address the complexity of ABO formation. The definition of ABO currently harbors a degree of ambiguity, describing any soluble A<sup>β</sup> structures greater than monomers, ranging from dimers to multimers above 1MDa (Glabe and Kayed, 2006). A diverse range of ABOs have been studied and implicated to be neurotoxic: AB dimers up to dodecamers, amyloid diffusible ligands (ADDLs), globulomers,  $A\beta$ \*56, amylospheroids,  $\beta$ -sheet intermediates, and protofibrils (Saez-Orellana et al., 2020 and Benilova et al., 2012). A growing body of literature illustrates a potential alternative AB aggregation pathway, and this "off" pathway leads to the production of large, globular aggregates, such as amylospheroids, instead of forming Aβ fibrils (Roychaudhuri et al., 2009). Furthermore, the process of Aß aggregation may also occur differently from the canonical models, with the recent discovery of paranuclei—which are Aß pentamers and hexamers forming the basic units of aggregation and which combine to form higher-order structures (Wolff et al., 2016). This hypothesis is corroborated by structural observation of protofibrils resembling beads-on-a-string, as well as many of the well-studied A $\beta$ O, such as ADDL, A $\beta$ \*56, and globulomer, being multiples-of penta- or hexamers (Roychaudhuri et al., 2009). The non-linearity of Aß aggregation has been a major obstacle in the study of A $\beta$  biology, compounded by the various extraneous factors, such as pH, temperature, salt concentration, and lipid presence, adding further complexity to Aß aggregation (Benilova *et al.*, 2012). Finally,  $A\beta Os$  exist as metastable formations in equilibrium, with various transient species likely to exist simultaneously and thus render the characterization of any specific form difficult. The permanence of the insoluble A<sup>β</sup> fibrils has also been brought into question by recent literature, which suggests that fragments may break off and re-enter solution to cause further aggregation.

# 1.4.3 Amyloid Hypothesis and Current Understanding

The elucidation of A $\beta$  peptide as the major component in neuritic plaques by Glenner and Wong opened the way to the study of AD pathology and eventually gave birth to the amyloid cascade hypothesis. The original amyloid cascade hypothesis described deposition of Aß peptide into neuritic plaques as the causative pathway to AD, building on the observations made by Alois Alzheimer and by Glenner and Wong. Glenner and Wong initially isolated plaque material from brains of Down's Syndrome patients, who displayed presenile, AD-like symptoms and plaque deposition (O'Brian and Wong, 2011). Subsequent identification of the amyloid precursor protein (APP) and its position on chromosome 21 led to the hypothesis that overproduction of Aβ peptide and plaque deposition was the cause of neurodegeneration in AD. The lack of presenile neurodegeneration in patients with Down's Syndrome missing the segment possessing the APP gene on chromosome 21, but the expression of this condition in patients with duplication of the APP gene segment corroborated with the hypothesis (Selkoe and Hardy, 2016). While the amyloid cascade hypothesis had called into question in light of the lack of correlation between plaque deposition and disease progression and increased understanding of the contribution of tau pathology in AD, which correlated better with the progression of disease symptoms, a host of literature supports the place of  $A\beta$  as the causative agent in AD. In addition to the discovery of the effect of trisomy 21, the strong genetic linkage between early-onset familial AD (EOAD) and highly-penetrant autosomal-dominant mutations of genes involved in APP processing pathways—APP, PSEN1, and PSEN2—robustly support the causative role of A $\beta$  (Ridge *et al.*, 2013). PSEN1 and PSEN2 code for two isoforms of presenilin, which constitutes the active site of  $\gamma$ -secretase, and mutations of PSEN1 represent the most common genetic cause of EOAD, with more than 150 mutations found to date (Ertekin-Taner, 2007).

PSEN1 mutations directly result in increased production of A $\beta$ 1-42 by reducing the endocarboxypeptidase activity of presenilin. The consequence of PSEN2 mutations is more variable compared to PSEN1, and genetic linkage between AD and PSEN2 is rarer, with 11 mutations associated with disease heredity (Ertekin-Taner, 2007). APP mutations represent the second most prevalent genetic cause of EOAD, with more than 20 variances associated with familial AD. These mutations occur around secretase cleavages sites, such as the Arctic mutation E693G, which disrupt  $\alpha$ -secretase cleavage site, the London mutation V717I and V717L, which affect  $\gamma$ -secretase cleavage and increase A $\beta$ 42/A $\beta$ 40 ratio, and the Swedish mutation KM670/671NL, which increases  $\beta$ -secretase cleavage and A $\beta$  production (Rosenberg *et al.*, 2016 and Breijyeh and Karaman, 2020).

The second piece of evidence in support of the amyloid hypothesis comes from recent studies establishing the precedence of pathology in AD. Studies in transgenic AD mouse models showed overexpression of human tau protein without overexpressing human APP was insufficient for instigating the development of AD pathology and symptoms, suggesting that the pathological role of A $\beta$  peptide lie upstream of hyperphosphorylated tau. This line of reasoning is supported by similar animal experiments in which A $\beta$ 42 overexpression in the absence of tau alleviates A $\beta$ -induced deficits (Ballatore *et al.*, 2007). In humans, mutations of the tau gene MAPT lead to frontotemporal dementia (FTD) with parkinsonism but not AD. Application of exogenous A $\beta$  aggregates termed oligomers (A $\beta$ O) leads to hyperphosphorylation of tau and causes death in rat primary neuron culture, and the presence of A $\beta$  antibody abrogates this effect (Hardy and Selkoe, 2016). GSK3 $\beta$  and cdk5 are kinases involved in tau hyperphosphorylation ad can be activated by A $\beta$ Os (O'Brian and Wong, 2011). Lastly, a seminal paper by Aoyagi *et al.* in 2019 shows that the abundance of APP and A $\beta$  declines with age, while the formation of insoluble tau neurofibrillary tangles increases with age, illustrating an earlier chronological order of  $A\beta$  pathology in the development of AD.

Finally, the final piece of evidence came as a result of a paradigm shift in the amyloid hypothesis, first presented by Hardy and Selkoe in 2002, which states that soluble  $A\beta O$ —the intermediate between individual Aβ monomers and insoluble fibrils—is the major neurotoxic agent. This modification came as a response to the poor correlation between Aβ plaque pathology with disease symptoms, and in lieu of the growing body of literature showing, both in vitro and in vivo, soluble, low-order ABOs mediated a host of downstream pathways leading to AD progression. The modified amyloid hypothesis addresses two main questions unanswered by the initial iteration: the agent responsible for the earliest symptoms of AD and the mechanism for the progression of AD, which begins with a deficit in the hippocampal function and spreads to the frontal cortex before developing into systemic pathology. Shankar et al. carried out a study in 2008 looking at the effect of human AD brain-derived material on synaptic transmission and showed that ABOs as small as dimers exist in AD patient brains and exert a significant effect over synaptic transmission when applied onto mouse hippocampal slices. The experiment revealed that insoluble plaques did not impair synapse, nor increase and decrease LTD and LTP, respectively, as did small soluble ABOs, but the solubilization of fibrils into smaller aggregate led to a similar effect as A $\beta$ Os. A later study by Yang *et al.* in 2017 showed that larger, high molecular weight (HMW) A $\beta$ Os consist large portions of A $\beta$  species in AD brain, but these oligomers display less neurotoxic effect compared to low molecular weight (LMW) ABOs as previously shown. This line of evidence illustrates that the earliest cognitive deficit in AD occurs as a result of small A $\beta$ Os, which can be composed of as few as two A $\beta$  peptides. The conformational change AB undergoes and subsequent aggregation into toxic oligomers resemble

the biology of prions, and the study by Aoyagi *et al.* in 2019 shows A $\beta$ O does propagate disease pathology in a prion-like manner, where soluble aggregates disperse from foci of genesis and seed aggregation of A $\beta$  monomers in distal sites. Evidence of iatrogenic transmission exists for A $\beta$  from cases of cadaveric growth hormone extract injection and dura mater graft transplant that were contaminated with A $\beta$  aggregates (Lauwers *et al.*, 2020). Cases have also been reported of A $\beta$  iatrogenic transmission through contaminated neurosurgical equipment and potentially through blood transfusion.

## **1.5 AD Treatments**

# 1.5.1 Current Treatments and Therapies

Currently, available protocols of care for Alzheimer's disease patients are limited to treatments and pharmaceutical agents aimed at slowing down cognitive deterioration and alleviating symptoms associated with AD, as well as management of wellbeing in an attempt to improve quality of life for both patients and caregivers (Yiannopoulou and Papageorgiou, 2020). Approaches to maintaining patient quality of life consist of a behavioral component, focusing on simplification of the living environment, the establishment of consistent routine and environment, and pleasurable activities, and a communication component, such as building open exchange between doctors, patients, and caregivers, and using calm, simple speech pattern with patients. Caregiver training and education represent another important aspect of current AD therapy in preparation for inevitable cognitive and psychiatric dysfunctions, which also emphasizes the need for regular relief of duty and support network for caregivers (Yiannopoulou and Papageorgiou, 2020). Non-therapeutic interventions for AD patients focus on alleviating or improving modifiable, non-genetic risk factors of AD, such as diet and exercise. Patients may be placed on cognitive-behavioral and music therapy to stimulate the brain and slow down cognitive decline (Yiannopoulou and Papageorgiou, 2020). Exercise therapy is carried out in order to alleviate the onset of physical disability due to difficulty with motor functions and increased sedentariness, as well as to allay cognitive decline. As sleep disruption and depression frequently occur as co-morbidities in AD, light therapy, along with exercise, can be administered in an attempt to assuage these symptoms (Yiannopoulou and Papageorgiou, 2020). Diet also represents an important component in AD caregiving, especially for patients with difficulty masticating and swallowing (Breijyeh and Karaman, 2020). As most AD patients become bedridden with disease progression, adequate intake of essential nutrients and calories needs to be monitored and administered while preventing the occurrence of weight gain and other cardiovascular and metabolic conditions. With the onset of dementia and associated psychiatric symptoms, antidepressants, such as selective serotonin reuptake inhibitors, and, in circumstances where the patient's behavior can put caregiver at risk of harm, antipsychotics may be the treatment of choice (Yiannopoulou and Papageorgiou, 2020). In particular, antipsychotic agents with low anticholinergic effects are preferred, as drug administration would not exacerbate AD severity.

The preeminent AD therapeutics prescribed today mainly target the cholinergic pathway in the brain. According to the cholinergic hypothesis of AD developed in the 1970s, cognitive decline in AD was found to be caused by degeneration of the cholinergic pathways and resultant decrease in synthesis of acetylcholine (Breijyeh and Karaman, 2020). Acetylcholine is a neurotransmitter involved in memory, attention, sensory information, learning, and other critical cognitive pathways. Later studies established the connection between acetylcholine deficits and  $A\beta$ , with the discovery of  $A\beta$ O binding with acetylcholinesterase (AChE) and downregulation of a host of presynaptic receptors in cholinergic neurons due to  $A\beta$  (Breijyeh and Karaman, 2020).

More recent experiments show that  $A\beta Os$  also directly affect synapse and dendrite density, as well as the cytotoxic effect of  $A\beta$  aggregates. A major mechanism by which  $A\beta O$  causes cell death, particularly for excitatory neurons involved in (LTP), is through binding to NMDA receptors (NMDAR) and leading to over-activation and an excessive influx of calcium ions (Breijyeh and Karaman, 2020). Calcium dyshomeostasis in neurons leads to excitotoxicity and cell death, representing a significant mechanism in the pathophysiology of AD.

Currently, four small-molecule therapeutics are approved by FDA for use that targets the disease mechanism: three of which aim to alleviate cholinergic deficit by inhibiting AChE or stimulating choline reuptake, and one acts as NMDA antagonist to suppress excitotoxicity (Breijyeh and Karaman, 2020). The first FDA-approved AChE inhibitor (AChEI) is tacrine, which acts on muscarinic neurons, but its use was discontinued soon after introduction to the market due to frequent hepatotoxicity and lack of efficacy. Donepezil, serving as the leading drug for AD, is a reversible second-generation AChEI specific for AChE prescribed for all stages of disease (Yiannopoulou and Papageorgiou, 2020). Unlike tacrine, donepezil displays high therapeutic efficacy, with significant cognitive and behavioral improvements, while being reasonably well tolerated. Transient cholinergic side effects occur in 5-20% of patients arising from cholinomimetic action of AChEIs, which affect the gastrointestinal tracts and nervous system and include nausea, diarrhea, and vomiting (Yiannopoulou and Papageorgiou, 2020). More severe adverse effects may occur on the cardiovascular system, leading to arrhythmia, bradycardia, and syncope. AChEIs prescriptions are withheld for patients with peptic ulcers, gastrointestinal bleeding, and seizures, and slow titration to prescribed dose is usually administered to allow patients to build up tolerance and for safety. Rivastigmine, another drug prescribed for mild to severe AD, acts as a pseudo-irreversible inhibitor of both AChE and

butyrylcholinesterase (BuChE), an enzyme found on glial cell, is responsible for only 10% of AChE activity in normal brain but is increased to 40-90% in AD (Yiannopoulou and Papageorgiou, 2020). Unlike donepezil, which is metabolized in the liver and intestine, rivastigmine is metabolized at the synapse, and its slow dissociation rate compared to Ach leads to pseudo-irreversible inhibition of AChE and BuChE. A special feature of rivastigmine is the option for transdermal delivery in the form of a patch for slow release of the drug (Breijyeh and Karaman, 2020). This is particularly useful for AD patients with difficulty swallowing and frequent forgetfulness for medication, as well as reducing the potential adverse effect that may lead to patient refusal of the drug. Galantamine, another first-line AD therapeutic prescribed in mild to moderate cases, acts to competitively inhibit AChE and activate nicotinic acetylcholine receptors (Breijyeh and Karaman, 2020). Similar to donepezil, galantamine is metabolized in the liver and intestine and displays high therapeutic efficacy and tolerability. A number of groups seek to develop a delivery system for improved uptake by the brain with galantamine using nanoparticles of various compositions through different routes. The last and most recently approved AD therapeutic is memantine, an uncompetitive, low-affinity antagonist of NMDAR prescribed for moderate to severe AD (Breijyeh and Karaman, 2020). This drug is safe and welltolerated, as it only transiently blocks excitatory synaptic transmission before quickly displaced by the high glutamate concentration, alleviating excitotoxicity and associated cognitive deficits without impairing learning and memory functions. Memantine can be administered alone or in combination with AChEIs, as the two agents display complementary and additive therapeutic effects without adverse consequences, and data show combination therapy, even in advanced AD, can have benefits on cognitive functions.

The key shortcoming of all current AD therapeutics is the inability to stop disease progression and underlying central causative mechanisms. While studies have shown that AChEIs and memantine attenuate cognitive decline in AD patients for at least the first year of treatment and improve measures of daily activity, cognitive decline invariably occurs (Yiannopoulou and Papageorgiou, 2020). Furthermore, gastrointestinal and nervous system side effects associated with AChEIs, in addition to being contraindicating in patients with cardiac arrythmia, seizure, peptic ulcer, and gastrointestinal bleeding, also necessitate long titration periods for safety concerns. Similar to AChEIs, memantine treatment can also lead to adverse effects, including somnolence, weight gain, confusion, hypertension, nervous system disorders, and falling (Yang *et al.*, 2013). The non-specific nature of the current AD therapeutics, targeting important components of CNS activity to alleviate disease symptoms, and their inability to modify disease pathology render them inadequate.

### 1.5.2 Future of AD Therapeutics

Since the discovery that A $\beta$  represented the major component of AD neuritic plaques by Glenner and Wong in 1984, a massive effort has been devoted to developing a curative agent for AD (de la Monte and Wands, 2005). However, despite increasing understanding of the disease pathophysiology, the pipeline of AD treatments is lined with many failures. The most recent FDA approval of a pharmaceutical agent for AD, memantine, took place in 2003, and none of the treatments on the market today target the mechanism of action (MOA) in AD for disease modification (Graham *et al.*, 2017). Initial search for an AD-curative agent in the early-90's focusing on  $\alpha$ ,  $\beta$ , and  $\gamma$ -secretases, as the former leads to the non-amyloidogenic processing of APP while the latter two are responsible for the production of A $\beta$  peptides.  $\gamma$ -secretase is the earliest therapeutic target explored for AD treatment; inhibitory compounds directed against  $\gamma$ -

secretase elicit a robust reduction in A $\beta$  production within hours of a single administration (Graham et al., 2017). However, the discovery that Notch-1, a receptor essential for development in the early embryonic stage and immune cell differentiation and maturation, requires  $\gamma$ -secretase cleavage for function. The multiplicity of ligands y-secretase acts on renders it a difficult target for effective therapy, as exemplified by the recent failure of semagacestat in phase 3 clinical trial, where treated patients show an increased occurrence of cancer and infection, along with greater cognitive decline (Yiannopoulou and Papageorgiou, 2020). Similarly, therapeutics targeting  $\beta$ -secretase BACE1 also proved unfruitful, with the recent termination of phase 3 clinical trials for lanabecestat, verubecestat, and atabecestat. All three of these agents resulted in a significant and dose-dependent reduction in CSF Aβ42, but no cognitive and functional improvement was observed in the patients (Yiannopoulou and Papageorgiou, 2020). Poor tolerability and significant risk of adverse side effects, such as the liver and neuropsychiatric symptoms, led to discontinuation of the trials. Development of therapeutics targeting  $\alpha$ -secretase is the most recent of the three secretases and is built upon the hypothesis that potentiation of  $\alpha$ secretase-mediated non-amyloidogenic pathway will reduce Aß concentration (Yiannopoulou and Papageorgiou, 2020). However, the main signaling pathway of  $\alpha$ -secretase is currently unclear, with current literature generally assuming that phosphatidylinositol 3-kinase (PI3K)/Akt pathway to be responsible and may be stimulated by GABA receptor signaling. Etazolate, a selective modulator of GABA receptors, advanced as a stimulator of  $\alpha$ -secretase into phase 2 clinical trial, showing good tolerability and safety in mild to moderate AD patients but failed in phase 3 due to lack of efficacy.

The advent of the amyloid hypothesis also instigated the development of therapeutic agents that aim to prevent or reduce the aggregation of or clear the aggregated forms of,  $A\beta$ .

Among these three treatment methodologies, the most effort went into developing agents for clearing AB aggregates, as the understanding of that time associated formation of AB plaques to AD pathogenesis. Within this push for discovery, the field of immunotherapy development has created the greatest optimism and disappointment so far. Immunotherapy, which can be separated into active and passive immunization, relies on the activity of phagocytic immune cells in the brain to remove A $\beta$  through the use of antibodies specific to A $\beta$  —identical mechanism to pathogen opsonization (Graham et al., 2017). An alternative pathway, termed "peripheral sink effect," also clears antibody-bound A $\beta$  through the activity of the BBB and vasculature. The earliest immunotherapy adopted the strategy of active immunization, which works identically to vaccination, and proved possible when, in 1999, exposure to synthetic human A\beta42 peptide reduced plaque formation and prevented memory deficit in AD animal models (Graham et al., 2017). However, this strategy of active immunization, termed AN-1792, failed phase 2 clinical trial due to 6% of patients developing severe meningoencephalitis (Breijyeh and Karaman, 2020). The result of the AN-1792 study and revelation of the possible complications associated with introducing A $\beta$  into the brain for active immunization led the field to turn its attention onto passive immunization, where antibody specific for  $A\beta$  was administered. Bapineuzumab, a humanized mouse IgG1 monoclonal antibody (mAb) targeting the N-terminal sequence of  $A\beta$ , was the first passive immunization agent to enter clinical trial after AN-1792 (Graham et al., 2017). However, despite showing significant A $\beta$  plaque clearance and proving to be generally safe in phase 2, phase 3 clinical trial for bapineuzumab was terminated due to unmet clinical endpoints, as well as the occurrence of edema and microhemorrhages in some of the patients (Citron, 2010). Subsequent immunotherapies followed the pattern observed for AN-1792 and bapineuzumab, and the constant failure of these therapeutics—which often showed stellar results

in animal models—in human trials led to increasing doubt about the validity of  $A\beta$  as the target to AD treatment and even the amyloid hypothesis as the causation of AD. The final straw that broke the camels back seemed to have arrived when phase 3 clinical trial for aducanumab and crenezumab, the latest in the line of therapeutic antibodies, were halted when interim analysis showed a lack of benefit or "futility" in March and September of 2019, respectively (Aisen *et al.*, 2020).

However, a surprising turn of events came in October 2019, when analysis of a larger data set showed that aducanumab does indeed slow cognitive decline in the group of mild AD patients carrying ApoE4 allele administered a higher dosing regiment (Aisen et al., 2020). At the time of the announcement, two other antibodies, BAN2401 and gantenerumab, are also in phase 3 clinical trials, showing a significant reduction in cerebral amyloid plaque and CSF biomarkers (Tolar et al., 2020). Preliminary data on BAN2401, in particular, shows significant clinically meaningful benefits in mild AD patients (Aisen et al., 2020). This reversal in the field of AD immunotherapy can be understood from several paradigm shifts that occurred within the last two decades of research. The most important tenet leading up to this turnabout came as a result of the understanding that soluble A $\beta$ Os are the main neurotoxic form of A $\beta$  and the causative agent in AD pathophysiology (Aisen et al., 2020). A large and expanding body of literature has investigated and shown strong evidence of the correlation between ABOs and AD, as well as the various mechanisms by which pathogenesis occurs. Past active and passive immunization therapeutics that failed in clinical trials mainly targeted A<sup>β</sup> plaque or A<sup>β</sup> monomer, which correlated poorly with AD disease progression and, in the case of A $\beta$  plaques and fibrils, may even possess neuroprotective functions. Solanezumab, a mAb with high reactivity to  $A\beta$ monomer, recently failed phase 3 clinical trial due to lack of efficacy in lowering AD biomarkers and slowing cognitive decline (Aisen et al., 2020). All three of the current antibodies entering or currently progressing in phase 3 clinical trial-BAN2401, aducanumab, gantenerumab-show greater reactivity toward oligometric forms of A $\beta$  over fibrils or monomer. The second tenet was the discovery that edema and microhemorrhage observed in patients administered therapeutic Aß antibodies can be attributed to clearance of vascular plaques, which adversely affected BBB integrity (Aisen et al., 2020). This phenomenon is exacerbated in carriers of ApoE4 alleles, which often showed greater vascular plaque depositions due to impaired A $\beta$  transport and clearance in a dose-dependent manner—with homozygous carriers showing the greatest exacerbation. The nature of immunotherapy, which relies in part on Fc-receptor-mediated phagocytosis by microglia, also entails increased neuroinflammation and resultant vascular disturbances. New understanding of the causation of AB antibody-associated edema and microbleed now transforms what was previously considered an unavoidable consequence of immunotherapy to a manageable safety concern addressable through careful target selection and antibody design (Aisen et al., 2020). This shift also led to the third tenet in the current state of immunotherapy, which is the acknowledgment of the need and implementation of higher dosing concentration, a strategy previously held back by safety concerns and lack of knowledge on safer antibody design. The last tenet is the re-evaluation of the current interim and futility analysis design for A $\beta$  immunotherapeutic clinical studies (Aisen *et al.*, 2020). The misleading result of the futility analyses for aducanumab represented only the latest of a string of inaccurate conclusions derived from the execution of the current study design. While interim and futility analyses were set in place for the protection of enrolled patients from unnecessary risk of ineffective treatment, the characteristics of immunotherapy necessitate closer inspection of data to prevent premature termination of study. In the aforementioned antibodies in clinical trials,

both aducanumab and gantenerumab require a 5 to 6-month titration period for concentration in the brain to reach peak therapeutic level. Furthermore, changes in patient populations due to new enrollment or dropout, along with alteration in the treatment regimens, affect the outcome of the study that may not be reflected in futility analysis, which fundamentally assumes the group analyzed is representative of the whole study population (Aisen *et al.*, 2020).

The current landscape for AD disease-modifying therapeutics has expanded dramatically since its start more than two decades ago. In 2020, a survey of all AD treatments in the pipeline shows a total of 121 agents currently in various phases of clinical trial (Cummings *et al.*, 2020). Aside from novel agents targeting A $\beta$  production and aggregation, various other pathways, such as inflammation, metabolism, vasculature, synaptic plasticity, and tau, are also targets of treatment. The complexity of AD pathophysiology intuitively necessitates a combinatorial approach to disease modification (Cummings *et al.*, 2019). However, the host of evidence indicating A $\beta$  as the most upstream pathogenic agent in AD, mediating downstream pathways either directly or through tau, underscores its therapeutic value and the importance of therapeutics targeting crucial A $\beta$ -associated mechanisms of action in AD. With new insights and anticipation from aducanumab and BAN2401, this new direction in the development of safer A $\beta$  oligomer-specific antibodies will be imperative toward the future of AD treatment.

### 1.6 Challenge in Identifying Aβ Oligomer Target

In light of the paradigm shift in the amyloid hypothesis—moving away from amyloid plaque as the causative agent to A $\beta$ O, the new generation of mAbs, including aducanumab and BAN2401, are designed to target the disease-relevant soluble A $\beta$  aggregates. Whereas former antibodies, such as bapineuzumab, solanezumab, and crenezumab, are sequence-specific and show no cognitive benefit despite effective A $\beta$  clearance, aducanumab and BAN2401 are not designed with Aß sequence in mind (Graham et al., 2017). BAN2401 is first generated by mice injected with protofibrils isolated from patients carrying the Arctic mutation, and subsequent studies show the humanized mAb indeed reacts to large Aβ aggregates of greater than 100kDa, but not to fibrils or monomers. Aducanumab is a fully human IgG1 found through a screen of healthy elderlies with no cognitive deficit thought to have naturally generated mAb against  $A\beta$ (Graham et al., 2017). The move away from sequence specificity to structure specificity is logically sound, as the target in question originates from a constitutively expressed protein and exists in a highly complex continuum of aggregation states, forming a heterogeneous population in solution. However, despite the appropriate shift in design focus from sequence selectivity to aggregation state selectivity, the next-generation antibodies have yet to completely remedy the shortcomings present since the inception of A $\beta$  immunotherapy (Tolar *et al.*, 2020). Administration of aducanumab and BAN2401, to a lesser extent, still causes edema and microhemorrhage due to cross-reactivity to A $\beta$  fibrils. A comparative study of aducanumab and BAN2401 in relative binding affinity to  $A\beta O$  and fibril shows BAN2401 possesses a 10-fold higher specificity to ABO compared to aducanumab (Tolar et al., 2020). As a result of the shortcomings in current design strategies, a shift from simple aggregation state selectivity toward even more stringent target selection will be necessary to achieve minimal fibril reactivity.

One such novel antibody is PMN310, a mouse mAb designed utilizing molecular dynamic simulation and computational modeling toward a unique cyclic structural epitope present only on toxic AβOs (Gibbs *et al.*, 2019). Surface plasmon resonance (SPR), a label-free microfluidic analytic technique capable of monitoring biomolecular interaction in real-time, shows that PMN310 preferentially binds to AβOs, with minimal binding for monomers. Immunohistochemical staining of AD brain sections further revealed minimal plaque reactivity

by PMN310, as opposed to robust binding by aducanumab and bapineuzumab. In in vitro experiments, PMN310 inhibits A $\beta$ O-mediated propagation of A $\beta$  aggregation and abrogates A $\beta$ O toxicity, while in vivo experiments reveal a reduction in synaptic loss and inflammation, as well as alleviation of cognitive and memory disruption. The most significant finding is that PMN310 binds preferentially to LMW fraction of clarified AD brain homogenate over high molecular weight (HMW) fraction, as opposed to aducanumab, which showed no preference in reactivity. It was further revealed that PMN310 binds only to a subpopulation of targets bound by aducanumab, and a subsequent competition assay, where the first and second ligand is PMN310, shows only one unique epitope is present in bound A $\beta$ Os. PMN310 represents the next evolution in A $\beta$  immunotherapy, where design is carried out by computational calculation not just to aggregation state but to unique conformation epitope to achieve greater A $\beta$ O specificity.

One major question often remains in the characterization of A $\beta$  antibodies, and that is the specific A $\beta$  species being targeted. Thus, we set out to answer this question using PMN310, an antibody with well-characterized activity and epitope specificity, not only to add to the therapeutic profiling of PMN310 but also to contribute to the ongoing investigation for the A $\beta$ O species most responsible for AD pathogenesis.

#### 1.7 Rationale, Hypotheses, and Objective

### 1.7.1 Isolation and Collection of Heterogeneous Populations of AβOs

The canonical technique in the characterization of Aβ aggregates is SDS-PAGE, which separates protein mixture present in a sample according to mass. However, this method necessitates preparing two aliquots of a sample to allow for functional analysis, which introduces potential confounding factors. Furthermore, a large body of evidence reveals that SDS alters the Aβ oligomerization states in samples, breaking down large AβOs while promoting small AβOs and monomers to aggregate (Pujol-Pina *et al.*, 2015 and Wildburger *et al.*, 2016). Techniques, such as nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FTIR), electron microscopy, atomic force microscopy, mass spectrometry (MS), limited proteolysis, and chemical modifications, have all been employed to probe the structure of A $\beta$  aggregates, but the conditions for sample preparation and equipment involved in these approaches still prevent the simultaneous use of single sample preparation for both characterization and functional assay (Carulla *et al.*, 2010). The prolonged assay time will also affect the presence of transient small A $\beta$ O species. The ideal technique should allow for rapid characterization of A $\beta$ Os with minimal sample manipulation and sufficient sensitivity to detect minor populations in solution while simultaneously permitting the collection of analyzed A $\beta$ Os for subsequent experiments with PMN310.

Size exclusion chromatography (SEC) fulfills all the criteria ideal for A $\beta$ O characterization, and recent advances in matrix material and construction, as well as automation of the process, allow for highly accurate and reproducible experimentation, along with broad buffer solution compatibility. For the purpose of determining A $\beta$ O targeting by PMN310, we will carry out SEC on samples prepared using synthetic A $\beta$ 42 peptides incubated for different periods of time. We hypothesize that populations of A $\beta$  will change with incubation time, with a shift toward HMW A $\beta$ O and fibrils at longer time points. Homogenates of AD and healthy human brains will also be analyzed and prepared for subsequent testing. We hypothesize that the majority of soluble proteins present within brain homogenate will be HMW species, with a subpopulation of A $\beta$ Os existing in a continuum from HMW to LMW. As the concentration of soluble A $\beta$ Os concentration decrease with AD progression and makes up only a minor portion of

brain homogenate, we hypothesize that the healthy control and AD brain homogenates will be similar in profile (Aoyagi *et al.*, 2019).

### 1.7.2 Examining Seeding Activity and PMN310 Reactivity of Different ABO Species

Enzyme-linked immunosorbent assay (ELISA) represents the technique of choice, along with western blot, for biochemical analysis of antibody binding, with widespread use within the literature. In both methods, a protein analyte is deposited and fixed onto a surface: in the case for western blot, samples are transferred onto a membrane—its performance and chemical profile vary by polymer used and pore size, and in the case for ELISA, antibodies are fixed onto a matrix to capture the analyte. Both methods then proceed similarly with the addition of a primary antibody, followed by a detector—usually an enzyme-conjugated antibody—that processes detection reagents to allow for colorimetric or fluorometric quantifications. However, long assay time, frequent washing steps, and changing chemical conditions render ELISA a suboptimal technique for analysis of  $A\betaOs$ .

Dot blotting, a western blot technique that omits the usual SDS-PAGE step, is suitable for the purpose of this study due to its ease of executing and comparatively rapid assay time from sample deposition to antibody binding. We will be employing a modified version of dot blot, termed filter trap assay, in which vacuum is used to facilitate sample deposition onto the membrane. This technique remedies the shortcoming of dot blot, which is the limit of sample loading volume to several microliters and allows sample volume up to hundreds of microliters. Both AβO generated from synthetic Aβ42 and brain homogenate, fractionated by SEC, will be tested. We hypothesize that PMN310 will show greater reactivity for individual fractions of low molecular weight in both preparations, as Gibbs et al. reported a preferential binding of PMN310 to LMW brain homogenate fraction.

The ability of A $\beta$ Os to promote aggregation by acting as nucleation seeds constitutes a major part of the neurotoxicity and pathogenicity of A $\beta$ Os. Canonically, thioflavin-T (ThT) assay is used to study the process of aggregation. ThT was first used as a fluorescent probe for amyloid fibrils in tissue samples, and later usage in the quantification of fibrils in solution showed a tight correlation with fluorescent intensity, leading to widespread gold standard measure for fibril formation. As a fluorescent dye, ThT binding to  $\beta$ -sheet-rich structures leads to increased fluorescence and blue shift in the emission spectrum (Gade Malmos *et al.*, 2017). In a protein-only solution, ThT binds to fibrils with high specificity. We will subject synthetic A $\beta$ O and brain homogenate fractions to ThT assay, and we hypothesize that LMW A $\beta$ Os that show prominent PMN310 reactivity will show the greatest seeding activity. This hypothesis is built upon studies showing that LMW A $\beta$ Os are responsible for the pathogenesis and propagation of disease in early AD.

# 1.7.3 Analysis of Antibody-ABO Binding Using Surface Plasmon Resonance

Common, widespread techniques for characterizing antibody reactivity include immunohistochemical assays on biological samples and immunoassays such as ELISA and western blots. Even in light of recent advances in technologies expediting the experimentation process, these methods for antibody profiling remain unable to achieve instantaneous detection of binding activity. This is due, in part, to the reliance on labels for detection and quantification of antibody-binding, necessitating multiple wash steps and changing chemical conditions, unavoidably introducing potential errors. Furthermore, the complexity of these techniques prevents complete automation, hampering advances for reproducibility.

Surface plasmon resonance (SPR) technology represents the next-generation approach for therapeutic target discovery and validation. The optical interferometric basis of SPR allows for

fully automated, label-free detection of biomolecule interaction in solution real-time. SPR is proven to be highly sensitive for analytes in low abundance even within complex mixtures, which is beneficial for studying transient A $\beta$ Os in the heterogeneous population. Recent studies of A $\beta$ O antibodies, such as aducanumab, gantenerumab, and BAN2401, have employed SPR for affinity testing and comparison (Tolar *et al.*, 2020). Gibbs *et al.* employed SPR for testing the affinity of PMN310 against brain homogenates and showed differential binding to HMW and LMW fractions. In line with the experimental methodology, we will also employ SPR to examine PMN310 binding to recombinant A $\beta$ O incubated for different periods of time. This experiment will serve as corresponding reactivity characterization by PMN310 to the SEC timecourse experiment with recombinant A $\beta$ 42. We hypothesize that PMN310 will display robust reactivity to recombinant A $\beta$ 42 preparation incubated for the shortest period of time, as PMN310 binds preferentially to soluble, LMW A $\beta$ O (Gibbs *et al.*, 2019).
# **Chapter 2: Method and Materials**

#### **2.1 Aβ Monomer Preparation**

Hexafluoroisopropanol (HFIP)-treated and lyophilized recombinant A $\beta$  peptides were purchased from BACHEM. 6M guanidine hydrochloride (GnHCl) solution was prepared with double distilled water and filtered with a 0.22µm syringe filter. The filtered 6M GnHCl solution was then added to the A $\beta$  peptides and vortexed until complete reconstitution of the peptide. Dissolved A $\beta$  peptides were centrifuged with a benchtop centrifuge to remove insoluble material, and the resulting supernatants were aliquoted and stored at 4°C until use. A $\beta$ 1-40 (A $\beta$ 40) was prepared to 200µM. A $\beta$ 1-42 (A $\beta$ 42), A $\beta$ 42 with reversed and scrambled sequences were prepared to 100µM.

#### 2.2 Recombinant Aβ1-42 Oligomer Preparation

HFIP-treated and lyophilized recombinant  $A\beta42$  peptide was purchased from rPeptide. Anhydrous dimethyl sulfoxide (DMSO) purchased from Thermo Fisher was used to reconstitute  $A\beta42$  peptide to a stock concentration of 5mM. Peptide reconstitution was carried out in a cell culture hood to prevent particulate contamination. The stock solution was sonicated in a waterbath sonicator, with a mixture of ice and water to lower temperature, at high setting for 3 minutes. The  $A\beta$  stock was then diluted in ice-cold phenol red-free F12 medium (Thermo Fisher) to a final concentration of 100µM and stored at 4°C for incubation, and the  $A\betaO$  was then stored at -80°C following completion of incubation. For the 1- to 7-day time-course experiment, one stock of 100µM  $A\betaO42$  preparation was incubated at 4°C, and aliquots were withdrawn in cell culture hood with the stock preparation resting on ice to be injected onto the SEC for analysis at respective time points. For the 0- to 96-hour time-course experiment, one stock of 100µM AβO42 preparation was split into 2 aliquots and placed in 2ml deep-well plates (Greiner Bio-One), covered by plate sealer, and kept within the SEC and SPR devices at 4°C.

## 2.3 Brain Homogenate Preparation

Brains of AD patient and healthy individual were weighed before suspension in ice-cold TBS containing complete protease inhibitors (Roche) at a 10% weight-to-volume ratio. The tissue was homogenized at 4°C using a probe sonicator until the tissue was completely dissolved. The resulting lysates were centrifuged at 21,000×g for 15 min to remove cellular debris and large, insoluble material. Supernatants were aliquoted and stored at -80 °C until further use.

#### 2.4 Size Exclusion Chromatography

#### 2.4.1 Superdex 200 10/300

The Superdex 200 increase 10/300 GL column (GE Healthcare) was connected to the Dionex UltiMate 300 HPLC system from Thermo Fisher. The overall column volume (CV) was 24ml, and the system was equipped with a 250µl injection loop. PBS running buffer was prepared by diluting 10x PBS stock (Thermo Fisher) with double distilled water. Globular protein molecular weight standard was purchased from Bio-Rad and prepared according to protocol. Briefly, 0.5ml of double distilled water was added to reconstitute the lyophilized proteins and incubated on ice for 2-3 minutes. The standard was further diluted 1:5 with double distilled water, filtered with a 0.22µm syringe filter, and 250µl was injected onto the column, with a flow rate of 1ml/min for a run time of 30 minutes. At least one run of PBS was allowed to flow through the column prior to and after sample injection for baseline equilibration and cleaning, respectively. All samples for injections were kept at 4°C in the sample department of the Dionex system. For monomer determination, 100µl of 200µM Aβ40, 100µM Aβ42, and 100µM Aβ42 with reversed sequence were injected and eluted at a flow rate of 1ml/min. One injection of PBS was inserted between each sample injection to remove trace materials from the prior run and to re-equilibrate the column. For A $\beta$ O time-course experiment, 250 $\mu$ l of 100 $\mu$ M A $\beta$ 42 incubated for 2 days and 7 days were injected at 1ml/min flow rate. All SEC runs were monitored through built-in UV spectrometer by the absorbance at 280nm. Superdex 200 column was cleaned at the end of every week with at least 2 injections of double-distilled water, followed by at least 2 injections of 0.5M NaOH, and finished with at least 2 injections of double-distilled water.

#### 2.4.2 Superdex 75 10/300

The Superdex 200 increase 10/300 GL column (GE Healthcare) was connected to the Dionex UltiMate 300 HPLC system from Thermo Fisher, with identical CV and system setup. Injection of globular protein molecular weight standards prepared identically to Superdex 200 run was carried out with a flow rate of 0.5ml/min for the duration of 50 minutes. Additional standards—conalbumin at 75kDa, cytochrome c at 12.3kDa, and aprotinin at 6.5kDa—were prepared by reconstituting lyophilized protein with double-distilled water and 0.22µm syringefiltered before injection. For the 1- to 7-day time-course experiment, 250µl of 100µM Aβ42 incubated for 1 to 7 days were injected onto the column. Injections of 250µl of 100µM Aβ42 with a scrambled sequence (BACHEM) and 100µl of 100µM Aβ42 dissolved in 6M GnHCl were carried out for monomer determinations. For 0- to 96-hour time-course experiment, 100µl of 100µM Aβ42, incubated within the sample department in 2ml deep-well plate, was injected at respective time points. For filter trap assay fraction preparation, 250µl of 100µM AβO42 incubated for 1- to 3-days, and brain homogenates were injected and collected by the minute over the course of the entire column elution into 2ml deep-well plate. The collection plate was kept at 4°C in the sample compartment and kept on ice after sample collection to be used immediately.

# 2.4.3 Protein Concentration Determination

Protein concentration of recombinant A $\beta$ O42 SEC fractions was quantified using the Pierce micro-bicinchoninic acid (microBCA) from Thermo Fisher, following the microplate protocol.

# 2.4.4 Data Analysis and Graph

SEC run data were converted into excel format and, along with BCA results, entered into GraphPad Prism 5 for graphic comparison.

# 2.5 Filter Trap Assay

# 2.5.1 Recombinant AβO Dot Blot

Figure 2.1 showed the filter trap apparatus and method of assembly. In brief, 10x TBS was diluted with distilled water and served as assay buffer. Nitrocellulose membrane was equilibrated in the buffer for 10 minutes prior to assay, and filter papers were wetted just prior to apparatus assembly. The nitrocellulose membrane was placed upon 2 filter papers, and all the components were fitted and tightened with sealing screws.  $100\mu$ l of TBS was pipetted into each well, and the vacuum manifold was connected to the vacuum, with the flow valve closed off to the ambient atmosphere. A full vacuum was pulled, and sealing screws were quickly tightened once more. Subsequently, the vacuum was released by opening the flow valve to the atmosphere, and  $100\mu$ l of TBS was pipetted for a second time before pulling a partial vacuum to reduce buffer volume in the sample template. Once sufficiently reduced, the vacuum was turned off, and flow valve opened, and SEC fractions were loaded into the sample template. For recombinant A $\beta$ O42 dot blot, 100 $\mu$ l and 200 $\mu$ l of each fraction were loaded for 6E10 and PMN310 dot blot,

respectively. TBS was added to make up the difference in volume, and unused wells were filled with 200µl of TBS. A partial vacuum was used to slowly deposit sample onto the membrane, and TBS was added to wells as needed throughout the deposition process to prevent desiccation. Once all solutions were pulled through and samples deposited, the membrane was sectioned and incubated in 5% milk weight-to-volume in 0.01% TBS-T for one hour at room temperature for blocking. Both 0.45 and 0.1µm pore size nitrocellulose membranes (GE Healthcare) were used with the identical protocol.



Figure 2.1 Diagram of the assembly of the filter trap apparatus

Pan-A $\beta$  antibody 6E10 (Biolegend) was diluted 1:10000 from stock 1mg/ml concentration to 0.1µg/ml with 5% milk for use as primary antibody. Mouse PMN310 (ProMIS Neuroscience) was diluted 1:955 from stock 1.91mg/ml to 2µg/ml with 5% milk. Dot blots were incubated in primary antibody overnight at 4°C, and they were subsequently washed 3 times at

10 minutes each with TBS-T. Both blots were incubated in HRP-conjugated anti-mouse IgG1 secondary antibody (Thermo Fisher) at 1:5000 dilution from stock 1mg/ml concentration for one hour at room temperature. The blots were again washed 3 times at 10 minutes each with TBS-T, and the blots were treated with Femto Sensitivity Substrate ECL (Thermo Fisher) for 5 minutes before imaging with ChemiDoc Imaging System (Bio-Rad). 6E10 and PMN310 dot blots were exposed for one and 60 seconds, respectively, for optimal spot intensity.

#### 2.5.2 Brain Homogenate Dot Blot

Identical protocols used for recombinant AβO42 were applied with brain homogenate SEC fractions. 0.1µm nitrocellulose membrane was the only membrane tested, and hIgG1 (Biolegend) was added to the panel of primary antibodies examined. hIgG1 was diluted 1:5000 from the stock 1mg/ml to 0.2µg/ml for primary antibody incubation.

#### 2.5.3 Data Analysis

Dot bot intensities were quantified using ImageJ. The results were compiled and presented using GraphPad Prism 5. The total number of AβO42 seeds was calculated by extrapolating the average molecular weight of each fraction from molecular weight standard curve to correlate the recombinant AβO42 dot blot results to the abundance of AβO42 loaded. Molecular weight standard curves were generated by carrying out a linear fit of the log of molecular weight in Dalton (Da) to elution time. The microBCA-quantified concentration was divided by the estimated molecular weight of SEC fraction, multiplied by the Avogadro's number and filter trap assay loading volume, to determine seed abundance. The data were presented using GraphPad Prism 5.

# 2.6 Aggregation Seeding Analysis

#### 2.6.1 Thioflavin-T Assay

Thioflavin-T (ThT) (Sigma-Aldrich) was prepared to 200μM stock concentration using 50mM Tris-HCl with 1mM EDTA, which was prepared by dissolving Tris-HCl BioUltra tablet (Sigma-Aldrich) pH 7.4 in 499ml of double distilled water and 1ml of 500mM EDTA. The solution was vortexed and stored in the dark at 4°C, and it was filtered with 0.22μm syringe filter before use. 50µl of SEC fractions kept on ice, and Tris-HCL buffer—with 100µl of buffer added to the blank well—were added to a black-walled 96-well microtiter plate (Greiner Bio-One) and stored at 4°C to be used shortly after. Aβ42 peptide was reconstituted to 500µM stock concentration in 10mM NaOH—which was also filtered before use. 50µl of Aβ42 monomer was added to the plate, and 5.5µl 200µM ThT was added immediately after using a multichannel pipet. A sealer was placed on top of the plate, and the plate was tapped for gentle sample mixing. ThT fluorescence was measured every hour (excitation at 440 nm, emission at 486 nm) with Wallac Victor3v 1420 Multilabel Counter (PerkinElmer) at 25°C for 72 hours. The final assay results were subtracted against the buffer-only and respective control wells.

# 2.6.2 Data Analysis

The final buffer- and control-subtracted results were inserted into GraphPad Prism 5. Repeats of each fraction were analyzed separately, and the data were fitted using nonlinear Boltzmann sigmoidal regression analysis. Lag times of A $\beta$ 42 monomer alone and SEC fraction additions were calculated from the maximum and minimum signal, the slope of the elongation phase, and V50 of the curve.

#### 2.7 Surface Plasmon Resonance Assay

#### 2.7.1 Monitoring of Amyloid Beta Aggregation

All experiments were performed using a Molecular Affinity Screening System (MASS-2) (Bruker Daltronics), an analytical SPR biosensor that utilizes high-intensity laser light and highspeed optical scanning to monitor binding interactions in real-time. PMN310 and 6E10 were diluted to 40ug/ml in sodium acetate pH 4.5, then injected and covalently coupled to 2 separate spots on the sensor chip surface by amide bonding. Residual unreacted sites were blocked with 1M ethanolamine-HCl pH 8.5. An adjacent reference spot was similarly derivatized with mouse IgG1 isotype (Biolegend) to account for non-specific binding. Approximately 12000 resonance units (RU) of antibodies were immobilized.

Freshly prepared A $\beta$ O42 monomers incubated for 0, 1, 2, 3, 4, 5, 6, 12, 24, and 48 hours were injected over the immobilized antibodies for 300 seconds followed by a dissociation phase of 180 minutes during which only buffer was injected over the surface. After each analytical cycle, the antibody surfaces were regenerated with Glycine-HCl ph1.5 (Bruker Daltronics).

#### 2.7.2 Data Analysis

The resultant sensorgrams (binding responses over time) were double-referenced by subtraction of binding on the mIgG1 reference surface and also the F12-DMSO diluent used to reconstitute the A $\beta$  monomer. Binding report points were then obtained 30 seconds post-injection of amyloid beta during the dissociation phase.

# Chapter 3: AβO42 and Brain Homogenate Characterization Using Size-Exclusion Chromatography

# **3.1 Introduction**

SEC is the current method of choice for the separation of molecules in solution on the basis of size. The first instance of liquid chromatography utilizing a size-exclusion mechanism is the separation of peptides from amino acids using a starch matrix based on the "molecular sieve" effect (Hong *et al.*, 2012). The advent of synthetic porous beads from crosslinked dextran, trademarked as Sephadex, revolutionized SEC, as optimal size resolution range can now be controlled through the manufacturing process (Bouvier and Koza, 2014). The increased mechanical strength and chemical stability led to a dramatic decrease in assay time to mere hours, down from weeks as formerly required. The use of Sephadex and other polymer-based beads in SEC became widespread, attributable in large part to its minimal non-specific interactions. Following Sephadex, the development of silica beads raised the performance of column SEC to new heights. Silica-based beads possess greater mechanical strength, withstanding greater pressure before the collapse, further reducing run time, and increased chemical stability, minimizing the effect of varying buffer conditions on matrix integrity—a problem plaguing polymer-based beads. Silica beads can also be produced at smaller particle sizes, improving separation efficiency by allowing tighter matrix packing within a column. Separation of molecules through the matrix is achieved by trapping molecules in intraparticle pores, and the probability of molecules retained during travel depends upon its Stokes radius, which changes according to its shape and the buffer condition (Hong et al., 2012). Stokes radius correlates positively with molecular size and weight, thus changes in retention time approximate particle mass, which can be estimated by running globular protein standards with known mass

for comparison. While this method only allows approximation of size and requires additional techniques, such as sedimentation velocity analytical ultracentrifugation (SV-AUC), to achieve true quantitation, it is sufficient for the purpose of fractionating samples for analysis.

SEC has proven to be an effective method for characterizing and separating ABOs from synthetic peptide preparations and biological sources (Jan et al., 2010). The rapid aggregation kinetics of ABO poses a major concern in characterizing distinct ABO populations as intermediates along the aggregation pathway. Application of highly amyloidogenic Aß proteoforms, such as Aβ42 and Aβ40 carrying the Arctic mutation, to neuron cultures lead to perturbation in 7 hours and cell death within 24 hours. While SEC cannot detect subtle changes in ABO population happening on the scale of minutes, it is able to isolate LMW ABOs such as dimers and trimers from HMW ABOs, consisting of protofibrils and amorphous aggregates, with high purity. In fact, studies utilizing photo-induced cross-linking of unmodified proteins (PICUP) have shown that with correct column selection and buffer conditions, SEC is capable of separating these LMW ABOs individually (Bitan et al., 2001). Current SEC columns are frequently equipped with filters that can capture insoluble materials from the injected sample, ensuring the fractionated ABOs contain no fibrillar seeds (Jan et al., 2010). The capability of modern SEC technology to rapidly separate transient and unstable protein complexes, such as ABOs, from complex mixtures in detergent-free solution not only solidifies its place as the preparative technique of choice but also harbors potential as a qualitative analytic technique for studying A $\beta$ O changes over time.

The experiments in this section aim to compare the suitability of two SEC columns, Superdex 200 GL 10/300 and Superdex 75 GL 10/300, for separating and collecting A $\beta$ O samples for subsequent analysis. We will utilize SEC as a qualitative technique to test the hypotheses that synthetic  $A\beta Os$  exist in a continuum of aggregation states that varies with time and that brain homogenates of healthy individuals and AD patients will be similar in profile.

# 3.2 Result

#### 3.2.1 Superdex 200 GL 10/300 Result

Injected globular protein molecular weight standards eluted at 8.993 minutes for 670kDa marker to 20.468 minutes for 1.3kDa marker, with 158, 44, and 17kDa markers eluting at 12.025, 14.685, and 17.169 minutes, respectively. At 1ml/min flow rate, this corresponds to elution volume of 8.993, 12.025, 14.685, 17.169, and 20.468ml for the five markers over the 24ml of the column. Figure 1.1 shows the linear fit of the logarithm of molecular weight to its elution time, with an r-squared value of 0.98. A 670kDa marker indicates the exclusion limit of the column, where molecules larger than the upper-resolution limit of the matrix pass through the column with minimal retention, and 1.3kDa indicates permeation limit, where molecules smaller than the lower resolution limit of the matrix get retained and behave identically. The exclusion of the 1.3kDa marker improved the r-squared value, showing a better linear fit of the data (Figure 3.2). This phenomenon indicated the permeation limit of the column was earlier than determined by the 1.3kDa marker.



Figure 3.1 Molecular weight standard for Superdex 200 10/300 at 1ml/min flow rate



**Figure 3.2** Molecular weight standard for Superdex 200 10/300 at 1ml/min flow rate. The permeating limit of the column was before the elution time of the 1.3kDa marker.

A $\beta$  peptides dissolved in 6M GnHCl are injected to determine monomer elution time. A $\beta$ 40 and A $\beta$ 42 with reversed sequence serve as controls, as they are less amyloidogenic than A $\beta$ 42, minimizing the probability of aggregation over the course of SEC assay. All three A $\beta$ peptides displayed a major peak eluting at 17.78 minutes, corresponding to approximately 11.9kDa (Figure 3.3). Minor peaks appear at earlier and later elution time, and a set of prominent peaks of similar intensities appear after the 1.3kDa marker for all injected samples.



# AB42 in 6M GnHCI Superdex 200

Figure 3.3 A $\beta$  monomer molecular weight determine on Superdex 200 10/300 column at 1ml/min flow rate. A $\beta$  peptides eluted at 17.78 minutes, corresponding to 11.9kDa.

Aβ42 incubated at 4°C for 2 days and 7 days appeared as a complex mixture of oligomers upon injection (Figure 3.4). AβO42 generated after 2- day incubation displayed a significant peak at 17.74 minutes, corresponding to 12.1kDa. A minor peak eluted earlier at 15.36 minutes, corresponding to 35.6kDa. A dispersed peak ranging from 8.61 minutes to 13.71 minutes, peaking at 10.52 minutes, preceded the 35.6kDa peak, a range of 75kDa up to the exclusion limit of the column, with a maximum at 320kDa. The 2-day AβO42 also included aggregates eluting beyond the exclusion limit. AβO42 generated at 7-day incubation displayed a significantly diminished peak at 17.74 minutes, as well as the dispersed peak observed for the 2-day AβO42. The peak at 15.36 minutes was absent in the 7-day AβO42. A significant and sharp peak appeared in 7-day AβO42 beyond the exclusion limit, at a higher intensity compared to 2-day AβO42. Both samples showed a set of prominent peaks from 18.88 minutes to 22.32 minutes, followed by a peak at 24.63 minutes. The first set of peaks ranged from 7.2kDa to beyond the permeation limit, and the second peak eluted beyond the end of the column elution volume.



Aß42 Oligomers Superdex 200

**Figure 3.4** A $\beta$ 42 incubated at 4°C for 2 days vs. 7 days compared using Superdex 200 10/300 column at 1ml/min flow rate

#### 3.2.2 Superdex 75 GL 10/300

Identical molecular weight standards as used in Superdex 200 were injected, with the 670kDa marker eluting at 15.687 minutes and the 1.3kDa marker eluting at 37.65 minutes. Intermediate markers eluted at 16.513, 20.44, and 25.903 minutes, corresponding to 158, 44, and 17kDa, respectively. Additional protein standards marking 75, 12.3, and 6.5kDa were injected, eluting at 18.45, 27.913, and 32.763 minutes, respectively. At 0.5ml/min flowrate, these molecular weight standards eluted at 7.844, 8.257, 9.225, 10.22, 12.952, 13.957, 16.382, and 18.825 minutes. Figure 3.5 shows the linear fit of the logarithm of molecular weight to its elution time for all markers from 670kDa to 1.3kDa, with an r-squared value of 0.9281. Figure 3.6 shows the linear fit of the same values excluding 670kDa, with an r-squared value of 0.9805.



**Figure 3.5** Molecular weight standard for Superdex 75 10/300 at 0.5ml/min flow rate. 670 kDa standard deviated from the trend line due to the standard surpassing the exclusion limit of the column matrix.



**Figure 3.6** Molecular weight standard for Superdex 75 10/300 at 0.5ml/min flow rate, with 670kDa standard excluded

A $\beta$ O42s incubated at 4°C for one to 7 days are injected, and resulting profiles are compared. Figure 3.7 shows the A $\beta$ O42 chromatograms overlayed, with all injections displaying a set of prominent peaks eluting from 34.5 to 45.83 minutes and an additional peak eluting at 49.09 minutes, corresponding to 3.14kDa to beyond the permeation limit of the column. A $\beta$ 42 with scrambled sequence dissolved in 6M GnHCl was injected as monomer reference, eluting at 28.89 minutes, corresponding to 9.9kDa. Figure 3.8 shows a close-up of the chromatograms, focusing on the peaks corresponding to A $\beta$ O42. A $\beta$ O42 formed from 1 to 4-day incubation displayed similar profiles, with one prominent peak eluting at 28.74 minutes, corresponding to approximately 10.21kDa. Minor peaks eluted earlier at 26.14, 24.6, and 21.96 minutes, corresponding to 17.4, 23.8, and 41kDa, respectively. A significant peak, spanning from 12.1 to 19.8 minutes, with the maximum at 15.65 minutes, was present in A $\beta$ O42 injections, corresponding to 63.78kDa to beyond the exclusion limit of the column. No significant difference was present between 1- and 2-day A $\beta$ O42 at 15.65 and 28.74 minutes, with decreases occurring at all peaks between these two times. Significant decrease at the 28.74 minutes peak occurred 3- and 4-day A $\beta$ O42, with complete absence in 7-day A $\beta$ O42. Intermediate peaks decreased from 1- to 7-day incubation, with a minor difference in the 15.65 minutes peak. An additional peak appeared in 7-day A $\beta$ O42 at 13.86 minutes. A $\beta$ 42 dissolved in 6M GnHCl was injected as monomer determination, eluting at 28.85 minutes, corresponding to 9.98kDa.



**Figure 3.7** A $\beta$ 42 incubated at 4°C from 1-7 days compared using Superdex 75 10/300 column at 0.5ml/min flow rate. No significant difference in A $\beta$ O42 population occurred until 3-day post-incubation. With a 7-day incubation, the majority of LMW A $\beta$ O42 aggregated into HMW species, in agreement with the Superdex 200 result. Monomer comparison used a scrambled sequence of A $\beta$ 42 dissolved in 6M GnHC1.



**Figure 3.8** Closer inspection of Aβ42 incubated at 4°C from 1-7 days compared using Superdex 75 10/300 column at 0.5ml/min flow rate. Monomer comparison was made using Aβ42 dissolved in 6M GnHCl. Aβ42 monomer eluted at 28.85 minutes, corresponding to approximately 10 kDa.

Shorter incubation lengths at 4°C were carried out with A $\beta$ 42, and resulting A $\beta$ O42 were compared (Figure 3.9). All injections displayed a peak at 28.81 minutes, corresponding to 10kDa. The intensity of this peak was greatly diminished only at 96 hours, or 4 days, of incubation, with 0-48 hours showing no significant deviation in intensity. All injections, including at 0-hour, showed a peak at 15.76 minutes, eluting at the exclusion limit of the column, with a range from 15 to 18.92 minutes. The intensity and dispersity of the peak increased with incubation time and progressed in distinct stages: with 0-hour at the lowest, followed by 2, 4, and 6-hour at the same level, and 24, 48, and 96-hour at the highest with minimal difference. An additional peak appeared in 96-hour A $\beta$ O42 at 18.25 minutes, corresponding to 87.6kDa. A

prominent shoulder peak was also present for all A $\beta$ O42 timepoints, eluting at 26.51 minutes and corresponding to 16.1kDa. Another shoulder peak at 27.9 minutes, corresponding to 12.1kDa, was also present in all A $\beta$ O42 timepoints except 96-hour.



Aß42 0-96 Hours Incubation

**Figure 3.9** Close inspection of A $\beta$ O42 incubated at 4°C from 0-96 hours compared using Superdex 75 10/300 column at 0.5ml/min flow rate. No significant change in population from 0hour to 48-hour is observed, most likely indicating a near-instantaneous aggregation of A $\beta$ 42 in solution even at low temperature.

Clarified brain homogenates from healthy individual and AD patient were injected for comparison on Superdex 75 column (Figure 3.10). Both samples presented similar profiles, with prominent peaks eluting early in the run, followed by peaks appearing at and after the permeation limit of the column, with the most significant positioned at 37.87 minutes. AD brain homogenate displayed higher intensity compared to healthy brain over the course of the run, with significant deviations up to 20.6 minutes and after 35 minutes. In between these two time points, two peaks appeared with similar intensity between the two injections at 20.79 and approximately 25.83 minutes, corresponding to 52.1 and 18.5kDa, respectively. At earlier elution time, AD brain homogenate displayed distinct peaks at 13.67, 15.87, 17.19, and 18.22 minutes, with the first two peaks eluting beyond the exclusion limit and the last two peaks corresponding to 108.89 and 88.17kDa, respectively. The corresponding peaks were also observed in healthy brain homogenate at similar times, though at a lower intensity.



Brain Homogenates Superdex 75

Figure 3.10 Brain homogenates compared using Superdex 75 10/300 at 0.5ml/min

# 3.3 Summary

Results from these experiments showed a difference in the separation range between Superdex 200 and Superdex 75. The permeation limit of Superdex 200 column was above the 1.3kDa marker, showing diminished resolution at separating lower molecular weight range, while Superdex 75 showed diminished resolution at high molecular weight, with 670kDa marker beyond its exclusion limit. Superdex 75 was selected as the column of choice for its resolution at lower molecular weight range. No distinct peak corresponding to Aβ42 monomer was found in AβO42 preparations. Aβ42 dissolved in chaotropic solution eluted at greater molecular weight than monomer in both columns. Aβ42 forms a heterogeneous oligomer population as early as 0hour. Varying length of incubation changed AβO42 populations, with a decrease in LMW population and a corresponding increase in HMW population. AD brain homogenate showed greater abundance at the HMW range and permeation limit relative to healthy brain homogenate. AD brain homogenate also began eluting earlier than healthy brain homogenate, showing significantly higher absorbance at 280nm. The intermediate region of both homogenates displayed similar profiles and absorbance.

# **Chapter 4: ABO42 SEC Fractions Dot Blot and ThT Assay**

# **4.1 Introduction**

ThT assay represents the canonical method for analyzing aggregation of A $\beta$  in solution. Along with Congo Red and Thioflavin S, ThT is an aromatic fluorescent probe of amyloid structure (Nilsson, 2009). Vassar and Culling first used ThT in fluorescence microscopy, examining amyloid deposits in tissue samples in 1959 (Gade Malmos et al., 2017). The establishment of ThT as an agent for detecting aggregation came in 1989, where Gade Malmos et al. showed that ThT fluorescence intensity accurately quantitated increasing fibril formation in *vitro*. ThT shows high specificity toward amyloid fibrils even in the presence of other proteins and other non-fibrillar aggregates. Other biomolecules, such as DNA and polysaccharides, are also able to interact with ThT and lead to ThT fluorescence, precluding the use of ThT in a mixed biological sample. Studies on ThT-amyloid interaction revealed binding of the fluorescent probe to pockets along the surface of  $\beta$ -sheets forming the fibril, with the long axis of the molecule parallel to the length of the fibril (Krebs et al., 2005). Steric interactions between ThT and side-chain residues of the  $\beta$ -sheets stabilize the probe, leading to a characteristic increase in fluorescent intensity upon binding to amyloid fibrils. This functional elucidation of ThT, extending to other amyloid probes, also explains the increased fluorescence observed with DNA and other biopolymers, which also possess highly-structured pockets that stabilize ThT upon binding. Derivatives of ThT have been developed to serve as a diagnostic tool for imaging amyloid deposits in patients, with the most notable of which being Pittsburg Compound-B (Nilsson, 2009).

A feature of amyloid protein, such as  $A\beta$ , is the ability for aggregate to act as seeds to propagate aggregation at a distal site in a prion-like mechanism (Aoyagi *et al.*, 2019). Studies

showed that A $\beta$ Os possess significantly greater potency at seeding compared to insoluble fibrils, serving as major pathogenic agents in neurodegenerative diseases (Sengupta *et al.*, 2016). One of the most widespread biochemical assays for detecting A $\beta$ Os is the western blot. However, the use of detergent often used in the preceding steps to western blots, such as SDS in denaturing gel electrophoresis, has been shown to dramatically affect the aggregate population (Pujol-Pina *et al.*, 2015). Hence, in the scenario of a pure protein sample where separation is unneeded, a dot blot can be performed, where the sample is directly deposited onto a membrane for immunoassay (Kayed *et al.*, 2003).

In the following experiments, we aim to analyze the reactivity of PMN310 to A $\beta$ O42s fractionated by SEC utilizing a modified dot blot technique, termed filter trap assay. The filter trap assay remedies the major caveat of conventional dot blot, namely the minuscule sample loading volume, through the use of a loading apparatus connected to a vacuum, allowing much greater sample volume. The same SEC-fractionated A $\beta$ O42s will simultaneously be examined using ThT assay to determine seeding activity. Concentration of A $\beta$ O42 will be quantified using bicinchoninic acid (BCA) assay. Brain homogenates will also be subjected to identical experimental procedures to analyze PMN310 reactivity and seeding activity of each SEC fraction.

#### 4.2 Result

# 4.2.1 Synthetic AβO42 Dot Blot

A $\beta$ O42 incubated from one to three days was injected onto Superdex 75 column for fractionation, and the chromatograms were overlayed for profile comparison (Figure 4.1). All three injections displayed a set of prominent peaks from 32 minutes to the end of the run. This range of time corresponded to elution beyond the permeation of the column. A peak eluting at

approximately 28.8 minutes was present for all three injections, with the highest intensity shown by 1-day A $\beta$ O42 and the lowest by 3-day A $\beta$ O42. Minor decrease in intensity occurred for 2-day A $\beta$ O42 relative to 1-day A $\beta$ O42. All three injections displayed a peak eluting at approximately 14.8 minutes, corresponding to elution beyond the exclusion limit of the column. The intensity of this peak increased with longer incubation time, with the most significant increase occurring between 1- and 2-day A $\beta$ O42 and a minor increase from 2- to 3-day A $\beta$ O42. Two intermediate peaks were observed for 1- and 2-day A $\beta$ O42, eluting at approximately 22.1 and 25 minutes, corresponding to approximately 40 and 22kDa, respectively. These two peaks were absent in the 3-day A $\beta$ O42.



**Figure 4.1** Comparison of AβO42 1-3day incubations. The result corroborated with Aβ42 time course study, with minimal changes in AβO42 population between 1- and 2-day incubation. Both AβO42 1- and 2-day incubation display two predominant populations—one at the exclusion limit, likely consisted of protofibrillar species (HMW), and one corresponding to monomerdimer equilibrium (LMW)—with two minor populations at approximately 22.1 and 25 minutes, respectively. These corresponded to approximately 40 kDa and 22 kDa, respectively.

1-, 2-, and 3-day AβO42 SEC fractions were loaded onto nitrocellulose membranes with 0.45µm pore size, and the resulting filter trap assay dot blots using 6E10 antibody were placed alongside SEC chromatograms for comparison. 6E10, a pan-Aβ antibody, showed strong reactivity from fractions 16 to 18, as well as fractions 29 and 30 for 1-day AβO42 (Figure 4.2). 6E10 reactivity diminished from fraction 19 to 20, followed by a slight increase in signal to reach a moderately strong reactivity to fraction 23. The signal once again diminished from fraction 31,

despite the absence of a clear peak at this position. Of note, 6E10 did not show increased signal for fractions 25 and 26 corresponding to the 22kDa peak. Similar 6E10 reactivity pattern was observed for 2-day A $\beta$ O42 SEC fractions (Figure 4.3). An observable decrease in dot blot intensity occurred at fractions 29 and 30 relative to 1-day A $\beta$ O42. Significant deviations in 6E10reactivity were observed for 3-day A $\beta$ O42 SEC fractions (Figure 4.4). Signal intensity further diminished at fractions 29 and 30 in 3-day A $\beta$ O42, with no signal from fractions immediately before and after the peak. No antibody reactivity was present for intermediate fractions, and the signal diminished for fraction 18. Fraction 15 showed moderate antibody reactivity in 3-day A $\beta$ O42 that was absent at shorter incubation periods. None of the A $\beta$ O42 injections displayed antibody reactivity for any fraction eluted after 32 minutes, despite intense peaks in this region of the chromatograms.



**Figure 4.2** AβO42 1-day incubation dot blot on 0.45μm pore size nitrocellulose with 6E10 pan-Aβ antibody. Robust reactivity was observed for the HMW and LMW populations, with moderate reactivity to the 40 kDa peak. Minimal reactivity is observed for the 22 kDa peak.



**Figure 4.3** AβO42 2-day incubation dot blot on 0.45μm pore size nitrocellulose with 6E10 pan-Aβ antibody. LMW population signal decreased compared to 1-day incubation.



**Figure 4.4** A $\beta$ O42 3-day incubation dot blot on 0.45 $\mu$ m pore size nitrocellulose with 6E10 pan-A $\beta$  antibody. LMW population further decreased compared to 1- and 2-day incubation, with a concomitant absence of the intermittent A $\beta$ O42 species and appearance of spots for fraction 15 not present at shorter incubations.

New 1-day AβO42 was prepared and SEC-fractionated to be deposited onto nitrocellulose membrane with 0.1 μm pore size. The dot blots were imaged using 6E10 and PMN310 as primary antibodies. The new 1-day AβO42 displayed a similar profile to previous preparation, with peaks eluting at the column exclusion limit and at approximately 28.8 minutes (Figure 4.5). Peak eluting at 25 minutes previously observed was absent in this preparation, with an increase in intensity for the peak at 22.1 minutes. Strong 6E10 reactivity was observed for fractions 16 to 18, similar to previous preparation, followed by diminishing signals for subsequent fractions until fraction 23, showing a moderate signal. Fraction 29 and 30 also showed 6E10 reactivity, as well as a minor signal for fraction 31, which was also present in previous preparation. Slight reactivity to fraction 15 was present in this 1-day AβO42 preparation. Figure 4.6 shows PMN310 reactivity to the same 1-day AβO42 SEC fractions. Fractions 16 and 17 showed a robust reaction to PMN310, with low to moderate signal for fraction 23 and fraction 29 to 30, respectively. Slight reactivity by PMN310 was present for fractions 15 and 31. PMN310 reactivity diminished more rapidly after fraction 17, compared to 6E10 reactivity. Fractions eluting after 31 minutes display no reactivity to either 6E10 or PMN310.



**Figure 4.5** A $\beta$ O42 1-day incubation dot blot on 0.1 $\mu$ m pore size nitrocellulose with 6E10 pan-A $\beta$  antibody. Differences from previous preparation include increased intensity for the HMW peak and 40kDa peak, as well as the absence of peak corresponding to 22kDa.



**Figure 4.6** A $\beta$ O42 1-day incubation dot blot on 0.1 $\mu$ m pore size nitrocellulose with PMN310 antibody. PMN310 was diluted to 2 $\mu$ g/ml for use as primary antibody for dot blot, with 200 $\mu$ l loading volume for each fraction.

# 4.2.2 Synthetic AβO42 1-Day Incubation BCA Assay

The concentration of the 1-day A $\beta$ O42 SEC fractions used in the filter trap assay was determined using micro-BCA assay (Figure 4.7). The assay results agreed with observations made in the dot blots. Fractions 15, 20, 21, 24, and 25 fell beneath the lower detection limit of the assay and presented as absence of protein. Fraction 29 and 30 displayed much greater protein concentration relative to fraction 16 to 18, despite the dot blot result suggesting otherwise. The average molecular weight of each fraction was calculated by comparing the midpoint of each fraction collection time range to molecular weight standard to elucidate the relative binding of each antibody to the estimated number of A $\beta$ 42 oligomer molecules present (Figure 4.8). Fractions 15 and 16 were arbitrarily set at 670kDa, as the fractions before or at the 670kDa

marker, indicating column exclusion limit, respectively. Using calculated concentration and average molecular weight of A $\beta$ O42 present in each fraction, the number of A $\beta$ O42s, or seeds, was determined and compared to antibody reactivity, indicated by dot blot signal intensity (Figure 4.9). Both 6E10 and PMN310 displayed robust reactivity to fractions 16 to 18. Fractions 16 to 18 showed greater or comparable dot blot signal for both antibodies to fraction 29 and 30, despite the fact that two orders of magnitude fewer seeds were loaded onto the membrane. Some minor reactivity was observed for fractions with protein concentration below the assay detection limit, with PMN310 reactivity only observed in fraction 15, 20, 21, and 24, while 6E10 reactivity was also present for fraction 25. Interestingly, fractions 25 to 28 showed an absence of PMN310 reactivity, despite microBCA confirmed detectable presence of protein. Fraction 23 displayed greater reactivity to both 6E10 and PMN310 when compared to fractions 29 to 21.



**Figure 4.7** microBCA result of 1-day AβO42 SEC fractions used in filter trap assay. Fraction 15, which showed 6E10 and PMN310 reactivity, was assayed as devoid of protein and may be a result of falling beneath the lower detection limit of the assay. Similarly, fractions 20, 21, 24, and 25, which displayed antibody reactivity, were shown as devoid of protein.

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**Figure 4.8** Average molecular weight of each SEC fraction. Fractions 15 and 16 were arbitrarily set as 670kDa for eluting at or before the 670kDa marker, indicating column exclusion limit. Average molecular weight was approximately by taking the midpoint of the fractionation time range to be compared to molecular weight standards.



Figure 4.9 Calculated Aβ42 seeds relative to dot blot signal intensity

# 4.2.3 Brain Homogenate Dot Blot

Brain homogenates from healthy individual and AD patient were SEC-fractionated and loaded onto three 0.1µm nitrocellulose membranes to be tested with 6E10, PMN310, and human IgG1 (hIgG1) antibodies. Figure 4.10, 4.11, and 4.12 illustrated comparison of dot blot intensity to the chromatogram of healthy brain homogenate for 6E10, PMN310, and hIgG1, respectively. 6E10 showed the greatest reactivity to fraction 16, which eluted at the column exclusion limit, and reactivity declined with each successive fraction. Fraction 21, consisting of a portion of the peak eluting at approximately 20.8 minutes—corresponding to 52kDa, represented the exception to the trend, showing moderately robust reactivity to 6E10, similar in magnitude as fraction 18. Peaks eluting at 25.83 and 37.87 minutes did not show particular reactivity. Fraction 15, despite being the most prominent peak of the chromatogram, only displayed minor reactivity with 6E10. Dot blot intensities for each fraction detected with different antibodies were compiled and juxtaposed in figure 4.16.



Figure 4.10 Healthy brain homogenate filter trap assay dot blot detected using 6E10

Fractions 16 and 17 of healthy brain homogenate showed the greatest reactivity to PMN310, with fractions 18 and 19, corresponding to the two peaks eluting at 17.19 and 18.22 minutes, respectively, displaying weaker reactivity. Fraction 21 showed moderate reactivity similar in magnitude to fraction 19, which fell off for fraction 22. Interestingly, despite the absence of a prominent peak, fraction 23 showed reactivity to PMN310, with greater dot blot intensity than fraction 21. The subsequent fraction 24 also showed a signal in a pattern akin to fractions 21 and 22. Minimum reactivity for PMN310 was present from fraction 25 onward.


Figure 4.11 Healthy brain homogenate filter trap assay dot blot detected using PMN310

Healthy brain homogenate displayed minimal to moderate reactivity to hIgG1. Only fractions 17, 21, and 22 showed significant signals, with minor binding to hIgG1 also observed for fractions 15, 16, 18, and 23. No reactivity was present from fraction 24 onward.



Figure 4.12 Healthy brain homogenate filter trap assay dot blot detected using hIgG1

In contrast to healthy brain homogenate, AD brain homogenate reacted weakly to 6E10 overall (Figure 4.13). The earliest fraction to show binding of 6E10 was fraction 16, and signal intensity diminishes with each successive fraction until fraction 19. Signal rose moderately for fraction 20 and reached the greatest level for fraction 21 and 22—representing the most prominent reactivity—before falling for fraction 23 and 24. Minimal 6E10 binding was present for fractions 25 to 28 before a complete absence of reactivity occurred from fraction 26 onward. Interestingly, AD brain homogenate chromatogram indicated elution of materials from 12 minutes onward, well beyond the exclusion limit of the column, but fractions 13 to 15 showed no 6E10 reactivity.



Figure 4.13 AD brain homogenate filter trap assay dot blot detected using 6E10

PMN310 showed the strongest reactivity to fraction 21 and 23 in AD brain homogenate, corresponding to an estimated average molecular weight of 55 and 37kDa, respectively (Figure 4.14). Of note, the intermediate fraction 22 did not show similar reactivity to PMN310 as its flanking fractions. Fraction 24 displayed moderate PMN310 signal, and subsequent fractions showed minimal to no antibody reactivity. Fraction 16 represented the earliest fraction showing PMN310 binding, displaying moderate reactivity. A slight increase in signal intensity was observed for ensuing fractions 17 and 18, followed by greatly diminished reactivity for fractions 19 and 20. Fractions 13 to 15 once again showed no antibody binding, similar to AD brain homogenate with 6E10.



Figure 4.14 AD brain homogenate filter trap assay dot blot detected using PMN310

Fraction 17, which eluted at approximately the same time as the 158kDa marker, of AD brain homogenate showed robust hIgG1 reactivity (Figure 4.15). Fractions 16 and 18, which eluted before and after, also showed strong binding with hIgG1. Minimal reactivity was present for fractions further left and right from fraction 17. Interestingly, fractions 14 and 15, eluting beyond the column exclusion limit, showed weak but detectable hIgG1 reactivity. Fraction 21 and 22, which together captured the peak eluting at approximately 20.8 minutes, showed reactivity to hIgG1 on a similar magnitude as fraction 16 and 18. Fractions 23 and 24 showed weak hIgG1 binding, and no signal was detected for subsequent fractions.



Figure 4.15 AD brain homogenate filter trap assay dot blot detected using hIgG1



**Figure 4.16** Compiled dot blot intensity vs. SEC fraction comparisons for healthy and AD brain homogenate

4.2.4 Synthetic AβO42 and Brain Homogenate ThT Assay

The ability of 1-, 2-, and 3-day A $\beta$ O42 SEC fractions used in filter trap assay to seed aggregation was examined in ThT assay. Figure 4.17 showed the compilation of graphs derived from the analysis of ThT assay for all three A $\beta$ O42 preparations. The slope of aggregation curve indicated the speed of the increase in ThT fluorescence at the elongation phase of A $\beta$ fibrillization. The maximum signal represented the highest recorded fluorescence intensity at the plateau phase of A $\beta$  fibrillization. No significant difference in the slope of the elongation phase was found between SEC fractions of one A $\beta$ O42 preparation, as well as between all three preparations. The maximum signal at the plateau phase showed a general increase with SEC fraction additions, with the effect being more pronounced for HMW fractions. A $\beta$ 42 monomeronly maximum signal trended lower than with A $\beta$ O42 seeding in all three assays, with the difference being significant in the 3-day A $\beta$ O42 assay. The addition of 3-day A $\beta$ O42 fractions to A $\beta$ 42 monomer did not result in a significant reduction in the lag phase of aggregation when compared to monomer alone in the same assay. A trend toward a decrease in lag time was observed in 2-day A $\beta$ O42 for fractions 16 to 18, 23, and 29 to 32 when compared to A $\beta$ 42 monomer alone, but the difference was not statistically significant. 1-day A $\beta$ O42 showed a significant reduction in lag phase at fractions 17 and 23, with fraction 23 showing the most pronounced reduction. Fraction 15, 16, 18 to 20, and 29 to 31 also trended toward a reduction in lag time, but the difference was not statistically significant when compared to A $\beta$ 42 monomer alone.



**Figure 4.17** Compilation of graphs comparing aggregation of Aβ42 monomer and monomer with the addition of AβO42 SEC fractions using ThT assay. Left: 1-day AβO42. Middle: 2-day AβO42. Right: 3-day AβO42

Identical ThT assay methodology was applied to brain homogenates of healthy individual and AD patients. Figure 4.18 showed the compilation of graphs comparing healthy brain homogenate to AD brain homogenate. Fraction 14 and 15 from both homogenates reduced the lag phase of A $\beta$ 42 aggregation, with the AD homogenate showing greater potency. Fraction 13 of AD homogenate also showed seeding activity that was absent in healthy homogenate. A general reduction in the slope of elongation was observed with the addition of homogenate fractions for both homogenates, with the greatest reduction occurring with the addition of fractions 21 and 23. The maximum signal of the plateau phase increased with the addition of fractions 24 to 30 for both homogenates when compared to Aβ42 monomer alone. The addition of fractions eluting at an earlier time generally reduced the maximum signal at plateau. Fraction 17 decreased maximum signal, with diminishing effect for successive fractions until fraction 20. Both fraction 21 and 23 led to the reduction of maximum signal, with fraction 23 showing greater potency, and the intermediate fraction 22 showed minimal to no effect on plateau phase fluorescent intensity.



**Figure 4.18** Compilation of graphs comparing aggregation of Aβ42 monomer and monomer with the addition of brain homogenate SEC fractions using ThT assay. Left: healthy control. Right: AD patient

## 4.3 Summary

A $\beta$ O42 formed by 1- to 3-day incubations showed a mixture of LMW and HMW populations, with only 1- and 2-day A $\beta$ O42 showing intermediate A $\beta$ O42 populations, with an

average weight of 22 and 40kDa, which were present at 1- and 2-day incubations. LMW population diminished as the length of incubation increased, with a concurrent increase for the HMW population. Robust 6E10 reactivity was observed for both HMW and LMW fractions, while only the 40kDa-peak, but not the 22kDa-peak, showed increased binding to 6E10. All peaks eluting after 31 minutes showed no antibody reactivity, strongly suggesting that non-A $\beta$ materials eluted at this range. 1-day AβO42 was selected for further experiment due to the presence of multiple oligometric populations showing robust antibody reactivity. 6E10 reactivity in filter trap assay using nitrocellulose membrane with smaller  $0.1 \mu m$  pore size was comparable to the previous assay using 0.45µm pore size and was selected moving forward. PMN310 reacted strongly with HMW peak, followed by moderate binding to LMW and 40-kDa fractions. The number of Aβ42 oligometric seeds, calculated from the BCA assay, showed robust antibody binding to both 6E10 and PMN310 by the HMW population. The 40-kDa fraction showed greater reactivity to both antibodies relative to LMW fractions when total A $\beta$ 42 seeds were compared. The addition of 1- to 3-day ABO42 SEC fractions to AB42 monomer in ThT assay did not significantly affect the rate of elongation and maximum signal at plateau. 1-day ABO 42HMW and 40-kDa fractions significantly reduced lag time, with the 40-kDa fraction showing greater reduction, indicating a stronger seeding effect by this ABO42 population. An identical, though non-significant, trend was also present for 2-day A $\beta$ O42, with complete ablation of significant seeding by 3-day A $\beta$ O42.

SEC fractions of healthy brain homogenate bound to both 6E10 and PMN310 while only reacting weakly to hIgG1. Fractions showed antibody reactivity in two distinct groups: HMW fractions from 15 to 18 and intermediate fractions from 21 to 24—ranging from 61 to 27kDa. All antibodies showed robust binding to HMW fractions, with moderate binding to fractions 21 and

24. No antibody reactivity was observed at fractions before fraction 15 and after fraction 25. In contrast, AD brain homogenate displayed weak 6E10 and strong PMN310 and hIgG1 reactivity. A similar reactivity profile to healthy homogenate was present in AD homogenate. Intermediate fractions showed stronger PMN310 reactivity than HMW fractions, peaking for fractions 21 and 23. Fraction 22 showed weak PMN310 reactivity, a trend also observed in healthy brain homogenate. hIgG1 antibody reacted very strongly to fraction 17, showing four times greater signal intensity than the identical fraction in healthy brain homogenate. Comparatively, intermediate fractions displayed weaker hIgG1 reactivity, peaking at fraction 21. Despite strong absorbance, antibodies did not react to fractions 12 and 13 and only weakly to 14 and 15. Fractions 13 to 15 of both brain homogenates to  $A\beta42$  monomer in ThT assay moderately reduced lag phase, with AD homogenate showing more pronounced effect. A moderate reduction in the slope of elongation phase and the maximum signal was observed for the addition of intermediate fractions from both homogenates, with fraction 23 showing prominent plateau phase maximum signal reduction.

# Chapter 5: Analysis of Antibody Binding to AβO42 at Different Incubation Lengths Using Surface Plasmon Resonance

## **5.1 Introduction**

SPR holds great potential as the next-generation approach in the study of Aβ and screening of anti-Aβ therapeutics. In this system, ligands are immobilized onto a sensor chip coated with a refractive metal, with gold nanolayer deposited upon glass prism being the most common (Homola, 2003). When two phases with different refractive indexes are present, light of a specific wavelength shone at a specific angle onto the metal will cause the absorption of the light and result in the phenomenon of the surface plasma wave. This phenomenon occurs near the interface between the two phases, and it is highly sensitive to changes in the refractive index. Binding of an analyte to ligand—in this case, protein to antibody—will cause such change, forming the basis of SPR detection. With the advent of fully automated instruments combining microfluidic systems and SPR, rapid, reproducible, and label-free detection of biomolecular interaction can be carried out in real-time in solution.

An expanding body of literature testifies to the effectiveness of SPR for characterizing  $A\beta$ . Frenzel *et al.* showed, in a study conducted in 2014, that immobilization of  $A\beta$  monomer, oligomer, and fibril onto a SPR sensor chip is possible and outlined necessary alteration in the assay methodology to account for the instability of  $A\beta$  oligomer. They subsequently confirmed the binding of  $A\beta$  to the sensor chip using a single-chain antibody fragment targeting the N-terminal sequence of  $A\beta$ . The feasibility of this procedure opens up new experimental avenues without necessitating antibody capture of  $A\beta$ , allowing direct interaction studies. A study by Silverman *et al.* utilized this method in 2018, where  $A\beta42$  and  $A\beta42$  with scrambled sequence were subjected to oligomer-forming process and immobilized onto SPR sensor chip to test binding of an oligomer conformation-specific antibody to  $A\beta$ . The assay showed that minimal interaction occurred between  $A\beta42$  scrambled and the antibody and robust interaction with  $A\beta42$ . Gibbs *et al.* also showed the potential of SPR for detecting ligand-analyte interaction in complex mixtures, such as brain homogenates, where they characterize the specificity of PMN310 to HMW and LMW brain homogenate fractions. They subsequently carried out a sandwich assay, in which a secondary  $A\beta$  antibody was added to bind to the analyte captured by PMN310 to confirm binding of  $A\beta0$ .

In the following experiment, we aim to examine PMN310 reactivity to A $\beta$ Os formed from different incubation lengths as a complement to SEC characterization. PMN310 reactivity will be compared to pan-A $\beta$  antibody 6E10.

## 5.2 Result

Figure 5.1 depicted sensorgrams of binding by 6E10 and PMN310 to A $\beta$ O incubated for 0- to 48-hour. The greatest binding response for both antibodies was reported for 0-hour A $\beta$ O, with a successive decline in binding response with longer incubation time. Figure 5.2 illustrated an alternative presentation of binding response data. Binding response declined with longer incubation, with the greatest change occurring at earlier durations. PMN310 displayed lower binding responses to A $\beta$ O42 than 6E10, and binding responses of PMN310 declined more dramatically, plateauing from 4-hour onward. 6E10 binding response declined moderately over time but did not stop even up to 48-hour.

#### 5.3 Summary

A $\beta$ O42 showed decline in binding responses to both 6E10 and PMN310 with increased length of incubation. PMN310 presented lower binding responses to A $\beta$ O42 at all time points compared to 6E10.

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**Figure 5.1** Representative sensorgrams were showing A $\beta$ O42 incubated for 0, 1, 2, 3, 4, 5, 6, 12, 24, and 48 hours binding to immobilized 6E10 and PMN310. A $\beta$ O42 samples were injected at 10ul/min for 300 seconds, followed by dissociation for 180 seconds, during which only buffer flowed over the sensor chip surface.



**Figure 5.2** Binding responses of A $\beta$ O42 binding to immobilized antibodies. Binding report points were collected 30 seconds post-injection stop of A $\beta$ O42 during the dissociation phase.

# **Chapter 6: Discussion and Conclusion**

#### 6.1 Discussion

The result of experiments in this thesis characterized, for the first time, the specificity of PMN310, an oligomer-specific antibody that targets a unique conformation epitope, to A $\beta$ O species identified by its size and toxicity—measured by its ability to seed aggregation. By varying the length of incubation in oligomer-generating conditions with Aβ42, changes in AβO populations were demonstrated through SEC, showing the existence of specific intermediate A $\beta$ Os unique to shorter incubations. The data showed that PMN310 reacted to soluble A $\beta$ Os of all aggregation states present in synthetic peptide preparations, as determined by Superdex 75 SEC column, with greater specificity for HMW species. One specific fraction containing intermediate ABO at approximately 40kDa, that bound to PMN310, showed significant seeding activity in ThT assay. Experiments using brain homogenates from healthy individual and AD patient showed differential reactivity profile to PMN310, with a shift toward lower, intermediate molecular weight fractions in AD homogenate. Once again, the fraction corresponding to 40kDa showed increased PMN310 binding. While ThT assay result showed no significant seeding activity for this intermediate ABO, with lag time reduction mainly achieved by HMW ABOs, the 40kDa fraction from both homogenates showed a significant reduction in maximum signal at the end of the assay.

In conducting this study, we first examined two size exclusion chromatography columns, Superdex 200 and Superdex 75, for their suitability in separating A $\beta$ Os. By running a set of globular molecular weight standards and plotting by linear fit, the elution time for the 670kDa and 1.3kDa markers deviated from the trend in Superdex 75 and Superdex 200 respectively. This phenomenon was the result of the protein marker not eluting within the optimal resolution range of the column. The estimated resolution range for Superdex 200 was 10-600kDa, whereas, for Superdex 75, the resolution range was 3-70kDa (Selkoe *et al.* 2017). We then injected A $\beta$ peptide dissolved in a chaotropic solution of 6M GnHCl for monomer determination. SEC traces showed different peak magnitudes for injections of A $\beta$  monomers at different concentrations, reflecting the link between absorbance and concentration. For both Superdex 200 and Superdex 75, A $\beta$  monomer eluted as a peak corresponding approximately to 10-12kDa. This may be explained by the rapid equilibrium between A $\beta$  monomer and small aggregates, such as dimers and trimers, over the course of SEC fractionation, precluding isolation of individual species (Bitan *et al.*, 2001). Selkoe *et al.* also showed, in 2017, that A $\beta$  monomer eluted in fractions at approximately 10kDa range on Superdex 75 column. As Gibbs *et al.* showed that PMN310 bound more robustly to LMW brain homogenate fractions of up to 70kDa, we selected Superdex 75 for its resolution for low molecular weight.

A $\beta$ 42 incubated for up to seven days were initially analyzed using both columns. Twoday A $\beta$ O42 was injected onto Superdex 200 column, with a flow rate of 1ml/min, and the chromatogram showed the elution of a HMW population beyond the column exclusion limit and a LMW population corresponding to 12kDa, along with a range of intermediate species—most prominently a broad peak with a maximum at 320kDa and an approximately 36kDa peak. By 7day incubation, the LMW and 36kDa peaks disappeared, along with a decreased absorbance of the 320kDa peak, to the appearance of a sharp peak beyond the exclusion limit. An identical experiment, with the inclusion of 1-, 3-, and 4-day A $\beta$ O, was carried out using Superdex 75 at a flow rate of 0.5ml/min for better separation between peaks. The result illustrated a similar trend, with a successive decline of the LMW peak, eluting at 10kDa in Superdex 75, with incubation length, until complete absence at 7-day. Along with the change in LMW population, a decline in the intermediate peaks, along with an increase of the HMW population, was observed. 1- and 2day ABO displayed minimal difference at HMW and LMW peaks, though a decrease across the intermediate-range was observed. HMW peak declined with incubation length, with 4- and 7-day showing similar magnitude, with the difference of the appearance of a peak eluting even earlier for 7-day A $\beta$ O. At all time points, a set of peaks eluted after approximately 19 and 34 minutes in Superdex 200 and Superdex 75, respectively, and most likely represent F12 media and DMSO used in oligomer formation. The data thus far corroborated the understanding of A $\beta$  aggregation, with a shift from LMW AβOs to larger aggregates. The decline in HMW peak observed in the 1to 7-day ABO Superdex 75 analysis can be explained by the appearance of an even larger species, indicated by the additional peak in 7-day ABO that may be too small to be resolved at 4day period. An additional study was carried out on ABO formed at even earlier time points, starting from 0-hour, with a smaller injection volume, in an attempt to minimize the concentration-related effect on A $\beta$  aggregation (Nick *et al.*, 2018). Data showed the formation of the HMW and LMW populations by A $\beta$ 42 immediately after resuspension in buffer, a phenomenon also reported in the literature (Bitan and Teplow, 2005). Under this experimental methodology, intermediate species eluted at smaller molecular weights than the previous assay, but the trend of decline with time for these species, along with the LMW peak, remained identical. HMW peak increased with incubation length, with the first increase occurring at 2hour, followed by another increase at 48-hour. An additional peak appeared at 4-day incubation, eluting at approximately 88kDa. Data of ABO formed at shorter incubations agreed with conclusions established by data at longer time points, though minor differences existed, which may be due to the higher concentration of A $\beta$ 42 injected onto the column obscuring small-scale changes.

Human brain homogenates from healthy individual and AD patient were also analyzed by SEC. In contrast to synthetic A $\beta$ O42, no peak corresponding to the LMW population was observed. The chromatogram for both homogenates mainly composed of HMW and intermediate peaks, followed by a group of peaks eluting at and after the column permeation limit. AD brain homogenate began eluting earlier than healthy homogenate and displayed greater absorbance in this region up to approximately 21 minutes, with the peaks up to approximately 15 minutes being more than double the magnitude. The abundance of material showing absorbance at 280nm eluting for the AD homogenate may represent very large aggregates not present in healthy homogenate. The brain homogenate chromatograms acquired in this study presented similarities with the data illustrated in the report by Gibbs *et al.*, with the elution of peaks at approximately 17 and 44kDa, as well as a peak eluting between 18 to 19 minutes, corresponding to approximately 88kDa. Differences in the SEC profiles between the two studies can be explained by natural variability in tissue samples.

SEC fractions of synthetic A $\beta$ Os and brain homogenates were tested with 6E10 and PMN310 using filter trap assay, a modified dot blot method. 1- and 2-day A $\beta$ O displayed four distinct peaks—a HMW population at and beyond the column exclusion limit, two intermediate populations with averaged molecular weights of 22 and 40kDa, and a LMW population. The two intermediate populations were absent at 3-day A $\beta$ O, and increase and decrease of HMW and LMW populations occurred with incubation length, consistent with a progressive aggregation of A $\beta$ 42. Fractions eluting after 31 minutes displayed no antibody reactivity, confirming the identity of the peaks as the mixture of F12 media and DMSO was used to generate the A $\beta$ O. Testing PMN310 to A $\beta$ O fractions necessitated doubling the sample volume and vastly greater antibody concentration compared to 6E10. This can be explained by the specificity of PMN310

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for a structural epitope unique to  $A\beta O$ , and this epitope likely existed only transiently and in low abundance. PMN310 showed reactivity to three peaks presented in 1- and 2-day A $\beta$ O—the HMW population, LMW population, and an intermediate  $A\beta O$  eluting approximately 40kDa, and upon subsequent concentration quantification by BCA assay and calculation of the number of A $\beta$  seeds present, the data seemingly indicated a specificity toward HMW A $\beta$ Os. This result, which contradicted previously established conclusions by Gibbs et al., may be explained by two possibilities: that large protofibrillar aggregates fragmented, in a similar manner as fibrils in amyloid plaque generate soluble A $\beta$ O, over the course of experimentation into smaller oligomers, or that ABOs larger than a specific size eluted as one peak at the exclusion limit (Koffie et al., 2009). The second possibility held the most merit, as the exclusion limit of 70kDa—corresponding only to a 16- to 17-mer—for Superdex 75 may preclude resolution of even moderately large ABOs into distinct peaks. ABO42 fractionation with Superdex 200 column supported this theory, as the resulting chromatogram showed that HMW aggregates existed at low quantities relative to LMW species over a wide range of molecular weight above 100kDa (Sebollela et al., 2014). Interestingly, while PMN310 showed binding to the 40kDa population, no reactivity to 22 kDa was observed, despite similar abundances. In 2003, Kayed et al. also reported binding by an ABO-specific polyclonal serum to SEC fraction of soluble ABO42 corresponding to around 40kDa. Whether this roughly octameric aggregate represented the same AβO as observed in this study will require further investigation, but prior data of interest existed for this  $A\beta O$  species.

Dot blot comparison between AD brain homogenate to healthy homogenate depicted three main differences: an overall decrease in 6E10 and increase in hIgG1 reactivity, as well as a shift in PMN310 reactivity from HMW to intermediate AβOs. Increased hIgG1 reactivity toward AD brain homogenate concurred with the current understanding of inflammation as a hallmark of AD pathology (Heneka et al., 2015). Decreased 6E10 reactivity in AD brain homogenate may be a result of binding of A $\beta$ Os with other proteins, such that the sequence targeted by 6E10 became unavailable. The increased hIgG1 signals in many of the same fractions supported the explanation of ABO sequestration by proteins, preventing 6E10 detection. PMN310 showed strong and moderate reactivity to HMW and intermediate fractions in healthy brain homogenate, respectively. A study by Yang *et al.* in 2017 showed that large A $\beta$ O derived from AD brain exerted less cytotoxicity relative to smaller A $\beta$ O, and these HMW species may more likely be sequestered by plaques in the brain, further attenuating its toxic activity. It was not surprising, then, to find elevated PMN310 binding to HMW fractions in healthy homogenate, which suggested a greater abundance of large oligomers with less neurotoxicity. On the other hand, AD homogenate showed stronger reactivity to PMN310 at lower molecular weight fractions. Specifically, PMN310 showed elevated binding to fractions 21, 23, and 24, corresponding to a range of 27 to 50kDa. These fractions thus encompassed A $\beta$  hexamers up to dodecamers, and hexamers had also been shown to act as paranuclei—important basic building blocks for protofibrillar formation (Roychaudhuri et al., 2009). Previous reports had found dodecameric oligomers in AD brains, and many of these oligomers, such as ADDL and A $\beta$ 56, caused cytotoxicity *in vitro* and memory impairment in animals (Sengupta *et al.*, 2016). Interestingly, despite the fact that AD homogenate chromatogram showed intense absorbance for fractions 13 and 14, minimal antibody reactivity was observed. The identity of material in these fractions will require further investigation, but the data showed these fractions did not bind with A $\beta$  antibodies.

The addition of A $\beta$ O fractions from synthetic A $\beta$ 42 preparations to A $\beta$ 42 monomers did not significantly alter the slope of ThT assay aggregation curve. As the slope of ThT curve was indicative of the speed of fibril elongation, canonically assumed to proceed through monomer additions, the result suggested that A $\beta$ Os examined in this study did not alter the kinetics of aggregation (Gade Malmos et al., 2017). Maximum signal of ThT assay showed a general increase with an A $\beta$ O addition when compared to A $\beta$ 42 monomer alone, which can be explained by the presence of A $\beta$ O increasing the total concentration of A $\beta$  present for fibrillization, thus increasing final ThT fluorescence. Inter-assay comparison showed minimal differences for fractions of different A $\beta$ O preparations except at the very HMW population. At this range, a trend toward an increase in maximum signal was observed at longer incubation length, which can be accounted for by the increased abundance of large aggregates present in the 3-day A $\beta$ O, compared to 1- and 2-day ABO. Intra-assay difference between monomer alone and ABO addition in the 3-day ABO experiment seemed significant, but this difference was caused by the low variability between repeats for A $\beta$  monomer only. A $\beta$ O exerted the greatest activity in reducing lag phase—a period of time where the number of aggregative seeds remained below the threshold, with 1-day A $\beta$ O showing the most potent seeding activity. HMW, LMW, and the 40kDa fractions showed seeding activity, which diminished at 2-day incubation and finally disappeared at 3-day incubation. These data are in agreement with the hypothesis that ABO formed early in the aggregation pathways showed the most potent seeding activity (Aoyagi et al., 2019). More significantly, the 40-kDa AβO seeded more potently than the HMW and LMW which corresponded to monomers to trimers--fractions, supporting the current literature on paranuclei as important intermediates in the aggregation pathway.

ThT assay results with healthy and AD brain homogenate fractions were more ambiguous compared to synthetic A $\beta$ Os. Only the HMW fractions from both homogenates led to lag time reduction when added to A $\beta$ 42 monomers, with AD homogenate showing greater potency.

Notably, the addition of fractions 14 and 15 led to the shortening of the lag phase, despite the fact that neither homogenate showed robust antibody reactivity at this elution range.

Furthermore, fraction 13 of AD homogenate, which showed no antibody binding, also resulted in lag time reduction. In contrast, the intermediate fractions showed minimal seeding activity. This collection of results, including data that these HMW fractions also reduced slope of elongationindicating a slowed fibrillization rate, may be a result of the high concentration of other materials that existed in the complex mixture of brain homogenate interfering with normal aggregation. This was observed in the chromatogram of healthy and especially AD brain homogenate, showing strong 280nm absorbance at this elution range. Intermediate fractions also depressed the slope of elongation, with fractions 21 and 23 showing the most pronounced effects. Interestingly, the addition of these two fractions, along with fraction 17, to  $A\beta 42$  monomers resulted in a significant reduction in the maximum signal at plateau phase. Fraction 23 displayed the greatest potency in these two measures, in contrast to identical fractions from synthetic A $\beta$ Os, which had no effect on slope of elongation and a moderate effect on increasing maximum signal. The characteristics of fraction 23, which decreased the rate of elongation and suppressed maximum ThT signal, pointed to an ABO population with activity in facilitating non-fibrillogenic or offpathway. A review by Benilova et al. illustrated the complexity of Aβ aggregation pathway and that paranuclei can lead to the off-pathway, forming annular protofibrils and amylospheroids, which remain in solution and cause cytotoxicity and LTP impairment and instigate tau hyperphosphorylation. Alleviation of memory deficit in mice by PMN310 may be due to binding to A $\beta$ Os present in fraction 23, preventing further neurotoxic activities by these aggregates (Gibbs et al., 2019).

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SPR assay with A $\beta$ O at different incubation time points corroborated the SEC and filter trap assay results. Binding response was at its highest for 0-hour and declined with time, with the 6E10 binding at nearly twice the magnitude as PMN310 at 0-hour to ten times the magnitude by 24-hour. Weaker PMN310 binding response to A $\beta$ O was consistent with the structural epitope constraint, which SEC data showed to be most abundant at early incubation time points. The decline in binding response over time may be due to aggregation of A $\beta$  sequestering binding site of 6E10 and PMN310, as well as formed fibrils falling out of solution, lowering total soluble A $\beta$ concentration.

The employment of a combinatorial approach, utilizing experimental methods with rapid assay or preparation length such as SEC and filter trap assay, allowed for simultaneous characterization of multiple aspects to a single sample, minimizing the potential changes to protein mixture and inter-experiment sample variability. A major shortcoming of this study was the limitation on the accuracy of experimental techniques, such as SEC—which estimated molecular size through the use of globular protein standards—and ThT assay—in which detection of Aβ aggregation can be affected by buffer conditions and stochastic activity of Aβ, rendering it to mostly qualitative analysis without additional measures. The stability of  $A\beta O$ present within samples may also be brought into question, despite the great effort undertaken to ensure sample integrity throughout the course of experimentation. Photoinduced Cross-Linking of Unmodified Proteins (PICUP) represented a popular method to stabilize A $\beta$ O in solution (Bitan and Teplow, 2004). However, artificially crosslinking Aβ may produce aggregates unrepresentative of natural A $\beta$ Os, and PICUP was associated with potential confounding factors of its own (Bitan et al., 2001). However, the nature of this study, which aimed to examine PMN310 reactivity to the full continuum of A $\beta$ O species, minimized the impact of potential

variability in sample integrity and diminished the need for accurate orthogonal methods, such as SV-AUC. This study presented one such method, surface plasmon resonance, and its capability for rapid antibody binding analysis on the order of hours, with potential for resolution down to order of minutes. The inability to completely separate the complex mixture of proteins in brain homogenates—seen in dot blot, where a fraction can show reactivity to antibodies targeting different proteins-represented another shortcoming of this study. The effect of fractions 13 to 15 on the aggregation of A $\beta$ 42 in ThT assay added another problem in brain homogenate analysis. In regard to the ThT assay, the result can be partially corroborated by filter trap assay, showing that fractions with increased PMN310 binding exhibited a greater effect on A $\beta$ aggregation. Filter trap assay also showed an absence of protein in fractions 13 to 15, suggesting that the observed effect on ThT assay was an artifact of a high concentration of nonproteinaceous substances in these fractions. Lastly, SEC fractions added to Aβ42 monomers in ThT assay were not normalized by concentration nor by the number of ABO seeds. Thus, the result may not accurately reflect the activity of A $\beta$ Os present in these fractions. Additional manipulation of the fractions for normalization will increase assay time, negatively impacting the ABO integrity. As a number of fractions presented minimal to no protein content by microBCA, normalization could not be carried out without setting an arbitrary point of standard, which may also negatively impact the result of the assay. The microBCA result also affected determination of total A $\beta$ 42 seeds loaded in filter trap assay, rendering detailed quantitative comparison of PMN310 reactivity to different ABO fractions challenging. However, the robust activity of the lowabundance 40kDa fraction in comparison to HMW fractions at greater abundance provided strong evidence for the validity of the result.

The two major obstacles in the development of AD immunotherapy, in light of the current understanding of the amyloid hypothesis, are target specificity and selection of the optimal ABO target. While antibody designs have moved away from sequence specificity toward the novel paradigm of structure specificity, exemplified by aducanumab and BAN-2401, which show therapeutic and disease-modifying effect, ambiguity remains as to the pathogenic activity of targeted ABOs and their relevance to AD, a task complicated by the transience of ABOs. The aforementioned antibodies target aggregated  $A\beta$ , with BAN-2401 designed against protofibrils, contradicting the evidence in literature showing LMW A $\beta$ Os as major pathogenic species (Tolar et al., 2020). PMN310, which further refined the paradigm of structure specificity by targeting a unique conformation epitope, showed preferential reactivity to a transient population of ABO consisting of hexamers up to dodecamers, resembling paranuclei (Roychaudhuri et al., 2009). This paranuclei population of A $\beta$ O possessed more significant seeding activity in the presence of A $\beta$  monomers over other populations, pointing to the possibility that paranuclei, intermediates in protofibril formation, may be the therapeutic target of PMN310, corroborating with evidence demonstrating the pathogenicity of LMW ABO. PMN310 also showed a time-dependent decrease in reactivity to the heterogenous ABO population, suggesting that PMN310-reactive A $\beta$ O could be formed at the earliest stage of AD pathology. The results of this study carry several implications: ABO antibodies currently in clinical trials may be acting on downstream aggregates of the smaller A $\beta$ Os that represent the major pathogenic species, and more stringent epitope selection will allow for targeting of these ABOs. The importance of moving from oligomeric structure specificity toward unique conformation specificity cannot be overstated, as precise targeting of LMW ABO will decrease cross-reactivity to fibrils, a limiting factor to the safety and therapeutic efficacy of AD immunotherapy, as observed for aducanumab and BAN-

2401 (Aisen *et al.*, 2020). Such progress cannot be achieved, however, without better characterization of A $\beta$ O targets, and therapeutic antibodies themselves may serve as the most optimal tool for this purpose. As demonstrated by this study, simultaneous screening of antibody reactivity to various A $\beta$ Os and assaying of multiple aspects of targeted A $\beta$ Os, employing techniques that allowed for real-time observation of activity along with traditional biochemical assays, linked antibody binding profile to target activity for not only target elucidation, but also relevance of target in disease. While additional work will be necessary to address the shortcomings presented within this study, which may not be overcame without further technological advances, the methodology employed here may serve as a template for the field moving forward.

## 6.2 Conclusion

The study of this thesis examined the aggregation timeline of synthetic Aβ42 and showed, using SEC, AβO formed immediately upon resuspension and shifted toward HMW species over time, as well as the presence of transient intermediate species existing only at short incubation time points. Identical analysis carried out on both healthy and AD human brain homogenates showed similar profiles, with the majority of proteins populating the HMW range, along with lesser populations in the intermediate range. No LMW population corresponded to what was seen in synthetic AβOs. Using filter trap assay, PMN310 tested on SEC fractions of synthetic AβOs with different incubation timepoints showed reactivity to HMW, LMW, and an approximately 40kDa peak. Due to the resolution limit of Superdex 75, the HMW fractions may represent a continuum of smaller AβOs. Determination of the number of AβOs present showed greater specificity by PMN310 toward the 40kDa population compared to the LMW population. Filter trap assay conducted with PMN310 on brain homogenates showed increased binding at HMW and intermediate fractions in healthy and AD homogenates, respectively. Notably, the 40kDa fraction displayed the strongest PMN310 binding in AD homogenate. ThT assay examined the seeding activity by synthetic A $\beta$ O and brain homogenate fractions, and the data showed the synthetic ABO 40kDa fraction most significantly reduced lag time to aggregation, while brain homogenate 40kDa fraction most significantly reduced speed of elongation and maximum signal. This result represented two different activities-one propagating aggregation, while the other promoted progression of the non-fibrillogenic pathway-that represented major pathogenic mechanisms in AD. SPR experiment with PMN310 corroborated with SEC results, showing the highest binding response to the shortest ABO incubation. This study identified a potential target for the therapeutic effect of PMN310: an intermediate, 40kDa ABO that existed in synthetic peptide preparation with short incubation and brain homogenate. Further study will be needed to validate the activity of this intermediate  $A\beta O$  species and its binding with PMN310. Clarification on the structure and toxicity of this ABO could elucidate the mechanism of action of PMN310 and provide insight that will further advance the field of A $\beta$  immunotherapy toward curing AD.

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