CHAPTER 1

Introduction

1.1 Historical Background

An estimated 35.6 million people worldwide suffer from dementia, and the number of people affected is expected to increase to more than 48.1 million, 65.7 million, 90.3 million and 115.4 million in 2020, 2030, 2040 and 2050, respectively (1), making it a pressing global health concern. Alzheimer's disease (AD) is the most common type of dementia; approximately one in eight people over the age of 65 years are at risk of developing AD (2). AD is an age-related neurodegenerative disorder characterized by a progressive loss of memory and cognitive functions, thus interfering with daily life (3).

The number of AD cases worldwide tends to increase every year. An estimated 5.4 million Americans had AD in 2012 (4). This includes 5.2 million people age 65 and older (5) and 200,000 individuals under age 65 who have younger-onset AD (6). In 2050, this number will increase by 3-fold, to 13.2 million, with a high proportion of those being people over age 65 (5). The cost of long-term care for people with AD and other dementias is projected to increase from $200 billion in 2012 to $1.1 trillion in 2050. The increasing number of people with AD will thus have an impact on healthcare systems, families and caregivers. In addition, AD is becoming a more common cause of death in the United States and other countries. While deaths from other major causes continue to significantly decrease, those from AD have continued to rise. Between 2000 and 2008, deaths from AD increased 66 percent, while the number-one cause of death, heart disease, decreased 13 percent (7, 8). In Thailand, the prevalence of AD depends on the population and area surveyed. The Health Information System Development Office (HISO) listed 229,000 persons in 2005, and the number will continue to increase, to 450,000 and 1,200,000 persons by 2020 and 2050, respectively (9). The cost of health care for AD increased from 2007 to 2011 (10). For these reasons, early diagnosis before symptoms develop is important.

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Evaluation of cognitive functions and the clinical diagnosis of AD are based on a medical history combined with neuropsychological tests and a clinical examination of symptoms according to diagnostic criteria (11, 12). The following is a brief review of the current state of knowledge regarding biomarkers in mild cognitive impairment (MCI) and AD, including neuroimaging, cerebrospinal fluid (CSF), plasma and serum biomarkers. A variety of imaging markers support clinical diagnosis of MCI and AD, including brain volumetric measures using magnetic resonance imaging (MRI), and positron emission tomography (PET) (13). Neuroimaging studies have found some interesting results; however, compared to CSF or plasma biomarkers, these approaches are expensive, and not widely available. Thus, biomarkers in body fluids such as CSF, plasma and serum can be utilized to increase the accuracy of diagnosis for cognitive decline and prediction of MCI and AD progression. As previously mentioned, amyloid-beta (Aβ) is a proteolytic product of amyloid precursor protein (APP) cleavage produced in large amounts during the progression of AD, eventually accumulating as the component of senile plaques in CSF. In particular, cleavage of APP by both β-secretase (N-terminus) and γ-secretase (C-terminus) enzymes results in the formation of $A\beta_{40}$ and/or $A\beta_{42}$, which are associated with neuronal cell death. In particular, it is the longer, more hydrophobic $A\beta_{42}$ that is toxic in AD (14, 15). The observation that $A\beta_{42}$ is abundant in amyloid plaques led to the development of assays for this specific Aβ form. Recently many studies have reported lower CSF $A\beta_{42}$ levels in AD patients compared to age-matched healthy individuals (16). Lower $A\beta_{42}$ levels are suggested to reflect $A\beta_{42}$ in senile plaques or the formation of soluble Aβ oligomers, as has been detected by enzyme-linked immunosorbent assay (ELISA) (17, 18). While CSF is useful for research into neurodegenerative diseases, it is limited by the invasiveness of the procedure for obtaining a specimen (lumbar puncture), particularly in elderly people, making it unsuitable for routine laboratory testing.

Currently, plasma and serum biomarkers are used to measure concentrations of Aβ specific to AD pathology and progression. In humans, CSF are absorbed into the bloodstream daily (19), making blood a suitable source of neurodegenerative disease biomarkers. The simplicity of venipuncture as compared to lumbar puncture makes

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blood tests suitable for biomarker investigation of AD patients. Previous studies have reported that plasma Aβ_{42} levels may be elevated many years before the onset of sporadic AD (20). One study suggested that an increased level of Aβ_{42} in the plasma of AD patients may be involved in the pathology of AD in the brain (21). Besides Aβ, other plasma proteins have been reported to be associated with AD. For example, proteins in the apolipoprotein family, such as apolipoprotein E (ApoE) and apolipoprotein J (ApoJ) or cluterin are expressed in both brain and plasma and bind to Aβ with high affinity. In AD brains, clusterin is found to be associated with amyloid plaques and is expressed by astrocytes and hippocampal neurons (22, 23). In vitro and experimental animal models have demonstrated that clusterin is able to inhibit Aβ aggregation and facilitate its clearance across the blood brain barrier (24, 25). In addition, a recent study shows that increased plasma clusterin concentration was correlated with the development, severity and progression of AD (26). Thus, study of the relationships of Aβ and clusterin in MCI and AD patients may be useful for diagnosis of AD. Melanotransferin (MTf) or p97 have also been reported to be related with AD. Levels of p97 in the serum of AD patients are often higher than those in non-dementia elderly controls or in patients with other dementias (27, 28).

Recent biomarker studies have moved away from the traditional approach of investigating levels of a single or several biomarkers implicated in the pathogenesis of AD, and are instead focusing on profiling of human sera in an attempt to discover novel biomarkers. Proteomics is a powerful tool for biomarker discovery. General methods in proteomic studies typically include protein separation by two-dimensional gel electrophoresis (2-DE), followed by mass spectrometry (MS) or tandem MS and database searches to determine protein identity. Many such studies have constructed panels of proteins for the discrimination of AD from normal cohorts. For example, using 2-DE and tandem MS a panel of 23 protein spots was generated, one that differentiated AD from non-AD with a sensitivity and specificity of 94% and a predictive error rate of 5.9% (29).

The aims of this study are to determine the levels of four protein biomarkers that are present in serum of MCI and AD patients, and to search for novel serum markers by

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the proteomic method. This may then be used as a diagnostic tool for AD in the future. The use of both biological markers and proteomic markers in MCI and AD patients may improve the accuracy of the early diagnosis of AD. An ideal biomarker should reflect the neuropathology of AD and can be used in monitoring the progression of the disease and the efficacy of therapy for AD.

1.1 Objectives

1.2.1 To compare serum protein levels, including $A\beta_{40}$, $A\beta_{42}$, clusterin and p97, between normal cognitive subjects, MCI and AD patients for diagnosis and identification of AD progression.

1.2.2 To investigate serum protein expression profiles in MCI and AD subjects using proteomic methods.

1.3 Literature Review

1.3.1 Prevalence of Alzheimer's disease

Alzheimer's disease, recognized as the most common form of dementia, is a progressive neurodegenerative disorder characterized by memory dysfunction and cognitive impairment (3). The proportion of elderly people with AD is increasing, and the prevalence of AD has been shown to increase exponentially with age, doubling every 5 years after the age of 65 (1). The number of Americans with AD and other dementias will grow each year as the size and proportion of the U.S. population age 65 and older continue to increase. On average, 15% of 65 year-olds have developed AD, whereas approximately 44% of 75 year-olds and 38% of 85 year-olds are affected (30) as shown in **Figure 1.1**.

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The prevalence of AD refers to the proportion of people in the population who have AD at a given point in time. Prevalence and the number of prevalent cases describe the magnitude of the burden of AD on the community and the health care system, but do not provide an estimate of the risk of developing the disease. An estimated 5.2 million Americans of all ages had AD in 2014. This includes an estimated 5 million people age 65 and older, of which 3.2 million were women and 1.8 million

were men (30). Approximately 200,000 individuals under age 65 have younger-onset AD (31), and the number is expected to reach 13.2 million by 2050 (4, 5). In Thailand, estimates of the number of AD patients in 2008 found 10,683 AD cases, including both male AD and female AD at 39.9% and 60.1%, respectively (9).

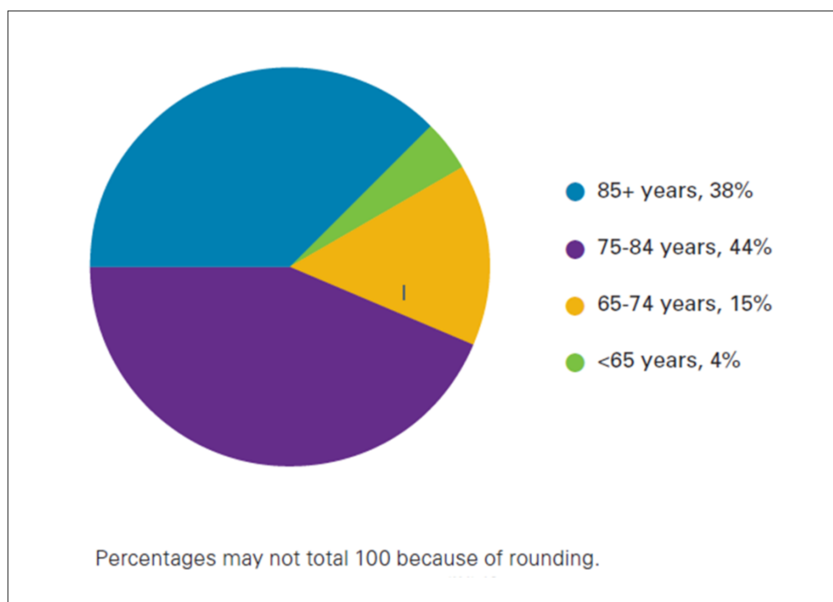

Figure 1.1 Age-specific proportions of people with Alzheimer's disease in the United States (30)

1.3.2 Risk factors for Alzheimer's disease

Alzheimer's disease is most likely a disease with a multifactorial origin, where combinations of genetic and environmental factors lead to disease development. Getting old is the major risk factor for AD and the number of cases markedly increases with advancing age. The prevalence is 10 % among people aged 70, and increases to 50 % at the age of 85 (32). Gender differences exist as well; the ratio of affected women to men is 1.2:1 to 1.5:1 (33). Genetic risk factors for late-onset AD have been identified. The most important type of these is the apolipoprotein e4 (ApoE4) allele. This is a cholesterol-bearing protein which increases the probability of developing AD and decreases the age at onset. The lifetime risk of AD for an individual without the ApoE-4 allele is approximately 10%, whereas the lifetime risk for an individual carrying at least one allele is 30%. Determining the apolipoprotein genotype cannot be regarded as a

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diagnostic test for AD, since some individuals who do not bear the risk allele develop the illness and some who have the allele are spared the disease (34). However, several gene mutations have been suggested to increase risk for AD. These mutation are transmitted as an autosomal dominant in the APP gene (chromosome 21), presenilin 1 gene (chromosome 14) or presenilin 2 gene (chromosome 1), all of which are involved in Aβ production in familial AD (35). Even though AD cases may be considered familial, most do not have an identified genetic cause. There are rare cases of families in which an autosomal dominant inherited point mutation has lead to AD, accounting for approximately 5% to 10% of all AD cases (11). Among possible disease causing environmental factors, head trauma, depression, obesity, high cholesterol levels, diabetes and low educational status have been associated with increased risk of developing AD (36). Conversely, high consumption of fruits, vegetables and omega-3, a moderate coffee intake and a rich social life have been suggested to lower disease risk (37).

1.3.3 Molecular mechanisms of Alzheimer's disease pathogenesis

The exact mechanism of AD pathogenesis is unknown, but it may be linked to the accumulation of two types of insoluble fibrous materials, which are extracellular Aβ peptide deposited in senile plaques and intracellular neurofibrillary tangles (NFTs). A hallmark neuropathological feature of AD is the accumulation of extracellular amyloid plaques. Aβ is a 4 kDa protein fragment which is formed after sequential cleavage of the APP by β- and γ-secretases, resulting in Aβ peptides of 38-43 amino acids (38). The peptide has the ability to aggregate and build up soluble oligomers, which can proceed to insoluble fibrils that are the main component in senile plaques. $A\beta_{42}$ is the species most prone to aggregate and is the main component in amyloid plaques, whereas $A\beta_{40}$ is the main form of Aβ deposited in cerebral blood vessels (39, 40).

A second neuropathological hallmark of AD is the accumulation of intracellular NTFs composed primarily of tau protein. The tau protein is a microtubule-associated protein (MAPs) located in the neuronal axons binding to tubulin in the microtubules, thereby promoting axonal transport, microtubule assembly and stability (41). Tau

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expression is high in non-myelinated cortical axons, especially in the regions of the brain that are involved in memory consolidation, such as the limbic cortex (42). The hyperphosphorylation of tau causes disassembly of microtubules, thereby disrupting axonal transport. Furthermore, hyperphosphorylated tau becomes more prone to aggregate into insoluble fibrils, which can form larger aggregates and become NFTs (43). The loss of microtubule stabilization and NFT formation leads to neuronal and synaptic dysfunction. However, whether hyperphosphorylation and aggregation of tau is a cause or a consequence of AD pathology is still unknown.

1.3.3.1 APP processing leads to formation of Aβ peptides

Amyloid precursor protein (APP) is a trans-membrane glycoprotein that is ubiquitously expressed and encoded by a gene located on chromosome 21 (39, 44). The physiological role for APP remains unclear. A characteristic feature of APP is its proteolytic cleavage by secretases (**Figure 1.2**) (45). The differential action of these secretases leads either to a non-amyloidogenic or an amyloidogenic pathway.

In the non-amyloidogenic pathway, APP is cleaved by α-secretase in the location between amino acids 16 and 17 within the Aβ region. That produces a soluble, secreted αAPP (αAPPs) and the 83 amino acid C-terminal fragment (C83). The C83 fragment is retained in the membrane. Then it is subsequently cleaved by γ-secretase, which produces a fragment called p3 and the APP intracellular C-terminal domain (AICD) (46, 47). Importantly, since cleavage by α-secretase occurs within the Aβ regions, formation of Aβ is precluded. AICD can form multi-protein complexes, which are transported to the nucleus and may be involved in intracellular signaling events and gene transcription (48). Although it was initially thought that p3 is non-toxic, recent studies have shown that this fragment interacts with the membrane and forms Zn-dependent channels (49).

The amyloidogenic pathway, on the other hand, leads to Aβ generation. The initial cleavage by β-secretase (BACE) at residues 671 and 672 of APP, results in the release of soluble βAPP (βAPPs) into the extracellular space. The 99 amino acid C- terminal fragment (C99) that remains bound to the membrane is cleaved by γ-secretese to generate AICD and Aβ fragment (50, 51). The variability in the cleavage site of

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γ-secretase creates several Aβ peptide forms, with lengths varying from 38 to 43 amino acids (38). Among them, $Aβ_{40}$ and $Aβ_{42}$ are the most common forms and are present in amyloid plaques. While $Aβ_{40}$ is much more abundant, $Aβ_{42}$ is much more amyloidogenic, which means it more prone to aggregation and more toxic than $Aβ_{40}$ (40, 52).

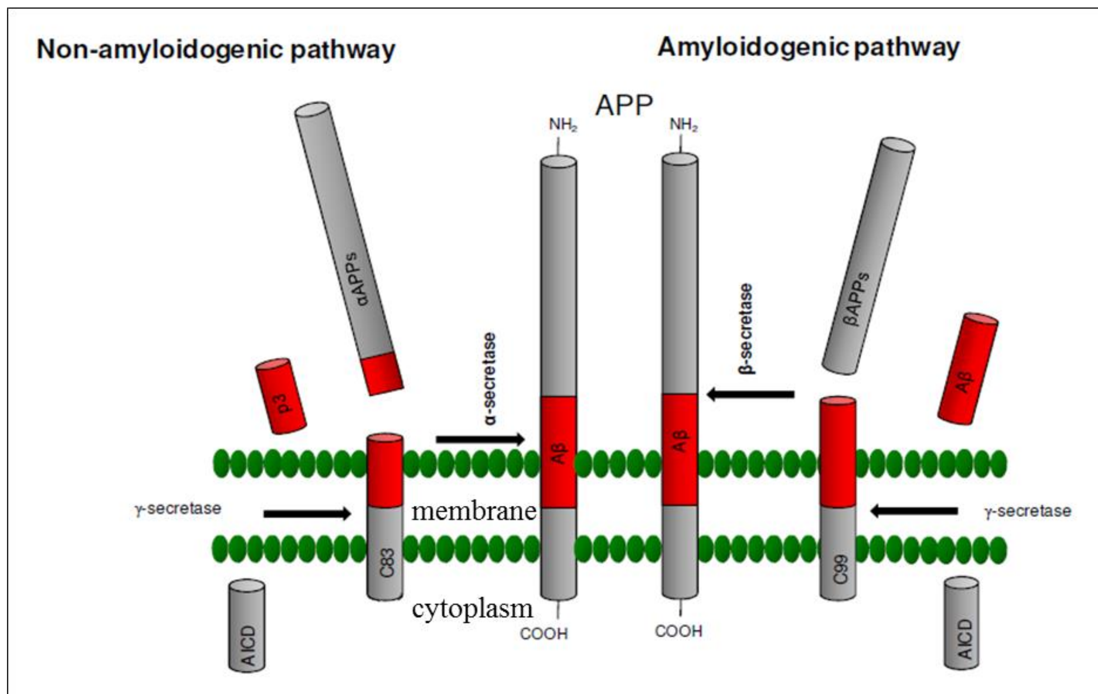

Figure 1.2 APP processing in the non-amyloidogenic (left) and the amyloidogenic (right) pathways. APP is cleaved by α-secretase in the non-amyloidogenic pathway resulting in the C83 peptide, which undergoes cleavage by γ-secretase producing the p3 peptide, AICD and soluble sAPPα fragment. In the amyloidogenic pathway, APP is cleaved by β-secretase resulting in the C99 peptide. Subsequent cleavage by γ-secretase results in the production of Aβ and AICD (47).

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1.3.3.2 Aβ aggregation and amyloid plaques

As previously described, much of the Aβ found in senile plaques is $A\beta_{42}$, which is particularly prone to aggregate (39, 40). Aβ fibrillization is a multistep process. The conformation of Aβ is shifted from mostly α- helical and randomly coiled to a β-sheet structure that is more prone to aggregate (**Figure 1.3**). Many of the plaques found in the limbic and association cortices, but also in the thalamus, caudate nucleus and putamen and cerebellum, show relatively light amorphous Aβ immunoreactivity that occurs without a compact fibrillar center (53, 54). These lesions are referred to as diffuse plaques. Diffuse plaques contain mainly $A\beta_{42}$ species and provide little or no $A\beta_{40}$, neuritic dystrophy, neuroglia cell changes or paired helical filaments (53, 54). It has been suggested that diffuse plaques may represent precursors of the senile plaques (55), which aretoxic to neurons. The body's immune response to remove senile plaques by microglial activation and production of arachidonic acids induces brain tissue inflammation (56). This hypothesis is best demonstrated by studies of individuals with Down's syndrome (who have trisomy of chromosome 21, which encodes APP) who have little or no Aβ deposition in the first decade of life, but by around 12 years begin to display diffuse plaques containing $A\beta_{42}$ and more such plaques during the second and third decade of life. After the age of 30 years, amyloid fibril formation in senile plaques is present with associated microglia, astrocyte and neuritic dystrophy in people with Down's syndrome (57).

1.3.3.3 The amyloid cascade hypothesis

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The amyloid cascade hypothesis proposes that the pathogenesis of AD (both familial and sporadic AD) is triggered by changes in the steady-state levels of the Aβ in the brain (58, 59). There is evidence for three mechanisms of Aβ accumulation: overproduction of Aβ, production of longer forms of Aβ (which are more prone to aggregation) and impaired clearance of Aβ. All these mechanisms result in the increased concentration of the Aβ within the brain, its subsequent deposition into Aβ plaques, triggering tau pathology, synaptic dysfunction and neuronal loss (**Figure 1.4**). It is understood that during the conversion of the Aβ monomers into the Aβ fibrils and Aβ plaques, several Aβ intermediates, such as low and high molecular weight oligomers

and protofibrils form. Initially, the amyloid cascade hypothesis proposed the insoluble Aβ fibrils and plaques as the main toxic peptide moiety. However Aβ fibrils and plaques are not specific to AD and have been observed in older patients free from AD symptoms (60). This finding was explained by showing that Aβ oligomers and protofibrils are more toxic than Aβ fibrils (61, 62), while plaques have been proposed to be inert. Indeed, the formation of plaques has been suggested as a neuroprotective pathway for sequestering toxic oligomers.

The Aβ oligomers have been proven to be neurotoxic by a variety of mechanisms, including permeabilization of the cell membranes, disruption of the cytosolic Ca^{2+} ion homeostasis and formation of reactive oxygen species (ROS) (60, 63). In addition, binding of Aβ oligomers to a variety of proteins, such as mitochondrial alcohol dehydrogenase, causes their dysfunction (64). At synapses, the oligomers interaction with tau protein and several kinases resulting to the hyperphosphorylation of the tau, which affects its role in maintaining axon structure and eventually results in the death of neurons (65).

1.3.3.4 Neurofibrillary tangles and tau protein

Neurofibrillary tangle (NTF) formation is generally believed to be a secondary event preceded by Aβ pathology. The tau protein is associated with microtubules, with three or four microtubule binding repeats. Tau facilitates polymerization of tubulin into microtubules and stabilizes them. When tau becomes hyperphosphorylated, it has low affinity for microtubules, leading to destabilization of microtubules and impaired axonal transport. The phosphorylated tau can aggregate into paired helical filaments and form intracellular NFTs in the entorrhinal cortex, spread to the hippocampus and eventually result in the death of neurons (43).

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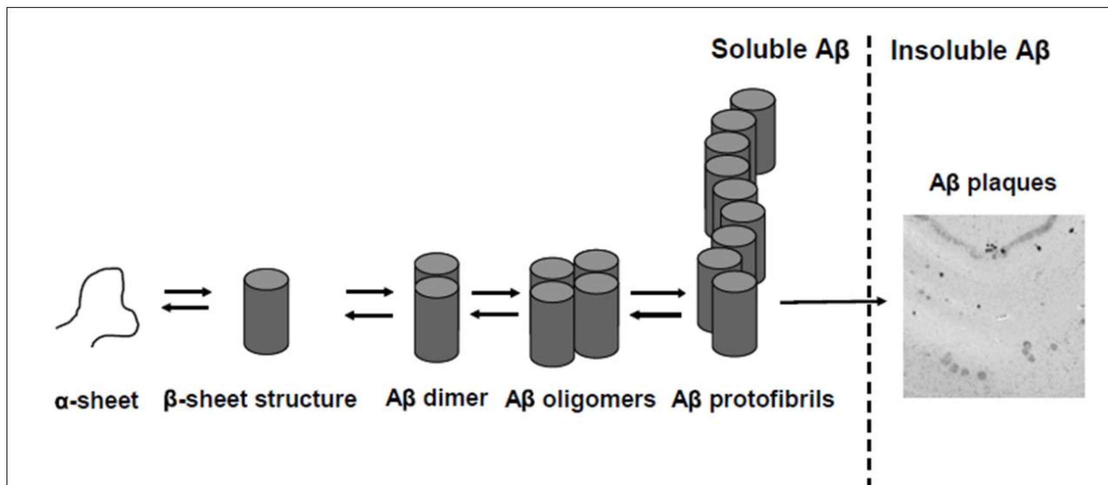

Figure 1.3 Building of Aβ fibrillization. Aβ fibrillization is a multistep reaction in which the conformation of Aβ which shifted from α-helical to a β-sheet structure, which is more prone to aggregate (53).

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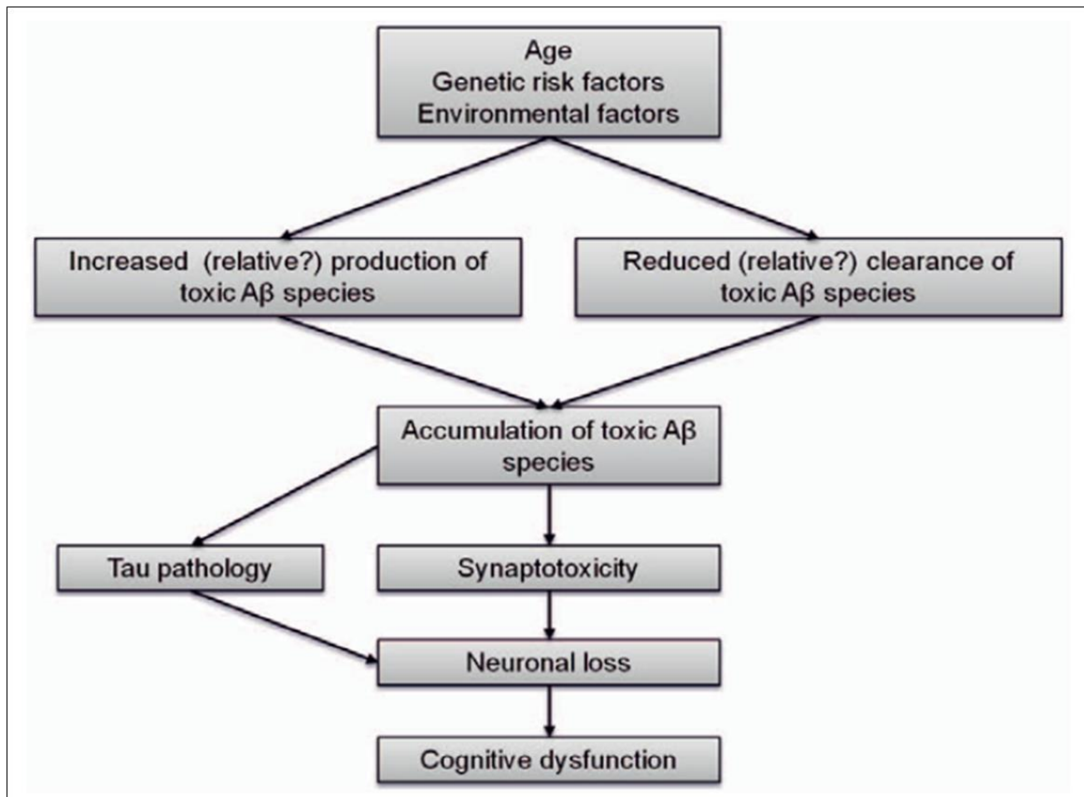

Figure 1.4 The amyloid cascade hypothesis. Possible relations between risk factors, Aβ pathology, tau pathology and cognitive dysfunction. The hypothesis proposes that there is a primary imbalance between Aβ production and decreased Aβ clearance, leading to inflammation and oxidative stress caused by the aggregation and depositing of Aβ. These processes combine to impair neuronal and synaptic function with resulting neurotransmitter deficits and cognitive symptoms. Tau pathology with tangle formation is regarded as a downstream event, but could contribute to cognitive dysfunction (58).

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1.3.4 Diagnosis of Alzheimer's disease

The diagnosis of AD in general practice is based on a careful medical history, usually extended by a caregiver; a battery of neuropsychological tests, and a thorough clinical examination of symptoms according to diagnostic criteria. Brain imaging techniques and determination of AD biomarkers in CSF are used as a complement to support the diagnosis of AD.

1.3.4.1 Clinical diagnosis

There are several guidelines for the clinical diagnosis of AD. The National Institute of Neurological Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) and the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) criteria have been most widely used in research, as they are well validated and provide high diagnostic accuracy. A comparison of the NINCDS-ADRDA and the DSM-IV criteria for AD (11, 12) is presented in Table 1.1.

The NINCDS-ADRDA criteria divide AD into three categories with increasing reliability of the diagnosis, including possible AD, probable AD and definite AD. The patient has probable AD when dementia is characterized by gradual onset and progression, when deficits are documented by examination and testing in two or more cognitive areas, and when other disorders that could cause dementia are absent. The onset should be between the ages of 40 to 90 years, and no disturbances of consciousness should be present. The probable AD diagnosis is strengthened by a positive family history of dementia, normal findings in routine CSF analysis, atrophy in brain imaging, impaired activities in daily life and a change in behavior. Histopathological changes in autopsy or biopsy of a probable AD patient confirm the diagnosis of definite AD. Possible AD is diagnosed when the patient has another potentially dementing disorder that is not considered to be the primary cause of dementia, or when the patient has variations in the presentation of dementia. The accuracy of the clinical diagnosis of AD using NINCDS-ADRDA criteria is over 80% (66, 67). In these studies, the sensitivity has been better than the specificity, and the

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follow-up of patients has improved the diagnostic accuracy. Disorders that may overlap with AD include depression, normal pressure hydrocephalus, dementia with Lewy bodies, vascular dementia, and frontotemporal dementias (11).

The DSM-IV offers a stepwise diagnosis of AD. The initial diagnosis is memory loss, followed by the occurrence of at least one other cognitive deficit, such as aphasia, apraxia, agnosia, or a disturbance in executive functioning. As a result of any one of these deficits, either occupational or social performance should be impaired at a level that represents a decline from a previous level of functioning (2). Disturbances in executive functioning are also common in AD. The DSM-IV defines executive functioning as the ability to think abstractly, planning, initiate, sequence, monitor, and stop complex behavior (68). An individual with a disturbance in executive functioning often finds situations where new information that needs to be processed is difficult, and will try to avoid such situations. Several oral tests for executive function exist, such as asking the individual to recite the alphabet, count to 10, and state as many animals as possible in one minute. It is also often reported by the individual or the individual's caregivers that they have difficulty with day to day activities such as the ability to work, plan and budget (2). The DSM-IV stresses that other dementia etiologies, such as delirium, vascular dementia, HIV infection, encephalitis, stroke, substance withdrawal, or intoxication, must be ruled out to reach a diagnosis of probable AD provided the history includes gradual onset and continuing decline (2, 68).

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Table 1.1 Comparison of the NINCDS-ADRDA and DSM-IV criteria for AD.

NINCDS-ADRDA	DSM-IV
Probable AD - Deficits in two or more domains of cognition - Progressive decline of memory and other cognitive functions - Preserved consciousness - Onset between ages 40 and 90 - Absence of systemic or any other brain disease that could account for symptoms **Possible AD** - Atypical onset, presentation or clinical course of dementia - Presence of another illness capable of producing dementia **Definite AD** - Clinical criteria for probable AD fulfilled - Tissue diagnosis by autopsy or biopsy	• Insidious onset with progressive decline of cognitive function resulting in an impairment of social or occupational functioning from a previously higher level • Impairment of recent memory in at least one of the following cognitive domains: - Aphasia - Apraxia - Agnosia - Executive functioning (planning, abstracting) • Cognitive deficits are not due to other neurological, psychiatric, toxic, metabolic or systemic diseases • Cognitive deficits do not occur solely in the setting of a delirium

1.3.4.2 Neuropsychological tests

Neuropsychological deficits in AD include changes in a variety of cognitive functions, such as memory loss, language, executive abilities, attention, and visuoperceptual processes. Neuropsychological examinations may be used to identify cognitive symptoms. The most commonly administered test is the Mini-Mental State Examination (MMSE) score (69). The MMSE is a tool that can be used to systematically and thoroughly assess mental status. It is an 11-question measure that tests five areas of cognitive function: orientation, registration, attention and calculation, recall, and language. The maximum score is 30. A score of 23 or lower is indicative of cognitive impairment. In general, scores of 27 or above (out of 30) are considered normal. However, a score below this does not always mean that a person has dementia. Their

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mental abilities might be impaired for another reason, or they may have a physical problem such as difficulty hearing, which makes it difficult for them to take the test (69-71). On average, people with AD lose 2 to 4 points each year on the MMSE (72). The MMSE takes only 5-10 minutes to administer and is therefore practical to use repeatedly and routinely. As a screening test for dementia, the sensitivity of the MMSE has ranged from 56-90 % and its specificity has varied from 85 % to 95 % (73).

1.3.4.3 Brain imaging

A variety of imaging strategies support clinical diagnosis of MCI and AD, including brain volumetric measures using magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), positron emission tomography (PET), and computed tomography (CT). Brain imaging techniques are used as a complement to support the diagnosis of AD and are utilized to exclude other disorders that may be causing dementia-like symptoms, such as tumors or malnutrition. The accuracy of the clinical AD diagnosis can be up to ~90 % when compared to neuropathological confirmed AD (74, 75). The medial temporal lobes, especially the hippocampus and entorhinal cortex, are among the areas involved in AD (76). MRI has shown decreased hippocampal and entorhinal cortex volume in patients with AD compared with age-matched control subjects (77). The F-18 fluorodeoxyglucose (F-18 FDG) PET scans of AD patients demonstrated reduced glucose metabolism in the parietal and superior/posterior temporal regions (78). The SPECT scans also demonstrated temporoparietal hypoperfusion or hypometabolism in patients with AD, as well as posterior cingulate pathology (79). The CT scan provides the physician with information about the density of tissues in the body. This technique is most often used to identify the NTFs and amyloid plaques seen during advanced stages of AD (74). Neuroimaging studies have found some interesting results; however, these approaches are generally expensive, and availability of instrumentation is not widespread.

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1.3.4.4 Biological fluid studies

Biomarkers in body fluids such as CSF, plasma and serum can be utilized to increase the accuracy of diagnosis for cognitive decline and prediction of MCI

progression. In routine clinical practice the analysis of CSF $A\beta_{42}$, total tau protein (T-tau) and phosphorylated tau protein (P-tau) levels in CSF is a well-established test used in dementia assessment. Across a large number of studies, CSF $A\beta_{42}$ concentrations have been consistently and significantly reduced in patients with AD, by approximately 50% when compared with healthy control subjects (16, 80) making CSF $A\beta_{42}$ a neurochemical marker of AD. The sensitivity and specificity of CSF $A\beta_{42}$ for distinguishing between healthy, elderly control subjects and AD patients has been found to range from 80% to 90% (81). Tau protein has been quantified in the CSF of AD under the hypothesis that it is unlikely to be able to bind and stabilize microtubules, possibly leading to axon degeneration, and so the increase in CSF tau would be due to the release of tau from degenerating neurons and its subsequent diffusion into the CSF (82). Using both CSF P-tau, and $A\beta_{42}$ could increase the sensitivity and specificity of differentiating AD from other forms of dementia. Blood (plasma or serum) contains proteins produced in the periphery that may affect brain processes, but blood also contains proteins/peptides exported from the brain. Blood biomarkers for AD would be ideal because of the ease and non-invasive process of sample collection. Blood biomarkers have been identified as indicators of dysregulation of Aβ metabolism and senile plaque formation, for example plasma $A\beta_{40}$ and plasma $A\beta_{42}$ (83).

1.3.5 Mild cognitive impairment (MCI)

Mild cognitive impairment (MCI) is defined as a stage of cognitive decline intermediate between normal health and dementia. MCI is classified as a cognitive state in which there is evidence of memory impairment, but the symptoms do not fulfill the criteria of dementia. MCI patients may progress to AD, vascular disease and other kinds of dementia, or may remain stable, and sometimes they revert to normal cognitive function (84). There are two broad subtypes of MCI: amnestic and non-amnestic MCI. Amnestic MCI, the more common of two, is clinically significant memory impairment that does not meet the criteria for dementia. Patients and their close contacts initially notice increasing forgetfulness. However, other cognitive abilities, such as executive function, use of language and visuospatial skills, are less impaired or intact (85). Non-amnestic MCI is characterized by a subtle decline in functions not related to

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memory, affecting attention, use of language, or visuospatial skills and is less common than the amnestic MCI. Nonamnestic MCI may also be the pre-clinical stage of dementias unrelated to AD, such as frontotemporal lobar degeneration or dementia with Lewy bodies (86). In clinical trials involving patients with amnestic MCI, more than 90% of those with progression to dementia had clinical signs of AD (87).

Additionally, one longitudinal study showed that people with MCI were 6.7 times more likely to develop AD than cognitively normal individuals (88). According to varied studies, the rates of annual conversion from MCI to dementia range from 2.7% to 10-15% (89). In one study, 56% of MCI patients proceeded to dementia during a follow up period of 4 years (90). However, not all subjects with MCI progress to dementia. There is currently a great need for diagnostic tools for the identification of people with MCI who will subsequently develop dementia caused by AD pathology (91). The diagnosis of MCI requires considerable clinical judgment, including clinical observation, neuroimaging, blood tests and neuropsychological testing in order to rule out an alternate diagnosis. The most commonly used instrument available is the MMSE score. Subjects with MCI often score in the 26 to 28 range on the MMSE, which is typically reported as cognitively normal (92). Another diagnostic tool for MCI is the Clinical Dementia Rating (CDR), with a score of 0.5 as a clinical criterion for MCI. The main outcome of these patients is AD, but also vascular dementia and dementia with Lewy bodies may develop. In many studies, this criterion for MCI is divided into domain specific subgroups, depending on what cognitive domain is affected, for example amnestic or executive subtype. The problem with this definition is that the groups of patients with a CDR score of 0.5 include both patients with MCI and patients with very mild dementia (93).

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1.3.6 Biomarkers of Alzheimer's disease

A biomarker may be defined as a physiological, biochemical or anatomical variable that indicates specific features of disease-related pathological changes. Many studies have reported on potential biomarkers to identify AD patients and subjects with risk to develop AD. Major biomarkers include CSF biomarkers and blood biomarkers (plasma/serum). Briefly, CSF biomarkers include Aβ, T-tau, and P-tau (93). The blood

biomarkers include plasma Aβ, serum Aβ, anti-Aβ antibody, and oxidative stress. Current evidence suggests that Aβ and oligomeric forms of Aβ are potentially the most promising candidate biomarkers. Importantly, an ideal biomarker should reflect the underlying pathology and correlate well with the clinical severity of a disease and should identify AD cases at a very early stage of the disease, before the cognitive symptoms are found in neuropsychological tests. Finally, the ideal biomarker would be measurable in an easily accessible tissue of the patient and inexpensive, with no need for specialty clinics.

1.3.6.1 CSF biomarker

CSF is the fluid surrounding the central nervous system (CNS). CSF is in direct contact with the extracellular space of the brain and may therefore reflect biochemical changes that occur in the latter. For these reasons, CSF is considered the optimal source of AD biomarkers (81, 94). However CSF is a useful resource for research into neurodegenerative diseases; CSF sampling is more complex, particularly in elderly populations, and is expensive. Thus, CSF analysis of AD patients may be unsuitable for routine application.

The most widely accepted CSF biomarkers for AD are CSF $A\beta_{42}$, T-tau and P-tau. The first studies on CSF Aβ as a biomarker for AD gave disappointing results. Studies showed that CSF Aβ levels were decreased (95), increased (96) or not changed at all in AD patients (97). After the discovery that the two main types of Aβ peptides in CSF are $A\beta_{40}$ and $A\beta_{42}$, it was found that CSF $A\beta_{42}$ levels were consistently lower in AD (97). Subsequently it was established that not only CSF $A\beta_{42}$ was lower in AD, but it also could distinguish patients with AD from healthy controls with reasonable accuracy, even in patients with mild AD. The sensitivity and specificity of CSF $A\beta_{42}$ for distinguishing between healthy, elderly control subjects and AD patients has been found to range from 80% to 90% (81, 94). In addition, a recent meta-analysis revealed that MCI subjects have similar reductions in CSF $A\beta_{42}$ concentrations to AD patients. Also, MCI subjects with later conversion to AD have lower CSF $A\beta_{42}$ concentrations at baseline when compared with non-converters (98).

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T-tau found in CSF has been studied for over a decade as a possible biomarker for AD. Studies have shown that CSF T-tau may be significantly elevated in AD patients. However, the sensitivity and specificity levels tend to vary. The sensitivity levels range between 40% and 80%, and specificity varies between 65% and 85 %. There is also a link between T-tau and age related increases in non-demented persons (13).

1.3.6.2 Blood biomarker

Blood biomarkers would be a very convenient method for early AD detection. Although CSF is a good resource for biomarker research in AD, it is limited by the invasive procedure and the requirement of highly trained personnel to draw CSF. Biomarker research in serum and plasma has become more popular. Multiple lines of research suggest a role for many plasma or serum proteins such as Aβ, clusterin and p97 involved in AD pathogenesis.

$A\beta_{40}$ and $A\beta_{42}$

Many researches have focused on verifying Aβ peptides, with the result that $A\beta_{40}$ and $A\beta_{42}$ are effective biomarkers in MCI and AD plasma. In cross-sectional studies, there is conflicting evidence concerning whether plasma Aβ peptide is a useful diagnostic marker. Some early studies did not find that Aβ peptides in plasma differed significantly between AD patients and control groups (99-101). One study showed a significant increase of $A\beta_{40}$ in AD, but with an abundant overlap between AD and control groups (102). Some recent follow up studies have shown that plasma Aβ peptide levels may be related to disease progression. Mayeux *et al*. found that levels of plasma $A\beta_{42}$, but not $A\beta_{40}$, decreased over time in patients with newly acquired AD, and may be associated with mortality in AD patients, whereas plasma $A\beta_{40}$ was stable or increased in prevalent and incident AD cases (103). Plasma $A\beta_{42}$ levels declined with time in a four year longitudinal study, and high baseline $A\beta_{42}$ levels and a decrease during follow-up were associated with decline in MMSE scores (104). One study reported that high concentrations of $A\beta_{40}$ but not $A\beta_{42}$ at baseline were associated with an increased risk of AD, and individuals with increased $A\beta_{42}/A\beta_{40}$ ratio had a decreased risk of AD (105). Consistently low $A\beta_{42}/A\beta_{40}$ plasma ratio was associated with

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increased risk of AD. A possible factor that contributes to the association of low plasma $A\beta_{42}/A\beta_{40}$ ratio and risk of AD is that $A\beta_{42}$ deposits in the brain earlier than $A\beta_{40}$ (106).

In a recent longitudinal study, AD outpatients were followed up for more than four years, and low plasma levels of $A\beta_{40}$, $A\beta_{42}$ and high-sensitivity C-reactive protein were associated with rapid cognitive decline (107). In addition, some studies support high plasma $A\beta_{42}$ level as a risk factor for AD. It has been reported that increased plasma $A\beta_{42}$ predicted the conversion from normal cognition to MCI, but did not predict conversion from normal cognition to AD (108). One study found that subjects with normal cognition having high plasma $A\beta_{42}$ were twice as likely to develop AD as subjects having low plasma $A\beta_{42}$ group, and plasma $A\beta_{42}$ level was elevated in AD patients compared to controls (109). $A\beta_{42}$ level in plasma increases at an average rate of 9% per year in normal elderly controls but declines at an average rate of 12% per year in people with MCI (110). Another study showed that the increased $A\beta_{42}$ level was detected in women with MCI, but not men with MCI (111).

Clusterin

Clusterin, also known as ApoJ, is a ubiquitous multifunctional glycoprotein capable of interacting with a broad spectrum of molecules. The predominant clusterin isoform is a 75 to 80 kDa secretory protein, but smaller nuclear and cytoplasmic versions exist. The exact physiological role of clusterin remains uncertain, but it has been implicated in a variety of processes including apoptosis, lipid transport and complement regulation, and it also functions as a molecular chaperone (112). This diverse set of functions can be attributed to the existence of two alternatively spliced forms of the clusterin gene that encode secretory clusterin (sCLU) or nuclear clusterin (nCLU). The sCLU form seems to be cytoprotective (113) while nCLU migrates to the nucleus on cytotoxic stress to trigger cell death (114). As a chaperone, clusterin is involved in both promotion and prevention of Aβ aggregation, depending on the clusterin to Aβ ratio (112). Clusterin is a stress-induced protein that is increasingly expressed during certain disease states, including AD, and as a response to neuronal injury and degeneration (115, 116). Noteworthy is the fact that clusterin is found in amyloid plaques along with ApoE, and variants within both the *CLU* gene and the *ApoE*

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gene have been identified as susceptibility for AD in genome-wide association studies (117).

Recent investigations independently discovered a pathological role for plasma/serum clusterin levels with regard to AD. In AD, clusterin is present in amyloid plaques and cerebrovascular deposits but is rarely seen in NFTs-containing neurons (117). It has also been shown to play a role in maintaining Aβ soluble in AD (118) and is overexpressed in AD (23). Plasma clusterin was reported to be associated with brain atrophy, baseline disease severity, and rapid clinical progression in AD, suggesting its possible use as a biomarker for AD (26). In addition, association of plasma clusterin with AD has been recently reported by Schrijvers EM *et al.* (119). They screened 60 individuals with prevalent AD, a random sub-cohort of 926 participants, and an additional 156 participants diagnosed with AD during follow up. Among patients with AD, higher clusterin levels were associated with more severe disease. Plasma clusterin levels were not related to the risk of incident AD during total follow up. In 2012, Gemma M *et al*. investigated whether ApoJ levels were significantly altered in AD and MCI subjects as determined by ELISA. Analysis of these preliminary results indicated differences in plasma ApoJ levels between the three groups. Compared to controls, plasma ApoJ was increased by 14% in the AD group (p=0.002) and by 18% in the MCI group (p< 0.001) (120).

Melaotranferrin (p97)

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Melanotransferrin, also known as p97, is localized in capillary endothelial cells of the human brain. In brain tissue derived from AD patients, p97 was detected in a subset of reactive microglia associated with senile plaques, indicating that the iron uptake through this alternative pathway plays a role in AD (121, 122). Kennard *et al*. have demonstrated that p97 concentrations (mean ± standard deviation) are consistently elevated in the serum of AD patients (43.8 ± 11.6 ng/ml; n =17), compared with controls (7.0 ± 3.3 ng/ml; n =15) (123). There was no overlap between the groups, and the correlation between age and p97 serum concentration was not significant. However, a significant correlation was found between disease progression and increased p97

serum concentrations. Extrapolation of these data suggests that the p97 concentration may begin to increase about two years before the first clinical symptoms of AD. Quantitation of p97 in serum is a promising candidate as a biomarker for AD (123). In 2001, Kim DK and colleagues measured serum p97 concentrations in 211 subjects (71 patients with AD, 56 patients with non-AD-type dementia, and 84 normal control subjects). They showed that serum p97 concentrations were elevated 3-to 4-fold in AD as compared to non-AD dementia and normal. This elevation was significant at 13.54 ± 3.72 pg/μl, even in the 38 subjects with mild AD. These results further support the significance of high serum p97 levels in AD and its potential utility as a biological marker in AD (27).

1.3.7 Detection and measurement of proteomics based biomarker

Proteomics has been used extensively to discover new biomarkers for AD both in CSF as well as in blood. The core components of any proteomic method are effective separation of proteins from a complex mixture, the use of dedicated software applications to detect changes in the expression profile of specific proteins between patients and controls, and finally the identification of the proteins of interest.

1.3.7.1 Proteomic based plasma biomarkers

Identification of plasma biomarkers associated with AD from 10 pathologically diagnosed AD patients and 10 non-dementia control subjects has been analyzed by a combination of 2-DE and liquid chromatography tandem mass spectrometry (LC/MS/MS). Liao and colleagues detected over 900 spots from silver stained 2-DE gel images of plasma, and identified six potential plasma biomarkers: α-1-antitrypsin (AAT), vitamin D binding protein (VDBP), inter-α-trypsin inhibitor family heavy chain-related protein, Apo J precursor, cAMP-dependent protein kinase catalytic subunit α1, and orf (124). Some of these molecules are known to play important roles in CNS microglia activation, while others are involved in actin metabolism and fibrinolysis in the periphery (124). Elevated plasma AAT and Apo J levels in AD patients were further validated by ELISA, which showed that the plasma levels of AAT in AD were higher than those of controls, confirming the 2-DE findings. However, no

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difference in total Apo J concentration was observed between the AD and non-dementia groups. AAT has been shown to be present in NTFs and senile plaques (125), and as a serine proteinase inhibitor participates in the control of proteinases during inflammation, coagulation and fibrinolysis (126). AAT has also been reported at elevated levels in AD proteomics studies of CSF (127).

Excluding albumin and immunoglobulin fragments, Hye and colleagues identified 11 proteins in plasma which were significantly different between AD patients and age-matched controls subjects (19). They used image analysis of all identified proteins on 2-DE as predictors to differentiate disease cases and control subjects, achieving 56% sensitivity and 80% specificity. The Western blot techniques further confirmed elevated levels of complement factor H (CFH) and α-2-macroglobulin (A2M) in the plasma of AD patients. CFH and A2M have also been found in amyloid plaques in AD (128, 129). Another two novel biomarkers were recently reported in proteomics experiments. It was noted that serpin F1 (pigment epithelium-derived factor) and complement C1 inhibitor are down-regulated in plasma from AD patients, and these observations were confirmed by specific assays (130).

1.3.7.2 Proteomic based serum biomarkers

Proteomics methods have been used to mine biomarkers in human serum. Liu and colleagues collected serum from 10 AD patients and 10 sex and age-matched control subjects. They selected 9 differentially expressed spots on 2-DE gels for matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis, and found low serum levels of apolipoprotein A 1 (ApoA1) in AD patients (131). In this study, polymorphism of the ApoA1 gene was not associated with the occurrence of AD. ApoA1 is the major constituent of high-density lipoprotein, has an important role in cholesterol transportation out of cells and in the maintenance of lipid homeostasis, and was shown to reduce aggregation of Aβ via lipid homeostasis (132). However, apolipoproteins are difficult to detect in prefractionation proteomics studies, which may be due to the tendency of lipoproteins to adhere to plastic vials resulting in loss during sample transfer (133). By using a combination of multi-dimensional liquid

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chromatography (LC) and gel electrophoresis coupled to MALDI quadrupole/ time-of-flight (QqTOF) MS and ion trap LC/MS/MS, haptoglobin, haemoglobin, vitronectin, apolipoprotein B100, fragment of factor H, and histidine-rich glycoprotein were found to be elevated in the serum of AD patients compared with controls (134). A recent study used a similar method, identifying four monoisotopic peaks as potential serum markers that discriminate AD from Parkinson's disease and control subjects (135). However, a limitation of this type of analysis is the lack of sequence information, which precludes identification of the proteins from which these peptide peaks were derived.

1.3.7.3 Proteomic based biomarkers of MCI to AD progression

Proteomic analysis is also used to study the progression from MCI to AD. In one study, 17 potential protein biomarkers were differentially expressed in the CSF of MCI patients who progressed to AD (136). Five up-regulated proteins in this group were associated with AD neuropathology, including C3a anaphylatoxin des-Arg, C4a anaphylatoxin des-Arg, phosphorylated osteopontin C-terminal fragment, ubiquitin and β2-microglobulin (136). C3a anaphylatoxin des-Arg and C4a anaphylatoxin des-Arg are related to inflammation (137). Ubiquitin is implicated in protein degradation in brain, as well as in the formation of senile plaques and NTFs (138). Biomarkers which reflect longitudinal changes in disease may increase confidence in their diagnostic efficacy, but currently there is no published data relating to proteome based plasma biomarkers and the likelihood of progression from MCI to AD. Longitudinal studies utilizing discovery based approaches are therefore critically needed.

1.3.8 Proteomic technologies

Proteomics analysis is a powerful method for studying complex protein mixtures in microorganisms, tissue cells, body fluids and other biological samples. Large scale increase in diversity of proteins is speculated to be due to alternative splicing and post-translational modification of proteins. Gene expression alone is not enough to fully characterize the diversity of proteins. On the other hand, proteomics is a very useful tool for characterizing cells and proteins of interest. Proteomics helps to determine the

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structure of proteins; it helps to determine modifications, localizations, and protein-protein interactions in addition to protein expression levels. Proteomics relies greatly on 2-DE for separation of proteins and MS to identify the proteins of interest. 2-DE is actually a combination of two different types of protein separations. In the first, the proteins are dissolved on the basis of isoelectric point (p*I*) by isoelectric focusing (IEF). In the second phase, the focal proteins then are further resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), separating proteins according to their molecular weights (139) (**Figure 1.5**).

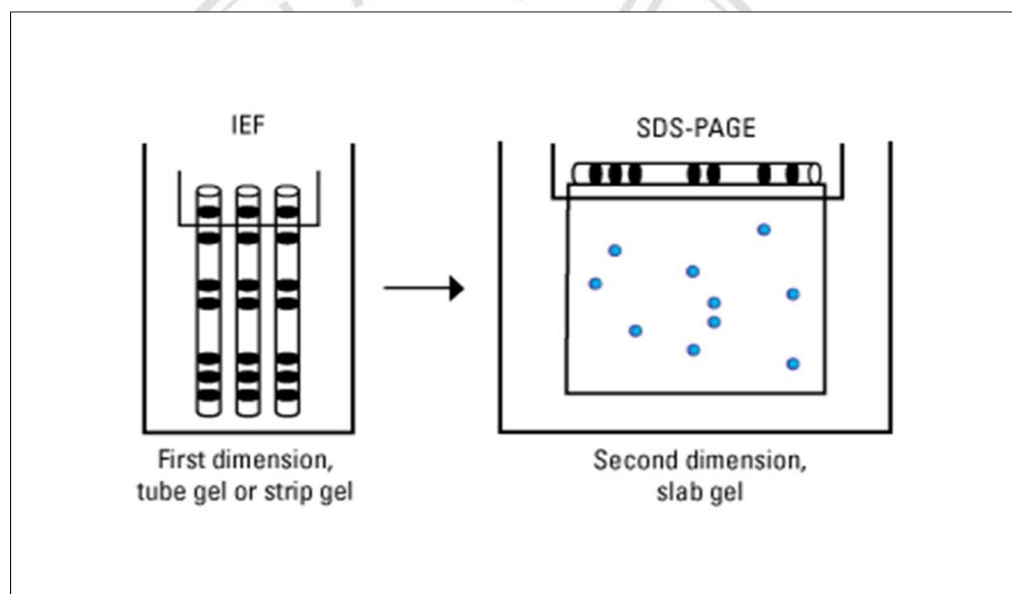

Figure 1.5 Two dimensional gel electrophoresis. In the first dimension (left), one or more samples are resolved by IEF, which separates proteins according to p*I* in separate tube or strip gels. In the second dimension (right), proteins are separated according to their approximate molecular weight using SDS-PAGE (140).

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1.3.8.1 Two-dimensional gel electrophoresis (2-DE)

A. **Sample preparation**

An appropriate sample preparation is the key to obtaining good results from 2-DE. The protein composition of samples will be displayed in the pattern on a 2-DE gel without losses or modifications. Thus it is important not to contaminate the sample with other proteins or peptides. Currently, it is important to define the kind of a proteome population intended to be studied, such as the proteome of one particular type of cell, a subcellular organelle, a cytosolic or a biological fluid. Understandably but unfortunately, there is no single powerful method for sample preparation that can be applied to all kinds of samples to be analyzed by 2-DE, since the chemical nature of proteins is extremely diverse in different tissues and localizations. The three fundamental steps in sample preparation are cell disruption, protein inactivation plus removal of interfering substances, and solubilization of proteins of choice. Additionally, in some cases, proteins need to be enriched or prefractionated. It is desirable that the sample preparation should be as simple and reproducible as possible (141).

B. **First dimension: Isoelectric focusing (IEF)**

Isoelectric focusing is the method to separate the protein samples according to their p*I* in a pH gradient generated by carrier ampholytes. Proteins are amphoteric molecules with acidic and basic buffering groups and carry either positive, negative or zero net charge depending on the environmental pH. The net charge of a protein is the sum of all the negative and positive charges of the amino acid side chains. The p*I* is the specific pH at which the net charge of protein is zero. Proteins present the positive charge at pH below their p*I* and negative charge at pH above their p*I*. Proteins with positive charge move toward the cathode, while, proteins with negative charge move toward the anode to a pH where proteins have no net charge (142).

In this step, proteins are mixed with rehydration buffer, which allows more dilute samples to be loaded and also allows large quantities of samples to be loaded and separated by preventing evaporation of proteins. The samples are placed on an immobilized proteins gradient (IPG) strip that is composed of a polyacrylamide matrix.

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The rehydration buffer consists of urea (chaotropic agent that denatures proteins), thiourea (denatures proteins), 3-(3-cholamidopropyl) dimethyammonio-1-propane sulfonate (CHAPS) (chaotropic agent that denatures proteins), dithiothreitol (DTT) (reduces disulfide bonds), bromopheonol (tracking dye) and ampholyte (helps to move protein based on their p*I*) (143). Thus the proteins are separated based on their p*I* in the IEF cells when electricity is applied.

C. **Equilibration**

Prior to the second dimension run, the IPG strips are equilibrated with a solution containing DTT, iodocetamide (IAA) and SDS. Equilibration is the process of preparing proteins for the second dimension. Proteins should be completely unfolded and carry negative charges. There are two steps during the process. DTT is added in order to cleave disulfide bonds between cysteine residues and complete unfolding of proteins. It reduces disulfide linkages to free sulfhydryl groups in proteins. To prevent the reforming of disulfide bonds, IAA is used as an alkylating agent. It binds covalently with thiol groups of cysteine, converting them to carbamidomethyl cysteine, thus preventing formation of disulfide bonds. Alkylation with IAA results in the covalent addition of a carbamidomethyl group and prevents disulfide bond formation. Proteins that were reduced, alkylated and saturated with SDS are ready for transferring to the second dimension.

D. **Second dimension: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

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In the second dimension, the proteins are separated based on their molecular weight. The strips are then loaded onto the gel. A marker is added for better comparison of migration rates of identified protein to those of known standards and a voltage is applied. High molecular weight proteins travel slower and low molecular weight proteins travel faster, and thus proteins become separated in the second dimension in the SDS-PAGE.

1.3.8.2 Visualization method

The detection method for 2-DE gel should be highly sensitive, with a wide linear range for quantitation, be compatible with MS, have low toxicity, be environmentally friendly and affordable. However, there is no method that has all these features together. The detection methods that are used in 2-DE laboratories are the following:

A. **Silver staining**

Silver staining technique is a sensitive non-radioactive method which can detect protein at amounts as low as 0.2 ng. This technique is based upon saturating gels with silver ions, washing the less tightly bound metal ions out of the gel matrix and reducing the protein-bound metal ions from metallic silver (144). There are two main types employed for 2-DE gels, silver nitrate in combination with formaldehyde developer in alkaline carbonate buffer and silver diamine in combination with formaldehyde developer in citrate buffer. The silver nitrate protocol is mostly preferred to the silver diamine protocol, because it needs only 10% of the amount of silver nitrate, and it is less apt to produce a silver mirror on the gel surface. Both methods are quite complex in that multiple steps must be stopped at some arbitrary time points in order to avoid over development; thus automation of the procedure is very helpful. It is important that staining is performed in closed trays to prevent keratin contamination (145).

B. **Coomassie Brilliant Blue (CBB) staining**

Coomassie brilliant blue stain is a steady state method providing good quantitative linearity, is inexpensive, and is easy to use, but has relatively low sensitivity. It is compatible with MS. Coomassie blue R-250 and G-250 dyes are two chemical forms of a disulfonated triphenylmethane compound that is commonly used as the basis of stains for detection of proteins in gel electrophoresis and Bradford-type assay reagents for protein quantitation. The R-250 (red-tinted) form lacks two methyl groups that are present in the G-250 (green-tinted) form, which is also called colloidal Coomassie blue dye.

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In classical Coomassie blue (R-250) staining, gels are saturated with dye that has been dissolved in an aqueous solution containing methanol and acetic acid, followed by destaining in a similar solution without the dye. Some proteins may lose the dye earlier than the background gel when being destained. In colloidal Coomassie blue staining (G-250), which contains alcohol, the presence of ammonium sulfate helps to increase the hydrophobic interaction between proteins and dye. Thus, modern protocols with G colloidal form were introduced for the purpose of avoiding the destaining problem. However, this procedure takes a long times and needs many steps. Coomassie R-250 has a sensitivity of 8-10 ng of protein, and 30-100 ng proteins for Coomassie G-250. Both dyes provide a linear response with protein amount over a 10-30 fold range of concentrations (144, 145).

C. **Fluorescence staining**

Fluorescence staining methods are less sensitive than silver staining, about 2-8 ng are required. But they have very wide linear dynamic ranges, approximately 10^4, and they are compatible with subsequent mass spectrometry analysis. Sypro Ruby is the most sensitive dye; other fluorescence dyes are Nile Red, Sypro Red, and Sypro Orange. Unfortunately, all these dyes are expensive and a fluorescence scanner or CCD camera is required (142, 144).

1.3.8.3 Image analysis

The protein pattern differences between gel images can be very subtle and difficult to detect by eye, therefore digital image analysis is a natural part of this process. Image analysis software also guides the excision of proteins from gels for further analysis, whether this is done manually or automatically with a spot-excision robot. The purposes of image analysis are comparison of 2-DE patterns of treated with non-treated samples, to construct average gels for replicate runs of the same sample, to detect novel, missing or modified proteins, to quantify protein spots, to detect up- or down- regulated proteins, to define spot positions for spot cutting, to detect and characterize protein families and networks, to statistically analyze experimental results,

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to enable database queries, to link the 2-DE data to MS data and to integrate the image data with a laboratory workflow system (145).

1.3.8.4 Mass spectrometry (MS)

Mass spectrometry is a key technique in proteomic analysis, providing accurate mass measurements, according to the mass-to-charge ratio *(*m/z*)*, of small quantities of proteins, peptides and peptide fragments. The latter provides information concerning the amino acid sequence and modifications. Three components are generally present in all mass spectrometers: an ion source, a mass analyzer, and a detector. Sample molecules are introduced into the ion source where they are converted into gas phase ions. The mass analyzer separates the ionized species according to their m/z ratio and the detector records an ion current of the separated analyzes. Results are then plotted in the mass spectra, as the ion current against m/z (145, 146).

The ionization methods currently most suitable for analysis of peptides and proteins are MALDI and electrospray ionization (ESI) since they enable production of intact gaseous ions of large biomolecules.

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