筑　波　大　学

博　士　（医　学）　学　位　論　文

Identification of serum complement C3, apolipoprotein A1 and transthyretin as useful biomarkers for assessment of risk in the progression of cognitive decline by longitudinal and cross-sectional cohort studies

（縦断ならびに横断コホート研究による認知機能の低下を評価する血液バイオマーカーの同定：血清補体タンパク質 C3、アポリポタンパク質 A1 ならびにトランスサイレチンの臨床有効性について）

２０１６

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Abbreviations

Aβ	amyloid-beta
AD	Alzheimer's disease
ADDLs	soluble amyloid Aβ-derived oligomers
ADNI	Alzheimer's Disease Neuroimaging Initiative
ApoA1	Apolipoprotein A1
ApoE	Apolipoprotein E
ApoJ	Apolipoprotein J
APP	Amyloid precursor protein
AUC	area under the curve
BBB	blood-brain barrier
BS	blood sugar
C3	Complement component C3
C4	Complement component C4
CI	confidence interval
CSF	cerebrospinal fluid
DECO	Deterioration de Cognitive Observe
DLB	dementia with Lewy bodies
FTD	frontotemporal dementia
HDS-R	Revised Hasegawa dementia scale
HDL	high-density-lipoprotein

LDL	low-density-lipoprotein
MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
MMSE	Mini-Mental State Examination
NDC	Non-demented disease control
NFT	Neurofibrillary tangles
PCR	polymerase chain reaction
rCBF	regional cerebral blood flow
ROC	receiver operating characteristic curve
ROI	region of interest
S.D.	standard deviation
SPECT	single-photon emission computed tomography
TTR	transthyretin
TC	total cholesterol
TG	triglyceride
VD	vascular dementia
VSRAD	Voxel-based Specific Regional analysis system for Alzheimer's disease
WAIS-R	Wechsler Adult Intelligence Scale-Revised

Preface

Dementia is progressive deterioration in memory and cognitive ability which particularly affects the older people. People with dementia have symptoms of memory loss, disorientation and decline of understanding, judgement and language, as a result, they have difficulties in normal daily life. The worldwide prevalence of dementia is predicted to be double at every 20 years and number of patients will be 81 million by 2040 (1). Ministry of Health, Labor and Welfare of Japan reported that 4.86 million people, 15% of individuals aged ≥65 years, were affected with dementia in Japan (Report of Health and Labor Sciences Research Grants [201218011A]).

It should be noticed that dementia has economic impact on health care system and also on regional society. People with dementia need health and social care services and also family's help to maintain their quality of life. In United Kingdom, dementia costs 23 billion pond in terms of health and social care, informal care, and productivity losses in the society (2).

Alzheimer's disease (AD) is the most common form of dementia which contributes to 60-80% of cases of dementia, and 46.8 million people are suffered from this neurodegenerative disorder worldwide (3). Mild cognitive impairment (MCI) is a pre-stage of dementia showing cognitive decline that is greater than that expected for an individual's age, and MCI is regarded as a risk group for dementia. MCI due to AD is considered as a high-risk condition developing to clinically probable AD. Without clinical intervention, 40% of patients with MCI may convert to AD within 4 years after diagnosis (4). To reduce the number of people with dementia, development of biomarker for cognitive decline is urgently needed, because early diagnosis by using biomarker and early intervention may delay progression of MCI and prevent AD.

It is likely that many factors contribute to AD pathogenesis, and among them, production and aggregation of amyloid-β peptide (Aβ) in the brain are thought to be

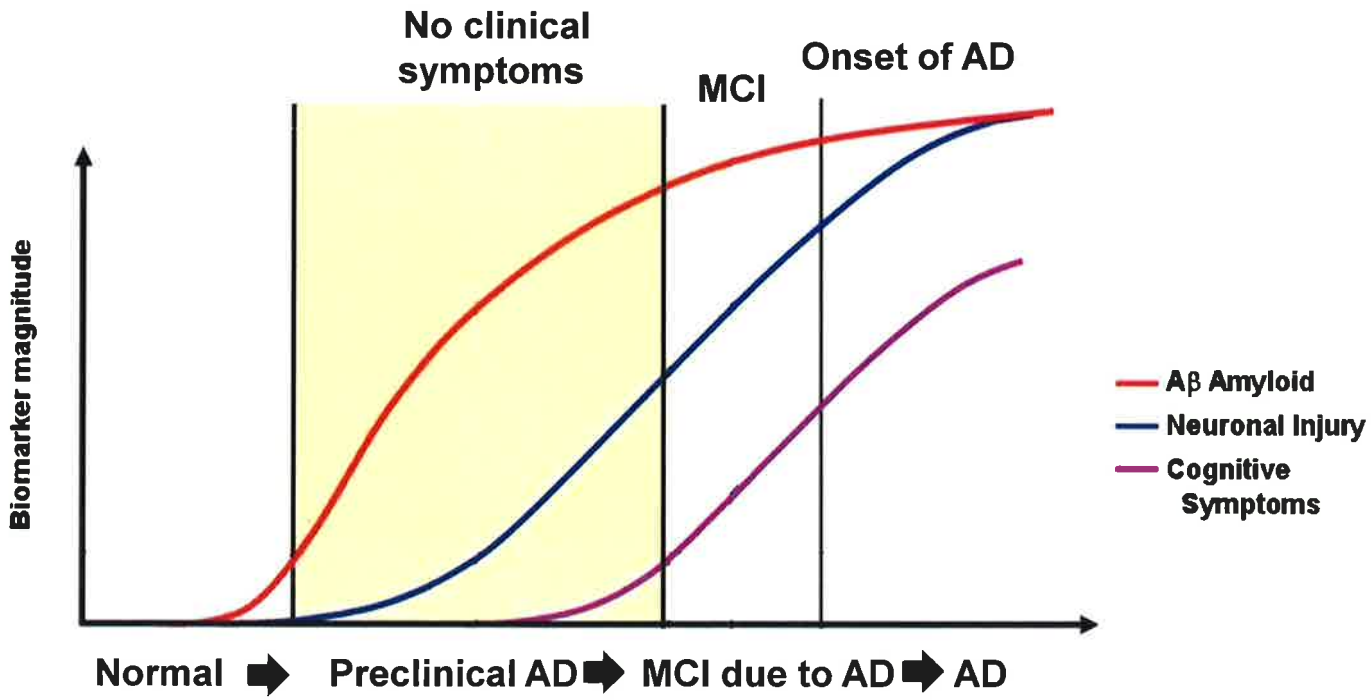
mainly involved in AD pathogenesis and consequently lead to synaptic dysfunction. Amyloid plaques, extracellular deposits of Aβ, and neurofibrillary tangles (NFT) composing the microtube associated protein tau in filamentous aggregates within cell, are main histopathological features of AD. Aβ is produced by proteolytic cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase. Soluble amyloid Aβ-derived oligomers (ADDLs) are also involved in the pathogenesis of AD. ADDLs target synaptic spines and disrupt synaptic plasticity leading to synaptic dysfunction and memory loss. Several reports indicate ADDLs cause abnormal spine morphology and significantly decreased in spine density (5, 6). Soluble forms of Aβ oligomers extracted from AD brains impaired memory of learned behavior in normal rats (7).

Magnetic resonance imaging (MRI) can detect the brain atrophy, however, it is difficult to monitor the onset of nascent dementia before clinically emergence of measurable cognitive impairments. If early interventions for dementia are applied at early stages of cognitive impairment or before onset of dementia, it is possible to prevent dementia and reduce the number of dementia patients.

Thus, development of biomarker for cognitive decline is needed for early diagnosis of AD and MCI. Interventions at early stages of cognitive decline should slow the disease progression, and ultimately reduce the prevalence of MCI and AD. Deposition of Aβ starts decades before clinical onset of dementia (**Figure 1**) (8). Blood-based biomarker which can monitor levels of Aβ accumulation in the brain and neurodegeneration must be useful to detect cognitive declines at the early stages of the disease.

Levels of Aβ accumulation may be determined by balance of Aβ production and clearance in the brain. In this study, we focused on proteins involved in 'Aβ sequestration' to identify biomarker for cognitive impairment and analyzed their peripheral levels in MCI, AD and non-demented control (NDC). 'Aβ sequestration' means both Aβ clearance and protection of neuron against Aβ neurotoxicity (**Figure 2**) (9). By using serum samples

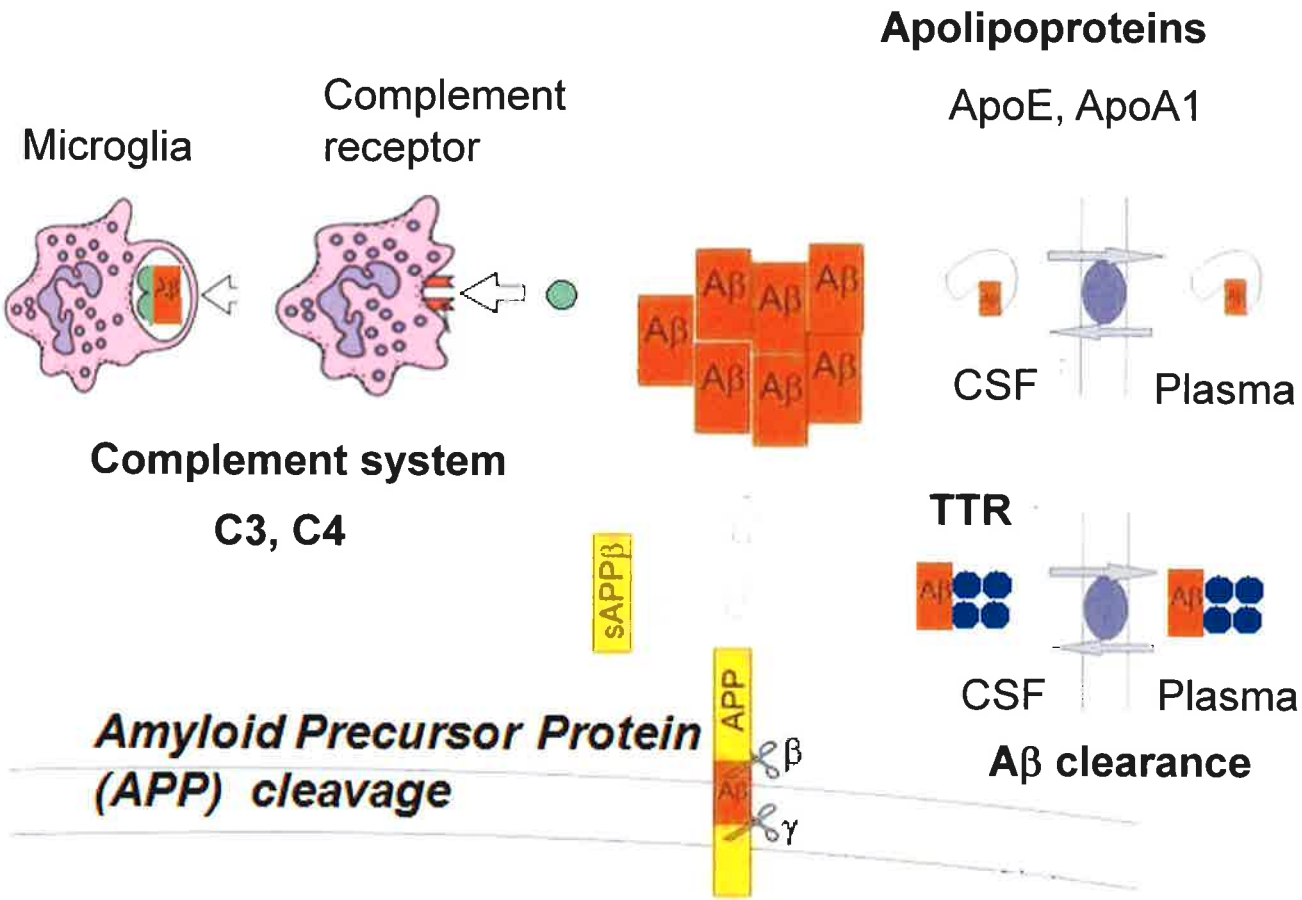
in the longitudinal and cross-sectional cohort studies, we analyzed clinical potential of several Aβ sequester proteins as potential blood-based biomarker for MCI and AD. Furthermore, using three of these proteins, we established assay system for clinical laboratory test and validated their clinical potential in discrimination of MCI from NDC.



Modified from Jack, C. R *et al.* (8)

Figure 1. Disease progression model of AD

More than 20 years prior to onset of AD, Aβ deposition starts in the brain followed by neuronal injury, memory loss, and finally daily living is affected in AD.



Referred from Kazuhiko Uchida *et al.* (9)

Figure 2. Sequester proteins and Aβ clearance

Accumulation of Aβ in senile plaques is the main pathologic features of AD. 'Sequester proteins' like apolipoproteins, complement proteins and transthyretin may involved in Aβ clearance from the brain and protection against Aβ neurotoxicity.

Chapter 1

Screening of blood-based biomarker for cognitive impairment by longitudinal and cross-sectional studies in the Tone cohort

1-1. Introduction

Deposition of Aβ in senile plaques is main pathophysiological features of AD. In healthy individuals, to prevent synapse damage by Aβ, Aβ is removed via clearance mechanisms. Accelerated clearance of Aβ may delay the disease progression (**Figure 2**).

Apolipoproteins (apolipoprotein E [apoE] and apolipoprotein A1 [apoA1]) bind to Aβ peptides, by which Aβ deposition is prevented and its neurotoxicity is reduced (10) (11). The apolipoproteins are plasma proteins involved in transportation of cholesterol. In the central nervous system, apoE is involved in growth during development and repair of the central nervous system after injury. ApoE has high avidity in binding to Aβ, but individuals with *APOE* ε4 allele have reduced biding activity of apoE to Aβ and are frequently observed in late-onset AD(12) (13).

The mRNA for all complement proteins in the classical pathway are detected in the brain tissues(14). Complement C3 and C4 mRNA were most abundantly observed in all areas of both normal and AD brain. In addition, in an APP transgenic mice model, C3 and C4 in classical complement pathway were activated. C1q, C3, factor H and factor B were substantially and significantly elevated in AD (15). Aβ is suggested to be pro-inflammatory agents and induce inflammatory components. Microglia and astrocytes in AD brain showed elevated levels of chemokines such as macrophage inflammatory protein 4 (MIP-4) (16). Schwarzman *et al.* identified transthyretin (TTR) as Aβ interacting proteins in cerebrospinal fluid (CSF) by incubation with synthetic Aβ. TTR suppresses neuro-toxicity of Aβ oligomers (17) (18).

Thus, the proteins involved in 'Aβ sequestration' may be involved in the disease processes of AD via both Aβ clearance and synaptic protective functions. In this study, we hypothesized that serum levels of these proteins could be biomarkers for assessment of cognitive impairment.

1-2. Materials and Methods

1-2-1. Participants

A longitudinal study of cognitive impairment, "Tone Project" was designed and held in Tone city, Ibaraki, by University of Tsukuba (**Figure 3**) (19). Participants were recruited to investigate the relationship between possible biomarkers and cognitive decline, and also to analyze preventive factors and risk factors. A total of 1,270 participants at age ≥65 years old were recruited at 2001. Cognitive normal in 2001 were set as the baseline. Assessment of cognitive function in participants and blood collection were conducted at every 3 or 4 years. In 2005 and 2008, 1,024 and 584 participants were followed up, respectively. Physical exercise and nutrition interventions were performed from 2003 to 2005(20). In this study, blood samples in 2005 and 2008 were used. We selected participant with MCI who was first diagnosed in 2005. This study was focusing on decline of cognitive functions. There was no exercise or nutrition intervention in these participants.

The protocol used for recruiting of participants and collection of blood sample was approved by the Ethics Committee of University of Tsukuba Hospital (Approval number: 140).

1-2-2. Procedures for clinical diagnosis

Participants were underwent a screening assessment test for cognitive decline named the 5-Cog, which used a set of tests measuring 5 cognitive domains; attention, memory, visuospatial function, language, and reasoning.

Japanese version of the set dependency activity (21) was used to assess alternating attention. This test was used to analyze participants shifting their attention toward a particular character in the correct row. Meanwhile, some of the characters were placed in

the incorrect rows. A Category Cued Recall test (22) was used to assess memory ability. After given a list of items to remember, the participants were asked to recall the items by the cue. In order to assess visuospatial function, the Clock Drawing test was used (23). In this test, participants were required to draw the clock depicted the time at 'ten after eleven'. Language ability was examined using a category frequency test (24). The participants were asked to write down as many examples as possible within 2 minutes from the semantic category 'animals.' The similarity subset of the Wechsler Adult Intelligence Scale-Revised (WAIS-R) was employed to assess abstract reasoning ability.

In the Tone cohort, NDC was defined as participant who had no evidence of cognitive impairment, and neurological or psychiatric disease, and who had no diagnosis of MCI or AD. Petersen criteria was used to define MCI (4). Medical, neurological, psychiatric and neuropsychological data were reviewed in the assessment. Subjects considered as MCI were required to have; **(A)** objective impairment in ≥ 1 cognitive domain based on the average of scores on neuropsychological measures within that domain 1.5 S.D. cutoff using normative corrections for age, years of education, and sex, **(B)** essentially preserved basic abilities in activities of daily living which were measured using Nishimura's Activities of Daily Living, **(C)** presence of memory complaints by Deterioration de Cognitive Observe (DECO) (25), **(D)** no diagnosis of dementia. Procedures of diagnosis were conducted by three doctors who are specialists in cognitive disorder.

The diagnosis of AD was made according to DSM-IV criteria; **(A)** Development of cognitive deficits in memory and with one or more following: cognitive disturbances aphasia, apraxia, agnosia and disturbance in executive functioning. **(B)** Gradual onset and continuing cognitive decline. **(C)** Cognitive deficits are not due to neurological or psychiatric disease. **(D)** Cognitive deficits do not occur during the course of delirium.

To analyze longitudinal change of cognitive decline, participants were categorized into 3 groups; **Group 1** NDC-NDC, **Group 2** NDC-MCI and **Group 3** MCI-MCI/AD. In

Group 3, participants with MCI in 2005 and MCI or AD in 2008 were selected. MCI or AD in 2008 have cognitive impairment at least for 3 years. We categorized these participants with MCI in 2008 as stable MCI (sMCI).

1-2-3. Serum sampling and multiple immunoassay

The blood samples were aspirated into collection tubes and placed on room temperature. Within 6 hours after serum separation, serum samples were aliquoted and stored at -80℃ until usage for immunoassay. Serum levels of sequester proteins were measured by multi-immunoassay, Luminex, which permitted multiplexing assays in a single assay (**Figure 4**), using assay kits of HNDG1-36K (C3, TTR, apoE, apoA1) and HNDG2-36K (C4, MIP-4) (EMD Millipore, Billerica, MA, USA).

In Luminex assay for apoA1, C3 and TTR, analytes bound to immunobeads were characterized by immunoprecipitation (IP)-Western blotting. IP-Western blot analysis showed that corresponding molecular weights of apoA1 and TTR were 27 kDa and 15 kDa, respectively. C3 indicated molecular mass that corresponded to a full-length C3, not the activated C3 fragments (**data not shown**).

In determination of serum levels of these proteins, quality control of each analyte was included in each assay. We also added standard serum sample as standard to check reproducibility in immunoassay.

1-2-4. Statistical analysis

In the longitudinal analysis, Wilcoxon signed-rank test was used to analyze for significant differences between individuals in 2005 and 2008. In statistical analysis in three groups, Kruskal-Wallis test was used and Bonferroni correction was applied when two groups were compared. A P value of 0.05 or less was considered significant. The software Origin

(ver. 7.5) (OriginLab Corp., MA, USA) were used to perform statistics analysis.

1-3. Results

1-3-1. Longitudinal changes of serum apoA1, apoE, C3, C4, TTR, and MIP-4 levels in the Tone cohort.

Out of 1,270 participants, 211 were diagnosed as MCI in 2005. In 2008, total of 584 participants were followed up, and among them, 92 were MCI. Based on diagnosis during follow-up, paired blood samples from the same participant were used for biomarker screening and categorized into 3 groups (**Table 1**). **Group 1**: NDC-NDC (n = 20), comprised individuals who had maintained cognitively normal from the beginning of the study in 2001, 2005 and 2008. **Group 2**: NDC-MCI (n = 9), comprised participants who had progressed from normal in 2005 to MCI in 2008. **Group 3**: MCI-sMCI/AD (n = 6), comprised participants who were diagnosed with MCI in 2005 and MCI or AD in 2008. We selected participants with MCI due to AD in 2005 and 2008, and excluded participants who progressed to other types of dementia than AD by diagnosis of these participants in 2012. There was no significant difference in age, education, body mass index, and geriatric depression scale (26) among the groups.

Longitudinal changes in serum levels of analytes were shown in **Figure 5** (the left panels). Serum apoA1 ($P = 0.00915$) and apoE ($P = 0.01285$) showed substantial decrease in **Group 2** (NDC-MCI: NDC in 2005 and MCI in 2008). Serum levels of apoA1 ($P = 0.03603$) and apoE ($P = 0.03603$) showed significant decrease in **Group 3** (MCI-sMCI/AD: MCI in 2005 and MCI in 2008). TTR concentrations were not changed in NDC-NDC group, and showed relatively decrease trend in both NDC-MCI and MCI-sMCI/AD groups. Serum C4 levels increased in all 3 groups. No significant changes were found in MIP-4.

1-3-2. Cross-sectional changes of serum apoA1, apoE, C3, C4, TTR, and MIP-4 levels in the Tone cohort.

Results of cross-sectional analyses in the Tone cohort were shown in the right panels of **Figure 5**. Significant decrease of TTR ($P = 0.01264$) and apoE ($P = 0.0278$) were found among the NDC, MCI and sMCI/AD groups. Between NDC and sMCI/AD, TTR levels showed significant difference ($P = 0.02025$).

1-4. Discussion

In this study, we tested the clinical potential of 'Aβ sequester protein' as biomarker candidates for cognitive impairment. These proteins may have neuroprotective functions against AD pathogenesis. We selected individuals with "MCI due to AD." We carefully checked the diagnosis of each participant in 2005-2008 and 2008–2012. We excluded individuals with MCI who developed other types of dementia such as dementia with Lewy bodies (DLB), vascular dementia (VD), and frontotemporal dementia (FTD). After the selection of MCI and AD, we randomly selected twenty age-matched NDC subjects to adjust the number of NDC with MCI.

Present study revealed that serum levels of apoE and TTR and were significantly lower in sMCI and AD in 2008. ApoA1 and C3 levels indicated decreasing trends among groups. Complement proteins are proinflammatory molecules and an integral part of the immune system, which sequestrate Aβ via an innate immune response in the brain (27). Recent studies also indicated that increased levels of CSF C3 and factor H in AD patients and correlated with severity of cognitive impairment in terms of Mini-Mental State Examination (MMSE) scores. These results suggested that C3 levels reflect extent of complement activation in disease progression (28).

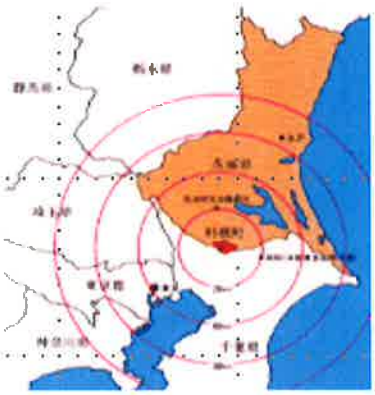
TTR was also known as prealbumin, and has a half-life of two days in plasma. It is shorter than that of albumin and more sensitive in reflecting of nutrition status. Peripheral TTR are affected by the overall nutrition, infection and inflammation (29) (30). TTR is produced not only in the liver but also in choroid plexus of the brain, and secreted in the blood and CSF (31). In AD patients, CSF levels of TTR are significantly lower than controls (32) (33). Recently, lower plasma TTR levels were observed in patients with cognitive decline and AD (34) (35).

In previous report, the ε4 allele of *APOE* was detected in 40-50% of all AD patients, and was risk factor in the pathogenesis of AD (36). Sasaki.*et al.* reported significant association between *APOE* ε4 allele and dementia (37), however, association of peripheral apoE protein levels and AD pathogenesis are controversial (38, 39). ApoE is involved in Aβ clearance via transports low-density-lipoprotein (LDL) receptor (40) (41). The ability of apoE to bind Aβ was affected by polymorphism of *APOE* gene, ε2 >ε3 >ε4 (42). Florbetair (^{18}F) PET demonstrated association of *APOE* ε4 with elevated levels of amyloid deposition in the brain and lower Aβ levels in CSF. *APOE* genotype screening is not sufficient for routine clinical diagnosis of AD (43).

To find the biomarkers for MCI and AD, we analyzed TTR, apoE, apoA1, C3, C4 and MIP-4, and did not test apolipoprotein J/clusterin (ApoJ) in this study. We performed apoJ ELISA in a pilot experiment and found that serum apoJ levels seemed to increase in AD, however, the results were not reproduced when independent sample sets were analyzed.

ApoJ is one of the known strong genetic risk factors of AD. ApoJ may cooperatively regulate Aβ clearance and aggregation with apoE (44). However, peripheral levels of apoJ in AD vary in literature (45, 46). In this study, it did not show clinical utility for the assessment of cognitive decline. We speculated that apoJ as well as apoE affect the progression of AD via structural alterations due to risk alleles, rather than protein levels.

In the next step, clinical potential of these sequester proteins were tested in independent clinical samples, and the results were described in **Chapter 2**.



Tone, Ibaraki

Neuropsychological assessment

2001 Normal → 2005MCI → 2008 sMCI/AD

2001 Normal → 2005 Normal → 2008 Normal

- The association between blood biomarkers and cognitive decline
- Identification of preventive factors for cognitive decline
- Check the risk factors like *APOE* ε4 and major depression

Figure 3. Longitudinal study of cognitive impairment in the Tone cohort

During follow-up period, cognitive function assessment and blood sampling were conducted at every 3 or 4 years since 2001.

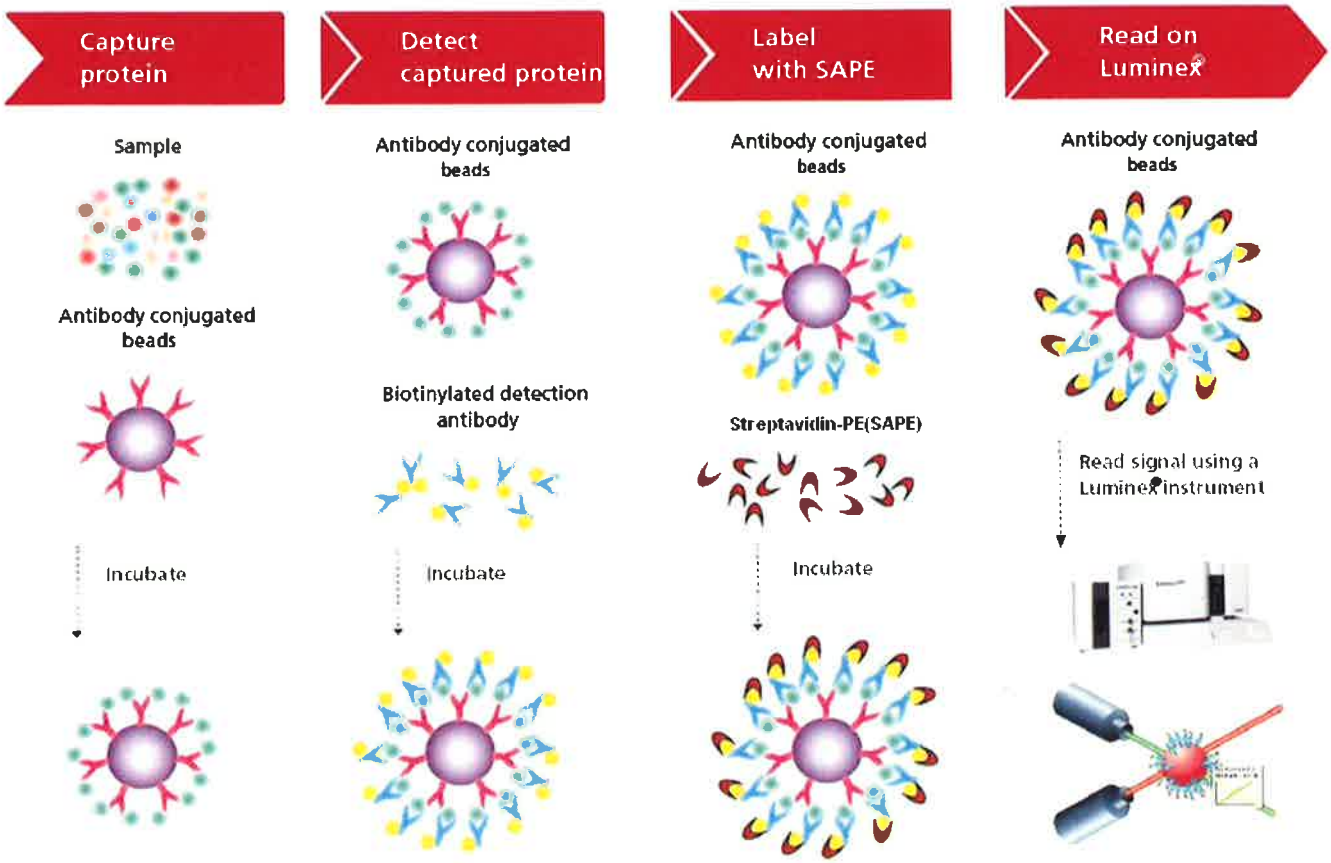
Table 1. Demographic characteristics of participants in longitudinal study of Tone cohort.

Characteristics	NDC during follow up (n = 20)	NDC to MCI (n = 9)	MCI to sMCI / AD (n = 6)	*P* value[‡]
Age in 2005	76.4 ± 5.8*	73.7 ± 6.4	74.7 ± 5.7	0.5231
Age in 2008	80.3 ± 5.8	77.6 ± 6.4	78.3 ± 5.7	0.43784
Male / Female	7 / 13	2 / 7	2 / 4	
Years of education	10.3 ± 2.4	9.8 ± 1.9	11.5 ± 2.9	0.4752
BMI[†]	22.6 ± 2.4	23.8 ± 5.0	24.3 ± 5.0	0.35639
GDS Score[†]	2.1 ± 1.7	3.6 ± 3.5	3.6 ± 2.6	0.31795
Cigarette smoking	4	1	2	
Alchohol drinking	7	1	2	
APOE ε4 carrier	3	0	2	
History of disease				
Cardiovascular disease	1	0	0	
Diabetes mellitus	1	0	1	
Hyperlipidemia	0	0	0	
Hypertension	2	2	1	

*mean ± SD

[†]Values in 2008

[‡]Kruskal-Wallis test. Significant differences among 3 groups are indicated.



http://www.xconomy.com/wordpress/wp-content/images/2013/12/luminex.jpg

Figure 4. Multiple immunoassay by Luminex permit the multiplexing assays within a single sample

Each bead is conjugated to antibody against a specific target analyte followed by binding with a biotinylated detection-antibody. The reaction mixture is then incubated with streptavidin-phycoerythrin. The beads are allowed to pass through capillary. Captured analytes on beads were visualized by laser excitation.

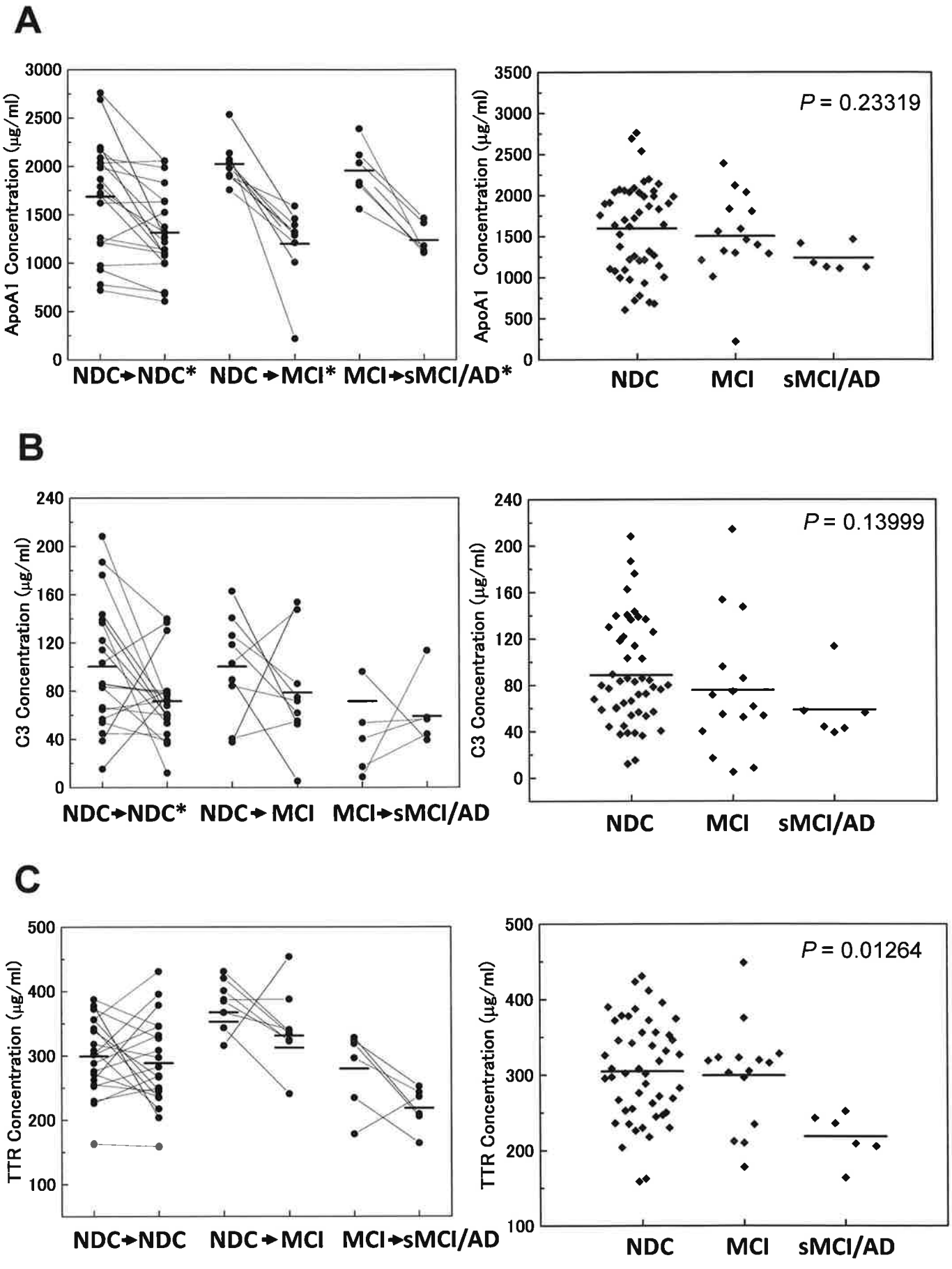

A
ApoA1 Concentration (μg/ml)
3000
2500
2000
1500
1000
500
0
NDC→NDC*
NDC→MCI*
MCI→sMCI/AD*
P = 0.23319
NDC
MCI
sMCI/AD
B
C3 Concentration (μg/ml)
240
200
160
120
80
40
0
NDC→NDC*
NDC→MCI
MCI→sMCI/AD
P = 0.13999
C
TTR Concentration (μg/ml)
500
400
300
200
100
NDC→NDC
NDC→MCI
MCI→sMCI/AD
P = 0.01264

Figure 5.

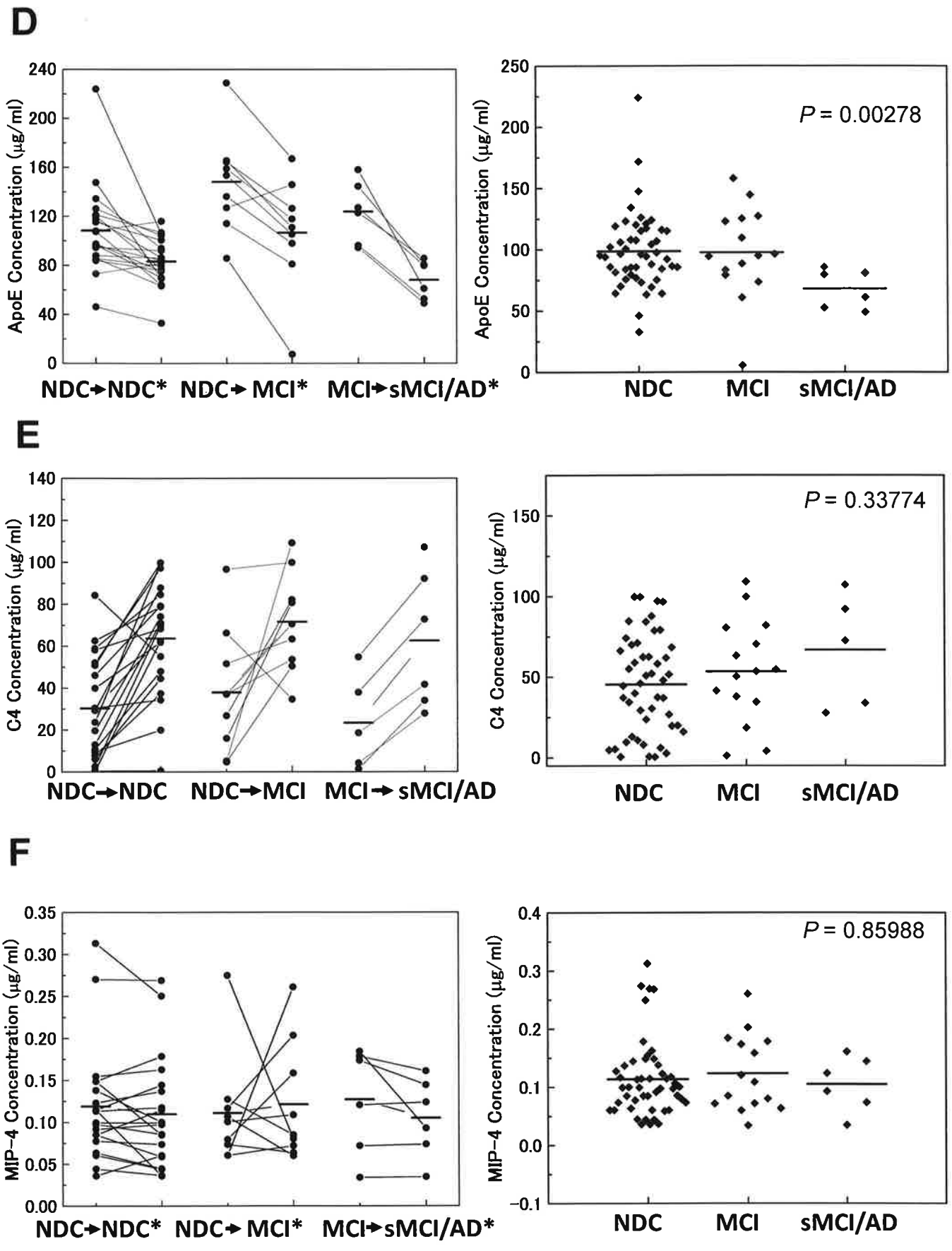

D
ApoE Concentration (μg/ml)
240
200
160
120
80
40
0
NDC→NDC*
NDC→MCI*
MCI→sMCI/AD*
ApoE Concentration (μg/ml)
250
200
150
100
50
0
P = 0.00278
NDC
MCI
sMCI/AD
E
C4 Concentration (μg/ml)
140
120
100
80
60
40
20
0
NDC→NDC
NDC→MCI
MCI→sMCI/AD
C4 Concentration (μg/ml)
150
100
50
0
P = 0.33774
NDC
MCI
sMCI/AD
F
MIP-4 Concentration (μg/ml)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
NDC→NDC*
NDC→MCI*
MCI→sMCI/AD*
MIP-4 Concentration (μg/ml)
0.4
0.3
0.2
0.1
0.0
-0.1
P = 0.85988
NDC
MCI
sMCI/AD

Figure 5.

Figure 5. Serum levels of apoA1, C3, TTR, C4 and MIP-4 in NDC, MCI, sMCI and AD in longitudinal and cross-sectional analyses in the Tone cohort

Serum levels of sequester proteins apoA1 (A), C3 (B), TTR (C), apoE (D), C4 (E) and MIP4 (F) during progression of cognitive impairment from 2005 to 2008 in longitudinal (left panel) and cross-sectional (right panel) studies from the Tone cohort.

In the left panel, asterisks indicate significant changes in protein levels between 2005 and 2008 participants. In the right panel, the bold solid bars within the dotplot represent the median abundance.

In longitudinal analysis, significant changes in protein levels between paired samples (Wilcoxon signed-rank test) are indicated by asterisk. ApoA1: NDC-NDC (P = 0.00109), NDC-MCI (P = 0.00915), MCI-sMCI/AD (P = 0.03603), apoE: NDC-NDC (P = 4.19E-04), NDC-MCI (P = 0.01285), and MCI-sMCI/AD (P = 0.03603).

Significant differences among three groups are indicated (Kruskal-Wallis test). In cross-sectional analysis, significant changes in protein levels between two groups (Bonferroni test) are likewise indicated by asterisk. TTR: NDC versus sMCI/AD (P = 0.02025).

Abbreviations: TTR, transthyretin; SD, standard deviation; NDC, nondemented disease control; MCI, mild cognitive impairment; sMCI, stable mild cognitive impairment; AD, Alzheimer's disease.

Chapter 2

Validation of apoA1, C3 and TTR as blood-based biomarker for cognitive impairment by cross-sectional study in the Tsukuba cohort

2-1. Introduction

To validate blood-based biomarker candidates for cognitive impairment, clinical samples were collected from independent cohort. MCI was defined as cognitive decline greater than expected for an individual's age and education level but that does not interfere notably with activities of daily living (47). Although MCI can present with a variety of symptoms, the clinical presentations of MCI can be classified according to amnestic MCI (aMCI) and non-amnestic MCI (naMCI). Individuals with aMCI have higher risk of progression to AD, and thought to be prodromal AD (48).

In **Chapter 2**, the results of validation of the sequester proteins as blood-based biomarker for cognitive impairment are described. In the Tsukuba cohort, we carefully assessed cognitive abilities: memory, attention, visuospatial function, language and reasoning. Memory impairment is the predominant defining feature in aMCI. In both aMCI and naMCI single or multiple cognitive domains are affected. Four subtypes of MCI are classified as follow, aMCI single domain; aMCI multiple domains; naMCI single domain and naMCI multiple domains. MMSE is well-known test in assessment of cognitive impairment (49). In this study, we carefully selected aMCI and AD samples to validate blood-based biomarkers screened by the studies described in **Chapter 1**, and analyzed serum levels for biomarker proteins to MMSE scores.

2-2. Materials and Methods

2-2-1. Participants

Cross-sectional study was performed using serum samples from Tsukuba University Hospital (Tsukuba, Japan) (Ethics Committee of University of Tsukuba Hospital: Approval number: 227; Ethics Committee of MCBI: Approval number: 2013-01). Total sample number was 441, which included AD, MCI, other types of dementia, and other psychiatric diseases. NDC was defined as had no evidence of cognitive impairment, and

neurological or psychiatric disease, and who had no diagnosis of MCI or AD. MCI was defined by Petersen criteria (4). Subjects considered as MCI were required to have; **(A)** objective impairment in ≥1 cognitive domain based on the average of scores on neuropsychological measures within that domain 1.5 S.D. cutoff using normative corrections for age, years of education, and sex, **(B)** essentially preserved basic abilities in activities of daily living which were measured using Nishimura's Activities of Daily Living, **(C)** presence of memory complaints by Deterioration de Cognitive Observe (DECO) (25), **(D)** no diagnosis of dementia. Among cognitive domains, participants whose memory loss as the main symptom is selected as aMCI. The diagnosis of AD was made according to DSM-IV criteria; **(A)** Development of cognitive deficits in memory and with one or more following: cognitive disturbances aphasia, apraxia, agnosia and disturbance in executive functioning. **(B)** Gradual onset and continuing cognitive decline. **(C)** Cognitive deficits are not due to neurological or psychiatric disease. **(D)** Cognitive deficits do not occur during the course of delirium. MMSE score were used as one of clinical characteristics to measure cognitive decline. In addition to grouping due to diagnosis, we categorized participants by MMSE score to analyze cognitive status.

2-2-2. Serum sampling and multiple immunoassay

All serum samples were collected and processed as described in **Chapter 1** with the informed consent of each patient, and were subsequently analyzed in accordance with procedures approved by the Ethical Committee. Within 6 hours after serum separation, samples were aliquoted and stored at -80℃, until usage for immunoassay. Protein concentrations were determined using HNDG1-36K (C3, TTR, apoE, apoA1) and HNDG2-36K (C4, MIP-4; EMD Millipore, Billerica, MA, USA) according to the manufacturers' instructions.

2-2-3 Statistical analysis

A p value of 0.05 or less was considered significant. For data consisting of more than two groups, Kruskal-Wallis test was used and Bonferroni correction was applied when two groups were compared. The software Origin (ver. 7.5) (OriginLab Corp., MA, USA) and were used to perform statistics analysis.

We used multivariable linear regression to analyze the relationship between peripheral sequester proteins levels and severity of cognitive decline. Logistic regression is generalized linear model, in logistic regression outcomes are categorical, such as 0 or 1. It estimates the probability of two states either normal or onset of the disease.

$$F=1/(1+e^{-(a0 + aApoa1 \cdot x1 + aC3 \cdot x2 + aTTR \cdot x3)})$$

Where a0 is constant, and a[Apoa1], a[C3] and a[TTR] are regression coefficients. They were calculated by MedCalc (ver. 9.3.9) (MedCalc Software, Mariakerke, Belgium). The area under the curve (AUC) of the receiver operating characteristic curve (ROC) was calculated by MedCalc software. The closest point to the upper left corner of the ROC curve gave the optimum sensitivity and specificity values.

2-3. Results

2-3-1. Cross-sectional analysis of serum apoA1, C3, and TTR levels in the Tsukuba cohort

No significant difference (P = 0.27876) in age was observed among NDC (n = 49), aMCI (n = 51) and AD (n = 42). Left panel of **Figure 6** presents the results of cross-sectional analysis. Lower levels of serum apoA1 (P = 4.44E-05), C3 (P = 0.0126) and TTR (P = 4.07E-05) in aMCI and AD were found. Significant differences between NDC and aMCI were observed in apoA1 (P = 0.00265), C3 (P = 3.74E-04) and TTR (P = 0.0634). ApoA1 (P = 1.98E-05) and TTR (P = 1.08E-05) levels were obviously lower in AD than NDC.

No statistically significant differences in apoE, C4, and MIP-4 levels were observed among the groups.

The results of *C*-statistics were shown in the right panel of **Figure 6**. Black line presented the aMCI vs. NDC; apoA1: AUC = 0.64 (sensitivity 78%, specificity 48%, P = 0.0124); C3: AUC = 0.67 (sensitivity 88%, specificity 48%, P = 0.0033); and TTR: AUC = 0.62 (sensitivity 80%, specificity 47%, P = 0.0298). Comparison in AD vs. NDC showed higher sensitivity and specificity in apoA1 (AUC = 0.76, sensitivity 74%, specificity 71%, $P < 0.0001$) and TTR (AUC = 0.76, sensitivity 74%, specificity 69%, $P < 0.0001$).

In this study, individuals were categorized into 4 groups according to MMSE score (score 27–30 [n = 71], 24–27 [n = 34], 20–23 [n = 17], and <20 [n = 20]) (**Figure 7**). No significant difference (P = 0.14277) in age was observed among groups. Individuals with lower MMSE score had significantly decreased levels of apoA1 (P = 0.00359) and TTR (P = 0.01894). Especially, comparing with MMSE score 27–30 group significantly lower levels of apoA1 (P = 0.0396) and TTR (P = 0.05074) were observed in MMSE score <20 group. C4 and apoE showed no significant difference among the groups categorized by the MMSE score.

2-3-2. Multivariable statistical analysis of biomarker proteins

Clinical potential of combination of apoA1 and C3 in discriminating aMCI from NDC, and combination of C3 and TTR in discriminating AD from NDC were obtained by multivariable analysis using logistic regression. The results of ROC analyses were showed in **Figure 8**. In NDC vs. aMCI, AUC value was 0.74 ($P < 0.0001$) with 78% sensitivity and 63% specificity. Constant a0 is 4.6667, and coefficient a[Apoa1] is -0.001653, a[C3] is -0.02567. In NDC vs. AD, AUC value was 0.73 ($P < 0.0001$) with 76% sensitivity and 61% specificity. Constant a0 is 4.5218, and coefficient a[C3] is -

0.0465, a[TTR] is -0.00564. By adding the MMSE score to the linear regression model improved AUC values up to 0.78 and 1.00 in aMCI vs. NDC and AD vs. NDC comparisons, respectively.

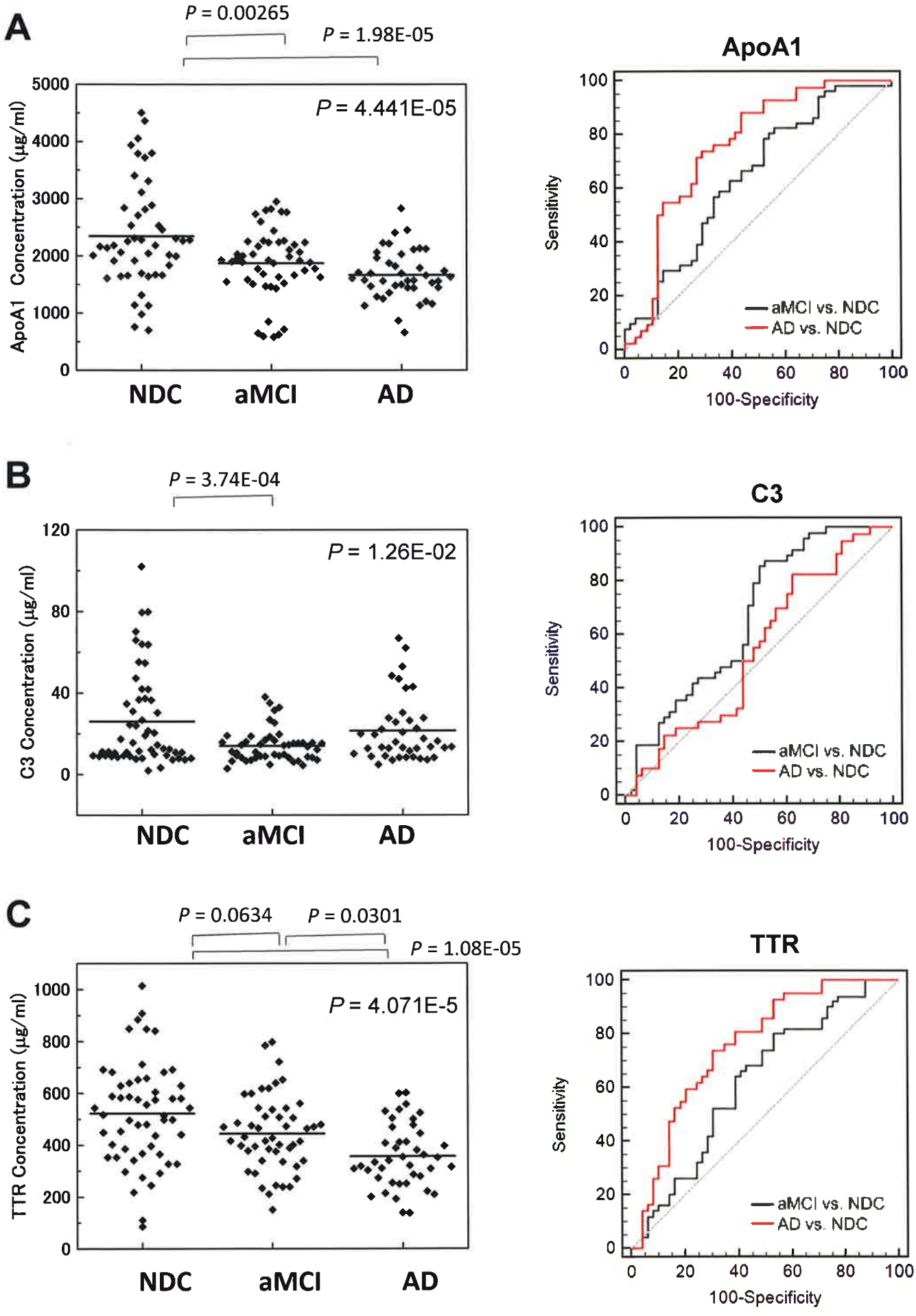
2-4. Discussion

Cross-sectional study in the Tsukuba cohort was performed to validate biomarker candidates described in **Chapter 1**. Serum levels of TTR and apoA1 were lower in aMCI and AD as compared in NDC. C3 levels tended to decrease at early stages of cognitive decline. Individuals were categorized according to the MMSE scores and decreased levels of apoA1 and TTR were found in individuals with lower MMSE score. ApoA1 and TTR showed similar decreasing trends with decline of cognitive functions. C4 is important components of complement system and *APOE* ε4 is critical risk factor in the AD patients. However, serum C4, apoE and MIP-4 levels did not show significant difference among the groups.

We focused on a set of apoA1, C3, and TTR in further ROC statistical analysis. Multivariable statistical analysis indicated that combination of apoA1, C3 and TTR have clinical potential for distinguishing aMCI and AD from NDC.

ApoA1 is the main component of high-density lipoproteins (HDL) and is well-known for its critical role in reverse cholesterol transport. Previous studies revealed that CSF levels of apoA1 is lower in the AD patients (50) (51). Interaction of apoA1 and Aβ affected the processes of amyloid aggregates. The binding between apoA1 and Aβ protected neurons from Aβ-induced neurotoxicity (11). Deduction of C3 may reflect accelerating dysregulation of Aβ sequestration via complement system in the brain. Lower CSF and serum levels of C3 were associated with cognitive decline (52) (53). Plasma TTR levels were found to be associated with disease severity(54). Taking together, decreased levels of serum apoA1, C3 and TTR may cause failure in functions of Aβ

sequestration that promote synapse damage by Aβ neurotoxicity in disease progression of cognitive impairment.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P = 4.441E-05
ApoA1 Concentration (μg/ml)
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4000
3000
2000
1000
0
NDC
aMCI
AD
ApoA1
Sensitivity
100-Specificity
aMCI vs. NDC
AD vs. NDC
B
P = 3.74E-04
P = 1.26E-02
C3 Concentration (μg/ml)
120
80
40
0
NDC
aMCI
AD
C3
Sensitivity
100-Specificity
aMCI vs. NDC
AD vs. NDC
C
P = 0.0634
P = 0.0301
P = 1.08E-05
P = 4.071E-5
TTR Concentration (μg/ml)
1000
800
600
400
200
0
NDC
aMCI
AD
TTR
Sensitivity
100-Specificity
aMCI vs. NDC
AD vs. NDC

Figure 6.

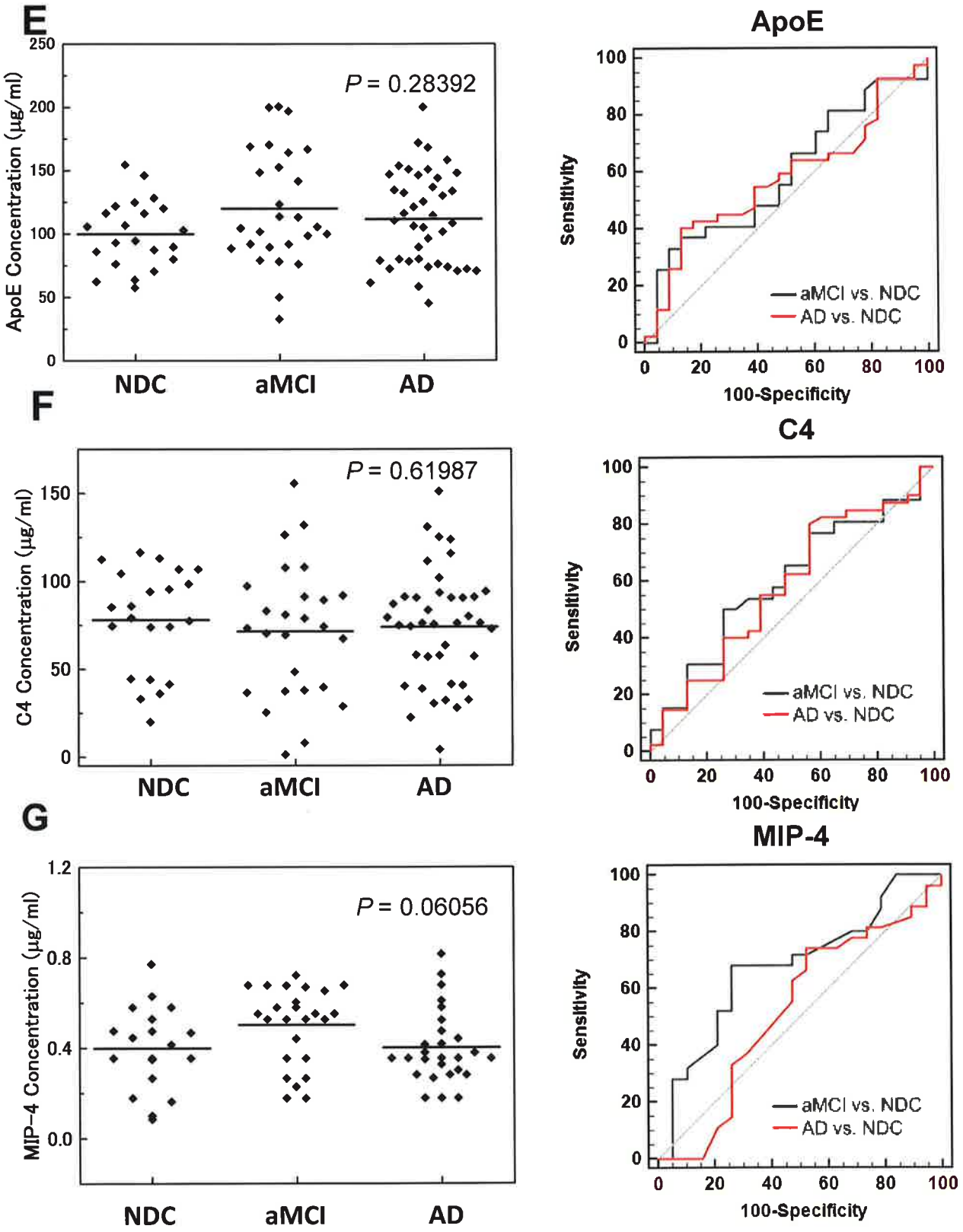
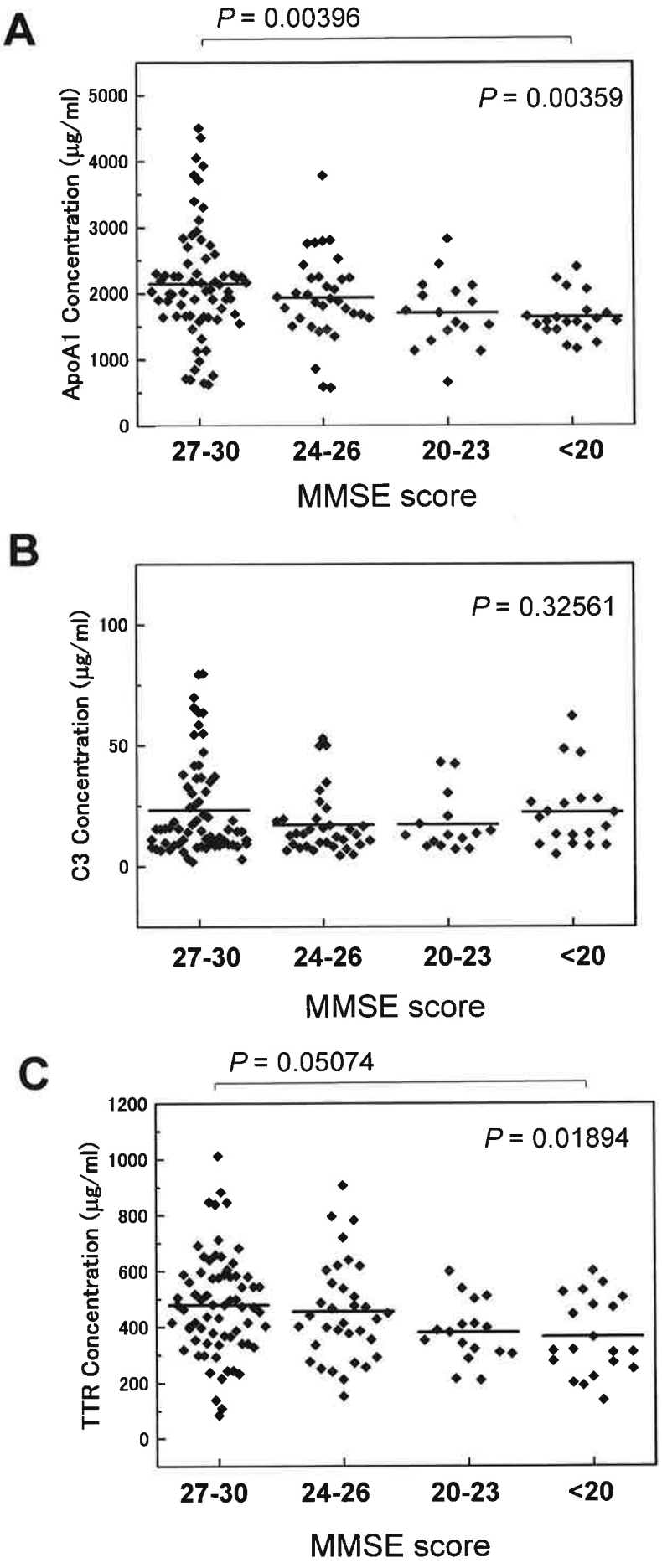


Figure 6. Serum levels of apoA1, C3, TTR, apoE, C4 and MIP-4 in NDC, aMCI and AD in cross-sectional study of the Tsukuba cohort

Serum levels of apoA1 (A), C3 (B), TTR (C), apoE (E), C4 (F) and MIP-4 (G) in NDC, MCI and AD (left panel) and *C*-statistics analyses for discriminating MCI (black solid line) and AD (red solid line) from NDC (right panel) are shown.


A
P = 0.00396
P = 0.00359
ApoA1 Concentration (μg/ml)
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4000
3000
2000
1000
0
27-30
24-26
20-23
<20
MMSE score
B
P = 0.32561
C3 Concentration (μg/ml)
100
50
0
27-30
24-26
20-23
<20
MMSE score
C
P = 0.05074
P = 0.01894
TTR Concentration (μg/ml)
1200
1000
800
600
400
200
0
27-30
24-26
20-23
<20
MMSE score

Figure 7.

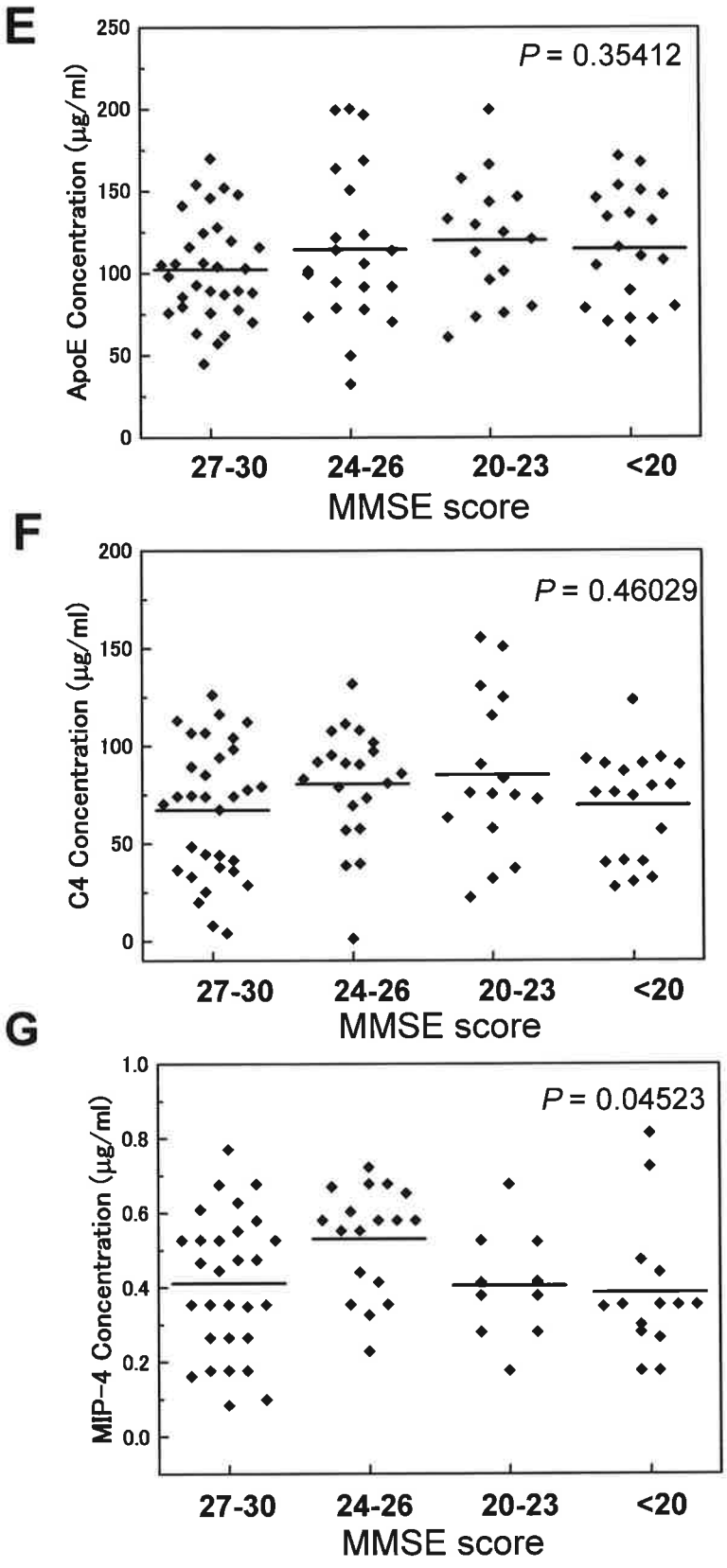

Figure 7. Relationship of serum levels of apoA1, C3, TTR, C4, apoE and MIP-4 to MMSE score in the Tsukuba cohort

Significant differences among three groups are indicated (Kruskal-Wallis test). Significant difference in protein levels between two groups are also indicated (Bonferroni test). MMSE score 27-30 vs. <20 were observed in apoA1 ($P = 0.0396$) and TTR ($P = 0.05074$).

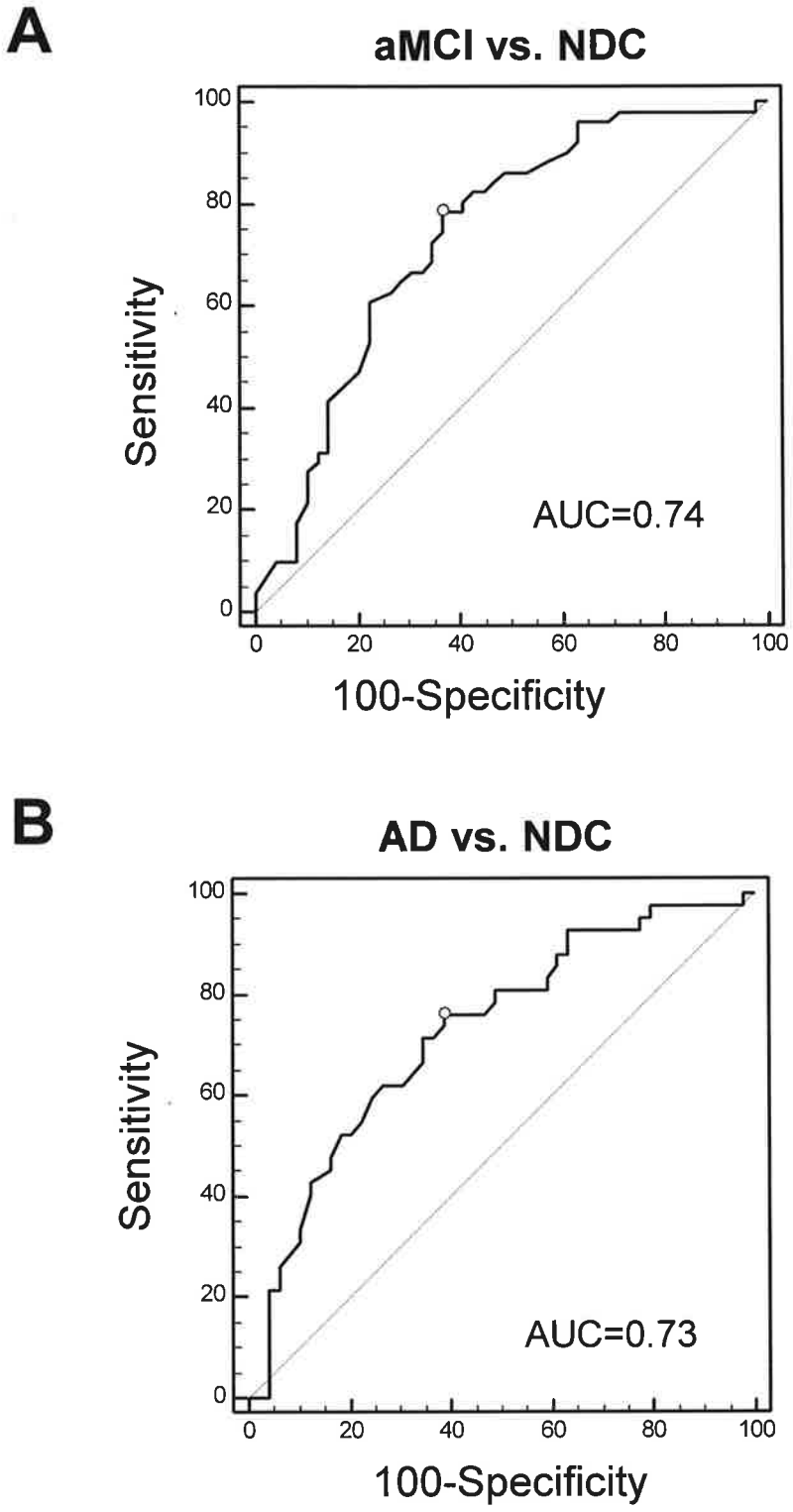

Figure 8. Evaluation of clinical potential of a set of serum apoA1, C3, and TTR in the cross-sectional study using logistic regression model

C-statistics analyses of combination of serum apoA1 and C3 as sequester proteins in aMCI vs. NDC (A), and combination of serum C3 and TTR in AD vs. NDC (B) are shown. aMCI vs. NDC; AUC = 0.74 ($P < 0.0001$), sensitivity 78%, specificity 63%. AD vs. NDC; AUC = 0.73 ($P < 0.0001$), sensitivity 76%, specificity 61%.

Chapter 3

Evaluation of apoA1, C3 and TTR as blood-based biomarker for cognitive impairment by multivariable regression in prospective study

3-1. Introduction

In validation of biomarker candidates, clinical performance of these biomarkers was analyzed by further clinical study using prospectively collected samples in independent cohort. Currently, the concept of preclinical AD was used to elucidate the link between the pathophysiological cascade of AD and the emergence of clinical symptoms (**Figure 1**) (55). Aβ accumulation, tau formation and neuronal injury results in emergence of clinical symptoms during progress of cognitive impairment. Subjects with preclinical stage AD are thought to have several pathophysiological changes, but cannot be diagnosed as MCI or dementia.

Although it is still lack of standardized clinical definition of preclinical AD, several pathophysiological events start in advance of AD onset. Duara *et al.* reported that a definable pre-MCI state may exist between MCI and NDC (56). The subjects with pre-MCI do not show obvious cognitive deficits and do not meet the criteria for MCI in neuropsychological cognitive test, but they indicate with hippocampus atrophy.

In this chapter, to assess the cognitive dysfunction in terms of time-course of disease progression, subjects who did not show predominant memory impairment, but indicated decreased cerebral blood flow or hippocampus atrophy were defined as 'pre-MCI'. In this study, we analyzed blood-based biomarkers for MCI and pre-MCI; relatively late stages in preclinical AD, i.e. precursor state to MCI in the point of view of continuum between NCI and MCI (56).

To confirm clinical utility of a set of biomarkers described in **Chapter 2** by multivariable analysis. In this study, prospectively collected samples including NDC, pre-MCI, MCI and AD were analyzed.

3-2. Methods

3-2-1. Participants

A total of 258 blood samples were collected prospectively in Uji Hospital (Kyoto, Japan) as Uji cohort study (Ethics Committee of Uji Hospital: Approval number: 2; Ethics Committee of MCBI: Approval number: 2013-01). In this study, statistical analysis was performed by using unlinked anonymous data from the Uji cohort which were provided by research division of MCBI, Inc. Among 258 participants, 64 were recruited for further clinical diagnosis. MMSE, interview to patients and their family, *APOE* genotyping, single-photon emission computed tomography (SPECT) imaging / MRI were performed.

MCI was diagnosed by Petersen criteria (4). Diagnosis of AD was made according to DSM-IV criteria. NDC was defined as cognitive normal, without neurological or psychiatric disease and no diagnosis of MCI or AD. In this study, pre-MCI stage was defined according to the progression of cognitive impairment. The subject with pre-MCI shows no obvious cognitive deficits, but may have decreased cerebral blood flow or hippocampus atrophy. Criteria of the clinical categorization is shown in the **Table 2**. Participants with any psychiatric illness according to DSM-IV criteria were excluded. Written informed consent was obtained from each participants, and all samples were rendered into anonymous prior to sending them to the laboratory for analysis.

In this study, MMSE score were used as clinical characteristics to measure cognitive decline and categorize possible intermediate state pre-MCI and severity in AD. Pre-MCI was defined as the participant with MMSE score of 27−29, or with atrophy of hippocampus by MRI scans, or with slight decreases of cerebral blood flow by SPECT imaging study. Early AD was defined as in those individuals with MMSE score above 21, both atrophy of hippocampus and decreased cerebral blood flow.

Participants were categorized into 4 groups in terms of brain atrophy in MRI: normal, mild, moderate and severe. Voxel-based Specific Regional analysis system for Alzheimer's disease (VSRAD) approach allows to measure atrophy of medial temporal

structures, especially entorhinal cortex, hippocampus and amygdala (57).

SPECT was used to analysis deduction of regional cerebral blood flow (rCBF) in the posterior cingulate gyrus, precuneus, and parietal cortex (58). The participants were categorized into 4 categories by deduction levels of rCBF in SPECT. **Normal**: No abnormality was observed in 2 region of interest (ROI) of posterior cingulate gyrus/precuneus or 2 ROI of parietal cortex. **Probable AD/DLB**: Decreased in 1 ROI of posterior cingulate gyrus/precuneus or 1 ROI of parietal cortex. **Suspected AD/DLB**: Decreased in 1 ROI of posterior cingulate gyrus/precuneus, and 2 ROI of posterior cingulate gyrus/precuneus and parietal cortex. **AD/DLB**: Decreased in 2 ROI of posterior cingulate gyrus/precuneus, and 3 ROI of posterior cingulate gyrus/precuneus and parietal cortex.

3-2-2. Serum sampling and multiple immunoassay

After coagulation of samples for 30 min at room temperature and centrifugation at 1,300 g for 15 min at 20℃, serum was transferred to 1.5 ml tubes (TreffLab, Degersheim, Switzerland). The samples were stored at –80°C until further use. Protein concentrations were determined by HNDG1-36K kit (C3, TTR, apoA1; EMD Millipore, Billerica, MA, USA) by researchers in MCBI, Inc.

3-2-3. Statistical analysis

A p value of 0.05 or less was considered significant. For data consisting of more than two groups, Kruskal-Wallis test was used and Bonferroni correction was applied when two groups were compared. The software Origin (ver. 7.5) (OriginLab Corp., MA, USA) and were used to perform statistics analysis.

We used multivariable linear regression to analyses the relationship between the

sequester proteins levels and severity of cognitive decline. Logistic regression is generalized linear model, in logistic regression outcomes are categorical, such as 0 or 1. It estimates the probability of two states either normal or onset of the disease.

$$F=1/(1+e^{-(a0 + aApoa1 \cdot x1 + aC3 \cdot x2)})$$

Where a0 is constant, and a[Apoa1], a[C3] are regression coefficients. They were calculated by MedCalc (ver. 9.3.9) (MedCalc Software, Mariakerke, Belgium). AUC of the ROC was calculated by MedCalc software. The closest point to the upper left corner of the ROC curve gave the optimum sensitivity and specificity values.

3-3. Results

3-3-1. Clinical performance of combination of sequester proteins as biomarker for cognitive decline

As shown in the **Table 3**, decreased apoA1 (P = 0.00411), C3 (P = 0.01699) and TTR (P = 0.03065) was observed among NDC, pre-MCI and MCI. Serum apoA1 (P = 0.02298) levels were lower in NCD than pre-MCI. And significant differences between NDC and MCI were observed in apoA1 (P = 0.03907) and C3 (P = 0.03305).

Furthermore, we used logistic regression equation derived from the Tsukuba cohort to assess the reproducibility of clinical potentiality of a set of sequester proteins in discrimination of MCI from NDC. **Figure 9** presented multivariable statistic results obtained from logistic regression. Significant differences were observed in NDC vs. AD (P = 0.01903), NDC vs. MCI (P = 0.0037) and NDC vs. pre-MCI (P = 0.00524). Surprisingly, logistic regression constant and coefficient derived by Tsukuba cohort in **Chapter 2** was sufficient in discriminating not only MCI but also pre-MCI from NDC with a high ROC value. (**Figure 10A**: AUC = 0.85, sensitivity 75% and specificity 80% in MCI vs. NDC; **Figure 10B**: AUC = 0.84, sensitivity 73% and specificity 90% in pre-

MCI vs. NDC; **Table 4**).

3-4. Discussion

As described in **Chapter 2**, combination of apoA1, C3, and TTR achieved high AUC value in discrimination between cognitively normal individuals and those with aMCI and AD by using serum samples in the Tsukuba cohort. To confirm their clinical utility, the samples collected form a prospective study in independent cohort were analyzed.

In this study, pre-MCI was defined in terms of MMSE score, decreased rCBF in the parietal lobe, posterior cingulate gyrus and precuneus on SPECT, and hippocampal atrophy on MRI. According to the Alzheimer's disease Neuroimaging Initiative (ADNI) criteria, the MMSE score for MCI is 24–30. We designated as pre-MCI those participants with MMSE score of 27–29, individuals with mild decrease of cerebral blood flow on SPECT images, or those with brain areas showing atrophy on MRI regardless of the MMSE score. Clinical potential of serum apoA1, C3, and TTR to discriminate early stages of cognitive impairment from NDC was tested. Combination of apoA1 and C3 differentiated both MCI and pre-MCI from NDC, and showed clinical potential for the assessment of cognitive decline.

The failure of Aβ clearance from the brain via the blood-brain barrier (BBB) may be the major factor in the accumulation of Aβ. Monitoring the proteins involved in protection of the synapse damage against Aβ toxicity during progression of cognitive impairment may help early intervention and more effective treatment.

We validated biomarker candidates by using amnestic MCI in the Tsukuba cohort. In Uji cohort, besides +MCI due to AD, MCI due to other type of dementia were possibly included. And a set of biomarker were useful for discriminating MCI from NDC. In this point of view, MCI in the Tsukuba and Uji cross-sectional studies may suggest that combination of three protein biomarkers might be useful for detection of other types of

MCI than MCI due to AD.

By cross-sectional and longitudinal analyses, combination of sequester proteins C3, apoA1 and TTR could be useful biomarker for screening high risk group proceeding to cognitive decline. Early detection of individuals with cognitive impairment may give us a chance to perform early intervention and to reduce MCI and AD.

Table 2. Clinical categorization criteria in the Uji prospective study

Stage	Clinical categorization criteria
NDC	MMSE = 30 and Atrophy of the hippocampus (-) and Decreased cerebral blood flow (±)
Pre-MCI	27 ≤ MMSE or Atrophy of the hippocampus (+) or Decreased cerebral blood flow (+)
MCI	24 ≤ MMSE and Atrophy of the hippocampus (+) and Decreased cerebral blood flow (+)
Early AD	21 ≤ MMSE and Atrophy of the hippocampus (+) and Decreased cerebral blood flow (+)
AD	16 ≤ MMSE and Atrophy of the hippocampus (+) and Decreased cerebral blood flow (+)
Severe AD	MMSE ≤15 and Atrophy of the hippocampus (+) and Decreased cerebral blood flow (+)

Table 3. Characteristics of paticipants with clinical diagnosis and serum C3, apoA1, and TTR levels in prospective study for MCI and AD

	NDC (n = 10)	Pre-MCI (n = 26)	MCI (n = 23)	Early AD (n = 5)	*P* value[†]
Age	62.6 ± 8.3*	65.5 ± 10.5	69.9 ± 9.6	77.0 ± 3.7	0.11499
Male/Female	2 / 8	7 / 19	14 / 9	1 / 4	
APOE e4 carrier, %	10.0	19.2	43.5	20.0	
ApoA1[‡]	2044.0 ± 235.9	1583.6 ± 470.4	1605.1 ± 381.4	1412.1.4 ± 520.4	0.00411[†]
C3[‡]	7.5 ± 2.3	5.7 ± 2.2	5.0 ± 2.2[¶]	6.2 ± 3.2	0.01699[†]
TTR[‡]	425.2 ± 104.2	339.8 ±144.9[§]	320.8 ± 126.0[¶]	373.2 ± 149.9	0.03065[†]

*mean ± SD

[†]Kruskal-Wallis test. Significant differences among 3 groups (NDC,Pre-MCI and MCI) are indicated.

[‡]μg/ml

[§]Bonferroni test. Significant differences between NDC and Pre-MCI were observed in apoA1 (P=0.02298).

[¶]Bonferroni test. Significant differences between NDC and MCI were observed in apoA1 (P=0.03907) and C3 (P=0.03305).

Table 4. Clinical potential of sequester proteins as biomarker for pre-MCI and MCI in the prospective study of the Uji cohort analyzed by logistic regression.

Disease	Biomarker	Sensitivity (%)	Specificity (%)	AUC	95% CI	*P* value	Criterion
MCI vs. NDC	ApoA1 C3	75	80	0.85	0.688 to 0.952	<0.0001	0.74
pre-MCI vs. NDC	ApoA1 C3	73	90	0.84	0.682 to 0.942	<0.0001	0.8

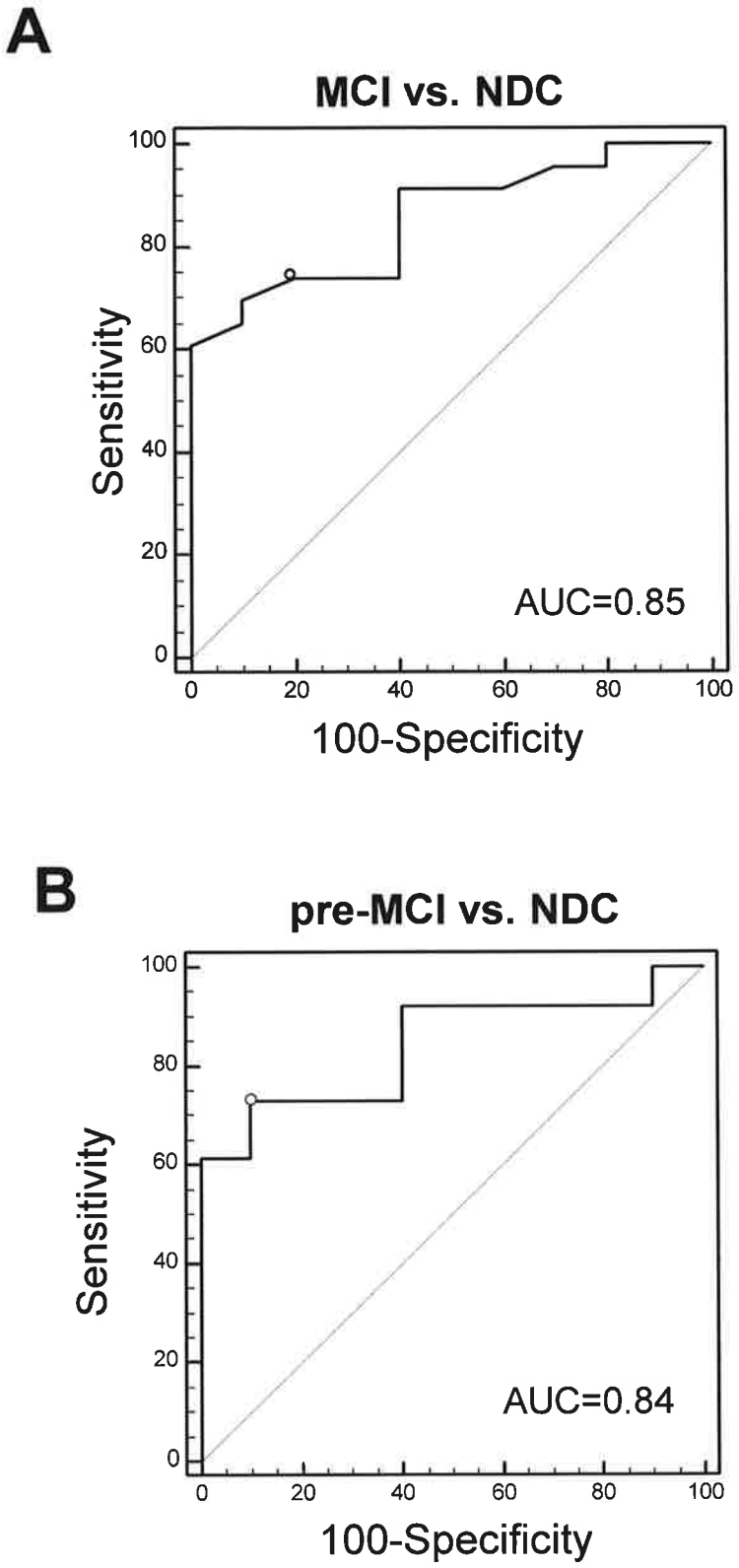

Figure 10. Evaluation of clinical potential of a set of serum apoA1 and C3 in prospective study using multivariable regression model in the Uji cohort

C-statistics analyses of combination of serum sequester proteins apoA1 and C3 in discriminating MCI and pre-MCI are shown in the (A) and (B). MCI vs. NDC; AUC = 0.85, sensitivity 75% and specificity 80%. pre-MCI vs. NDC; AUC = 0.84, sensitivity 73% and specificity 90%.

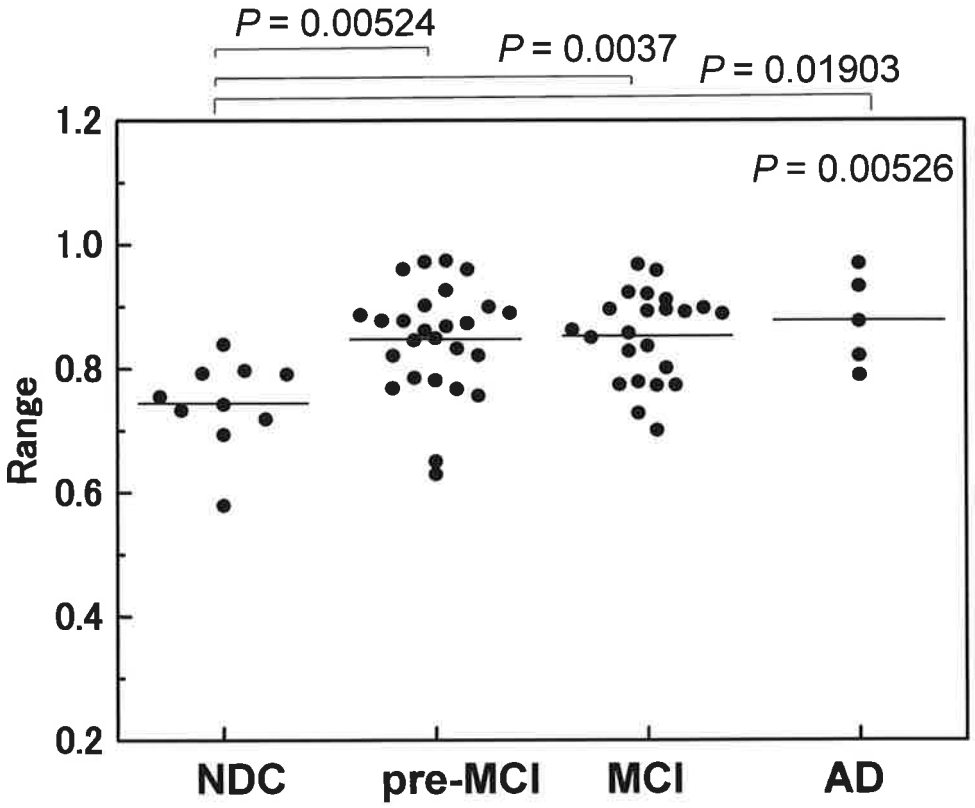

Figure 9. Analysis of clinical potential by multivariable statistic test in assessment of cognitive decline in the Uji cohort

Significant differences among groups are indicated ($P = 0.00526$). Between two groups significant differences were observed in NDC vs. AD (P = 0.01903), NDC vs. MCI (P = 0.0037) and NDC vs. pre-MCI (P = 0.00524).

Chapter 4

Establishment of the clinical laboratory assay for apoA1, C3 and TTR as risk assessment of cognitive decline

4-1. Introduction

As shown in the previous chapters, by longitudinal and cross-sectional analyses using independent cohorts, combination of sequester proteins; apoA1, C3 and TTR could distinguish cognitive impairment from NDC. To confirm clinical potential of these biomarkers for assessment of cognitive decline and to fulfill usefulness as routine clinical laboratory test, conventional immune-assay for the sequester proteins is required. In this chapter, clinical potential of apoA1, C3 and TTR in discriminating cognitive impairment from NDC was examined by using conventional immune-assay. High-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG), total cholesterol (TC), blood sugar (BS) and HbA1c were also analyzed.

4-2. Methods

4-2-1. Clinical data

64 participants with clinical diagnosis in Uji study were analyzed. The unlinked anonymous data from Uji cohort were obtained (**Table 3**). Written informed consent was obtained from each participants, and all samples were rendered anonymous prior to sending them to the laboratory for analysis.

4-2-2. Serum sampling and clinical assays

After coagulation of samples for 30 min at room temperature and centrifugation at 1,300 g for 15 min at 20℃, serum was transferred to 1.5 ml tubes (TreffLab, Degersheim, Switzerland). The samples were stored at –80°C for analysis. Quantification of serum samples was performed by Ikagaku. Inc, Kyoto, Japan. Concentrations of apoA1 and TRR were determined by turbidimetric immunoassay using Apo A-I Auto・N 'Dai-ichi' (Sekisui Medical, Tokyo) and N-Assay TIA Prealbumin Nittobo (Nittobo Medical,

Tokyo), respectively. C3 was quantified by enzyme-linked immunosorbent assay (Hokenkgaku Nisinihon, Kyoto). LDL, HDL, TC and TG were measured by enzymatic method using commercial kits (Sekisui Medical, Tokyo). Blood glucose and HbA1c were also measured by enzymatic method using Serotec GLU-L (Serotec, Sapporo) and CinQ HbA1c (LSI Medience Corp., Tokyo), respectively. The *APOE* genotyping was determined as follow (Ikagaku. Inc, Kyoto, Japan): after 40 cycle-amplification, the polymerase chain reaction (PCR) product was digested with the restriction enzyme *Hha*I, and subjected to electrophoresis in a 10% polyacrylamide gel.

4-2-3. Statistical analysis

A p value of 0.05 or less was considered significant. The Mann-Whitney and Kruskal-Wallis test were used in all cases to determine the statistical significance. Relationships between analytes and MMSE score were analyzed at bivariate correlation using the Pearson correlation coefficients. The software Origin (ver. 7.5) (OriginLab Corp., MA, USA) and were used to perform statistics analysis.

We used multivariable linear regression to analyses the relationship between the sequester proteins levels and severity of cognitive decline. Logistic regression is generalized linear model, in logistic regression outcomes are categorical, such as 0 or 1. It estimates the probability of two states either normal or onset of the disease.

$$F=1/(1+e^{-(a0 + aApoa1 \cdot x1 + aC3 \cdot x2)})$$

Where a0 is constant, and a[Apoa1], a[C3] are regression coefficients. They were calculated by MedCalc (ver. 9.3.9) (MedCalc Software, Mariakerke, Belgium). AUC of the ROC was calculated by MedCalc software. The closest point to the upper left corner of the ROC curve gave the optimum sensitivity and specificity values.

4-3. Results

4-3-1. Clinical performance of a set of sequester proteins as biomarkers for cognitive impairment

Figure 11 shows serum levels of the analytes in NDC and MCI. Lower levels of apoA1 ($P = 0.01513$), C3 ($P = 0.03277$), HDL ($P = 0.00264$) were observed in MCI group. *C*-statistics of apoA1, C3 and TTR in MCI vs. NDC showed following results; ApoA1: AUC = 0.77 (sensitivity 70%, specificity 80%, $P = 0.0011$); C3: AUC = 0.74 (sensitivity 74%, specificity 80%, $P = 0.0088$); TTR: AUC = 0.68 (sensitivity 70%, specificity 70%, $P = 0.0839$). Combination of apoA1 and C3 by logistic regression analysis showed that it had clinical potential in discriminating MCI from NDC with AUC = 0.82 (sensitivity 74%, specificity 90%, $P < 0.0001$) (**Figure 12**).

4-3-2. Levels of serum sequester proteins in terms of MMSE score of participants

We categorized participants according to MMSE score into 3 groups; groups with score 24-30 [n = 47], 20-23 [n = 20], and <20 [n = 19]. **Figure 13** summarized the results of relationship of analyte levels in serum and MMSE score. ApoA1 ($P = 0.01374$), HDL ($P = 0.00875$), TC ($P = 0.00306$) and TTR ($P = 0.09426$) indicated decrease trends in participants with lower MMSE score. Two groups' comparisons were conducted by Bonferroni test. Differences in MMSE score 24-30 vs. <20 were observed in apoA1 ($P = 0.00953$), HDL ($P = 0.01884$), TC ($P = 0.00215$) and TTR ($P = 0.05322$). Interestingly, TC levels were lower in group with MMSE score <20 comparing to group with 20-23 group ($P = 0.02498$).

4-3-3. Correlations of gender and age to serum levels of analytes

Eighty-six age-matched subjects (25 male and 61 female) were analyzed. The difference of analyte levels in gender was assessed by Mann-Whitney test. ApoA1 (P = 0.00625), HDL (P = 0.01943) and TC (P = 0.00383) levels in female serum were significantly higher comparing to those in male (**Table 5**). Correlation between age and decreased TTR were observed (r = -0.33, P = 0.00216) (**Table 6**).

4-3-4. Serum levels of analytes in *APOE* ε4-positive and-negative groups

Subjects were categorized into *APOE* ε4-positive and-negative groups. *APOE* ε4-positive group had lower serum apoE concentration comparing with *APOE* ε4-negative group. However, *APOE* ε4 did not affect serum levels of other analytes (**Table 7**).

4-4. Discussion

In previous chapters, multiple immunoassay indicated low levels of serum apoA1, C3 and TTR could be used as blood-based biomarkers for assessment cognitive impairment. In this study, routine clinical laboratory assays were applied to measure apoA1, C3 and TTR. Significant correlation between results from Luminex data and conventional assay data were obtained (**Table 8**). Combination of apoA1 and C3 discriminated MCI from NDC with high AUC value in ROC analysis.

ApoE is the major protein that functions as lipids transporter in the brain. People who develop AD are more likely to have an *APOE* ε4 gene polymorphism, which was considerate as risk-factor, because it favors the deposition of Aβ in the brain. We investigated the relationship between the presences of *APOE* ε4 and sequester proteins levels. Serum levels of apoE depended on gene polymorphism. However, sequester proteins apoA1, C3 and TTR did not changed in *APOE* ε4 positive and negative groups.

Lower levels of HDL were also associated with cognitive decline. HDL plays an

important role by removing cholesterol from peripheral tissues for hepatic degradation. Previous report indicated that individuals affected by dementia were significantly associated with lower HDL (59). Yasuno *et al.* reported that significant higher HDL concentration were associated with better cognitive function in the *APOE* ε4- group. In *APOE* ε4+ group, individuals with hypertension had lower cognitive score (60). In this study, decreased levels of apoA1, HDL and TC in participants with cognitive impairment were observed. Significant difference between genders were found, and female have higher apoA1, HDL, LDL and TC levels than male.

Lower levels of HDL is considered as a risk factor for atherosclerotic diseases and chronic hypertension, inducing cerebral hypoxia/ischemia, leading to brain damage. AD patients with cardiovascular comorbidities and risk factors showed lower levels of HDL (61). When either cardiovascular disease or stroke were excluded in the study design, higher levels of HDL were associated with better cognitive function (62). If AD and vascular dementia coexist in patients, it may affect its progression of disease and severity of cognitive decline in the early stage of disease.

In conclusion, combination of apoA1 and C3 measured by the routine clinical lab assays discriminated MCI from NDC with high ROC value. HDL and TC also indicated significantly lower levels in serum of participants with cognitive decline. Larger scale prospective cohort studies are needed to examine clinical utility of these sequester proteins as biomarkers for risk assessment of MCI and preclinical stage of AD.

Table 5. Correlation of gender and serum levels of analytes

Characteristics	Male (n=25)	Female (n=61)	*P* value[†]
Age	80.4 ± 7.4*	83.1 ± 5.2	0.1526
C3[§]	1180.9 ± 645.7	1017.7 ± 549.2	0.25177
C4[‡]	25.7 ± 4.3	25.1 ± 5.8	0.68749
TTR[‡]	23.2 ± 7.1	20.4 ± 5.6	0.09045
ApoA1[‡]	134.8 ± 25.5	153.1 ± 27.8	0.00625[†]
ApoB[‡]	90.4 ± 18.0	98.1 ± 21.1	0.25723
ApoE[‡]	3.6 ± 0.8	4 ± 0.9	0.19187
HDL[‡]	51.4 ± 13.7	61.5 ± 17.2	0.01943[†]
LDL[‡]	103.9 ± 22.7	116.4 ± 24.4	0.05814
TC[‡]	169.7 ± 28.4	193 ± 27.1	0.00383[†]
TG[‡]	140.4 ± 69.0	143.1 ± 78.2	0.83639
BS[‡]	129.1 ± 42.0	119.3 ± 45.0	0.22211
HbA1c, %	5.8 ± 0.6	5.7 ± 0.7	0.47739
MMSE score	22.5 ± 5.9	23.7 ± 5.6	0.35232

*mean ± SD

[†]Mann-Whitney test. Significant differences among 2 groups are indicated.

[‡]mg/dL

[§]Unit/μL

Table 6. Correlation of age and serum levels of the analystes

	Age	
Characteristics	coefficient r	*P* value[†]
C3	-0.04245	0.69795
C4	0.08373	0.47508
TTR	-0.32643	0.00216[†]
ApoA1	0.05636	0.60624
ApoB	0.23176	0.06322
ApoE	0.112	0.33874
HDL	-0.02097	0.85825
LDL	0.22615	0.05106
TC	0.15362	0.18822
TG	0.01545	0.89536
BS	0.02754	0.80962
HbA1c	0.03611	0.74133

[†] Pearson's correlation

Table 7. Serum levels of analytes in *APOE* ε4-positive and-negative groups

Characteristics	APOE ε4- (n=70)	APOE ε4+ (n=16)	*P* value[†]
Age	83.0 ± 5.6*	79.3 ± 6.9	0.03087[†]
C3[§]	1107.7 ± 612.9	879.2 ± 362.7	0.20581
C4[‡]	25 ± 5.5	26.5 ± 4.8	0.47738
TTR[‡]	20.9 ± 5.7	22.3 ± 8.1	0.65708
ApoA1[‡]	148.5 ± 27.1	144.9 ± 33.8	0.6732
ApoB[‡]	96.4 ± 20.7	93.7 ± 19.6	0.89555
ApoE[‡]	4.1 ± 0.8	3.3 ± 1.0	0.00619[†]
HDL[‡]	58.6 ± 16.1	59.1 ± 20.4	0.97886
LDL[‡]	113.1 ± 24.5	111.9 ± 29.1	1
TC[‡]	187.6 ± 26.1	182.3 ± 40.4	0.52915
TG[‡]	150 ± 10.0	111.5 ± 14.8	0.08506
BS[‡]	125.6 ± 46.4	109.3 ± 30.9	0.07188
HbA1c, %	5.8 ± 0.7	5.7 ± 0.4	0.88497
MMSE score	23.4 ± 5.7	23.3 ± 5.9	0.89374

*mean ± SD

[†]Mann-Whitney test. Significant differences among 2 groups are indicated.

[‡]mg/dL

[§]Unit/μL

Table 8. Correlation between measured values of analystes by Luminex and conventional clinical chemistry assays

Characteristics	Luminex and routine chemistry system coefficient r	*P* value[†]
C3	0.16243	0.0076[†]
TTR	0.43746	4.77E-14[†]
ApoA1	0.34785	3.49E-09[†]

[†] Pearson's correlation

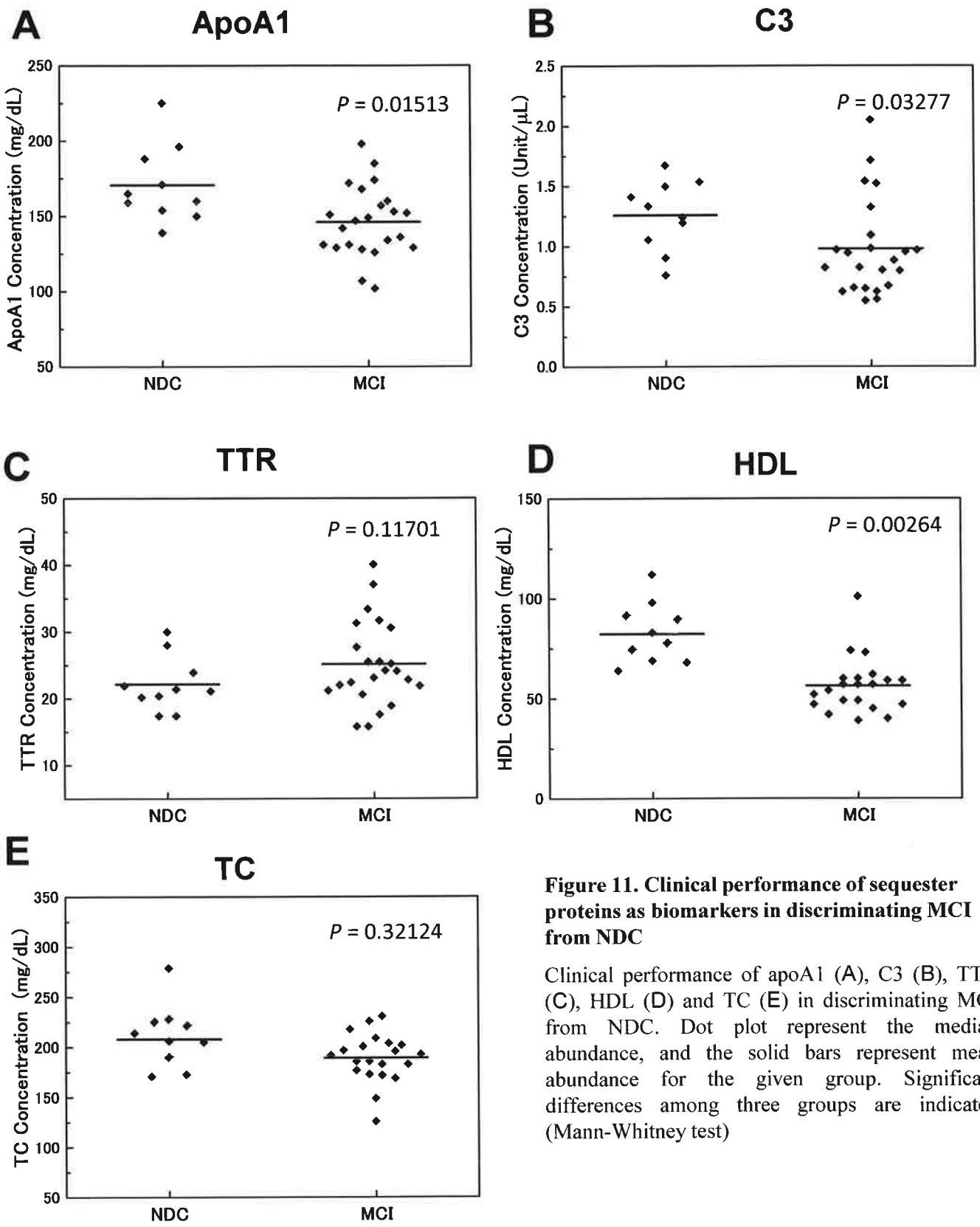

Figure 11. Clinical performance of sequester proteins as biomarkers in discriminating MCI from NDC

Clinical performance of apoA1 (A), C3 (B), TTR (C), HDL (D) and TC (E) in discriminating MCI from NDC. Dot plot represent the median abundance, and the solid bars represent mean abundance for the given group. Significant differences among three groups are indicated (Mann-Whitney test)

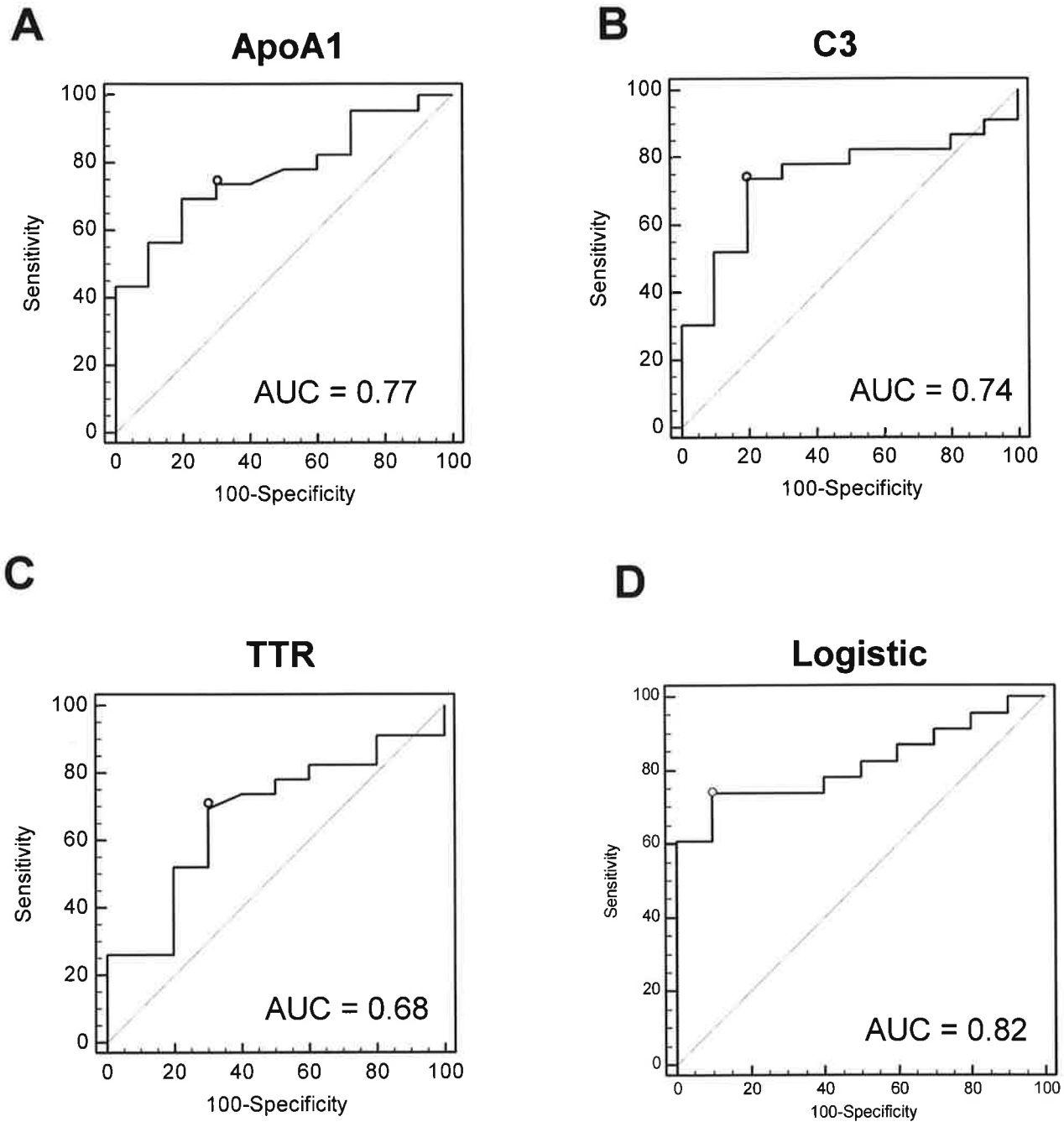

Figure 12. *C*-statistics analyses of apoA1 (A), C3 (B), TTR (C) in MCI vs. NDC

ApoA1: AUC = 0.77 (sensitivity 70%, specificity 80%, $P = 0.0011$); C3: AUC = 0.74 (sensitivity 74%, specificity 80%, $P = 0.0088$); TTR: AUC = 0.68 (sensitivity 70%, specificity 70%, $P = 0.0839$). Using logistic regression model, combination of apoA1 and C3 was sufficient in discriminating MCI from NDC with AUC = 0.82. (sensitivity 74%, specificity 90%, $P < 0.0001$).

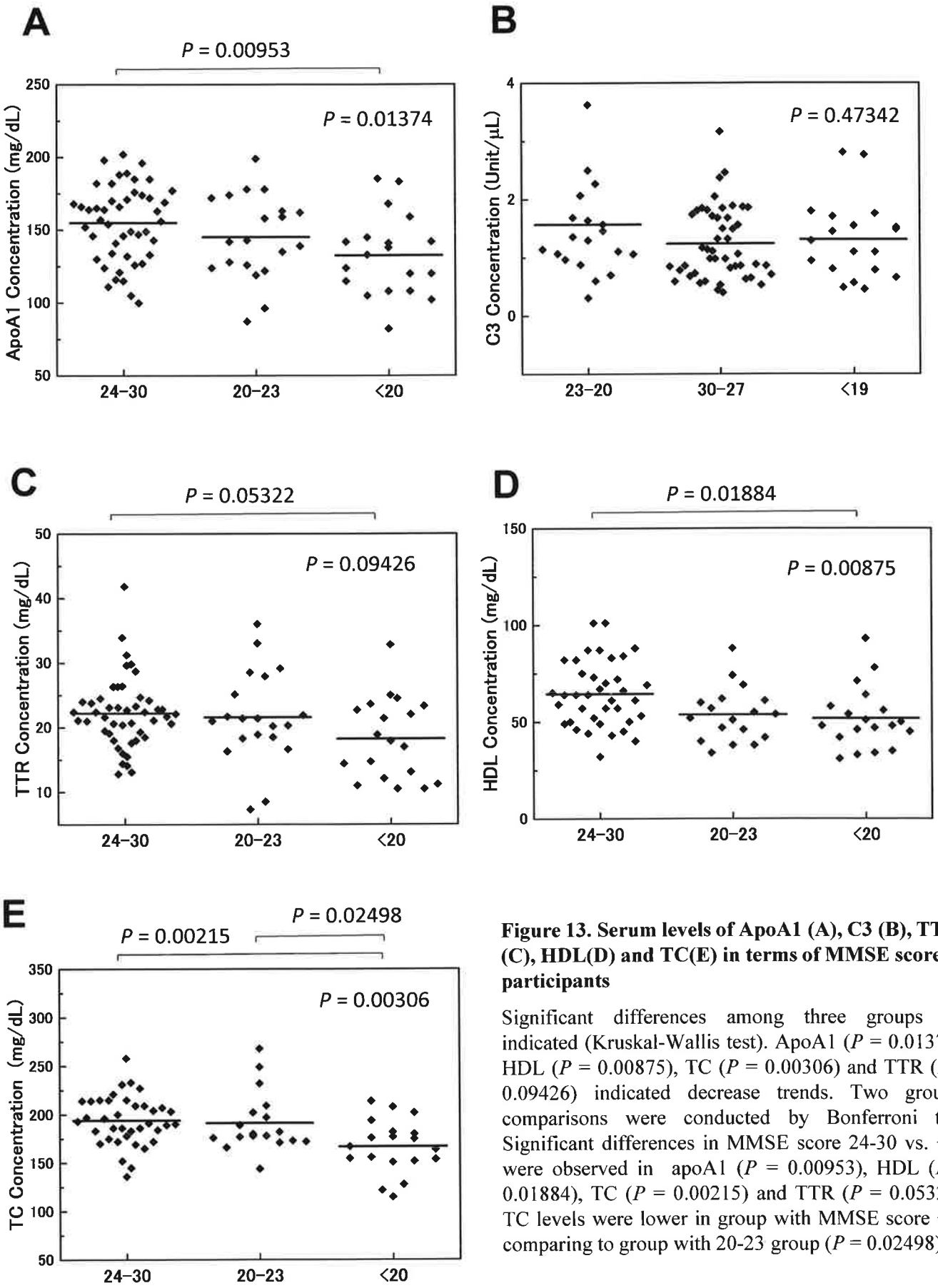

Figure 13. Serum levels of ApoA1 (A), C3 (B), TTR (C), HDL(D) and TC(E) in terms of MMSE score of participants

Significant differences among three groups are indicated (Kruskal-Wallis test). ApoA1 (P = 0.01374), HDL (P = 0.00875), TC (P = 0.00306) and TTR (P = 0.09426) indicated decrease trends. Two groups' comparisons were conducted by Bonferroni test. Significant differences in MMSE score 24-30 vs. <20 were observed in apoA1 (P = 0.00953), HDL (P = 0.01884), TC (P = 0.00215) and TTR (P = 0.05322). TC levels were lower in group with MMSE score <20 comparing to group with 20-23 group (P = 0.02498).

Conclusions

Jack *et al.* proposed a model of AD pathogenesis (8). During aggregation of Aβ in the brain, synapse damage gradually become prominent, and eventually neuronal dysfunction causes clinical symptoms of cognitive impairment. Prior to brain atrophy detected by MRI, series of pathologic changes in individuals occur. AD pathogenesis starts more than 10–20 years before the onset. Thus, in pre-clinical stages, biomarker which can detect the disease progression is crucial for early intervention and prevention of dementia.

Venipuncture is suitable and applicable for screening of AD and MCI in early stages, because it allows us large scale examination as routine health check. In this point of view, blood-based biomarkers have great advantage for early diagnosis and intervention in dementia. Discovery of blood-based biomarker by proteomics analysis must contribute to development of potential diagnostic tool in cognitive impairment.

This study indicates combination of apoA1, C3, and TTR is useful for the assessment of cognitive decline. Clinical utility of these blood-based biomarkers were confirmed by using conventional immune-assay which is available in most clinical chemistry laboratories. Screening and early detection of cognitive impairment by biomarkers identified in this study and early intervention might delay progression of cognitive decline and contribute to reduce the incidence of dementia.

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