CHAPTER 4

Discussion

Efforts to find reliable biomarkers for AD in peripheral blood have met with little success. Several candidate blood biomarkers have been proposed for establishing the usefulness of diagnosing and predicting AD. However, the findings from these studies have been contradictory and it is unclear whether changes in the peripheral blood reflect pathology within the brain (83, 148,149). In this study, we considered several markers that have been linked to AD, including $A\beta_{40}$, $A\beta_{42}$, clusterin and p97 in serum of AD, MCI, and NS subjects who were grouped based on neurological, physical and MMSE-Thai scores. Moreover, we also attempted to focus on the protein profiling in human serum for discovery of novel biomarkers by proteomic technology.

There are several general issues concerning the use of blood as a clinical sample for searching for biomarkers involved in MCI and AD. The search for protein markers in MCI and AD subjects studies used the plasma or serum fractions of blood. Serum is that part of blood which is similar in composition with plasma, but excludes clotting factors. The main difference between plasma and serum is the absence of fibrinogen, an important clotting factor, in the serum. Fibrinogen is removed by conversion into a fibrin clot together with the platelets. Thus, the decrease in fibrinogen content of plasma in the form of the fibrin clot makes serum protein concentration lower than that of plasma. Other proteins are eliminated by specific or non-specific interactions inside the fibrin clot (150). In contrast, plasma collection tubes contain anticoagulants such as EDTA, heparin, or sodium citrate. Each of these stabilizers can affect the protein composition in plasma, thus possibly impacting the use of plasma samples in proteomics investigations. Currently, there is no consensus on whether serum or plasma should be designated in proteomics for the discovery of biomarkers (151). However, the selection of a suitable sample type for protein investigation in MCI and AD may depend on the aim of the focal study.

4.1 Measurement of serum protein levels by ELISA technique

Plasma $A\beta_{42}$ measurement in AD has resulted in a variety of outcomes. Concordant with previous reports (102, 111), our results demonstrated that significantly higher $A\beta_{42}$ levels in serum were observed in AD and MCI subjects compared to NS subjects. Several studies claimed that the increase of $A\beta_{42}$ in AD could be useful in improving the progression of AD patients (105, 152). It has been reported that subjects with normal cognition having a high-plasma $A\beta_{42}$ level were twice as likely to develop AD as subjects having a low-plasma $A\beta_{42}$ level; and plasma $A\beta_{42}$ levels were elevated in AD patients compared to controls (109). An increase in the plasma $A\beta_{42}$ level could predict the conversion from normal cognition to MCI, but could not predict the conversion from normal cognition to AD (108). In addition, an increased $A\beta_{42}$ level was detected in women, but not in men, with MCI (111). From the mechanism of AD, accumulation of extracellular Aβ plaques in the brain, especially $A\beta_{42}$, is related to AD. CSF is absorbed into the bloodstream every day, and some exchanges of peptides occur. $A\beta_{42}$ may have the ability to pass the blood brain barrier, potentially allowing detection in serum or plasma of AD patients (153, 154), although the exact nature of this mechanism is unclear.

Additionally, the observed mean $A\beta_{42}$ level in MCI and AD subjects was lower in our study when compared with previous studies that used ELISA-based analyses. A potential factor in this variability may lie within the variability of ELISA, such as the avidity and concentration of capture and detection antibodies, incubation time and temperature, sample volume and dilution, enzyme and substrate types, and the quality of the detector. Therefore, quantifying $A\beta_{42}$ peptide levels in blood by ELISA varies between ELISA systems and protocols, which make data comparison difficult (155). Furthermore, presence of N-terminally truncated Aβ species in AD brains may create some problems for ELISA assays with N-terminal capture antibodies (156). One possibility for the broad overlap in serum Aβ measurements is that Aβ is capable of binding to plasma/serum proteins, including albumin (157), α2 macroglobulin (158), and various lipoproteins (159). Binding of $A\beta_{42}$ peptide to human serum protein can cause problems in measuring $A\beta_{42}$ levels and interfere with ELISA quantification (160).

By contrast, many recent results have demonstrated significantly decreased levels of $A\beta_{42}$ in the serum of AD patients compared to that of controls. One study showed that plasma Aβ levels may vary at different stages of AD. Compared with the early stages of AD, $A\beta_{42}$ values were found to be lower in AD subjects in moderate to severe stages of the disease (161). However, our study found that the difference in $A\beta_{42}$ levels between MCI to AD states or between mild to moderate to severe AD states was not significant. Several factors may explain the plasma amyloid results. Although $A\beta_{42}$ can cross the blood-brain barrier, Aβ in plasma and blood does not originate only in the brain. It is also the product of APP metabolism in skeletal muscle, pancreas, kidney, liver, vascular walls, lung, intestine, skin and several glands, and APP can be found in almost all peripheral cells (162). This may be one factor that affects Aβ plasma values. In addition, the effect of dementia treatment and other underlying diseases before AD should be considered.

Plasma concentration of clusterin is associated with changes in patients with AD and MCI. Our current observations provide evidence linking high clusterin levels to AD and MCI, as in previous studies (26). Thambisetty *et al*. (26) reported that high concentrations of plasma clusterin were correlated with the development of entorhinal cortex atrophy, and severity and progression of AD. Recently, high plasma clusterin levels in MCI were found to associate with slow rates of brain atrophy (163). In addition, associations of plasma clusterin with the risk of AD have been recently reported. Schrijvers *et al*. (119) suggested that increased plasma clusterin levels were associated with the prevalence and severity of disease, but not related to the risk of incident AD during follow-up. In addition, another study reported that the increase in plasma clusterin of MCI and AD subjects may occur in response to the disease process and would be predicted to increase binding capacity for Aβ peptides in plasma, enhancing their removal from the brain (164). Several protective effects of clusterin in the brain that may play a role in AD have been described in *in vitro* or *in vivo* studies. The major effect of clusterin on the risk of developing AD is *via* Aβ aggregation and clearance, influencing the onset of Aβ deposition. Clusterin inhibits the formation states of Aβ through interaction with Aβ (112). This complex formation significantly prevents

aggregation and polymerization of soluble Aβ. In addition, the binding to clusterin protects soluble Aβ from proteolytic cleavage (165). Furthermore, clusterin can enhance the clearance of Aβ peptides outside the brain (25). Clusterin is rapidly transported across the blood brain barrier via low-density lipoprotein receptor-related protein-2 (LRP2/megalin), and $A\beta_{42}$ clearance from the brain is significantly enhanced when the $A\beta_{42}$ is complexed with clusterin (25). The neurodegenerative changes that occur in AD may trigger an increased expression of clusterin. This is in line with our finding that plasma/serum clusterin was associated with AD diagnosis. Thus, $A\beta_{42}$ and clusterin in serum were considerably higher in AD and MCI patients than in cognitive normal subjects. This may be an indication of increased risk for developing AD.

In contrast, serum $A\beta_{40}$ levels in AD and MCI were not significantly different compared to NS. Although previous studies have reported elevated plasma $A\beta_{40}$ concentrations in patients with AD (102, 105), some reports suggested that $A\beta_{40}$ levels were not altered in AD and MCI, similar to our study. In a longitudinal study, it appeared that AD patients at baseline and those who developed AD during follow-up had significantly higher plasma $A\beta_{42}$, but not $A\beta_{40}$ levels, and plasma $A\beta_{42}$, but not $A\beta_{40}$ levels, declined over time in newly acquired AD (103). In addition, no difference in plasma $A\beta_{40}$ levels between patients with MCI who later developed AD and patients with stable MCI or healthy subjects has been reported. Currently, less is known concerning $A\beta_{40}$ in association with AD and MCI in plasma or serum than for $A\beta_{42}$. Although the $A\beta_{40}$ peptide is produced during molecular pathogenesis of AD and secreted into CSF, but amyloid plaques of AD contained mainly $A\beta_{42}$ species with little or no $A\beta_{40}$. In addition, $A\beta_{42}$ is more aggregate-prone 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). Therefore, plasma $A\beta_{40}$ level was not an optimal biomarker for AD (166).

Serum p97 is a secreted protein which is expressed in amyloid plaques and is associated with reactive microglia in AD. The p97 interacts with the transferrin receptor in brain capillary endothelium and could cross the blood brain barrier via the transferrin receptor (167). High serum p97 concentrations can be regarded as a substitutive marker

of AD. Kennard *et al*. have demonstrated that p97 concentrations are consistently elevated in the serum of AD patients, compared with controls (123). There was no overlap between the groups, and the correlation between age and p97 serum concentration was not significant. Kim DK *et al*. reported that serum p97 concentrations were elevated three to four fold in AD as compared to non-AD and normal controls (27). These results support the significance of high serum p97 levels in AD and its potential utility as a biological marker for AD. However, we found that p97 levels in serum of AD and MCI patients were not different from those of NS subjects. Two of the limitations of our study were its small sample size and the fact that many participants in AD and MCI groups were not newly diagnosed cases. Thus, the effect of dementia treatment may have affected the level of serum proteins in subjects, including p97.

4.2 Cut-off values of serum biomarkers by ROC curve analysis

To identify the optimal serum $A\beta_{42}$ and serum clusterin concentration for discriminating patients with cognitive impairment and cognitive normal subjects, ROC curve analysis was used in this study. Our results showed that a serum $A\beta_{42}$ level at a cut-off point of 0.49 pg/ml in cognitive impairment patients (AD and MCI) against NS had good sensitivity (84%) with low specificity (50%). However, the accuracy of diagnosis was poor (AUC of 0.685). For clusterin, we defined serum clusterin 80.23 ng/ml as the best cut-off point, which gives the same sensitivity (84%) as $A\beta_{42}$ and higher specificity (75%) than $A\beta_{42}$. Interestingly, the accuracy of diagnosis was good (e AUC of 0.814). Additionally, we attempted to differentiate patients with AD from MCI and NS. The same cut-off points of 0.49 pg/ml for serum $A\beta_{42}$ in AD patients against MCI and NS also had good sensitivity (88%), but low specificity (38.5%). The accuracy of diagnosis was poor (AUC of 0.603). Moreover, the same cut-off points of 80.23 ng/ml for serum clusterin showed 87% sensitivity and 49.2% specificity. The accuracy of diagnosis was poor (AUC of 0.687). In comparison with other studies, Chiu *et al*. (150) identified a cut-off point of 16.1 pg/ml for $A\beta_{42}$ to differentiate control subjects from patients (both AD and MCI) with 85.3% sensitivity and 88.5% specificity and found it a useful biomarker for AD. In addition, another study examined the accuracy of plasma $A\beta_{42}$ for developed AD that gives 86% sensitivity and 50% specificity (168).

Differences between study results may be related to sample size and the variety of ELISA and assay technique platforms available. There are several ELISA systems and protocols that have been used in measurements of Aβ peptide and which make data comparisons difficult. Sampling or timing of the sample collection in relation to the clinical period or stage of disease progression may be related. Furthermore, other data from animal models suggest that time after eating can significantly affect plasma Aβ level. Recent data demonstrated that plasma Aβ in a fed state was significantly higher than that in a fasted state (169). In accordance with our study, several reports have shown significantly increased serum clusterin levels in subjects with MCI and AD (26, 119). However, these studies have not addressed the optimal cut-off value, sensitivity and specificity of serum clusterin for distinguishing between cognitive impairment subjects and cognitive normal subjects, nor did they differentiate patients with AD from MCI and NS. An ideal biomarker should have a sensitivity of greater than 80% in detecting AD and a specificity of greater than 80% for distinguishing from other dementias (170). It is difficult to determine the exact normal range and cut-off points of blood biomarker levels that provide both high sensitivity and specificity. Many variable factors can impact the data, such as sample size, disease stage, race, therapeutic effects and the analytical process.

4.3 Two dimension gel electrophoresis and protein identification

Serum/plasma proteomics has been used to analyze many diseases, including AD. Today, discrimination of AD from controls and from other neurological diseases may be improved by the combination of immunoassays and proteomic methods. In this study, we attempted to focus the protein profiling in human serum between cognitive normal subjects and cognitively impaired (MCI and AD) for discovery of novel protein markers that may be involved in Alzheimer's disease.

In the 2-DE results, many protein spots were matched between cognitive impairment patients (AD and MCI) and the NS group with pH 3-10 and MW 11-245 kDa. Fourteen protein spot differences were matched in the set of MCI when compared with NS (6 spots having lower expression and 8 spots with higher expression when compared with NS) and 5 protein spot differences were observed in terms of

presence/absence and down/up regulated between MCI and NS (**Figure 3.6** and **Table 3.5**). In a comparison of AD and NS, 6 protein spot differences were matched. All of these protein spots showed low expression levels in AD. In addition, 9 protein spots were found only in AD subjects (**Figure 3.7** and **Table 3.6**). Moreover, 6 protein spots in MCI and AD predominated in the same area (lower expression of 3 spots and higher expression of 3 spots when compared with NS) (**Table 3.7**). In order to identify the protein profile related to AD from p*I* and MW range of selected spots of interest, the SWISS-2DPAGE database was used. We have classified the protein spots into 3 groups of interest. Firstly, in the 6 protein spots shared between MCI and AD, within p*I* range of 4-10 and the MW range of 11-35 kDa, 5 protein types were identified (**Table 3.8**). Secondly, 10 isolated spots were observed to be differentially expressed in MCI subjects compared with NS, within a p*I* range of 5-8 and the MW range of 35-48 kDa. Ten proteins were identified from this classification (**Table 3.9**). Finally, 9 specific protein spots that were present only in AD subjects when compared with NS within a p*I* range of 5-8 and the MW range of 25-63 kDa, 12 proteins were identified (**Table 3.10**).

After protein identification using the SWISS-2DPAGE database, we found a total of 22 proteins of interest, within a p*I* range of 5-7 and a MW range of 25-63 kDa, which were expressed in serum of AD and MCI subjects compared with NS. Of these, there were 9 proteins of interest which have been reported to be biomarkers of AD and MCI by proteomic technology. Up- regulated proteins in serum/plasma of AD proteomics studies are shown in **Table 4.1**. In addition, there are also 13 new expressed proteins were identified in serum of AD and MCI subject as shown in **Table 4.2**. However, association of these proteins with MCI and AD should be further studied in more details of mechanism pathogenesis and function to AD. With maybe useful to established a new biomarker for AD diagnosis and prognosis. The criteria of subject's selection such as underlining disease, drug therapy during treatment, development of disease, and onset of disease should be also estimated for actually correct in detection of protein identification by proteomic analysis. In addition, from a number of 22 identified proteins of interest by p*I* and MW (**Table 4.1** and **Table 4.2**), the $A\beta_{40}$, $A\beta_{42}$ and p97, except clusterin were not identified from selected proteins spot of interest in serum of AD and MCI subjects. Due

to $A\beta_{40}$ and $A\beta_{42}$ peptide is low-molecular weight protein which deposits mainly composed of 40- and 42-residue peptides ($A\beta_{40}$ and $A\beta_{42}$, respectively) (14). Detection of $A\beta_{40}$ and $A\beta_{42}$ was below at a lower limit of 2-DE analysis by MW 11-245 kDa. Moreover, effect from sample storage more than 12 months may affect to loss of p97 levels in serum.

To date, several proteins were discovered from proteomic technology both in plasma and serum such as apolipoprotein E (Apo E), apolipoprotein B100 (Apo B100) (171) and apolipoprotein J (Apo J) (124). However, the successful application and development of proteomic technology for AD diagnosis still appears very limited. It depends completely on the understanding of pathology, genetic and molecular mechanisms involved in the disease. Plasma proteomics analysis for potential biomarkers in the diagnosis of AD has been reported by Liao and colleague (124). They identified plasma biomarkers associated with AD from 10 pathologically diagnosed AD patients and 10 non-demented control subjects by using a combination of 2-DE and MS. Six plasma proteins (alpha-1-antitrypsin, vitamin D-binding protein, inter-alpha-trypsin inhibitor family heavy chain-related protein, Apo J precursor, cAMP-dependent protein kinase catalytic subunit alpha 1, and an orf) were different between the AD and non-demented control subjects. Due to alpha-1-antitrypsin and Apo J (clusterin) has been reported to be involved in the amyloid plaque formation in AD. Thus, alpha-1-antitrypsin and Apo J were further validated using either ELISA or Western blotting. The plasma level of alpha-1-antitrypsin in AD was higher than in controls, confirming the 2-DE findings. However, no difference in total Apo J concentration was observed between the AD and non-demented control subjects. (124) Conversely, Thambisetty *et al*. recently found that Apo J /clusterin separated from gel-based proteomic studies by LC-MS-MS in plasma proteins was associated with hippocampal volume of subjects with MCI, AD and those associated with fast AD progression (26). Plasma clusterin was specifically increased in AD subjects and may be associated with severity, pathology, and progression in AD (26). Recently, a combination of multidimensional liquid chromatography (LC) and gel electrophoresis coupled to MALDI-TOF and ion trap LC-tandem MS was used to identify protein

markers in serum of AD patients. Zhang R. reported that more than 36 proteins were affected in AD serum when compared with control serum (171). For example, Apo E, Apo B100, transthyretin, haptoglobin alpha-2chain, serum amyloid P-component and complement C4 were elevated in serum of AD patients (171). In addition, it has been suggested that Apo E can increase the risk of memory impairment in patients with AD or MCI if they have *ApoE* 4 allele defects (34). A separate report demonstrated that oxidized proteins including fibrinogen γ chain precursor protein and alpha-1-antitrypsin precursor protein were observed in plasma samples from both AD and non-AD subjects by MALDI-TOF/MS (172). However, the role of these proteins in pathogenesis of AD is still unknown.

A limitation of this study was identification of proteins from a broad range of p*I* and MW *via* the SWISS-2DPAGE database. In addition, one of the difficulties in performing proteomic analysis of serum is the abundance of proteins such as albumin, IgG, and transferrin which can interfere with low abundance proteins in serum (171). Although albumin and IgG were initially depleted, some remain in the serum. Approximately 85% of the albumin and 70% of the IgG in serum samples were removed. In addition, a larger sample size and 2-DE repeating may be required. Furthermore, confirming of protein identification by MS should be considered in the future for increasing the efficiency of novel biomarker discovery for AD.

Table 4.1 Up-regulated proteins in serum/plasma of AD proteomics studies (173, 174)

Identified Protein	**Function**	**Sample**
Apolipoprotein E	Lipid transport	plasma
Clusterin	Cell death, tumor progression, and neurodegenerative disorders.	plasma
Alpha-1-antitrysin	Proteolysis	plasma
Complement C4	Complement pathway	serum
Transthyretin	Thyroxine transport	serum
Serum amyloid P-component	Immune regulation	plasma
Haptoglobin alpha 2 chain	Inflammation	serum
Vitamin D-binding protein	Inflammation	plasma
Fibrinogen γ chain	Blood coagulation	plasma

Table 4.2 Expression of other proteins of interest in serum of MCI and AD subjects compare with NS by p*I* and MW, from the SWISS-2DPAGE database.

Accession number	Identified Protein	Function
P01008	Antithrombin-III	Blood coagulation
P01019	Angiotensinogen	Regulates blood pressure, fluid balance and electrolyte homeostasis.
P02753	Retinol-binding protein 4	Delivers retinol from the liver stores to the peripheral tissues
P04196	Histidine-rich glycoprotein	Immune complex and pathogen clearance, cell chemotaxis, cell adhesion, angiogenesis, coagulation and fibrinolysis
P05090	Apolipoprotein D	Lipid transport
P05156	Complement factor I	Complement pathway
P06727	Apolipoprotein A-IV	Lipid transport
P60709	Actin, cytoplasmic 1	Cell motility
P68871	Hemoglobin subunit beta	Oxygen transport
P99004	Possible apolipoprotein	-
P99007	Immunoglobulin light chain	Small polypeptide subunit of an antibody
P99008	Immunoglobulin heavy chain gamma (intermediate segment)	Large polypeptide subunit of an antibody (IgG isotype)
P99009	Immunoglobulin heavy chain mu (intermediate segment)	Large polypeptide subunit of an antibody (IgM isotype)