# MODULATORY EFFECT OF THICAPA ON THE AMYLOID PRECURSOR PROTEIN PROCESSING PATHWAYS OF FAMILIAL ALZHEIMER'S DISEASE USING FIBROBLAST CELL LINES

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**by**

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## LIST OF SYMBOLS AND UNITS

| | |
|---|---|
| % | Percentage |
| ± | Plus/minus |
| ~ | Approximately |
| ºC | Degree Celsius |
| > | More than |
| < | Less than |
| α | Alpha |
| β | Beta |
| γ | Gamma |
| Δ | Delta |
| $R^2$ | R-squared |
| × *g* | Gravitational force |
| $IC_{50}$ | Half-maximal inhibitory concentration |
| kDa | Kilodalton |
| kb | Kilobase |
| L | Litre |
| mM | Millimolar |
| mL | Millilitre |
| ng | Nanogram |
| nm | Nanometre |
| µM | Micromolar |
| pg | Picogram |
| RFU | Relative fluorescence unit |
| µL | Microlitre |
| µg | Microgram |
| v/v | Volume per volume |
| w/v | Weight per volume |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AD | Alzheimer's disease |
| ADAM10 | A Disintegrin and Metalloproteinase domain-containing protein 10 |
| APP | Amyloid precursor protein |
| Aβ | Amyloid beta |
| BACE1 | Beta-site amyloid precursor protein cleaving enzyme 1 |
| BBB | Blood-brain barrier |
| cDNA | Complementary DNA |
| CNS | Central nervous system |
| Cq | Quantification cycle |
| CT | Cycle threshold |
| CSF | Cerebrospinal fluid |
| CTFβ | C-terminal fragment beta |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| fAD | Familial Alzheimer's disease |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| IUPAC | International Union of Pure and Applied Chemistry |
| MCI | Mild cognitive impairment |
| MEM | Minimum Essential Medium Eagle |
| MRI | Magnetic resonance imaging |
| NEAA | Non-essential amino acid |
| NFT | Neurofibrillary tangles |
| NSAID | Non-steroidal anti-inflammatory drug |
| NTC | No template control |
| OD | Optical density |
| PBS | Phosphate-buffered saline |
| PET | Positron emission tomography |

| | |
|---|---|
| PSEN | Presenilin |
| qRT-PCR | Real-Time Quantitative Reverse Transcription - Polymerase Chain Reaction |
| RNA | Ribonucleic acid |
| ROS | Reactive oxidative stress |
| ROX | 6-carboxyl-X-rhodamine |
| sAPPα | Soluble amyloid precursor protein alpha |
| sAPPβ | Soluble amyloid precursor protein beta |
| SEM | Standard error mean |
| TBE | Tris/Borate/EDTA buffer |
| TBS | Tris-buffered saline |
| THICAPA | 3-[[(3S)-1,2,3,4-Tetrahydroisoquinoline-3 carbonyl]amino] propanoic acid |
| THIQ | Tetrahydroisoquinoline |
| UV | Ultraviolet |
| WHO | World Health Organization |

## LIST OF APPENDICES

**KESAN MODULASI THICAPA TERHADAP TAPAKJALAN PROSES PROTEIN PREKURSOR AMILOID PENYAKIT ALZHEIMER *FAMILIAL* MENGGUNAKAN TITISAN SEL FIBROBLAS**

## ABSTRAK

Penyakit Alzheimer *familial* (fAD) adalah gangguan neurologi yang diwarisi dan tidak dapat dipulihkan yang dicirikan oleh pengumpulan amiloid-beta (Aβ) toksik. Kehadiran simptom fAD adalah membimbangkan, kerana belum ada ubat tersedia untuk menyembuhkan penyakit ini. THICAPA adalah sebatian baru yang tergolong dalam kumpulan kimia jenis amina, iaitu tetrahydroisoquinoline (THIQ). Kajian THICAPA sebelumnya telah melaporkan kesan neuroprotektif dalam model transgenik *Drosophila melanogaster* (*D. melanogaster*). Kajian ini, menyiasat kesan modulasi THICAPA ke atas tapakjalan proses protein prekursor amiloid (APP) dengan menggunakan titisan sel fibroblas yang berasal dari kulit pesakit fAD (AG06840). Kajian ini menggunakan asai WST-1 untuk menilai sitotoksisiti dan pengebasan THICAPA, serta teknik qRT-PCR dan Western blot untuk menganalisis ekspresi gen dan protein, masing-masing. Selain itu, kuantifikasi protein dikaji menggunakan ELISA, dan tahap spesies oksigen reaktif (ROS) telah diukur menggunakan asai DCFDA. Asai sitotoksik THICAPA mendedahkan kesan ketoksikan yang tidak ketara terhadap AG06840 dan titisan sel fibroblas berasal dari kulit yang sihat (GM05879) pada tiga titik masa berbeza, 24, 48 dan 72 jam. Ujian pengebasan Aβ menunjukkan THICAPA mempunyai kesan penghapusan oligomer Aβ42 berumur yang tinggi pada 50 μM dalam titisan sel fibroblas GM05879. Seterusnya, THICAPA telah mengurangkan ekspresi gen APP dan nisbah ekspresi protein APP matang dan tidak matang (mAPP/imAPP). Kedua-dua gen dan ekspresi protein BACE1 dan PSEN1 dalam

tapakjalan amyloidogenik telah berkurangan, masing-masing menandakan pengurangan aktiviti enzim β- dan γ-secretase. Sebaliknya, ekspresi gen dan protein ADAM10 yang tinggi menandakan peningkatan aktiviti enzim α-secretase dalam tapakjalan bukan amyloidogenik. Kuantifikasi protein yang dihasilkan daripada tapakjalan proses APP telah menunjukkan pengurangan ketara sAPPβ, CTFβ, Aβ40, dan Aβ42 daripada tapakjalan amyloidogenik, dan peningkatan sAPPα daripada tapakjalan bukan amyloidogenik. Pengesanan ROS secara *in vitro* telah mendedahkan bahawa rawatan THICAPA mengurangkan pengeluaran ROS dalam titisan sel AG06840, berpotensi oleh amyloidogenesis yang dikurangkan. Ringkasnya, kajian ini menunjukkan THICAPA mempunyai potensi terapeutik untuk terus diformulasi sebagai terapi sasaran efektif untuk fAD dengan meningkatkan tapakjalan bukan amyloidogenik dan mengurangkan tapakjalan amyloidogenic.

**MODULATORY EFFECT OF THICAPA ON THE AMYLOID PRECURSOR PROTEIN PROCESSING PATHWAYS OF FAMILIAL ALZHEIMER'S DISEASE USING FIBROBLAST CELL LINES**

## ABSTRACT

Familial Alzheimer's disease (fAD) is a hereditary and irreversible neurological disorder characterised by the accumulation of toxic amyloid-beta (Aβ). The prevalence of fAD symptoms is concerning as there are no available therapeutic solutions to cure this disease. THICAPA is a novel compound belonging to the tetrahydroisoquinoline (THIQ) group of amines, which reported the neuroprotective effects on the AD transgenic *Drosophila melanogaster* (*D. melanogaster*) model. Therefore, this study investigated the modulatory effect of THICAPA on the Amyloid Precursor Protein (APP) processing pathway using the fAD patient skin-derived fibroblast cell line (AG06840). This study utilized WST-1 assay to assess cytotoxicity and scavenging potency of THICAPA, while qRT-PCR and Western blot to analyse gene and protein expressions, respectively. Additionally, protein quantification was performed using ELISA and intracellular reactive oxygen species (ROS) levels were measured using the DCFDA assay. The cytotoxicity assay recorded the insignificant toxic effect of THICAPA towards AG06840 and healthy skin-derived fibroblast cell line (GM05879) at three different time points of 24, 48, and 72 hours. The subsequent Aβ scavenging assay showed that 50 μM THICAPA exerted a potent scavenging effect on aged Aβ42 oligomers in the GM05879 fibroblasts cell line. THICAPA reduced the APP gene expression and the mature/immature APP protein (mAPP/imAPP) expression ratio. Both gene and protein expressions of BACE1 and PSEN1 in the amyloidogenic pathway were also diminished, indicating the

downregulation of the β- and γ-secretase activities, respectively. In contrast, the increased ADAM10 gene and protein expressions signify the upregulation of the α-secretase activity in the non-amyloidogenic pathway. In this regard, the quantification of proteins produced from the APP processing pathway displayed a significant reduction of sAPPβ, CTFβ, Aβ40, and Aβ42 from the amyloidogenic pathway and elevated sAPPα from the non-amyloidogenic pathway. The *in vitro* ROS detection revealed that THICAPA treatment mitigated the intracellular ROS production in AG06840 fibroblasts, potentially via downregulation of amyloidogenesis. In summary, this study revealed the therapeutic potential of THICAPA by upregulating the non-amyloidogenic pathway while suppressing amyloidogenic pathway and amyloidogenesis, attesting its effectiveness as a targeted therapy for fAD.

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Alzheimer's disease (AD) is an irreversible neurological disorder that primarily affects neurons and the brain. This disease develops slowly over time, marked by pathophysiological brain changes that occur before the onset of noticeable clinical manifestations. As such, AD is the most prevalent form of dementia detected in elderly individuals, contributing up to 80% of all dementia cases worldwide (Alzheimer's Association, 2021). This disease poses a significant healthcare and socio-economic burden, requiring over USD 179 billion for the clinical management of AD patients (Monfared *et al.*, 2022). According to the World Health Organisation (WHO) report in 2021, AD affects over 55 million people globally, with roughly 10 million new cases being diagnosed each year (WHO, 2021). It is estimated that the annual economic burden associated with the clinical management of AD patients will reach a staggering USD 2.62 trillion, with further projections indicating a ten-fold surge from 2021 to 2041 (Jun *et al.*, 2024). The deaths attributable to AD have also doubled, signifying a continuous upward trajectory, partially driven by the steady population growth and demographic shifts toward an ageing population.

Malaysia is experiencing an accelerated transition towards an ageing society driven by rapid demographic shifts. In view of this, there is a growing focus on AD in Malaysia, with projections suggesting that it will further worsen in the near future, highlighting the need for more comprehensive research and effective interventions. In Malaysia, approximately 260, 000 individuals, constituting roughly 8.5% of the elderly

population with dementia, require 24-hour care in the late stages of the disease (Alzheimer's disease Foundation Malaysia, 2020; WHO, 2021). Given that the elderly population in Malaysia is projected to triple by 2050, this increase is expected to lead to a 312% surge in the number of AD cases (Benoist *et al.*, 2022; Ong *et al.*, 2024).

Hypothetically, the neurodegenerative cascade in AD is associated with uncontrolled microglial activation, manifested by the build-up of amyloid-β (Aβ) plaques and Neurofibrillary Tangles (NFTs) due to hyperphosphorylated tau (Kamble *et al.*, 2024). Among them, the accumulation of Aβ plaques plays the dominant role in inducing tau pathology. This neuroinflammatory response involves the secretion of neurotoxins and inflammatory factors, which are believed to contribute to progressive neuronal loss, ultimately resulting in memory decline and cognitive dysfunction. This effect highlights the substantial and rapidly escalating healthcare challenges posed by AD patients.

There are two main categories of AD: familial AD (fAD) and sporadic AD (sAD). The Aβ peptides in fADs originate from a transmembrane protein called Amyloid Precursor Protein (APP) through β- and γ-secretase cleavage, demonstrating a robust genetic association (Cho *et al.*, 2022). Notably, a reduction in α-secretase activity promotes the formation of Aβ peptides, which potentially contributes to the accumulation of Aβ plaques, neuroinflammation, neuronal damage, and, ultimately, dementia (Kamble *et al.*, 2024). Genetic mutations in active gene coding of APP, Presenilin 1 (PSEN1), and Presenilin 2 (PSEN2) appear to increase the risk of fAD, thus strengthening their direct involvement in AD pathogenesis.

The fAD poses a greater threat to the healthcare system due to its inheritance, early onset and its potential to affect individuals as young as 20 years old. Moreover, fAD is

mostly detected when the patient has aged and the symptoms have worsened (WHO, 2021). Studies have reported that an upregulated amyloidogenic pathway produces Aβ isoforms, such as Aβ40 and Aβ42, which are highly toxic and tend to aggregate in neurons to form Aβ plaques (Ayton & Bush, 2021). Despite the fact that the Aβ pathology was identified in AD patients approximately 20 years before the clinical onset, there are yet any established drugs to cure or reverse the disease (Jia *et al.*, 2023). Concurrently, most of the drugs tested in clinical trials have failed to block the disease progression, underscoring the significant need to formulate effective treatments to reverse AD progression (Ord *et al.*, 2013).

In recent decades, research on 1,2,3,4-tetrahydroisoquinoline (THIQ) moieties has yielded several promising therapeutic candidates to treat diverse conditions, including neurological disorders, gout, pain, and cancer (Pyne & Martin, 2022). This unique heterocyclic scaffold belongs to the secondary amine class and is endogenously produced in the brain during the degradation of dopamine, which can readily cross the Blood-brain Barrier (BBB) (Hényková *et al.*, 2022). Studies on THIQ derivatives have demonstrated their ability to suppress Aβ production directly (Fang *et al.*, 2019), provide synaptoprotection against Aβ-induced toxicity (Wang *et al.*, 2020), and offer neuroprotective potential through the inhibition of Reactive Oxygen Species (ROS) production (Ting *et al.*, 2021). THIQ derivatives have also shown promising results in modulating APP proteolytic cleavage (Zhang *et al.*, 2011).

A recent study by Tan *et al.* (2023) highlighted the neuroprotective effect of a novel THIQ derivative known as THICAPA, with an IUPAC name of 3-[[(3S)-1,2,3,4-tetrahydroisoquinoline-3 carbonyl]amino] propanoic acid. The study revealed that

THICAPA alleviates AD-related defects in rat pheochromocytoma nerve cells (PC12) and transgenic *Drosophila melanogaster* (*D. melanogaster*) expressing human Aβ42. Although THICAPA treatment significantly boosted cell survival and enhanced cell growth when incubated with toxic Aβ42 peptides, the molecular interaction of THICAPA and its impact on human-derived cell lines has not been investigated. Given the significant reduction of Aβ42 aggregation by THICAPA in AD transgenic *D. melanogaster*, it is essential to explore the modulatory effect of THICAPA in the APP processing pathway, which mainly produces toxic Aβ peptides.

**1.2 Problem statement**

The surge in AD cases is alarming since 99.6% of AD drug testing in clinical trials has recorded the highest failure rates compared to all other diseases globally (Jabir *et al.*, 2021). There are also no effective treatments capable of curing or reversing AD progression. The available therapies only address the disease's symptoms, offering limited prevention or slowing down its progression (Kamble *et al.*, 2024). Hence, the inherent complexity and association with specific gene mutations of fAD present a significant burden to the healthcare system and economy.

Therapeutic strategies have focused on the neurotoxicity of Aβ accumulation as the primary target to alleviate AD pathogenesis. Notably, the APP processing pathway plays a crucial role in the production of Aβ peptides, where imbalances in the production and clearance lead to its accumulation (Delaby *et al.*, 2022). Nevertheless, therapeutic efforts on addressing the endpoint (Aβ inhibition) have failed to offer substantial success in AD treatments, warranting further assessment of the APP processing pathway. In light

of the absence of current therapies that can cure or reverse AD progression, the discovery of novel AD treatment drugs is paramount.

THICAPA has recently emerged as a promising AD therapeutic candidate in pre-clinical studies, evident by its ability to inhibit Aβ42 aggregation and extend the lifespan of transgenic AD *D. melanogaster* (Tan *et al.*, 2023). Despite its promising outcome, the modulatory effect of THICAPA on the APP processing pathway is still limited. Thus, a critical knowledge gap regarding its modulatory effect on AD pathogenesis exists, especially in human-derived cell lines, and needs to be explored. In particular, the key challenge in developing effective AD therapies is the lack of understanding of the influence of genes and proteins on Aβ production via the APP processing pathway, as well as the extent of THICAPA's modulatory effect on this pathway and its impact on disease progression. Thus, there is a need to investigate the modulatory effect of THICAPA in the APP processing pathway using the fAD patient-derived cell line to shed light on advancing targeted therapies and optimising drug formulations for AD treatments.

### 1.3 Research questions

Based on the arguments listed in the introduction and problem statement sections concerning the role of THICAPA in fAD pathogenesis, several research questions (RQs) were established as follows:

**RQ1:** Does THICAPA exhibit any toxicity and possess scavenging properties against aged Aβ42 oligomers-induced cytotoxicity to fibroblast cell lines?

**RQ2:** How does THICAPA impact the gene and protein expression levels involved in the APP processing pathway?

**RQ3:** How does THICAPA influence the proteins produced from the APP processing pathway?

**RQ4:** Does THICAPA exhibit any protective effect against ROS production by reducing amyloidogenesis?

### 1.4 Hypothesis and research objectives

This study hypothesises that THICAPA modulates APP processing by promoting the non-amyloidogenic pathway while suppressing the amyloidogenic pathway, thereby reducing intracellular ROS production in fAD pathogenesis. This study aims to elucidate the effect of THICAPA modulation in the APP processing pathway using the fAD patient skin-derived fibroblast cell line. The aim of this study is achieved by formulating the following specific objectives:

1. To assess the cytotoxicity of THICAPA in AG06840 and GM05879 cell lines and its scavenging effect against toxic Aβ42 oligomers in the GM05879 cell line using WST-1 assay.
2. To evaluate the effect of THICAPA on the genes and protein expression levels of APP, ADAM10, BACE1, and PSEN1 in the APP processing pathway using qRT-PCR and Western blot, respectively.
3. To quantify the sAPPα, sAPPβ, CTFβ, Aβ40, and Aβ42 proteins produced via the APP processing pathway using the sandwich ELISA method.
4. To validate the protective potential of THICAPA by evaluating ROS production associated with amyloidogenesis.

### 1.5 Research flow chart

Figure 1.1 outlines the research flowchart of this study.

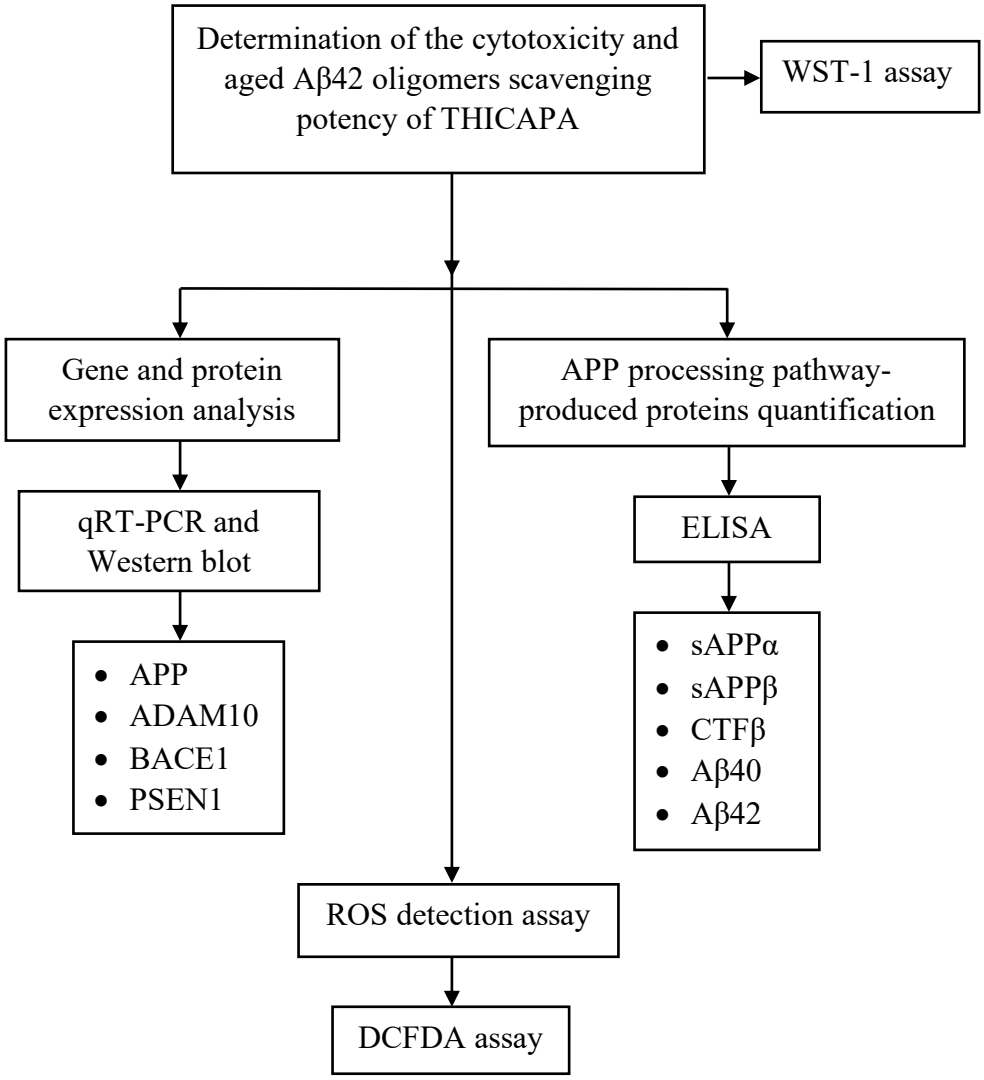


Figure 1.1 Research flowchart of the current study.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Alzheimer's disease (AD)

The umbrella term "neurodegenerative disease" encompasses an extensive range of chronic or progressive brain disorders known as dementia. This disease primarily affects the neurons, especially with higher prevalence in the ageing population (WHO, 2021). Hence, the global shift towards an increasingly ageing population has attracted significant attention worldwide, given its association with this particular health disorder. In particular, AD is the most prevalent form of dementia and accounts for nearly 80% of all reported dementia cases in the ageing population (Hou *et al.*, 2019). This disease disproportionately affects the elderly population, with an estimated prevalence exceeding 90% among individuals aged 65 and above (Lindemer *et al.*, 2017; Tarantini *et al.*, 2017). Moreover, recent reports have shown a rise in AD cases among young people as early as 20 years old due to the presence of genetic mutation, which accounts for less than 2% of all diagnosed AD cases (Jia *et al.*, 2023).

AD is also the fifth leading cause of death in the elderly population, comprising 4.4% of mortalities worldwide (Nichols *et al.*, 2019). The escalating trend in AD cases signifies a time-dependent exponential increase in AD patients, which doubles every five years (Moreno *et al.*, 2022). Geographically, it has become an alarming social and public health issue in developed countries (Trevisan *et al.*, 2019), with a higher reported prevalence among Europeans than Asians (World Alzheimer Report, 2021). In terms of gender, women are more susceptible to AD than men due to their longer life expectancy (Niu *et al.*, 2017).

In 2022 alone, the global prevalence of dementia was estimated at around 55 million people (Alzheimer's disease International, 2022; Ong *et al*., 2024). In addition, approximately USD 179 billion is spent annually on the clinical management of AD patients worldwide. In Malaysia, the prevalence of AD was approximated at 260, 000 people, while it stands at 108$^{th}$ position in the world ranking for the total number of AD cases diagnosed. It requires roughly MYR 1.2 million in allocations annually for the clinical management of AD patients (Ong *et al.*, 2024). This condition contributes significantly to the country's healthcare and economic burden. Statistically, the National Health and Morbidity Survey reported that 8.5% of Malaysians over 60 years old are affected by dementia (Ganapathy *et al.*, 2020). As Malaysia is expected to become an ageing nation by 2030, with 15% of its population being classified as senior citizens, the situation could be alarming. AD cases are projected to increase drastically, with the disease expected to develop every 33 seconds and reach 150 million cases by 2050 (National Institute of Aging, 2021).

## 2.2 Classification of AD

The International Statistical Classification of Diseases and Health-related Problems, 11$^{th}$ revision (ICD-11) (Harrison *et al.*, 2021) and the Diagnostic and Statistical Manual of Mental Disorders, 5$^{th}$ edition, text revision (DSM-5-TR) (American Psychiatric Association, 2021) have classified AD into two subtypes: familial AD (fAD) and sporadic AD (sAD). Over 90% of AD cases are classified as sAD, which is known as the late onset of the disease. In contrast, fAD is characterised by an early onset and comprises less than 10% of all AD cases. The fAD is a rare and inherited disease resulting mainly from genetic mutations of the APP, PSEN1, and PSEN2 (Deture & Dickson, 2019). The PSEN1 gene

exhibits the highest burden of reported mutations, exceedingly more than 360, followed by APP and PSEN2. It is commonly detected in individuals around the age of 35. However, a 19-year-old adolescent was reported as the youngest fAD patient in 2023, highlighting the severity of fAD, which can affect both elderly citizens as well as younger adults (Jia *et al.*, 2023). Conversely, the sAD is more common in individuals over 65 years old, manifested by complex risk factors that may overlap with fAD and are usually not caused by mutations. This underscores the heterogeneous nature and severity of fAD manifested across a wide range of age spectrum.

### 2.3 Symptoms

Among the earliest signs of AD is neuropsychiatric symptoms, which are often underdiagnosed. The symptoms can affect more than 97% of dementia patients and tend to progress over time (Heilman & Nadeau, 2022). The early onset of neuropsychiatric symptoms in AD patients varies widely, from anxiety and hallucinations to aggression, depression, and sleep disorders. Some patients show signs of impaired cognitive systems, such as poor motor disturbance, irritability, aberrant vocalisation, and delusions.

A recent Mild Cognitive Impairment (MCI) study reported that 59% of AD patients exhibited poorer psychosocial and cognitive function (López & Altuna, 2023). This condition prevents patients from living a normal life, such as remembering memories, performing problem-solving tasks, planning their daily activities, and communicating. Moreover, 67% of AD patients with psychotic symptoms reported an accelerated cognitive decline and an increase in mortality (Arendt *et al.*, 2015). Both hallucinations and delusions are also correlated in AD patients, where delusions are more often than hallucinations (Khanna *et al.*, 2022).

## 2.4 Diagnosis

The Alzheimer's Association and dementia experts of the International Working Group have employed unique biomarkers as an essential tool for the clinical diagnosis of AD patients (Alzheimer's Association, 2021; Dulewicz *et al.*, 2022). Various neuroimaging techniques, such as Positron Emission Tomography (PET) and Magnetic Resonance Imaging (MRI), are currently used to diagnose the abnormalities and *in vivo* AD biomarkers found in patients' brain sections (Deture & Dickson, 2019). In addition, PET tracers are implemented to detect the accumulation of fibrillar Aβ and cerebral tau proteins. Besides, these neuroimaging techniques are vital in diagnosing downstream AD biomarkers in both early and late-onset AD progression (Dulewicz *et al.*, 2022).

Furthermore, the Cerebrospinal Fluid (CSF) is used to detect key AD biomarkers in CSF include Aβ42 reflecting Aβ deposition, hyperphosphorylated tau reflecting NFT formation, and neurofilament as a neurodegenerative marker (Leuzy *et al.*, 2022). The use of Enzyme-linked Immunosorbent Assays (ELISAs) targeting Aβ42 has consistently revealed significant reductions in CSF Aβ42 levels in AD patients (Blennow *et al.*, 2015; Zetterberg, 2019). This decrease is inversely correlated with an increase in cortical plaque burden, as confirmed by post-mortem studies and supported by *in vivo* biopsy data.

Alternatively, blood-based biomarkers offer a promising solution to the inherent drawbacks associated with CSF sampling, with researchers uncovering increasingly reproducible changes in these biomarkers related to AD diagnosis. The quantification of the plasma Aβ42/40 ratio and concentration of Aβ isoforms have also emerged as a promising tool for identifying individuals with abnormal amyloid burden, as detected by CSF or PET, across the wide-range AD spectrum (West *et al.*, 2021).

## 2.5 Molecular pathogenesis

### 2.5.1 Pathological hallmarks

The most prominent pathological hallmark of AD is the aggregation of extracellular Aβ plaques in the brain section as a result of the proteolytic cleavage of APP. A growing amount of evidence suggests that the APP processing and oligomerisation of Aβ peptides induce fAD pathology, consequently leading to the formation of Aβ plaques (Lashley *et al.*, 2018). Thus, understanding the pathology of Aβ plaque formation and clearance is vital for elucidating the pathogenesis of AD.

Another pathological hallmark of AD is the formation of Neurofibrillary Tangles (NFTs) due to hyperphosphorylated tau. Tau is highly expressed in ocular neurons and the CNS (Muralidar *et al.*, 2020). It assists in stabilising the neuronal microtubules in dendrites and distal portions of axons (Pooler *et al.*, 2014). The abnormal folding, aggregation and oligomerisation of tau lead to the formation of hyperphosphorylated tau (Iqbal *et al.*, 2013; Wit & Ghosh, 2015). Subsequently, NFT is formed in AD patients due to the dysregulation of tau phosphorylation, characterised by the imbalance that favours excessive phosphorylation over dephosphorylation (Muralidar *et al.*, 2020).

Although both hallmarks hold a pivotal role in AD pathogenesis, AD research is more focused on investigating Aβ pathogenesis due to the perceived earlier onset compared to the formation of NFTs. It is relevant to highlight that the accumulation of Aβ plaques induces tau pathology in multiple APP transgenic animal models, but tau does not influence Aβ pathology (Guo *et al.*, 2020). Hence, the dominant pathological formation of Aβ plaques makes them and the APP processing pathway more compliant targets for therapeutic interventions aimed at alleviating AD development.

### 2.5.2 Amyloid Precursor Protein (APP)

The APP is instrumental in AD pathogenesis. This type-1 transmembrane glycoprotein contains an extracellular N-terminus that spans the membrane once (Zhao *et al.*, 2020). Located on chromosome 2 and encodes around 695–770 amino acids (Müller *et al.,* 2017; Tan & Azzam, 2017), the APP exhibits multifaceted roles in neuronal physiology, including transmembrane signal transduction, calcium metabolism, neuronal protein trafficking along the axon, cell adhesion, synaptogenesis, and neuronal growth, highlighting its significance that extends beyond amyloidogenesis (Zheng & Koo, 2006; Cho *et al.*, 2022).

At the gene level, the APP mRNA undergoes alternative splicing to produce various isoforms with distinct lengths and functions. For instance, the $APP_{695}$, $APP_{751}$, and $APP_{770}$ contain 695, 751, and 770 amino acids, respectively (Wang *et al.*, 2017). Whereas at the protein level, tightly regulated Post-translational Modifications (PTMs) determines the APP isoforms. For instance, N-glycosylation leads to immature APP (imAPP) formation and N- and O-linked glycosylation and phosphorylation leads to mature APP (mAPP) formation (Haass *et al.*, 2012; Gołacka *et al.*, 2021). The mAPP expressed at transmembrane undergoes proteolytic cleavage by α- and β-secretase at the plasma membrane and γ-secretase at the late secretory pathway in the APP processing pathway (Khambhati *et al.*, 2023).

### 2.5.3 APP cleaving proteases

Several key enzymes are involved in APP metabolism, such as α-secretase, β-secretase, and γ-secretase (Table 2.1). The α-secretase is a type-I transmembrane protein and a zinc metalloproteinase that cleaves APP in the non-amyloidogenic pathway (Guo *et al.*, 2020). It is also grouped in the A Disintegrin and Metalloproteinase (ADAM) family, encompassing several members with α-secretase-like activities, such as ADAM9, ADAM10, and ADAM17 (Zhang *et al.*, 2011). Studies have reported that the expression of ADAM10 is significantly reduced in the CSF of AD patients compared to healthy people (Hershkovits *et al.*, 2023), underscoring its inhibition in AD pathogenesis.

β-secretase is a type-I transmembrane aspartyl protease linked to the membrane near the C-terminus domain. BACE1, an active gene that codes for the protease of β-secretase (Kamble *et al.*, 2024), proteolyses APP at the N-terminus domain (Zhang *et al.*, 2019). The overexpression of BACE1 enhances the activity of β-secretase in the Golgi apparatus and endosomes, leading to over-proteolysis of APP. Although BACE2 is a homologue of BACE1 and is found in the critical region for Down's syndrome (Lashley *et al.*, 2018), it is not coded for β-secretase. Previous findings have suggested that inhibiting the BACE1 gene prevents the production of toxic Aβ peptides, restores memory functions and APP processing, and enhances insulin sensitivity in BACE1 knock-in mice (Dekeryte *et al.*, 2021).

The γ-secretase is an aspartyl protease comprising four complex transmembrane subunits, including nicastrin, anterior pharynx-defective-1 (APH1), presenilin enhancer 2 (PEN2), and presenilin (PSEN) (Hur, 2022). Both PSEN1 and PSEN2 are homologues of PSEN subunits that cleave their substrates at the transmembrane region. Mutations in the

chromosomes of PSEN1 (14q24.3) and PSEN2 (1q31–q42) result in numerous irregularities and dysfunctions, which lead to AD pathogenesis. PSEN1 mutation alone constitutes over 80% of all reported mutations in fAD. Among all the reported PSEN1 mutations, the A246E mutation gained pivotal focus which was first identified in 1995 (Sherrington *et al.*, 1995). The gain-of-toxic-function hypothesis suggests that mutations in fAD primarily enhance the production of aggregation-prone Aβ peptides and toxic oligomers implicated in AD (Selkoe & Hardy, 2016; Dehury *et al.*, 2020). Thus, therapeutic strategies targeting the inhibition of PSEN catalytic subunits should be highly specific (Hur, 2022).

Table 2.1 List of cleavage enzymes, isoforms, functions and its relevant information

| **Cleavage enzymes** | **Isoforms** | **Functions** | **Relevant information** | **References** |
|---|---|---|---|---|
| α- secretase | ADAM9 | Controlled proteolysis without producing sAPPα | Endoprotease bound to plasma membrane | Guo *et al*., 2020 |
| | ADAM10 | Cleaves APP at Aβ domain (Lysine 16 and Leucine 17), produces sAPPα & CTFα | Most active α-secretase isoform, precursor (~100 kDa), processed (~80 kDa), and active (~60 kDa) | Zhang *et al*., 2011; Hershkovits *et al*., 2023; Peron *et al*., 2018 |
| | ADAM17 | Key regulator of APP proteolysis | Dysregulation affects APP metabolism | Endres & Deller, 2017 |
| β- secretase | BACE1 | Cleaves APP at Methionine 596 and Aspartic acid 597, produces sAPPβ & CTFβ | Rate-limiting enzyme in APP metabolism, overexpression elevates β-secretase activity | Guo *et al*., 2020; Zhang *et al*., 2019; Kamble *et al*., 2024 |
| | BACE2 | Cleaves APP at θ-site (Phenylalanine 20) and prevents Aβ production | Less intense activity in AD pathogenesis | Lashley *et al*., 2018; Wang *et al*., 2019 |
| γ- secretase | PSEN1 | Cleaves APP at transmembrane domains at positions 6 and 7 | Mutations in PSEN1 (14q24.3) elevate Aβ42 and Aβ40 | Lin *et al*., 2021; Hur, 2022 |

| Cleavage enzymes | Isoforms | Functions | Relevant information | References |
|---|---|---|---|---|
| γ- secretase | PSEN2 | Cleaves APP at transmembrane domains | Mutations in PSEN2 (1q31–q42) cause AD | Sushma & Mondal, 2019; Zhou *et al*., 2020; AlzForum, 2023 |
| | Nicastrin | Active receptor interacting with APP sequestrates | Required for γ-secretase activity | Aßfalg *et al*., 2024 |
| | APH1 | Aids nicastrin localisation | Supports γ-secretase complex | Hur, 2022 |
| | PEN2 | Matures nicastrin, enables PSEN expression | Integral in γ-secretase function | Aßfalg *et al*., 2024 |

### 2.5.4 APP processing pathways

The APP metabolism takes place primarily via two proteolytic processing pathways: non-amyloidogenic and amyloidogenic (Figure 2.1) (Müller *et al.*, 2017). The APP is predominantly cleaved by α-secretase in the non-amyloidogenic pathway, producing a shorter C-terminal fragment-α (CTFα) and a longer soluble APP- α (sAPPα). Then, the γ-secretase complex further cleaves the CTFα to form an APP Intracellular Domain (AICD) and p3 peptide (Cho *et al.*, 2022). The sAPPα and CTFα fragments were reported to enhance synaptic plasticity, maintain neuronal excitability, and protect neurons against metabolic and oxidative injuries, memory, and learning (Folch *et al.*, 2018; Ribarič, 2018). The sAPPα inhibits BACE1 in the amyloidogenic pathway, reduces the formation induced by sAPPβ peptides, and restores the balance to the APP processing in pre-injury response for hypoxia (Libeu *et al.*, 2015).

In the amyloidogenic pathway, the APP is cleaved by β-secretase at the N-terminal of the Aβ domain, generating a C-terminal fragment-β (CTFβ) and soluble APP-β (sAPPβ) as the by-products (Lashley *et al.*, 2018; Cho *et al.*, 2022). The CTFβ fragments are further proteolyzed by γ-secretase to form varying lengths of Aβ peptides and AICD (Zhao *et al.*, 2020). As the APP accumulates, toxic Aβ peptides are produced in acidic neuronal compartments, such as lysosomes and late endosomes (Poulsen *et al.*, 2017). The mechanism of APP expression in AD pathogenesis remains obscure. Simultaneously, an increase in the production of toxic sAPPβ-induced stress levels in the ER (Botteri *et al.*, 2019). The overproduction of CTFβ also caused abnormalities in the endosomal Aβ production (Jiang *et al.*, 2019; Kwart *et al.*, 2019) and impaired neuronal functions (Takauji *et al.*, 2023).

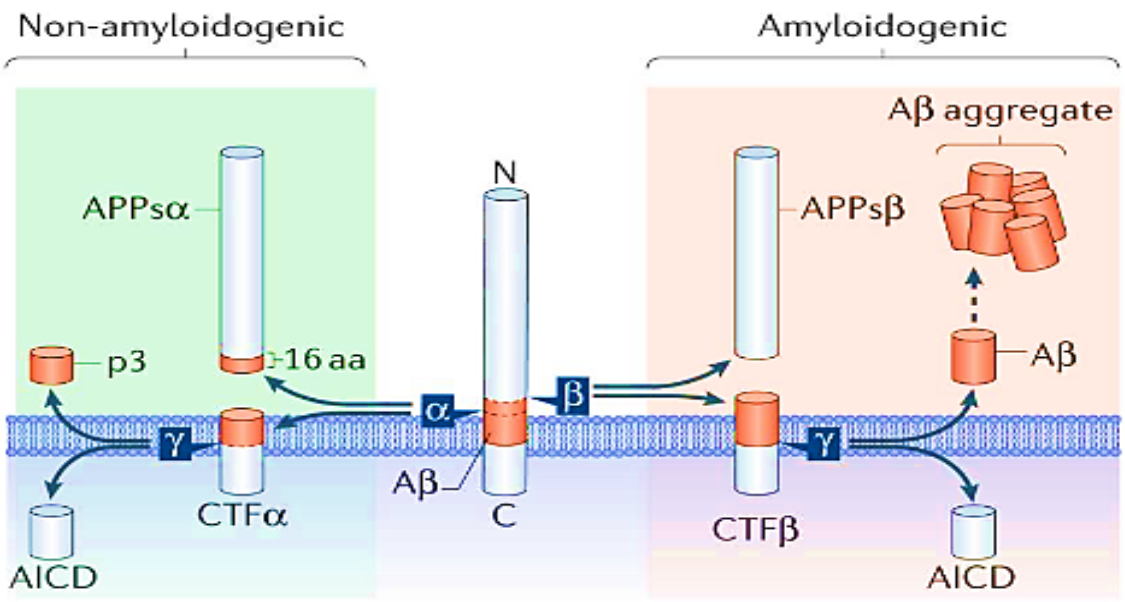


Figure 2.1 Schematic diagram of the APP processing pathway comprising the amyloidogenic pathway (pink background) and non-amyloidogenic pathway (green background). Adapted from Müller *et al.* (2017).

## 2.6 Amyloid-beta (Aβ)

### 2.6.1 Amyloid cascade hypothesis

Aβ peptides are one of the significant hallmarks of AD. Hardy & Higgins (1992) proposed the amyloid cascade hypothesis, which links the accumulation of Aβ peptides in the brain parenchyma to the primary event in AD pathogenesis. This hypothesis emphasises the critical imbalance between the production and clearance of Aβ peptides, ultimately leading to the complex neuropathology observed in AD. Despite over three decades of relentless studies on AD, researchers are unable to provide a convincing explanation of the etiopathogenic events involving the formation and advancement of disease apart from Aβ plaque aggregation.

### 2.6.2 Aβ conformations

The Aβ peptide comprises 37–49 amino acid residues and is formed through the proteolysis of APP by β-secretase and γ-secretase in the amyloidogenic pathway. The Aβ40 is the primary Aβ isoform, which is made up of 40 amino acids. However, the altered proteolysis of APP generates a minor isoform called Aβ42, which comprises 42 amino acids (Chen *et al.*, 2017). The two-residue variation between Aβ40 and Aβ42 leads to enormous differences in their clinical and biological actions.

Figure 2.2 shows the structural confirmation of Aβ peptides. It first exists in the form of monomers, which then accumulate to form oligomers in both normal and AD brains (Chen *et al.*, 2017). The Aβ fibrils are insoluble and large and tend to aggregate to form amyloid plaques. In contrast, Aβ oligomers are soluble, hydrophobic peptides and may rapidly diffuse through the cell membrane and the brain region. They can also rapidly aggregate into protofibrils and fibrils to form amyloid plaques. Thus, Aβ oligomers represent the most toxic confirmation of Aβ peptides (Bode *et al.*, 2017). Meanwhile, unbranched, long fibrils break down the myelin sheet of neurons, damage synaptic terminals, and cause neurodegeneration. Following the APP proteolysis, the newly produced Aβ peptides are secreted into the extracellular space or bound to the plasma membrane or other extracellular components, such as cellular membranes, receptors, and metal ions that offer binding sites for soluble Aβ (Chen *et al.*, 2017).

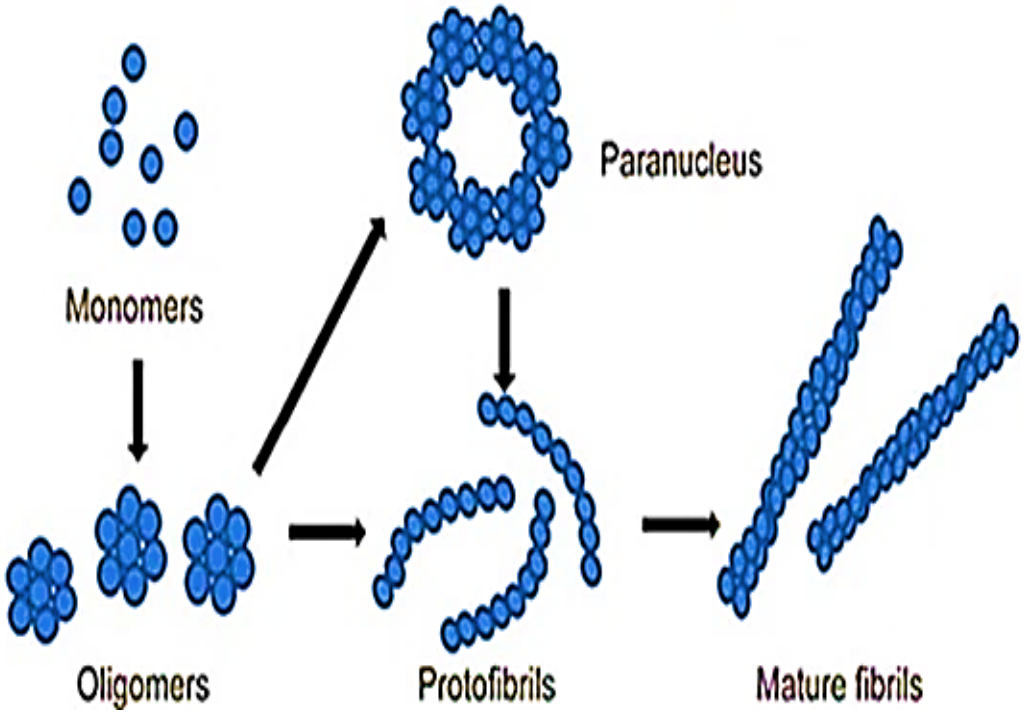


Figure 2.2 The structural confirmation of Aβ peptides. Adapted from Chen *et al.* (2017).

### 2.6.3 Aβ toxicity

The Aβ peptides are highly unstable, self-aggregating molecules that cause toxicity by dissociating into various isoforms. Lambert *et al.* (1998) published the first report on Aβ toxicity, which showed potent neurotoxin effects on the CNS. The oligomerisation of Aβ occurs mainly at the plasma membrane (Bode *et al.*, 2017), producing a wide range of Aβ oligomeric isoforms with different neurotoxicity levels and aggregation behaviours. The hydrophobic nature of Aβ oligomers makes them highly diffusible, leading to higher interactions at the phospholipid bilayer membrane of the cells and induce neuroinflammation (Salahuddin *et al.*, 2016; Yang *et al.*, 2017).

Decades of ongoing studies have revealed the harmful impacts of various Aβ isoforms, specifically Aβ40 and Aβ42, on the human brain (Matuszyk *et al.*, 2022). In particular, the Aβ40 can induce nuclear damage to the DNA in oligodendrocytes, which drastically reduces cell survival (Xu *et al.*, 2019). On the other hand, Aβ42 initiates the *in vivo* cascade of neuronal death in the brain's hippocampal region and loss of neuronal viability *in vitro* (Brouillette *et al.*, 2012; Matuszyk *et al.*, 2022). Numerous evidence also suggest that Aβ42 have a greater impact on synaptic losses and is the most cytotoxic compared to its Aβ40 counterpart (Yang *et al.*, 2017; Bate & Williams, 2018). This advantage prompted the preferential use of Aβ42 in neurotoxicity studies of potential AD therapeutics. Acknowledging the limited understanding of Aβ, more future studies are needed to address the potential knowledge gaps in AD.

## 2.7 Oxidative Stress (OS)

Reactive Oxidative Stress (ROS) are highly active oxygen metabolites in terms of oxidative modifications of cellular macromolecules (Cheignon *et al.*, 2018). However, when the biological defences of the cell are overwhelmed by pro-oxidants, such as ROS, this leads to Oxidative Stress (OS) (Singh *et al*., 2019). The OS hypothesis in AD suggests that mitochondria produce more ROS as they are damaged over time (Otín *et al.*, 2013). The high accumulation of neurotoxic Aβ peptides forms Aβ ion channels on the membrane, leads to a greater $Ca^{2+}$ influx into mitochondria (Salahuddin *et al.*, 2016). Hence, the disrupted $Ca^{2+}$ homeostasis and formation of Aβ ion channels compromise the physiological function of the organelles in neurons and peripheral cells.

OS in fAD is also attributed to the presence of mutations in APP, PSEN1, and PSEN2. These mutations were found to significantly elevate mitochondrial ROS production and trigger antioxidant enzyme dysfunction in triple transgenic mice carrying APP, PSEN1, and MAPT mutations (Han *et al.*, 2021). A positive feedback loop exists between Aβ and OS, where elevated levels of Aβ40 and Aβ42 led to an increase in OS in CNS and peripheral cells (Perluigi *et al.*, 2024). The increased accumulation of Aβ peptides induces lipid peroxidation on the neuronal and peripheral cell membranes. This process triggers higher OS, which results in membrane permeabilisation, leading to the formation of transmembrane pores. Thus, effective strategies must be implemented to prevent or reduce OS damage and provide therapeutic efficacy against AD. In this context, the viable strategy to overcome OS-caused abnormalities can be considered, including downregulating Aβ production, enhancing the antioxidant system, and improving mitochondria functionality.