CHAPTER 3

Results

3.1 Sample characteristic

One hundred fifty seven participants were included in this study. A summary of the demographic data is given in **Table 3.1**. According to their clinical assessment, individuals were grouped as AD (mild AD, moderate AD and severe AD), MCI and cognitive normal subjects (NS). Mean age and gender distribution were similar in all groups. In addition, significant decreases in MMSE-Thai score were found in AD and MCI groups compared with the cognitive normal subject group ($p < 0.001$) as shown in **Figure 3.1**

Table 3.1 Demographic characteristic of the subject groups

	Normal Subject	**MCI**	**Mild AD**	**Moderate AD**	**Severe AD**	***p*-value**
Total sample	32	33	30	30	32	-
Sex (M/F)	9/23	12/21	14/26	10/20	11/21	0.169
Age	74.9 ± 7.20	74.1 ± 7.05	76.3 ± 7.98	78.2 ± 8.17	76.4 ± (10.01)	0.633
MMSE-Thai Score	28.90 ± 1.04	25.77 ± 2.47	20.67 ± 2.91	14.72 ± 3.16	8.96 ± 4.03	$P < 0.001$

Note: Values are expressed as mean ± SD, M = male, F = female, $*p < 0.05$ was considered statistically significant

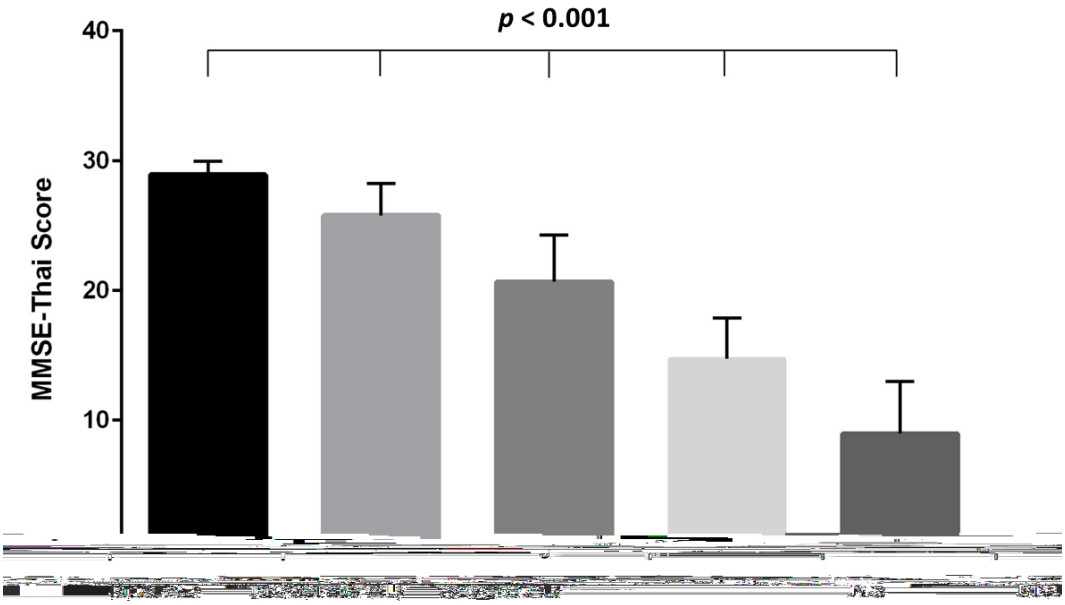

Figure 3.1 Comparison of MMSE-Thai 2002 values in Alzheimer's disease (AD), mild cognitive impairment (MCI) and cognitive normal subject (NS) groups. MMSE-Thai score are expressed as mean ± SD. There was a significant decrease in MMSE-Thai score of AD groups and MCI when compare with NS (*p<0.001*). Differences in means between each diagnostic group were investigated by One-Way ANOVA.

3.2 Measurement of serum protein levels by ELISA technique

Mean ± SD values for serum $A\beta_{40}$, $A\beta_{42}$, clusterin and p97 levels in AD, MCI and NS groups are summarized in **Table 3.2**. The AD patients were divided into three subgroups according to the type of the cognitive impairment, including mild AD, moderate AD and severe AD. There were no significant differences among all five groups in serum $A\beta_{40}$ and p97 levels. The mean serum concentration of $A\beta_{40}$ and p97 did not differ significantly between the MCI, AD patients and NS. On the other hand, there was a significant increase in serum levels of $A\beta_{42}$ ($p < 0.05$) and clusterin ($p < 0.001$) between AD groups and NS and also between MCI and NS. The mean concentration of $A\beta_{42}$ and clusterin in serum was significantly higher in MCI and AD patients than in NS. However, the differences of $A\beta_{42}$ and clusterin levels between MCI-to-AD groups were not significant (**Table 3.2** and **Figure 3.2** and **3.3**).

Table 3.2 Concentration of $A\beta_{40}$, $A\beta_{42}$, clusterin and p97 levels in subject groups

Tests	Normal Subject	MCI	Mild AD	Moderate AD	Severe AD
$A\beta_{40}$ (pg/ml)	14.37 ± 9.37	16.30 ± 14.22	15.10 ± 9.94	20.99 ± 16.09	15.58 ± 11.15
$A\beta_{42}$ (pg/ml)	1.26 ± 1.97	1.91 ± 2.13*	1.97 ± 2.02*	1.30 ± 1.44*	1.59 ± 1.81*
Clusterin (ng/ml)	83.25 ± 24.78	106.61 ±57.24**	121.91 ±70.94**	110.33 ±36.45**	113.73 ±62.93**
p97 (pg/ml)	1002 ± 599.82	995 ± 698.21	1114 ± 792.17	956 ± 720.67	1350 ± 899.87

Note: Values are expressed as mean ± SD, * $p<0.05$, ** $p<0.001$

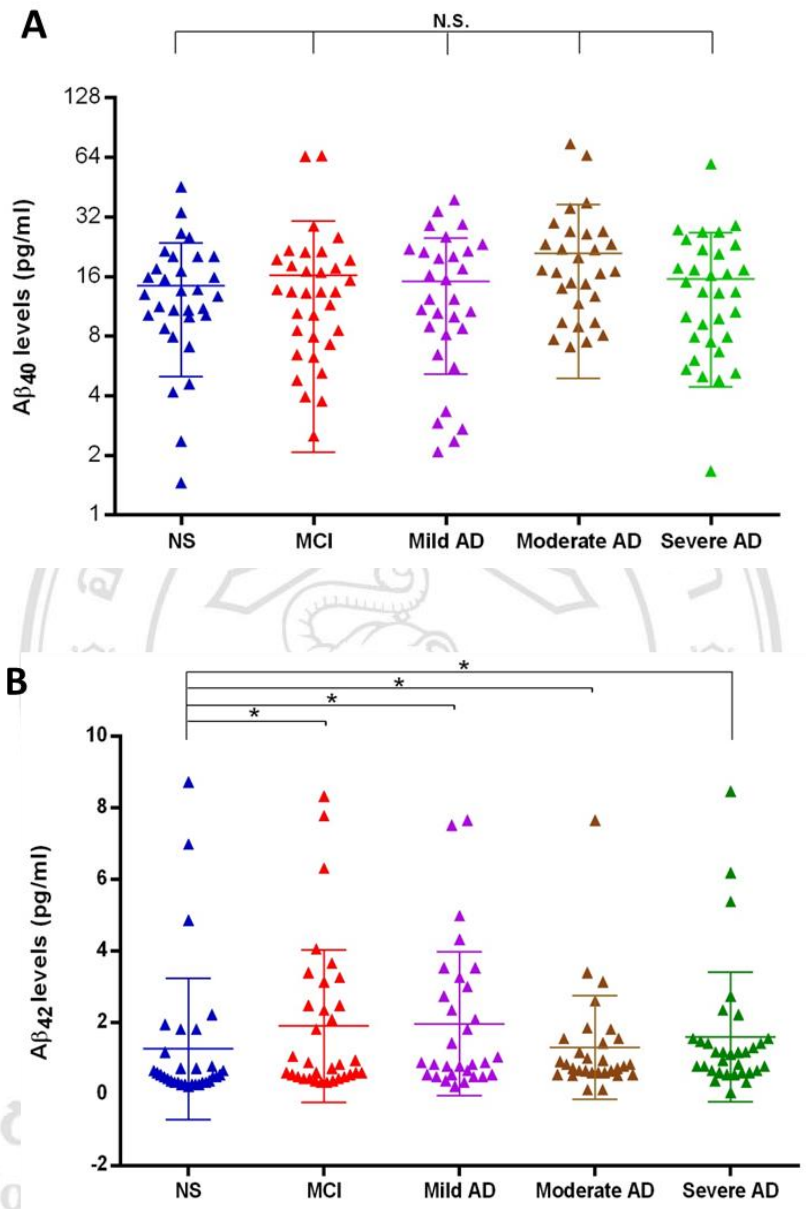

Figure 3.2 Scatter plot comparison of serum $A\beta_{40}$ and $A\beta_{42}$ levels in Alzheimer's disease (AD), mild cognitive impairment (MCI) and cognitive normal subject (NS) groups as measured by ELISA. Horizontal bars represent the mean ± SD values. **(A)** No significant difference in serum $A\beta_{40}$ level was observed in among all five groups. **(B)** Significant difference in high serum $A\beta_{42}$ levels of AD and MCI patients was demonstrated compared to NS ($p < 0.05$).

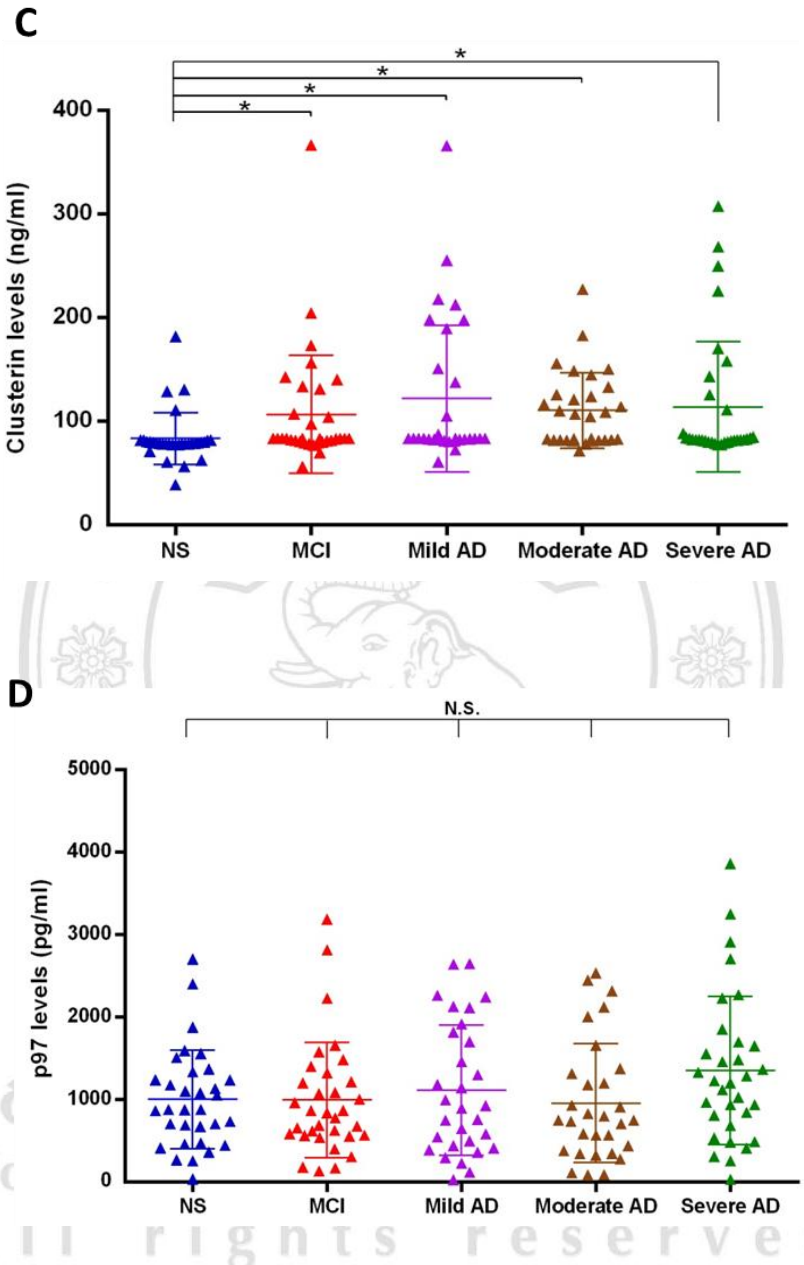

Figure 3.3 Scatter plot comparison of serum clusterin and p97 levels in Alzheimer's disease (AD), mild cognitive impairment (MCI) and cognitive normal subject (NS) groups as measured by ELISA. Horizontal bars represent the mean ± SD values. **(C)** Significant difference in high serum clusterin levels in AD and MCI patients was demonstrated compared to NS (*p<0.001*), **(D)** No significant differences in serum p97 level was observed in among all five groups.

3.3 Cut-off values of serum biomarkers for developing AD

The cut-off values for serum $A\beta_{42}$ and serum clusterin biomarkers for the diagnosis and monitoring the AD progression were determined by using ROC curve analysis. The subjects were divided into two groups, including the different groups of cognitively impaired patients (AD and MCI) versus NS, and AD patients were distinguished from the combination of MCI and NS.

3.3.1 Discriminating patients with cognitive impairment (AD and MCI) and cognitive normal subjects

The best cut-off value was the point on the curve that had the highest sensitivity and specificity. The optimal serum $A\beta_{42}$ and serum clusterin concentrations for discriminating patients with cognitive impairment and cognitive normal subjects were identified using ROC curve analysis. The AUC of serum $A\beta_{42}$ is 0.685 and serum clusterin is 0.814 (**Figure 3.4**). The optimal cut-off value of serum $A\beta_{42}$ was 0.49 pg/ml, which distinguished the AD and MCI patients from NS with a sensitivity of 84 % and a specificity of 50 %, while that of serum clusterin was 80.23 ng/ml with 84% sensitivity and 75% specificity as summarized in **Table 3.3**.

Table 3.3 ROC curve analysis of $A\beta_{42}$ and clusterin in serum for discriminating patients with cognitive impairment (AD and MCI) and cognitive normal subjects

Parameter	Area under the curve	Best cut-off point	Sensitivity %	Specificity %
$A\beta_{42}$	0.685	> 0.49 (pg/ml)	84%	50%
Clusterin	0.814	> 80.23(ng/ml)	84%	75%

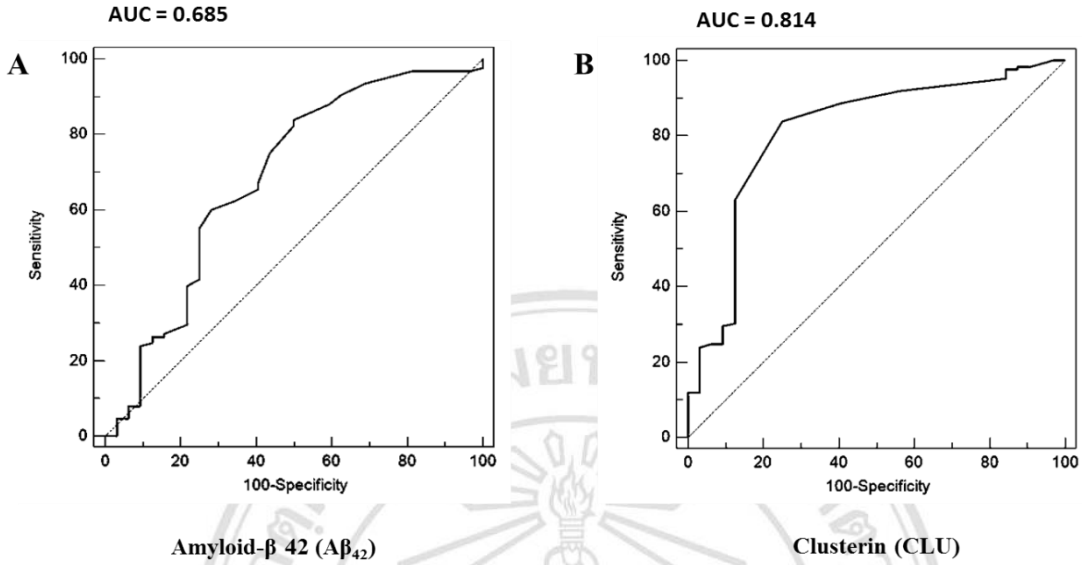

Figure 3.4 ROC curves analysis of $A\beta_{42}$ and clusterin for distinguishing between cognitive impairment subjects (AD and MCI) and cognitive normal subjects. **(A)** ROC curve analysis of serum $A\beta_{42}$. Cut-off point and area under ROC curve (AUC) of serum $A\beta_{42}$ was > 0.49 pg/ml and 0.685, respectively. **(B)** ROC curve analysis of serum clusterin. Cut-off point and AUC of serum clusterin was > 80.23 ng/ml and 0.814, respectively.

3.3.2 Discriminating patients with AD compare to MCI and NS

In addition, we determined the optimal serum $A\beta_{42}$ and serum clusterin concentrations for discriminating patients with AD compare to MCI and NS. The same cut-off point of 0.49 pg/ml for serum $A\beta_{42}$ to differentiate AD patients from MCI patients and NS provided 88% sensitivity and 38.5% specificity, and 80.23 ng/ml for serum clusterin with 87% sensitivity and 49.2% specificity (**Table 3.4**). The AUC of serum $A\beta_{42}$ is 0.603 and serum clusterin is 0.687 as summarized in **Figure 3.5**.

From ROC curve analysis, the diagnostic accuracy of serum $A\beta_{42}$ and serum clusterin were sufficient and very good, respectively. The MCI and AD subjects showed good sensitivity of serum $A\beta_{42}$ and serum clusterin, above 80%, but at this point the specificity is relatively low. Nevertheless, an optimal biomarker for AD and MCI, that has sufficient specificity and sensitivity as a biomarker for individual subject groups, still does not exist.

Table 3.4 ROC curve analysis of $A\beta_{42}$ and clusterin in serum for discriminating patients with AD compare to MCI and NS.

Parameter	**Area under the curve**	**Best cut-off point**	**Sensitivity %**	**Specificity %**
$A\beta_{42}$	0.603	> 0.49 (pg/ml)	88%	38.5%
Clusterin	0.687	> 80.23(ng/ml)	87%	49.2%

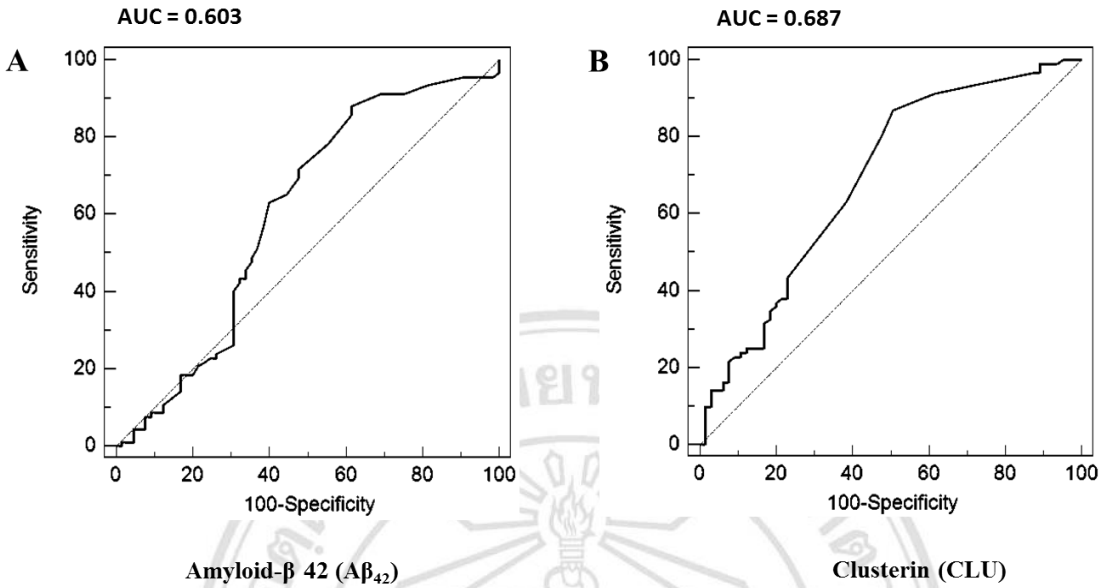

Figure 3.5 ROC curves analysis of $A\beta_{42}$ and clusterin for distinguishing AD from MCI and NS. **(A)** ROC curve analysis of serum $A\beta_{42}$. Cut-off point and area under ROC curve (AUC) of serum $A\beta_{42}$ was > 0.49 pg/ml and 0.603, respectively. **(B)** ROC curve analysis of serum clusterin. Cut-off point and AUC of serum clusterin > 80.23 ng/ml and 0.687, respectively

3.4 Proteomic based serum biomarkers by 2-DE

3.4.1 Detection and Matching of protein spots in 2-DE gel from subjects

The 100 μg of serum protein from subjects including MCI, AD and NS were separated by 2-DE, within the pH range of 3-10 and the MW range from 11-245 kDa. After running electrophoresis, the gels were stained with silver stain for protein visualization. The spot proteins were analyzed using Image Master 2D Platinum software. The detected spot proteins in subjects with MCI and AD were compared with those of the NS group. The volume of a spot was calculated as the volume above the spot outline, which is situated at 75% of the spot height (as measured from the peak of the spot). These protein spots were classified into 4 different types, namely the change of spot volume expression ratios, up-regulated, down-regulated and specific spots in NS, MCI or AD groups.

A comparison of 2-DE gel images from MCI and NS groups is shown in **Figure 3.6**. The numbers of matching 2D patterns in protein spots between MCI and NS groups is shown in **Table 3.5**. There are 14 spots that appeared in the same location both in MCI and NS groups. We found lower expression levels of spot volume in 6 protein spots and higher expression of spot volume in 8 protein spots. In MCI subjects, many of the protein spots in ID 881 were down-regulated and were up-regulated in ID 502. In addition, one protein spot in ID 26 was found in the NS group only, and there were 2 specific protein spots that were found only in the MCI group (ID 57 and ID 70, respectively). The p*I* and MW of protein spots were calculated from mean of p*I* and MW in 2-DE gel images.

A comparison of 2-DE gel images from AD and NS groups is shown in **Figure 3.7**. There were 6 spots that appeared in the same location both in AD and NS groups. The total volume of 6 protein spots from AD subjects was lower than that of the NS group. Moreover, there are 9 protein spots that appeared in only AD subjects, within p*I* range of 5.01-7.18 and an MW range of 24.9-55.7 kDa. The numbers of matching 2D patterns in protein spots between AD and NS groups is shown in **Table 3.6**.

Additionally, the comparison of matched protein spots in MCI and AD with NS groups revealed 6 spots that occurred in the same area, within a p*I* range of 4.62-9.44 and a MW range of 12.0-26.4 kDa. The peaks of spots were decreased in 3 spots (130, 131, and 134) and were increased in 3 spots (124, 125, and 501) between MCI and AD subjects compared with NS subjects. Comparable results of matching similar protein spots between MCI and AD subjects to NS subjects are shown in **Table 3.7**.

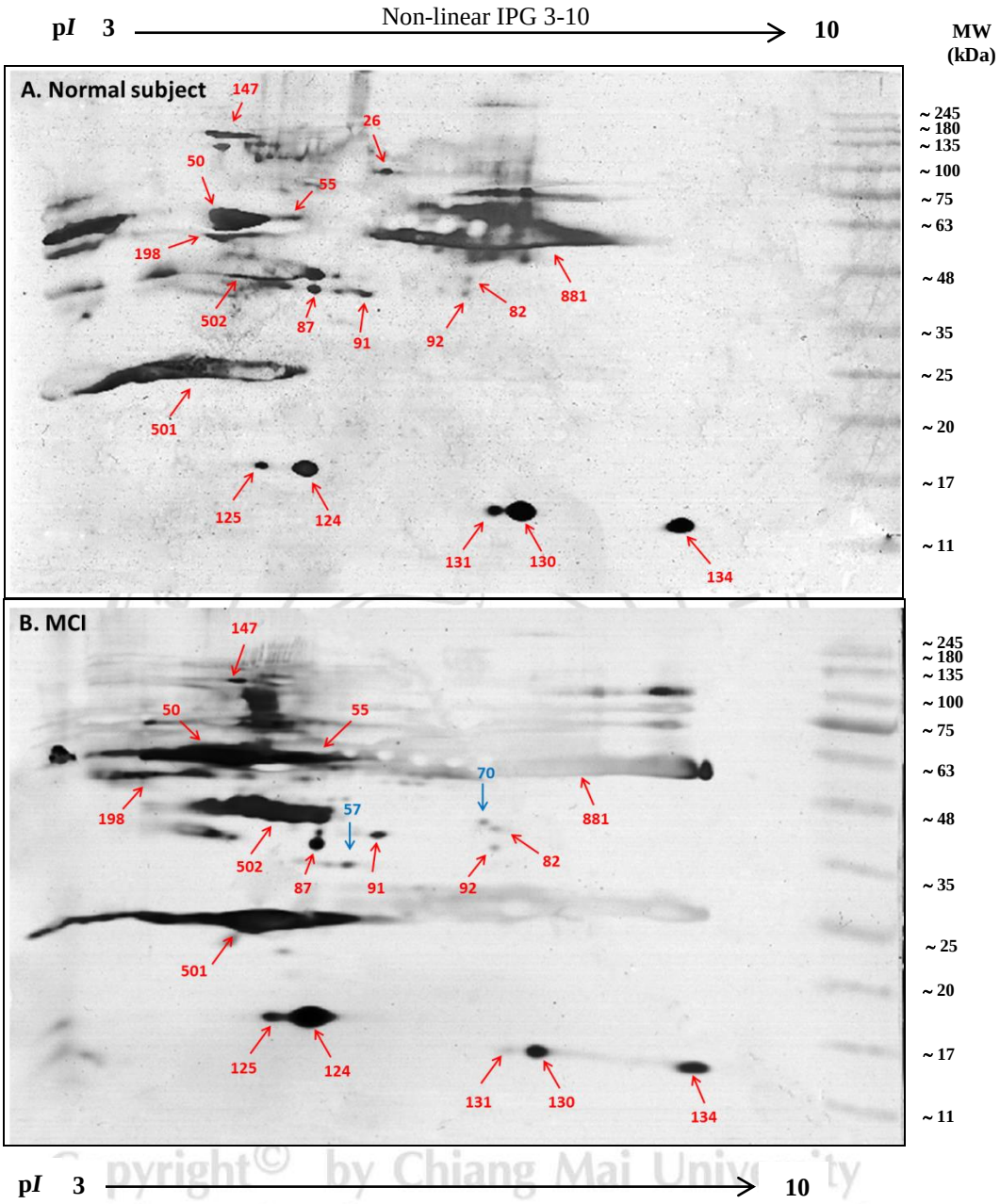

Figure 3.6 Two dimensional gel electrophoresis of serum proteins from cognitive normal subjects (A) compared with MCI subjects (B). The red arrows indicated the protein spots that were found to have different expression between MCI and NS groups. The blue arrows arrows indicated the protein spots that were only present in MCI subjects.

Table 3.5 Protein spot matched from 2-DE image analysis that has different expression levels between MCI and NS groups.

Spot ID	p*I*	MW (kDa)	Expression change of protein spots* (Peak of spots)	
50	4.57	64.8	7.10	⬆
55	5.89	65.0	2.13	⬆
82	7.31	45.2	0.32	⬇
87	5.69	43.7	1.21	⬆
91	6.28	44.4	0.62	⬇
92	7.35	42.9	0.16	⬇
124	5.78	17.1	1.88	⬆
125	5.30	16.2	1.23	⬆
130	7.84	13.3	0.27	⬇
131	7.57	13.8	0.10	⬇
134	9.44	12.0	0.44	⬇
147	4.96	181.7	0.19	⬇
198	4.17	59.5	2.42	⬆
501	5.12	26.4	0.87	⬇
26	6.52	105.5	Absence of spot in MCI	
57	5.97	39.3	Presence of spot in MCI	
70	7.28	46.1	Presence of spot in MCI	
502	5.29	48.9	Up-regulated in MCI	
881	6.15-9.24	64.0-110.7	Down-regulated in MCI	

* Expression change was the ratio of MCI to NS.

The red arrows indicated the protein spots that were more highly expressed in MCI.

The blue arrows indicated the protein spots that were less expressed in MCI.

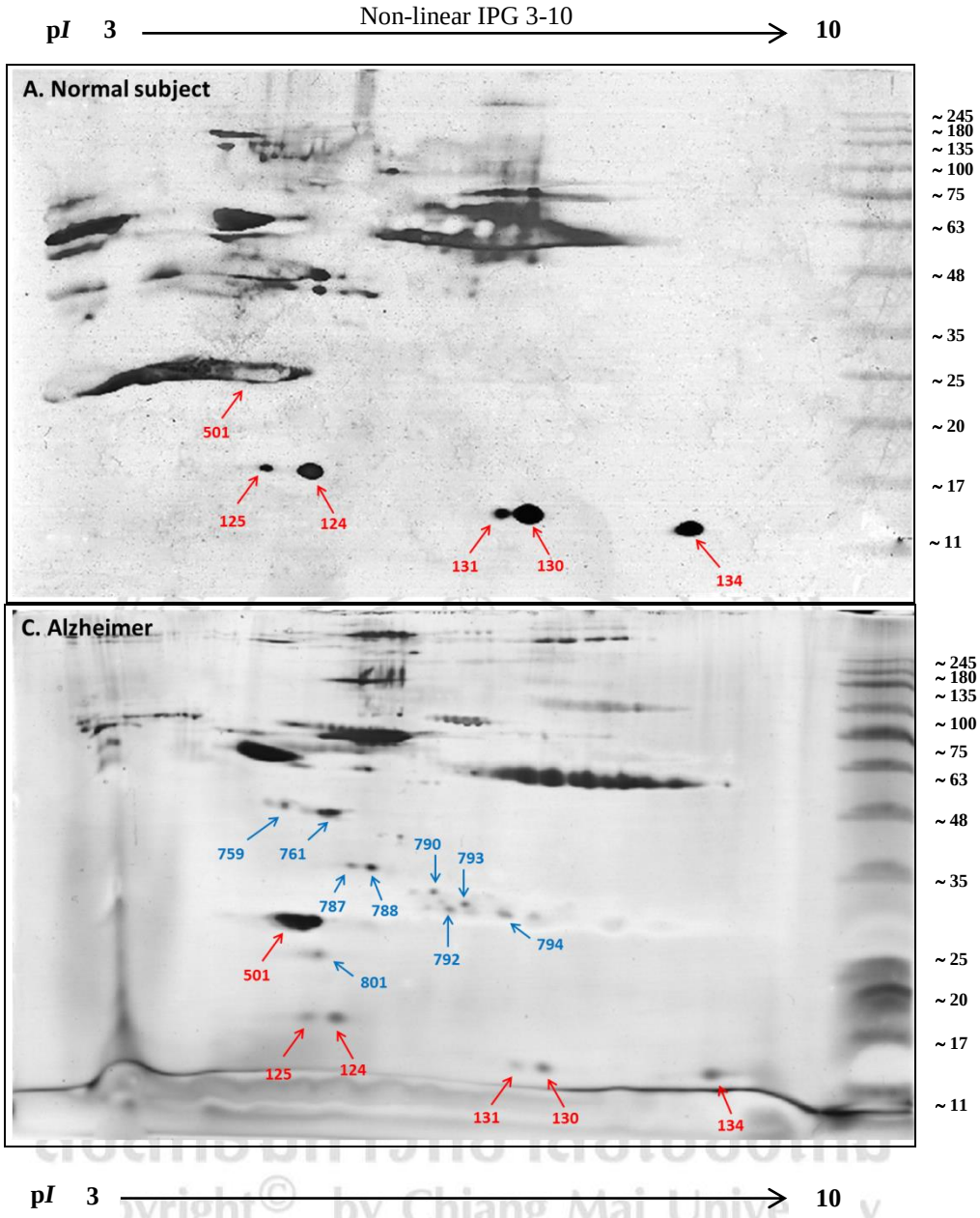

Figure 3.7 Two dimensional gel electrophoresis of serum proteins from cognitive normal subjects (A) compared with Alzheimer's Disease subjects (C). The red arrows indicate the protein spots that were found to have different expression levels between AD and NS groups. The blue arrows arrows indicate the protein spots that were only present in Alzheimer's subjects.

Table 3.6 Protein spots matching from 2-DE image analysis where there was differential expression between AD and NS groups.

Spot ID	p*I*	MW (kDa)	Expression change of protein spot* (Peak of spots)	
124	5.69	17.1	0.15	⬇
125	5.30	16.2	0.51	⬇
130	7.84	13.3	0.06	⬇
131	7.57	13.8	0.13	⬇
134	9.44	12.0	0.17	⬇
501	5.12	26.4	0.16	⬇
759	5.01	55.7	Presence of spot in AD	
761	5.35	50.0	Presence of spot in AD	
787	6.01	42.5	Presence of spot in AD	
788	6.33	41.2	Presence of spot in AD	
790	6.50	35.5	Presence of spot in AD	
792	6.83	33.2	Presence of spot in AD	
793	6.94	34.8	Presence of spot in AD	
794	7.18	31.7	Presence of spot in AD	
801	5.32	25.0	Presence of spot in AD	

*Expression change was the ratio of AD to NS.

The blue arrows indicate the protein spots that had lesser expression in the AD group.

Table 3.7 Comparable results of match similar protein spots between MCI and AD subjects to NS.

Spot ID	p*I*	MW (kDa)	Expression change of protein spots* (Peak of spots)	
			MCI	AD
124	5.69	17.1	1.88 ⬆	0.15 ⬇
125	5.25	16.2	1.23 ⬆	0.51 ⬇
130	7.84	13.3	0.27 ⬇	0.06 ⬇
131	7.57	13.8	0.10 ⬇	0.13 ⬇
134	9.44	12.0	0.44 ⬇	0.17 ⬇
501	4.62	26.4	2.12 ⬆	0.16 ⬇

*Expression change was the ratio of MCI and AD to NS.

The red arrows indicate the protein spots that were more highly expressed in MCI.

The blue arrows indicate the protein spots that had lowered expression in MCI and AD groups.

3.4.2 Protein identification by p*I* and MW

Protein identification by p*I* and MW was performed by using the SWISS-2DPAGE database for screening protein profiles of interest in AD and MCI groups. Based on the 2-DE gel images, comparative analysis of protein patterns was done between MCI and NS and between AD and NS groups. The protein spots of interest for identification by p*I* and MW were classified into 3 groups, including the same location of 6 protein spots between MCI and AD, within a p*I* range of 4-10 and a MW range of 11-35 kDa (**Table 3.7**). The numbers of 10 isolated spots were observed to be differentially expressed in MCI subjects compared with NS subjects, within a p*I* range of 5-8 and a MW range of 35-48 kDa. The nine protein spots that were only presence in AD subjects were compared with NS, occurring within a p*I* rage of 5-8 and a MW range of 25-63 kDa.

After searching the SWISS-2DPAGE database by p*I* and MW, 5 protein types were identified from the protein spots that were found in both MCI and AD subjects, as shown in **Table 3.8**. Ten proteins were identified from the isolated spots which were observed to be differentially expressed in MCI subjects compared with NS subjects, as shown in **Table 3.9** In addition, from specific protein spots that were only found in AD subjects compared with NS subjects (**Table 3.6**), the 12 proteins of interest are in **Table 3.10.**

Table 3.8 Identification of matched similar protein spots between MCI and AD subjects to NS by p*I* and MW, from the SWISS-2DPAGE database

Accession number	Protein name	Entry name	p*I*	MW (kDa)
P00738	Haptoglobin	HPT_Human	5.40 - 5.68	16.88 - 17.07
P02753	Retinol-binding protein 4	RET4_Human	5.24	19.76
P68871	Hemoglobin subunit beta	HBB_Human	7.05	10.53
P05090	Apolipoprotein D	APOD_Human	4.51 - 4.88	27.06 - 29.93
P99007	Immunoglobulin light chain	IGLC_Human	4.94 - 4.95	25.44 - 26.62

Table 3.9 Identification of the protein spots observed to be differentially expressed in MCI subjects compared with NS subjects by p*I* and MW, from the SWISS-2DPAGE database

Accession number	Protein name	Entry name	p*I*	MW (kDa)
P00738	Haptoglobin	HPT_Human	5.08 - 5.86	37.29 - 42.20
P02649	Apolipoprotein E	APOE_Human	5.24	35.32
P02679	Fibrinogen gamma chain	FIBG_Human	5.24 -5.65	44.59 - 49.57
P02766	Transthyretin	TTHY_Human	5.52	35.39
P05156	Complement factor I	CFAI_Human	5.03	37.90
P06727	Apolipoprotein A-IV	APOA4_Human	5.05 - 5.16	43.39 - 43.63
P10909	Clusterin	CLUS_Human	5.07	35.18
P60709	Actin, cytoplasmic 1	ACTB_Human	5.24 - 5.28	43.16 - 43.51
P99004	Possible apolipoprotein	NA3_Human	5.5 - 6.25	36.70 - 39.30
P99008	Immunoglobulin heavy chain gamma (intermediate segment)	IGSG_Human	6.74 - 7.32	37.52 - 38.05

Table 3.10 Identification of protein spots that only presence in AD subjects compare with NS by p*I* and MW, from SWISS-2DPAGE database.

Accession number	Protein name	Entry name	p*I*	MW (kDa)
P01008	Antithrombin-III	ANT3_Human	5.20 - 5.27	58.65 - 58.97
P01009	Alpha-1-antitrypsin	A1AT_Human	5.00 - 5.10	53.33 - 56.31
P01019	Angiotensinogen	ANGT_Human	5.07	58.97
P02679	Fibrinogen gamma chain	FIBG_Human	5.03 -5.65	48.11 - 51.35
P02743	Serum amyloid P-component	SAMP_Human	5.55	26.38
P02774	Vitamin D-binding protein	VTDB_Human	5.16 - 5.24	53.77 - 53.92
P04196	Histidine-rich glycoprotein	HRG_Human	5.31	52.9
P0C0L5	Complement C4-B	CO4B_Human	6.41 - 6.54	31.74 - 31.94
P99004	Possible apolipoprotein	NA3_Human	6.11 - 6.25	36.70 - 37.07
P99007	Immunoglobulin light chain	IGLC_Human	5.02 - 7.93	23.00 - 29.45
P99008	Immunoglobulin heavy chain gamma (intermediate segment)	IGSG_Human	6.74 - 6.98	37.82 - 38.05
P99009	Immunoglobulin heavy chain mu (intermediate segment)	IGSM_Human	5.90 - 6.15	48.50 - 48.90