CLEARANCE OF BETA-AMYLOID 42 (Aβ-42) AND TAU PROTEINS IN ALZHEIMERS DISEASE (AD)

By

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the main cause of dementia among the elderly worldwide. Despite intense efforts to develop drugs for preventing and treating AD, no effective therapies are available yet, posing a growing burden at the personal, medical, and socioeconomic levels. AD is characterized by the production and aggregation of amyloid β (Aβ) peptides derived from amyloid precursor protein (APP), the presence of hyperphosphorylated microtubule-associated protein Tau (MAPT), and chronic inflammation leading to neuronal loss. Aβ accumulation and hyperphosphorylated Tau are responsible for the main histopathological features of AD, Aβ plaques, and neurofibrillary tangles (NFTs), respectively. However, the full spectrum of molecular factors that contribute to AD pathogenesis is not known. Non-coding (nc)RNAs, including microRNAs (miRNAs), long noncoding RNAs (lncRNAs) regulate gene expression at the transcriptional and posttranscriptional levels in various diseases, serving as biomarkers and potential therapeutic targets. There is rising recognition that ncRNAs have been implicated in both the onset and pathogenesis of AD. Here, the ncRNAs implicated post-transcriptionally in the main AD pathways and discuss the growing interest in targeting regulatory ncRNAs therapeutically to combat AD by using a series of multifunctional molecules that contained APP, and Tau-recognition moieties and E3 ligase-binding moieties to enhance APP, and Tau degradation. The goals involve the exploration into non-invasive biomarker screening for cognitive dementia related to Alzheimer’s Disease (AD) and in canine blood samples. The specific aims are towards the identification and development of non-invasive AD-related biomarkers for detection screening before the onset of pathophysiologically symptoms related to cognitive deterioration seen in AD. The purpose of the project is concentration on the clearance of Aβ and Tau through the liver.
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1.1 – Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the main cause of dementia among the elderly worldwide. It has been estimated that the global prevalence of dementia has been estimated to be as high as 24 million and is predicted to double every 20 years until at least 2040. (Mayeux et al., 2012) A recent study suggests that the attributable risk of dementia increased with age, reaching 0.17 (95% uncertainty interval, 0.05 to 0.58) in the 95+ age group. We estimated there were 1.55 (0.35 to 4.54) million deaths globally due to dementia in 2019. Far more deaths occurred in women (1.02 million; 0.23 to 2.96) than men (0.54 million: 0.12 to 1.58), giving a female to male ratio of 1.90 (1.82 to 1.99). Due to population aging, the all-age mortality rates increased by 38.0%; 33.1 to 43.7 between 1990 and 2019. (Nichols et al., 2020) Despite intense efforts to develop drugs for preventing and treating AD, no effective therapies are available yet, posing a growing burden at the personal, medical, and socioeconomic levels. AD is characterized by the production and aggregation of amyloid β (Aβ) peptides derived from amyloid precursor protein (APP), the presence of hyperphosphorylated microtubule-associated protein TAU (MAPT), and chronic inflammation leading to neuronal loss. Aβ accumulation and hyperphosphorylated TAU are responsible for the main histopathological features of AD, Aβ plaques, and neurofibrillary tangles (NFTs), respectively.
1.2 Amyloid Precursor Protein (APP) and Aβ

Alzheimer’s disease (AD) results from the presence of neurotoxic beta-amyloid (Aβ) deposits in the brain. This article describes the generation of Aβ from amyloid precursor protein (APP). Aβ peptides are produced by the proteolytic cleavage of the transmembrane protein amyloid precursor protein (APP) by enzyme complexes α, β, and γ-secretases. APP cleavage occurs via two distinct pathways. The non-amyloidogenic pathway gives beneficial neurotrophic effects and the amyloidogenic pathway produces neurotoxic Aβ peptides. The Aβ peptides formed via the amyloidogenic pathway can misfold and aggregate to form deposits that contribute to AD pathology. The amyloidogenic pathway leads to neurotoxic Aβ generation. β-secretase (BACE1) mediates the first proteolysis step, which releases a large N-terminal ectodomain (sAPPβ) into the extracellular medium. A 99-amino acid C terminal fragment (C99) remains in the membrane. The newly exposed C99 N-terminus corresponds to the first amino acid of Aβ. Successive cleavage of this fragment by γ-secretase (between residues 38 and 43) releases the Aβ peptide. γ-secretase is a complex of enzymes consisting of presenilin 1 or 2 (PS1 and PS2), nicastrin, anterior pharynx defective (APH-1), and presenilin enhancer 2 (PEN2). Most of the Aβ peptides are 40 residues in length (Aβ1–40), with a small percentage containing 42 residues (Aβ1–42). Aβ1–42 is considered the more neurotoxic form because the extra two amino acids provide a greater tendency to misfold and subsequently aggregate. Elevated plasma levels of Aβ1–42 have been correlated with Alzheimer’s disease. The non-amyloidogenic pathway involves cleavage of APP by α-secretase to generate two fragments; an 83 amino acid C-terminal fragment (C83) that remains in the membrane and an N-terminal ectodomain (sAPPα) that is released into the extracellular medium. Three enzymes have been identified with α-secretase activity: ADAM9, ADAM10, and ADAM. Importantly, cleavage of APP by α-secretase occurs within the Aβ domain and consequently prohibits Aβ peptide production. Of note, the C83 membrane fragment can be subsequently cleaved by γ-secretase to produce a short fragment called P3 peptide and a C terminal fragment (CTF). The newly exposed C99 N-terminus corresponds to the first amino acid of Aβ. Successive cleavage of this fragment by γ-secretase (between residues 38 and 43) releases the Aβ peptide. γ-secretase is a complex of enzymes consisting of presenilin 1 or 2 (PS1 and PS2), nicastrin, anterior pharynx defective (APH-1), and presenilin enhancer 2 (PEN2).
1.3 TAU Protein

TAU protein is highly soluble and normally attached to axonal microtubules. TAU stabilizes the microtubules and makes them rigid. TAU interacts with actin in the cytoskeleton and neuronal outgrowth, anchors enzymes such as protein kinases and phosphatases, and regulates intracellular vesicle transport. TAU is phosphorylated by numerous serine/threonine kinases, including GSK-3β, PKA, PKC, CDK5, MARK, JNK, p38MAPK and casein kinase II. Neurofibrillary degeneration is characterized by the deposition in the neuronal body and processes of insoluble polymers of over-phosphorylated microtubule associated protein TAU. TAU aggregates as pairs of filaments that are twisted around one another (paired helical filaments). These deposits interfere with cellular functions by displacing organelles. By distorting the spacing of microtubules, they impair the axonal transport thus affecting the nutrition of axon terminals and dendrites. No mutations of the TAU gene occur in AD. Abnormal TAU first appears in the entorhinal cortex, then in the hippocampus, and at later stages in association cortex. Recent observations in transgenic mice suggest that the spread of the pathology to anatomically linked areas occur by passage of abnormal TAU across synapses. There are two main lesions in AD, senile plaques (SPs) (also called Alzheimer’s plaques) and neurofibrillary tangles (NFTs). SPs are spherical lesions in the cerebral cortex, measuring up to 100 microns. There are 2 kinds of SPs: diffuse Aβ plaques (AβPs) and neurotic plaques (NPs). AβPs are spherical extracellular Aβ deposits. NPs are AβPs containing degenerating neuronal processes with TAU paired helical filaments. NPs contain also reactive astrocytes and microglia. Aβ in NPs frequently forms a central core or small chunks, has a fibrillar fine structure and is Congo Red positive and birefringent, similar to other amyloids. TAU phosphorylation regulates both normal and pathological functions of this protein. We are interested in the roles of cytokine activity on the TAU protein. Microglia influences the inflammatory responses through interleukin-6 and pro-inflammatory interleukin-10 and propose that these two cytokines interfere with TAU metabolism as we propose that the development of the onset of pathophysiological diseases like Alzheimer’s, Parkinson’s, Huntington’s Disease, and ALS can be linked to the epigenetic modifications of specific sites on the TAU protein. TAU promotes microtubule assembly and stability and might be involved in the establishment and maintenance of neuronal polarity. The C-terminus binds axonal microtubules while the N-terminus binds neural plasma membrane components, suggesting that TAU functions as a linker protein between both.
Axonal polarity is predetermined by TAU/MAPT localization (in the neuronal cell) in the domain of the cell body defined by the centrosome. The short isoforms allow plasticity of the cytoskeleton whereas the longer isoforms may preferentially play a role in its stabilization. The goals of this proposal would use a PROTAC bi-directional molecule which would link the TAU proteins to the E3 Ligase for ubiquitylation for protein degradation using the proteasome pathway. The up-regulation of the UPS pathway would result in a decrease of TAU clearance in response to an increase of interleukin-10. We also are intrigued by the effects of down-regulation of the UPS using interleukin-6 and the cellular capacity to degrade TAU when exposed to the PROTAC molecule. The following PROTAC molecules use a bi-directional molecule which at one end has a sequence which is site-specific for TAU proteins and connected through a “linker” to another site-specific sequence that binds to the VHL motif of the L3 Ligase for ubiquitylation. We propose that there are two confounding influences which might significantly impair L3 Ligase mediated activation of the proteasome as the negative effects of the inflammatory cytokine – interleukin-6 and methylation of lysine residues responsible for ubiquitylation: as the two effects could render the PROTAC effectiveness as an option for degrading beta-amyloid 42 or TAU. The results might provide additional insight into the roles of methylation on the responsiveness of the ubiquitylation proteasome pathway with respect to cytokine activity as an epigenetic modulating side-effect that would impede the degradation of beta-amyloid 42 or TAU using PROTACs and their effectiveness of the treatment against beta-amyloid induced pathophysiological onset of Alzheimer’s Disease.

To achieve selective recognition of TAU, two peptides from α- and β-tubulin that were known to interact with TAU, α (430–441): KDYEEVGVSVE and β (422–434): YQQYQDATEDEQG (Maccioni et al., 1988, Rivas et al., 1988). Simultaneously, two peptides based on the substrates of two E3 ligases were chosen as moieties to tether E3 ligases. One peptide was DRHDS(p)GLDS(p)M, derived from IkBα, which bound to the Skp1-cullin-F box (SCF) E3 ligase (Sakamoto et al., 2001, Yaron et al., 1997, Yaron et al., 1998). The other was ALAPYIP, derived from a substrate of the E3 ligase, von Hippel-Lindau tumor suppressor protein (VHL) (Hon et al., 2002, Schneekloth et al., 2004). The TAU-recognition moieties were linked to the E3-ligase-binding moieties with short peptides, GSGS or GGSGG, to increase their flexibility (Taremi et al., 1998). Poly-arginine (D-Arg) was fused to the C terminus of the peptides to facilitate their penetration into cells (Schneekloth et al., 2004). We anticipated that these multifunctional peptides would penetrate the cell membrane and bind to intracellular TAU protein and E3 ligases.
1.4 Proteasome Ubiquitylation and Degradation through PROTACs

Targeted protein degradation using small molecules relies on using endogenous cellular processes for protein regulation. Every cell within all living organisms has evolved mechanisms to regulate protein concentrations in response to external stimuli to maintain homeostasis. Gene expression controls protein synthesis which uses the recycled amino acids generated from protein degradation. The ubiquitylation proteasome system (UPS) also prevents ontogeny diseases in which a misfolded protein can promote the etiology of disease and the pathophysiology responsible for the death – as protein aggregates cause cellular toxicity which triggers apoptosis via the p53 pathway. The transduction pathway for protein degradation is initiated by ubiquitin ligase complexes through the transfer of ubiquitin to the ε-nitrogen of a lysine residue on the target protein. The degradation process occurs once multiple ubiquitin molecules are added to form an oligomeric chain of ubiquitinated lysine residues which is then recruited by proteasomal polyubiquitin recognition domains. (Collins, Wang, Caldwell, & Chopra, 2017). Another strategy to induce protein degradation is to mimic protein misfolding by hydrophobic tagging (HyT). (Lebraud & Heightman, 2017) The use of an adamantyl group is used to tag a targeted protein which makes the hydrophobic properties of the protein “greasy” to trigger ubiquitylation for proteasome degradation. The tert-butyl carbamate-protected arginine (Boc3Arg) has been used to tag covalent inhibitors of GST and a non-covalent inhibitor of the Escherichia coli dihydrofolate reductase (DHFR), which were shown to induce degradation of GST and DHFR respectively. The significance of using the right inducing ligand depends on the pathway as the adamantyl group induces degradation which is dependent upon the ubiquitin-mediated pathway-promoting interactions between the target protein and ubiquitin ligase, as the resulting ubiquitination proceeds to degradation by the 26S proteasome. The Boc3Arg tag elicits degradation which is independent of ubiquitin but through the recruitment of the target protein directly to the 20S proteasome. (Lebraud & Heightman, 2017) To achieve selective recognition of TAU, we chose two peptides from α- and β-tubulin that were known to interact with TAU, α (430–441): KDYEEVGDSVE and β (422–434): YQQYQDATADEQG (Maccioni et al., 1988, Rivas et al., 1988). Simultaneously, two peptides based on the substrates of two E3 ligases were chosen as moieties to tether E3 ligases. One peptide was DRHDS(p)GLDS(p)M, derived from IkBα, which bound to the Skp1-cullin-F box (SCF) E3 ligase (Sakamoto et al., 2001, Yaron et al., 1997, Yaron et al., 1998).
Figure 1.1 The ubiquitin–proteasome system (UPS) and proteolysis-targeting chimera (PROTAC). For the degradation of a protein through UPS, the ubiquitin (Ub) is linked to an Ub-activating enzyme (E1) via a thioester bond in the ATP-dependent reaction. Next, activated Ub on E1 is transferred to an Ub-conjugating enzyme (E2) through trans-thioesterification. The Ub-ligase (E3) recognizes a degradation signal (degron) of an endogenous substrate and conjugates a Ub from E2 to a lysine residue of the substrate protein. The serial cascades of Ub-activation, Ub-conjugation, and Ub-ligation by E1, E2, and E3 polyubiquitylate the substrate or substrate-conjugated Ub. The 26S proteasome recognizes the polyubiquitin chains on the substrate, subsequently followed by deubiquitylation, unfolding, and degradation of the substrate. The PROTAC can hijack the E3 by its ligand and recruit a target protein for degradation through UPS. (Kim et al., 2022)
Figure 1.2 - Structures of compounds used for induced protein degradation technologies. Hydrophobic tags (HyTs) are utilized to recruit chaperones to a protein of interest (POI): adamantane and Arg-Boc3. E3 ligase recruiting ligands employed to hijack E3 ligases to ubiquitinate and degrade POIs. The wavy line illustrates the attachment point utilized in the studies discussed. The chemical structures of proteolysis-targeting chimeras (PROTACs) utilizing different E3 ligases to target the POIs for degradation. The POI ligands are shown in blue; the linkers are shown in black; and the E3 ligase recruiting ligands are shown in red. (Lai et al., 2016)
Figure 1.3 – Tau PROTAC VHL motif compound

PROTAC contains four motifs: (i) tau recognition motif, (ii) linker, (iii) E3 ligase-binding motif, and (iv) cell-penetrating peptide (CPP) motif. To select the optimal tau recognition peptide, three known peptides were evaluated and the peptide corresponding to the YQQYQDATADEQG sequence was determined to be the best in their study. Two E3 ligases, Skp1-cullin-F box (SCF) ligase and the von Hippel–Lindau (VHL) tumor suppressor protein, with binding peptide sequences of DRHDS(p)GLDS(p)M and ALAPYIP, respectively, were analyzed. The results showed that VHL is superior to SCF. With a short linker sequence (GSGS) between the tau-binding motif, E3 ligase-binding motif, and polyarginine CPP, the most active PROTAC compound, TH006, was established, which has a 32-amino-acid sequence. TH006 was demonstrated to successfully penetrate into cells and induce tau protein degradation by enhancing polyubiquitination by VHL E3 ligase. Moreover, in an AD transgenic mouse model, it was proven to reduce the neurotoxicity of Aβ through TU005-mediated lowering of the tau protein level. (Hyun et al., 2021)
The other was ALAPYIP, derived from a substrate of the E3 ligase, von Hippel-Lindau tumor suppressor protein (VHL) (Hon et al., 2002, Schneekloth et al., 2004). The TAU-recognition moieties were linked to the E3-ligase-binding moieties with short peptides, GSGS or GGSGG, to increase their flexibility (Taremi et al., 1998). Poly-arginine (D-Arg) was fused to the C terminus of the peptides to facilitate their penetration into cells (Schneekloth et al., 2004). We anticipated that these multifunctional peptides would penetrate the cell membrane and bind to intracellular TAU protein and E3 ligases. To investigate the ability of these multifunctional peptides to induce TAU degradation, we constructed a stable mouse neuroblastoma N2a-based cell line, which expressed TAU protein fused to EGFP (TAU-EGFP). These cells were treated with synthesized molecules (100 μM) for 24 hr. The intracellular EGFP fluorescence intensity was detected with flow cytometry. Among the molecules tested, TH006 appeared to induce the greatest reduction in EGFP. TAU is an unstructured, flexible protein (Tompa, 2002); thus, to facilitate the recognition and degradation of intracellular TAU, a series of multifunctional chimeric molecules comprised three parts:

(1) a moiety that could recognize TAU
(2) a moiety for binding to E3 ligase
(3) a cell-penetrating peptide.

To achieve selective recognition of TAU, we chose two peptides from a- and b-tubulin that were known to interact with TAU, a (430–441): KDYEEVGVDSE and b (422–434): YQQYQDATAEQG (Maccioni et al., 1988; Rivas et al., 1988). Simultaneously, two peptides based on the substrates of two E3 ligases were chosen as moieties to tether E3 ligases. One peptide was DRHDS(p) GLDS(p)M, derived from IkBa, which bound to the Skp1-cullin-F box (SCF) E3 ligase (Sakamoto et al., 2001; Yaron et al., 1997, 1998). The other was ALAPYIP, derived from a substrate of the E3 ligase, von Hippel-Lindau tumor suppressor protein (VHL) (Hon et al., 2002; Schneekloth et al., 2004). The TAU-recognition moieties were linked to the E3-ligase-binding moieties with short peptides, GSGS or GGSGG, to increase their flexibility (Taremi et al., 1998). Poly-arginine (D-Arg)8 was fused to the C terminus of the peptides to facilitate their penetration into cells (Schneekloth et al., 2004). They proposed that these multifunctional peptides would penetrate the cell membrane and bind to intracellular TAU protein and E3 ligases.
The epigenetic modulating side-effects could impede the degradation of TAU using PROTACs and their effectiveness of the treatment against TAU induced pathophysiological onset of Alzheimer’s Disease. Acetylation at Lys174 (K174) which hinders the interaction between TAU and microtubules. Acetyltransferase EP300 is the major acetylase of TAU at K174 implicated in its aggregation and neurodegeneration in AD. A dose dependent response of the UPS pathway with respect to the levels of interleukin-6 and interleukin-10 and hypothesize that post-translational medication activity is influenced by cytokine activity. Neurodegeneration, neurobehavioral disorder, and the accumulation of acetylated TAU in blood and brain of TBI mice. Acetylation was identified as a TAU PTM elevated at early Braak stages of AD and shown to positively regulate hyper-phosphorylated TAU levels and TAU aggregation in vitro. Deleting the deacetylase SIRT 1 elevated TAU acetylation, which suppressed poly-ubiquitination and subsequent protein turnover, providing additional evidence that TAU acetylation promotes AD progression. These findings further highlight the importance of PTM crosstalk in TAU regulation. Building upon these studies, Min et al. identified K174 as the specific lysine critical for TAU acetylation and defined the lysine acetyltransferase P300 as a regulator of TAU acetylation. K174 was acetylated in early and late Braak stages of AD. Importantly, the prescription drug, salsalate, which decreases P300 activity, reversed TAU-mediated memory impairments and hippocampal atrophy in a mouse Tauopathy model. Importantly, when salsalate was used on neurons expressing a TAU lysine acetylation mimetic, K174Q, it provided no significant benefit as shown by unchanged levels of total and phosphorylated TAU and atrophy of the hippocampus. These mutagenesis data provided additional evidence for a key role of acetylated TAU in AD progression. In AD patients with a history of TBI, the increase of acetylated TAU protein in the brain will be further enhanced. Patients receiving p300 / CBP inhibitors salsalate or diflunisal show a lower incidence of AD and clinical diagnosis of TBI.
TAU protein acetylation is a therapeutic target and potential blood biomarker of TBI, which may represent the pathological fusion between TBI and AD.

1: Reversing Acetylation of TAU using acetyltransferase inhibitors and Lysine acetyltransferase P300 inhibitors.

2: Stimulation of deacetylation of TAU using allosteric agonists as HDAC3 is recruited to the catalytic site of TAU protein, forming a TAU–HDAC3 complex and turning off TAU auto-acetyltransferase activity, therefore preventing TAU from having excessive acetylation at lysine 280.

3: Inducing ubiquitin-proteosome system pathway for TAU degradation using the E3-ligase (VHL) complex.

4. Cytokine activity on TAU post-translational modifying signaling molecules

Aβ binding to the microglial cell surface induces pro-inflammatory gene expression and results in the elevation of pro-inflammatory cytokine such as TNF-α, IL-1β, IL-6, IL-18, which lead to TAU hyperphosphorylation and neuronal loss through GADPH S-nitrosylation, Sirtuin1 deacetylation is prevented, which then activates p300/CBP acetyltransferase. This leads to TBI-induced TAU acetylation at Lys274 and Lys281. The modification of TAU at these lysine residues is also observed in AD, further suggesting the essential role of regulating induced PTMs to alleviate both aging and non-aging neurodegeneration. It would therefore be imperative to assess the potential of targeting S-nitrosylated GAPDH for proteolysis as a therapeutic approach for alleviating Tauopathy memory loss, AD, or other TAU-related NDDs. (Spratt et al 2021) TBI can induce TAU acetylation (ac-TAU), which also exists in human brains with AD. S-nitrosylated GAPDH, which can inactivate sirtuin1 deacetylase and activate p300/cbp acetyltransferase simultaneously and increase TAU acetylation in neurons. The dislocation of TAU protein leads to neurodegeneration and neurobehavioral disorder, as well as the accumulation of acetylated TAU in blood.
Epigenetic modifications take place in the nucleosome, the fundamental unit of the eukaryotic chromosome composed of 147 base pairs of DNA wrapped around an octamer of core histone proteins (two of each H2A, H2B, H3, and H4). On the amino terminal of each histone subunit, multiple sites exist for potential posttranslational modifications, including acetylation, methylation, phosphorylation, and ubiquitination. Modulation of epigenetic enzymes has been shown to be critically involved in behavioral and molecular responses involved in the development and progression of neurodegenerative disorders. RGFP-966, a brain-penetrant and selective HDAC3 inhibitor, or HDAC3 silencing, increases BDNF expression, increases histone H3 and H4 acetylation, decreases TAU phosphorylation and TAU acetylation at disease-associated sites. This becomes a unique opportunity to explore the epigenetic pathophysiology of Alzheimer’s disease with respect to lifestyle changes over time. Although, previous studies have suggested the use of a bi-functional PROTAC molecule that targets the toxic p-TAU for ubiquitin-induced degradation using the proteasome system (UPS) – eventually fails to achieve the necessary clearance of p-TAU as the aggregates clog the proteasome which halts protein degradation using ubiquitin proteasome pathway. p-TAU also impedes the molecular protein folding chaperones, which are responsible for maintaining protein conformation for optimizing protein functionality. We are curious about the role of the liver since it’s the primary detoxification site and protein degradation center which generates the proteomic catabolic waste by-product; urea which is excreted in urine through the urinary system. Liver diseases including cirrhosis and hepatitis result in a build-up of ammonia (NH3) in the blood eventually causes death from hepatic failure. p-TAU can be identified as a blood serum biomarker for AD development and likely causes dementia and cognitive impairment as TAU builds up in the brain during hepatic failure.
1.5 Role of the Liver in AD

Alzheimer’s Disease (AD), the most common form of dementia, is characterized by progressive deficits in cognitive function. Amyloid-beta (Aβ) peptides are believed to play a decisive role in the pathology of AD. Improving the clearance of toxic Aβ has, therefore, become a therapeutic strategy for AD. Unfortunately, almost all of the drug candidates tested for AD, including the Aβ centric therapeutic approaches, until now have failed to exhibit any efficacy. Previous evidence suggested that aerobic exercise training contributes to the improvement of cognitive decline and slows down the pathogenesis of AD; however, the exact mechanisms for this have not been fully understood. One of the most important beneficial effects of aerobic exercise on AD is modifying Aβ clearance. Accumulating evidence indicates that aerobic exercise not only upregulates the clearance of amyloid plaques and soluble Aβ in the brain but also increases its final removal from the periphery. But there are still many unanswered questions in this regard, including the proper timing of exercise interventions, optimal aerobic exercise mode, intensity, duration, and frequency as well as the possible effect of exercise on potential environmental Aβ-clearing agents, which should be considered in future studies. Several studies examined the association between altered liver enzymes and AD dementia diagnosis. There has been a strong association between elevated aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio (peripheral liver function markers), lower levels of ALT, and AD diagnosis. Lower levels of ALT were correlated with increased Aβ deposition and exacerbated brain atrophy, while higher AST to ALT ratios was significantly correlated with reduced glucose metabolism in some brain regions, including the bilateral frontal, parietal, and temporal lobes. Gaps remain in establishing the association of ALT and AST with the risk of dementia as low ALT and AST levels were found to be related to a higher risk of dementia. FGF21 has been shown to be an exercise-responsive factor; as circulating FGF21 elevates after different forms of exercise. The synthesis of FGF21 in response to exercise may mediate the multiple health benefits conferred by exercise. Previous studies have revealed that the protective effect of exercise on cardiac function was eliminated in liver-specific FGF21 knockout mice. FGF21 is protective against the development of AD; whether the induction of liver FGF21 is required for the beneficial effects of exercise for AD would be an issue worthy of further investigation. The liver is the principal organ responsible for Aβ peripheral clearance in normal physiological conditions.
Figure 1.5 - Chronic liver diseases may increase amyloid burden and Alzheimer’s pathology. This contribution results from an imbalance in peripheral amyloid-β (Aβ) clearance as a result of decreased LRP1 levels, general liver dysfunction, and chronic inflammation. These features may worsen blood-brain-barrier (BBB) impairment and contribute to a vicious cycle. As an example, the figure depicts fatty liver disease as a chronic liver condition. (Estrada LD et al., 2019)
When transported across the BBB from the brain, Aβ binds to other molecules. For instance, circulating Aβ can be transported to the liver via high-density lipoprotein (HDL) particles, and subsequently taken up into cells by hepatocyte low-density lipoprotein receptor-related protein-1 (LRP-1), where it is degraded or cleared through bile excretion. Abnormalities in these processes have been observed in both AD patients and animal models. Notably, an earlier study reported that the liver tissue from AD patients contains fewer Aβ molecules than that of healthy individuals, implying that the liver’s Aβ clearance function may be impaired in AD pathology. Indeed, it is increasingly recognized that a hepatic dysfunction is an early event in AD, preceding the pathological characteristics – thus supporting hepatic dysfunction as a risk factor in AD. The contribution of liver dysfunction to the pathogenesis of AD is due to LRP-1 - endocytic and signaling receptor expressed in multiple tissues, including LRP-1, which is a receptor required for the cerebral and systemic clearance of Aβ. In the brain, Aβ is combined with apolipoprotein E (ApoE) and transported to peripheral blood through LRP-1. Subsequently, most circulating Aβ binds to sLRP-1 to avoid excessive free Aβ concentrations in the blood. In the liver, Aβ can be absorbed by hepatocytes via surface LRP-1 and then is eliminated. Nevertheless, both LRP-1 expression in the liver and hepatic Aβ uptake can be significantly reduced in aging and AD animals, suggesting impaired Aβ peripheral transport and clearance. Regression analysis revealed a strong association between elevated aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio (peripheral liver function markers), lower levels of ALT, and AD diagnosis. Lower levels of ALT were correlated with increased Aβ deposition and exacerbated brain atrophy, while higher AST to ALT ratios was significantly correlated with reduced glucose metabolism in some brain regions, including the bilateral frontal, parietal, and temporal lobes. Similarly, in a longitudinal study designed to establish the association of ALT and AST with the risk of dementia, low ALT and AST levels were found to be related to a higher risk of dementia. Alzheimer’s Disease (AD), the most common form of dementia, is characterized by progressive deficits in cognitive function. The extracellular amyloid plaques formed by the deposition of Amyloid-Beta (Aβ) peptides are the specific hallmark of AD. The elevated level of Aβ as initiating factor in AD pathogenesis as well as its neurotoxic aggregates in the brain are believed to be associated with the perturbation of synaptic function and neural network activity leading to cognitive deficits and neurodegeneration.
1.6 Mass Spectrometer for Detection of Aβ-42 and TAU in AD Blood Samples

Mass spectrometry (MS) generates a molecular fingerprint for validating the presence of oligomers responsible for transmission of Aβ in AD and the correlation of the electrophysiological response through patch clamp with tandem mass spectrometry (MS/MS) provides as an oligomer fragmentation assay in real time. This will increase our understanding of the molecular changes responsible for cellular changes within the specific neurons as the change in firing activity induces anomalous synaptic plasticity and eventually network failures within specific regions of the hippocampus seen in clinical AD. No preliminary data is available this proposal builds upon the findings within the literature as there is a deficiency in our understanding of the etiology of AD and a lot of credulity due to the multitude of studies which have failed to resolve the discrepancies associated both the clinical diagnosis and prognosis of AD. We propose the use of nano-ESI tandem MS for the analysis of hippocampal cell lysate for the identification of molecular biomarker proteins. These biomarkers include α-synuclein, amyloid precursor protein (APP), and TAU promote neurotoxicity and the formation of neurogenerative plaques before they are clinically symptomatic. The ubiquitylation of these proteins using the proteasome might increase axonal clearance and resolve the pathophysiology by preventing neural death through synaptic plasticity recovery. We are intrigued by the possibility of the therapeutic nature of developing bi-functional linked ligands which selectively target the Aβ1–42 aggregates for degradation using the ubiquitylation proteasome system (UPS). Studies have suggested the prion mediated mechanism for transmission through direct contact in which prion seeding can occur though self-prorogating mechanisms (i.e., TNT and glial cells) which allow for prion migration throughout the brain. We seek to elucidate upon the causality schemes responsible for the formation of Aβ1–42 aggregates and migratory nature of such promiscuous Aβ fragments which interact with PrP pathway. Further research is to investigate the propensity with respect to prion-mediated pathways that are responsible for the pathophysiological lethality of diseases including CJD, Kuru, and SFE where the mortality rate is guaranteed with the onset of death occurs shortly after becoming clinically symptomatic, which exacerbates the epidemiology since exposure to prion like transmission during a typical latent phase with the hypothetical detection of α-synuclein, amyloid precursor protein (APP), and TAU proteins in future AD patients. The method development will include a matrix interference study along with a linearity study to determine accuracy and precision.
Samples will be ran using a bracketed sequence including the QA/QC controls: IB, MB, CCV (50 ppm), LCS, LCS-MS, LCS-MSD, CAL-1 (2.5 ppm), CAL-2 (5 ppm), CAL-3 (10 ppm), CAL-4 (25 ppm), CAL-5 (50 ppm), and CAL-6 (100 ppm). Concentrations will be compared to the values obtained from the ELISA values for validation. Several papers have shown that HPLC is an effective way to measure both AB 42 and TAU proteins in blood samples. However, it is very difficult to measure post-translational modification rates using HPLC-DAD and encourages the use of a mass spectrometer. Several types of mass spectrometers (MS) can be used to quantify proteomics with high resolution (m/z ratio). Libraries already exist for a large amount posttranslational modifications for the TAU protein and the M+, M+1, M-1 fragments can all be obtained using MALDI or ESI ionization source. Several mass spectrometers have been used including electromagnet (EM), Time-of-Flight (TOF), and tandem MS/MS scanning mass spectrometer with a triple quadrupole (QQQ). Regardless of the type of MS, the results have been published in several peer review journals and standards are available from multiple vendors. The use of trypsin breaks down proteins to expose their primary amino acid sequence and any modifications can be easily identified and cross-referenced for screening of epigenetic changes with respect to the onset of AD. Despite this, MS-based techniques can be usefully applied both to obtain structural information and to follow the complex heterogeneous early stages of aggregation of this amyloidogenic protein. (Phillips et al., 2015) The Aβ1-42 ions are formed by nano electrospray (nano-ESI) and injected through a small capillary into an ion funnel and are slowly dehydrated as they travel through the funnel to the entrance of the mobility cell where they are stored. Every 10-3 sec a pulse of stored ions is injected into the mobility cell filled to 5 Torr with helium gas. The ion pulse drifts through the gas under the influence of a weak electric field, exits the cell, passes through a quadrupole mass filter, and is detected as a function of time. The arrival time distribution (ATD) contains information regarding the shapes of the ions and whether more than one conformer is present in the mass-selected ion beam.

WT Aβ1-42 peptide: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
The ECD diagnostic ions [M + 2H - 60]+ and z6• - 57 were observed and successfully applied to differentiate the isomeric forms of the amyloid β tryptic peptide 17-28. Differentiation of aspartic and isoaspartic acid residues is also possible with EID using the same diagnostic ion z6• - 57 as in ECD. Amyloid β1-40 and β1-42 peptides can be analyzed by a top-down ECD approach without prior digestion and provide substantial sequence coverage. Amyloid β1-42 isomerized at residue 7 can be differentiated by ECD using the c6 + 57 diagnostic ion. Both ECD and EID can clearly define the presence and the position of isoaspartic acid residues in amyloid β peptides. The amyloid fragment β1-42 (Aβ42, which includes two more hydrophobic amino acids at the C-terminus compared to Aβ0, DAE...GAIIGLMVGGVVIA42) is the most abundant in the plaque deposits. Double deamidation was observed due to the presence of a second asparagine in the peptide sequence at position 27. Partial deamidation of Asn27 was also observed, which is explained by the fact that the rate of HNS deamidation is faster than the rate of SNK deamidation.
Noncoding RNAs (ncRNA) are functional RNA transcribed from DNA but fail to be translated into proteins. ncRNA, including transfer RNA (tRNA), ribosomal RNA (rRNA), microRNA (miRNA), and long noncoding RNA (lncRNA), are abundant, biologically active molecules that modify gene expression. MicroRNA are short (21–25 nucleotides), single-stranded RNA molecules that mimic small interfering RNA and function in RNA silencing and posttranscriptional regulation of gene expression. (Kuivaniemi, Ryer, & Elmore, 2016) One example of a lncRNA involved in neurodegeneration is BACE1-AS, a conserved antisense transcript that modulates BACE 1 gene expression and influences the pathogenesis of AD. β-Site amyloid precursor protein-cleaving enzyme 1 (BACE 1) cleaves amyloid precursor protein (APP) and leads to the formation of amyloid plaques in the brains of a patient with AD. BACE 1 AS is transcribed from the opposite strand of BACE 1 locus and is ~2-kb RNA. Studies have shown that BACE 1-AS and BACE 1 messenger RNA (mRNA) form a duplex, which in turn increases the stability of BACE 1 mRNA. As compared to control, AD patients show increased levels of BACE1-AS RNA in affected brain regions (Faghihi et al. 2008). Another lncRNA, BC200 is also linked to AD. BC200 levels were found to be increased in Brodmann’s area, a brain region that is mostly affected by AD (Mus et al. 2007). The BC200 plays an important role in regulating protein synthesis in dendrites and overexpression of this ncRNA in AD and in ageing can lead to synaptodendritic deterioration (Mus et al. 2007). Non-coding (nc)RNAs, including microRNAs (miRNAs), long noncoding RNAs (lncRNAs) regulate gene expression at the transcriptional and posttranscriptional levels in various diseases, serving as biomarkers and potential therapeutic targets. There is rising recognition that ncRNAs have been implicated in both the onset and pathogenesis of AD. ncRNAs are implicated post-transcriptionally in the main AD pathways and there are growing interest in targeting regulatory ncRNAs therapeutically to combat AD by using a series of multifunctional molecules that contained APP, and TAU-recognition moieties and E3 ligase-binding moieties to enhance APP, and TAU degradation. Therefore, partial reduction of TAU with such multifunctional peptides may provide a novel therapeutic strategy for AD treatment. miRNA, key players of post-transcriptional gene regulation, are approximately 20 nucleotides long non-coding RNA. An estimated 70% of miRNAs are expressed in the brain. They can be detected using methods such as real-time polymerase chain reaction (RT-PCR), and microarrays through deep sequencing technologies.
There is evidence showing that the changes at miRNA levels are associated with some parts of AD pathology, such as in the case of miR-16 which could potentially inhibit expression of amyloid precursor protein (APP) in age-related senescence-accelerated mouse prone 8 (SAMP8) mice. Several differently expressed miRNA in AD were identified, but these results have not yet been confirmed. There is still progressing to be made in continually monitoring the changes in the level of individual miRNA as biomarkers for AD. Liu et al., 2014) However, the α-synuclein found in Lewy bodies has condensed to fibrillar aggregates rich in β-sheet structure. The aggregation pathway has been described as containing multiple intermediate oligomer structures including spherical and ring-shaped forms and is susceptible to perturbation, through changes to environmental factors. These factors include agitation, temperature and pH. The transition from native conformation to these amyloid deposits is therefore not a single step, nor a single pathway, and hence in order to understand the mechanisms at play it is critical to identify the species present at each step. MS approaches have also been applied to study other aspects of α-synuclein for example to study the effect of fragments on aggregation and to characterize the influence of metal ion binding, on conformation and aggregation. MS has been used to examine post-translational modifications in α-synuclein, identifying phosphorylated species in CSF and to investigate the effect of such post translational modifications on the behavior of α-synuclein. (Phillips et al., 2015) Studies with AD patients revealed that miRNA, miRNA29 a/b is downregulated in a subset of AD patient that showed high BACE-1 expression level. miR 29 and miR 29 a/b targets BACE-1 mRNA. BACE 1 promotes formation of amyloidogenic peptide formation (Hebert et al. 2008). Other miRNAs that are downregulated in AD are miR-107, miR-15a, miR-16, miR-106a, miR-520c and miR-153 (Femminella et al. 2015). The importance of miRNA in regulation of certain proteins that are critical in AD pathogenesis (Femminella et al. 2015). Although much information is not available on ncRNA targets in UPS, there are reports of selective activation of small ncRNAs in affected brain regions in AD patients. In a recent study, HECTD1 and RNF8, two E3 ligases were identified as targets of ncRNA in the cortex and cerebellum of SCA1 patients and also of individuals with AD (Persengiev et al. 2012a, b). Regulating the expression levels of pathogenic ncRNAs (miRNAs) which promote the inability to remove Aβ1-42 aggregates by reducing the ubiquitylation proteasome pathway would impede axonal clearance resulting in the attenuation of synaptic potentials and the potentiation of network firing.
Figure 1.6, Formation of miRNA.

RNA translation occurs producing a pri-miRNA with a 5’ cap and poly adenosine tail. Drosha and Pasha cleave the 5’ cap and poly adenosine tail allowing for nucleus export to the cytosol. Dicer forms the miRNA duplex which becomes the mature miRNA fragment.
miRNA affects Tau clearance through several different means. miRNA can alter Tau mRNA and Tau protein transcription. miRNA can affect Tau phosphorylation through signaling kinases including AKT/PTEN, GSK3β, p38, ROCK, and CDK5. miRNA can alter Tau acetylation which impairs the UPS and chaperone systems through modulating SIRT1 and EP300.
The use of real-time PCR and traditional molecular biology techniques including western blotting (WB) and immunohistochemistry (IHC) assays will confirm the roles of ncRNA and the therapeutic efficacy of removing Aβ1-42 aggregates and might serve as a potential biomarker for the prediction of the onset of AD before clinical relevance manifests as a result of symptomatic behaviors from the permeant loss of brain regions which are key to longevity, survival, and quality of life. miR-132 regulates TAU phosphorylation (via direct targeting of GSK3β), acetylation (via a EP300), and cleavage (through calpain 2 and caspases-3/7), and it also reduces TAU mRNA via the direct targeting of the RNA-binding protein, Rbfox1. TAU hyperphosphorylation at PHF1 epitope, largely mediated by GSK3β, affects microtubule dynamics and NFT accumulation, which is considered hallmark cytopathology in AD and other Tauopathies. Since we validated both major TAU kinase GSK3β and acetylase EP300 as the direct miR-132 targets and additional TAU kinase CDK5 is also indirectly repressed by miR-132 via NOS1 signaling, thus, miR132 emerges as the major regulator of the post-translational modifications of TAU. miR-132 is regulated by the activity-dependent cAMP-response element-binding (CREB) transcription factor, and its expression pattern in the AD brain mimics that of the brain-derived neurotrophic factor and alterations in DNA methylation that affect gene expression and perhaps the onset of AD may play a role in miR-132 downregulation in neurons, as demonstrated for some cancer cells.
ncRNA like miR-132 regulates TAU phosphorylation (via direct targeting of GSK3β), acetylation (via a EP300), and cleavage (through calpain 2 and caspases-3/7), and it also reduces TAU mRNA via the direct targeting of the RNA-binding protein, Rbfox1. TAU hyperphosphorylation at PHF1 epitope, largely mediated by GSK3β, affects microtubule dynamics and NFT accumulation, which is considered a hallmark cytopathology in AD and other Tauopathies. Since we validated both major TAU kinase GSK3β and acetylase EP300 as the direct miR-132 targets and additional TAU kinase CDK5 is also indirectly repressed by miR-132 via NOS1 signaling, thus, miR132 emerges as the major regulator of the post-translational modifications of TAU. miR-132 is regulated by the activity-dependent cAMP-response element-binding (CREB) transcription factor, and its expression pattern in the AD brain mimics that of the brain-derived neurotrophic factor and alterations in DNA methylation that affect gene expression and perhaps the onset of AD may play a role in miR-132 downregulation in neurons, as demonstrated for some cancer cells. Exercise might provide a preventative means for lowering the age associated risk of developing AD-related cognitive dementia. Previous studies have shown that the clearance of AD-inducing proteins such as Tau could be an effective treatment option for patients which present pathophysiological clinical symptoms. There is no non-invasive screening method for detecting AD-inducing biomarkers in the clinical setting as the diagnosis depends on the pathophysiological symptoms which are present after significant brain deterioration has occurred due to the inability to detect the presence of AD-related biomarkers in a timely manner results in a poor treatment prognosis. The identification and development of non-invasive detection screening of AD-related biomarkers before the onset of pathophysiological symptoms related to cognitive deterioration seen in AD; which would improve patient prognosis and treatment responsiveness. There is a need for additional insight into the causality mechanisms which promote AD-related cognitive decline for biomarkers. Screening for a panel of biomarkers offers a risk assessment for potential subjects that would be likely to develop AD-related cognitive issues and would promote better prognosis rates as treatment intervention would occur prior to the onset of pathophysiological symptoms. Further insight into screening strategies would promote preventative measures as part of the risk assessment criteria for determining the appropriate course of treatment for improving patient prognosis.
Other factors like pro-inflammatory and inflammatory cytokines such as IL-1β and IL-10 have been shown to influence the progression of AD. Exercise can influence the cytokine signaling transduction pathway and might provide a preventive measure in which lifestyle changes lowers the risk of the pathophysiological development years before the onset of clinical symptoms. The evaluation of the liver in modulating the clearance of Tau and AB-42 in Alzheimer’s Disease (AD) is critical to understanding the peripheral mechanisms for regulating the bioaccumulation of AD-inducing proteins. Exercise significantly increases liver activity and might provide an increase in the rate of clearance from the body. There might exist a correlation between liver failure and the onset of cognitive dementia related to AD as a decline in hepatic clearance might exacerbate the build-up of Tau and AB-42 in the brain which results in neuronal toxicity due to the aggregation of phosphorylated Tau (p-Tau). Because liver activity decreases during age, increasing exercise activity during aging might slow the progression of AD-related cognitive decline and cytokine activity by promoting hepatic clearance from the blood. Criteria would be necessary to determine the risk assessment for AD development.
1.9 References


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