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Abstract

Alzheimer's disease (AD) accounts for around 60 to 70% of dementia cases and is a problem faced by societies throughout the world. Clinical trials of disease-modifying drugs target subjects with early-stage AD, hence blood-based biomarkers are needed for subject screening. We combined immunoprecipitation (IP) with MALDI-TOF MS to develop IP-MALDI-MS, an analytical technique that resulted in the first successful detection of plasma amyloid β ($A\beta$) by mass spectrometry. This technique was used to identify plasma APP669–711/ $A\beta$ 1–42 ratio, a novel biomarker that correlates with amyloid PET. Analysis of samples from subjects in Japan and Australia also revealed a high degree of concordance between amyloid PET and plasma APP669–711/ $A\beta$ 1–42 ratio, plasma $A\beta$ 1–40/ $A\beta$ 1–42 ratio, and a combination of these two biomarkers (a composite biomarker). As advances are made in disease-modifying drugs, blood-based biomarkers will become increasingly valuable due to their potential role for screening subjects and monitoring drug effects in clinical trials, and as adjuncts in the diagnosis of AD in clinical practice.

Keywords: Alzheimer's disease, Amyloid β , Blood-based biomarker, MALDI-TOF MS, Plasma

1. Introduction

This issue's theme of advanced technology development is covered in this article by focusing on blood-based biomarker measurement technology. The development of analytical techniques must be approached not only as a technical challenge for analytical chemistry, but also with an understanding of the social issues to be resolved. As such, this article starts by outlining the significance of biomarkers and the health care situation regarding dementia, a problem facing societies throughout the world. Around 55 million people currently suffer from dementia worldwide, a number that is predicted to rise to around 78 million by 2030 as the world's elderly population grows. The societal cost of dementia is also predicted to reach US\$ 2.8 trillion by 2030, and comprehensive initiatives encompassing diagnosis, prevention, treatment, care, and community services are urgently needed to deal with the disease.¹⁾ Dementia itself is a general term used to refer to diseases that cause a significant loss of nerve cells in the brain, leading to cerebral atrophy and decline in cognitive function. Alzheimer's disease (AD) is a type of dementia and accounts for 60 to 70% of all dementia cases. Drugs that are currently prescribed for AD, such as the acetylcholinesterase inhibitor donepezil,²⁾ inhibit the breaking down of the neurotransmitter acetylcholine but do

not stop the loss of nerve cells that produce neurotransmitters. Since a loss of nerve cells inevitably leads to reduced neurotransmitter production, inhibiting the breakdown of neurotransmitters only has a limited effect. As such, therapeutics for AD are merely symptomatic therapy and do not prevent the progression of AD itself. Therapeutics are needed that can prevent cognitive decline at a more fundamental level, or disease-modifying drugs are needed that slow down the speed of cognitive decline.³⁾ Drugs that treat the underlying disease have been tested in patients developing AD in clinical studies around the world, but almost all have ended in failure. The key to resolving this failure is found in biomarkers of AD, an area that has become increasingly important in recent years.

2. What is Alzheimer's Disease?

Histopathological hallmarks of AD include senile plaques formed by aggregation and accumulation of amyloid beta ($A\beta$) in the brain and neurofibrillary tangles formed by Tau protein aggregation. A definitive diagnosis of AD is reached based on these pathological features in

addition to cognitive symptoms. Since a pathological autopsy cannot be performed on a living person's brain, AD is diagnosed based on clinical symptoms using neurophysiological tests and other tools, and the term "probable AD" is used to indicate a diagnosis of AD that is not quite definite.⁴⁾ Recent years have seen the emergence of amyloid PET, an imaging technique capable of visualizing the abnormal accumulation of A β in the brain that provides a highly accurate estimate of whether a living patient is positive or negative for A β accumulation. This breakthrough technology revealed there is a 30% discrepancy between conventional clinical-based diagnoses of AD and actual AD pathology.⁵⁾ A DIAN study in the USA also showed that A β starts to accumulate in the brain more than 20 years before the onset of clinical symptoms of AD.⁶⁾ Based on these findings, biomarkers for AD pathology are now considered an essential element of accurate diagnostic criteria. The ATN system was established as a biomarker-based attempt to classify disease type and progression.^{7),8)} The framework is classified based on the presence or absence of A β accumulation (A), Tau accumulation (T), and neurodegeneration (N). The typical course of AD progression begins with gradual accumulation of A β , followed by accumulation and spread of Tau protein that induces neurodegeneration, resulting in the manifestation of cognitive decline as clinical symptoms (**Fig. 1**). This course of progression is called the AD continuum, and many researchers now consider A β accumulation as the start of AD. AD is perceived differently in clinical practice and research field, and understanding this difference is important. In clinical practice, AD is diagnosed after clinical symptoms appear, but in research field, AD is considered to start from an earlier phase of disease when A β accumulation begins. Many pharmaceutical companies have recently been allocating significant resources to developing disease-modifying drugs that target A β .⁹⁾

However, though A β is a factor in AD pathology, whether A β is a cause of AD is still a matter of debate. Familial AD is a form of AD that tends to develop genetically. Given these patients have genetic mutations in the amyloid precursor protein (APP) and in γ -secretase, which is an enzyme involved in the production of A β , the current prevailing theory is that A β is a cause of AD.¹⁰⁾

Why have researchers failed to develop a drug that treats underlying AD? A possible reason for this failure may be because clinical studies have recruited patients that progress to moderate or severe AD already. The AD continuum begins with A β accumulation, but preventing A β accumulation after cognitive decline is apparent does not prove effective as the neurodegenerative process cannot be stopped once it begins. Put simply, screening subjects in clinical studies was not appropriate. Recognizing this, pharmaceutical companies have adjusted their approach and are recruiting patients with early-stage AD in clinical trials. However, early-stage AD is difficult to diagnose based only on clinical symptoms as they are so mild, and it is becoming clear that AD pathology cannot be determined accurately based on clinical diagnosis alone. This makes it essential to have biomarkers that can reveal AD pathology. The gold standard methods used to detect biomarkers of AD pathology are amyloid PET and an immunoassay for A β 42 in cerebrospinal fluid (CSF). There are various different A β species with amino acid sequences of different lengths. In this article, these different A β peptides are generally represented by specifying the C-terminus residue, such as A β 42 or A β 40, or as A β 1-42 or A β 1-40 when the full length of the peptide is characterized. Because amyloid PET requires a large PET system and is expensive, and due to the invasiveness of collecting CSF to measure CSF A β 42, there was a demand for blood-based biomarkers that are simple to measure.

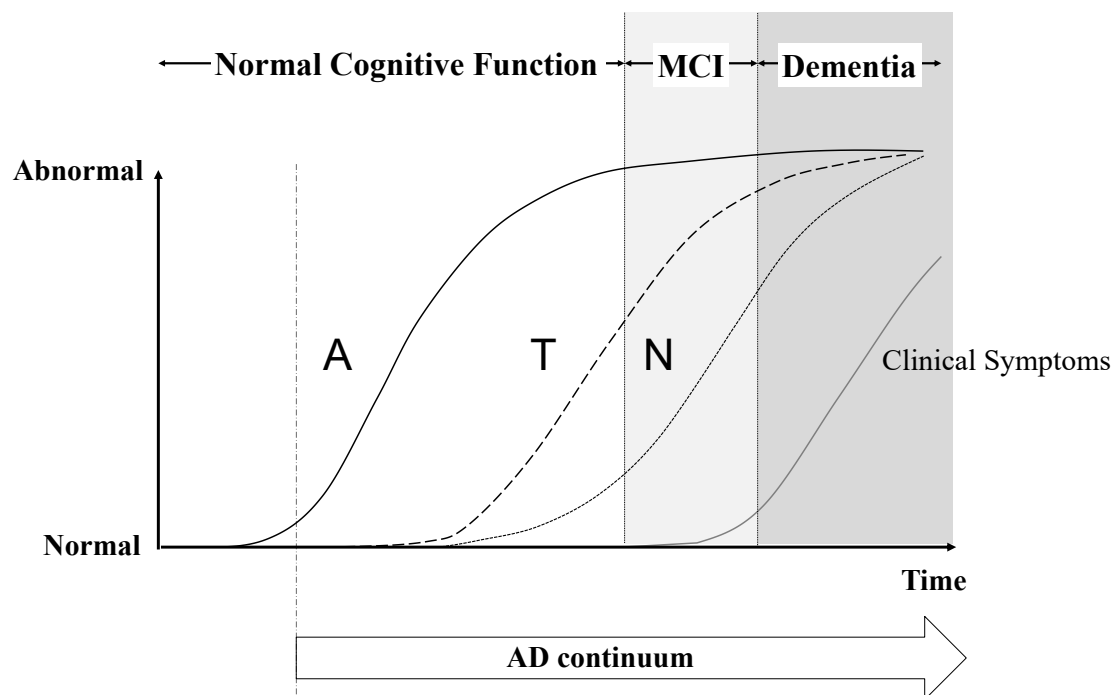


Fig. 1 Hypothetical biomarker-based model of AD progression
A: A β accumulation, T: Tau accumulation, N: Neurodegeneration

3. Blood A β Analysis: IP-MALDI-MS

A β peptides are important molecules in AD and have long been targeted as a blood-based biomarker. Although various studies on biomarkers have been reported by various research groups, the data is inconsistent and produced varied results, creating an inclination among researchers to dismiss A β 42 as a blood-based biomarker.¹¹⁾ Despite this, we set out to research and develop a method of measuring A β in blood by mass spectrometry. At the time, blood A β had only ever been evaluated using immunoassay methods, and no studies had used mass spectrometry to investigate A β as a blood-based biomarker. This omission was probably due to an absence of prior studies demonstrating the detection of low A β levels in blood by mass spectrometry. As such, this was the first challenge to be addressed.

Mass spectrometry is an analytical technique used in a variety of different fields and known to be capable of measuring minute quantities of analytes with high sensitivity. Mass spectrometry is also frequently used in proteomics and metabolomics as it offers the benefits of comprehensive analysis in simultaneously detecting analytes of different masses. Conversely, when a sample contains large amounts of impurities, the strong signal from these impurities drowns out the signal from analytes only present in small amounts, and blood plasma samples typically contain large amounts of impurities.¹²⁾ This has prevented the use of mass spectrometry to measure biomarkers in plasma samples with high sensitivity. Sensitivity can be improved by removing proteins that are present in high concentrations or by separating analytes by liquid chromatography, but these methods do not achieve the sensitivity required to detect A β , which is present at several pM. Sufficient sensitivity was probably prevented by inadequate separation of impurities or because A β was

absorbed to impurities during separation. We succeeded in selectively separating and concentrating only A β by introducing an antibody-based immunoprecipitation (IP) step into the sample preparation process. This analytical technique is typically referred to as immunoprecipitation-mass spectrometry (IP-MS). The method we developed also uses MALDI-TOF mass spectrometry, hence was named IP-MALDI-MS (**Fig. 2**). An IP-MS method for detecting A β was already reported in 1996 and could readily detect A β in samples of cell culture supernatant and CSF,¹³⁾ but blood has many more impurities and lower A β concentrations than culture supernatant and CSF, preventing the detection of A β in blood by IP-MS. To overcome these issues, we optimized the conditions used during IP, examining the preparation of antibody-coated beads, surfactant and eluate compositions during IP, and identifying a matrix solution composition suitable for samples after IP to establish a set of IP-MS conditions tailored to plasma A β analysis. This resulted in the first successful detection of endogenous A β 1-42 and A β 1-40 in human plasma by mass spectrometry¹⁴⁾ and simultaneously produced other discoveries only available using mass spectrometry. Specifically, mass spectrometry revealed the presence of many A β -related peptides other than A β 1-42 and A β 1-40 in human plasma, including A β species with an N-terminus elongated beyond A β 1-x (**Fig. 3**).

4. Amyloid Beta (A β)

This section describes the A β production pathway. A β is generated when the integral membrane protein APP is cleaved at N-terminus residue 672 (β -site) by β -secretase and subsequently released by γ -secretase cleavage at the C-terminus site (**Fig. 3**). γ -secretase can cleave at multiple sites, producing A β s of different lengths including A β species with a C-terminus at residue 38th, 40th, or 42th.¹⁵⁾ By contrast, β -secretase only cleaves at the β -site

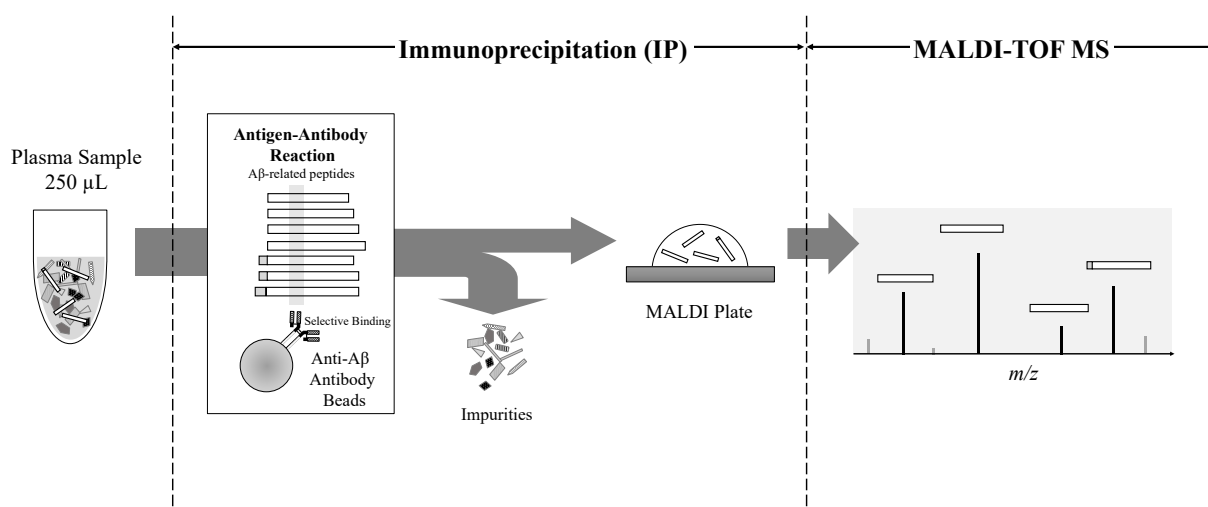


Fig. 2 Schematic diagram of IP-MALDI-MS

and β '-site (N-terminus residue 11 th) and is not known to cleave at other sites. Accordingly, until performing our research, the presence of A β species in human blood with an N-terminus cleaved outside the β -site was unknown. Our IP-MALDI-MS method was the first to detect A β species cleaved several residues beyond the β -site towards the N-terminus. In this article, the A β species cleaved three residues beyond the β -site towards the N-terminus possessing the same C-terminal residue as A β 1-40 is called APP669-711. The previous chapter mentioned that mass spectrometry offers the advantage of comprehensive analysis due to its ability to simultaneously detect molecules of different masses. Although the sensitivity of immunoprecipitation was improved for plasma samples by focusing on A β , using mass spectrometry as a detection method also allowed the discovery of APP669-711, which was previously unknown in plasma. Beyer et al. then suggested that proteases other than β -secretase were involved in N-terminal cleavage of APP669, though the protease responsible for this cleavage has yet to be identified.¹⁶⁾ Research is currently underway to identify the protease that cleaves APP669 and reveal the mechanism of APP669-711 production.

5. Blood-Based Biomarker Discovery Study

The next phase of research was to search for a blood-based biomarker. This required clinical specimens and medical knowledge, hence the search for an A β biomarker by IP-MALDI-MS commenced in 2013 in cooperation with the National Center for Geriatrics and Gerontology.

At the time, almost all blood-based biomarker studies used clinical symptoms as indicators to classify subjects into AD, mild cognitive impairment (MCI), and cognitive normal control, and performed measurements and analyses aimed at discriminating these clinical stages.¹⁷⁾ Amyloid PET showed that A β accumulation appear earlier than clinical symptoms, and revealed a certain number of cases of discrepancy between A β accumulation and the clinical diagnosis, which was a novel finding at the time. Therefore, our biomarker discovery study aimed to find a marker for A β accumulation in the brain rather than a marker for clinical symptoms, using the results of amyloid PET as an indicator. This biomarker discovery study using IP-MALDI-MS and amyloid PET (62 cases) discovered a higher ratio of APP669-711 to A β 1-42 (APP669-711/A β 1-42) in plasma from amyloid PET-positive patients (Fig. 4). Receiver operating characteristic (ROC) analysis of the APP669-711/A β 1-42 ratio for amyloid PET-positive patients also showed a high area under the curve (AUC) value of 96.9%. The APP669-711/A β 1-42 ratio was also significantly correlated with the standardized uptake value ratio (SUVR) of amyloid PET, with correlation coefficient of 0.687.¹⁸⁾ Based on this data, a correlation between plasma APP669-711/A β 1-42 ratio and amyloid PET was reported for the first time in 2014.

6. Validation Study

Insufficient reproducibility is a common issue with newly discovered blood-based biomarkers that often leads to their abandonment.^{19), 20)} Potential reasons for



Fig. 3 Plasma A β -related peptides detected by IP-MALDI-MS

abandonment include the reproducibility of the analytical method, the instability of target species in the sample, and markers specifically for the specimens used in the discovery study that are not generalizable. Therefore, the blood-based biomarker needed to be validated using specimens that were entirely independent of those used in the discovery study. A validation study was conducted in two datasets from Japan's National Center for Geriatrics and Gerontology and the Australian Imaging, Biomarker & Lifestyle Flagship Study of Aging (AIBL).²¹⁾ ROC analysis of the APP669-711/A β 1-42 ratio for amyloid PET-positive cases (using a PIB tracer) revealed a high

AUC value of 92.3% in the Japanese dataset (121 cases) and 89.5% in the Australian dataset (111 cases) (**Table 1**). A composite biomarker created by combining APP669-711/A β 1-42 ratio and A β 1-40/A β 1-42 ratio (an well-known biomarker) also improved AUC values to 96.7% in the Japanese dataset and 94.1% in the Australian dataset. The concentration of A β 1-42 in CSF is known to decrease when A β accumulates in the brain, a phenomenon that was similarly identified in our data, with reduced plasma levels of A β 1-42 in amyloid PET-positive patients. This change in A β 1-42 concentration may be caused by A β 1-42 being trapped in the brain, which lowers the

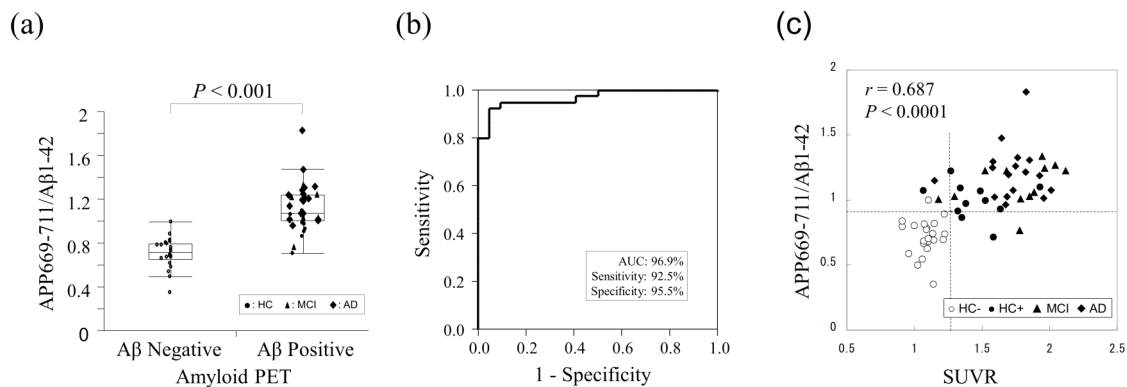


Fig. 4 Biomarker evaluation of plasma APP669-711/A β 1-42 ratio

(a) Box plot (A β positive vs. A β negative)

(b) ROC analysis

(c) Correlation with quantitative findings of amyloid PET (SUVR)

Biomarker	Evaluation Indicator	National Center for Geriatrics and Gerontology (n=121)	AIBL (n=111)
A β 1-42	AUC	87.2%	75.7%
	Sensitivity	74.0%	78.3%
	Specificity	88.7%	66.7%
	Accuracy	82.6%	73.0%
APP669-711/A β 1-42	AUC	92.3%	89.5%
	Sensitivity	68.0%	86.7%
	Specificity	91.5%	74.5%
	Accuracy	81.8%	81.8%
A β 1-40/A β 1-42	AUC	96.7%	88.9%
	Sensitivity	96.0%	90.0%
	Specificity	87.3%	70.6%
	Accuracy	90.9%	81.1%
composite biomarker	AUC	96.7%	94.1%
	Sensitivity	86.0%	91.7%
	Specificity	88.7%	82.4%
	Accuracy	87.6%	87.4%

Table 1 Evaluation data for each biomarker in validation study

The cut-off values for each biomarker are the same as those used by the National Center for Geriatrics and Gerontology and AIBL.

amount of A β 1-42 available to enter bodily fluids. ROC analysis of the blood concentration of A β 1-42 alone also showed AUC values of 87.2% (Japanese dataset) and 75.7% (Australian dataset), indicating A β 1-42 alone was inadequate as a biomarker, though these AUC values increased when A β 1-42 concentration was taken as a ratio of other A β species. This increase in AUC is probably due to individual variations in the total concentration of all A β species in the blood, which lowers the effectiveness of using absolute A β 1-42 concentration alone as a biomarker. Taking A β 1-42 as a ratio of another A β species may suppress the influence of this individual variation in total A β . A report showing a high degree of concordance between plasma A β measured by IP-MALDI-MS and amyloid PET served as a trigger for the recognition of blood A β as a useful biomarker by researchers worldwide.²²⁾

7. Current Status of Blood-Based Biomarkers of Dementia

In recent years, more studies have shown the utility of plasma A β 42/A β 40 ratio as a biomarker using highly sensitive digital ELISA and electrochemiluminescence immunoassay (ECLIA) methods, both based on the sandwich immunoassay.^{23), 24)} Note that A β 42/A β 40 ratio is often used synonymously with A β 1-40/A β 1-42. A research group at Washington University also reported a significant correlation between amyloid PET and A β concentrations measured by an IP-LC-MS method that used IP to concentrate A β , enzymatically digested A β , then used LC-MS/MS for measurement, and is now applying for FDA approval.^{25), 26)} Other research groups have also reported that A β measured by LC-MS methods without enzymatic digestion or IP correlated with amyloid PET or CSF A β .^{27), 28)} Meanwhile, in Japan, an IP-MALDI-MS-based assay system was approved for use as a medical device in 2020. Although various different plasma A β assays have recently emerged on the market, correlation between these assay methods is actually weak.²⁹⁾ Possible reasons for this include the assay system being affected by impurities in plasma and differences between antibody clones. Nonetheless, correlation between IP-MS assays is stronger than between other immunoassays. A head-to-head comparison of plasma A β assays by Janelidze et al. found that IP-MS assays showed higher concordance with amyloid PET and CSF A β compared to sandwich immunoassays, suggesting IP-MS is fundamentally more appropriate to measuring plasma A β .³⁰⁾ However, A β is not the only biomarker of AD. Numerous reports are also emerging that show the usefulness of another AD pathology, the Tau protein, as a blood-based biomarker. CSF phosphorylated Tau181 (p-Tau181) was already known to be a specific biomarker for AD, but a recent report used a highly sensitive immunoassay to show blood p-Tau181 was also a biomarker for Tau pathology.^{31), 32)} p-Tau217 has also been described as a better biomarker for detecting disease earlier than p-Tau181.^{33), 34)} Seven years have passed since the report was published in 2014 showing a correlation between plasma APP669-711/A β 1-42 ratio and amyloid PET findings. Research has progressed rapidly throughout the world since the potential offered by AD biomarkers was recognized, and measuring plasma levels of A β and p-Tau biomarkers is no longer an uncommon technique. Plasma levels of A β and p-Tau are also now known to vary in parallel with the lower tracer uptake in amyloid-PET and Tau-PET induced by disease-

modifying drugs, and furthermore with suppression of cognitive decline, hence these biomarkers are now being used to enroll subjects in clinical trials and monitor drug efficacy.^{35), 36)}

The development of disease-modifying drugs is now receiving significant attention thanks to the FDA's recent accelerated approval of aducanumab, the first Alzheimer's drug to receive approval in 18 years.³⁷⁾ Phase 2 clinical trials of lecanemab and donanemab are also showing a significant reduction in clinical symptoms.^{38), 39)} Biomarkers of clinical symptoms but also of AD pathology are already essential to these clinical trials. As a coincidence, biomarker measurement technology has also developed rapidly in parallel with the increasing necessity of biomarker, and is beginning to be used in clinical trials. Today, AD research has entered a new phase and disease-modifying drug development is finally proving fruitful after a long series of failures. Nonetheless, these disease-modifying drugs are criticized as being very expensive and having a limited pool of indicated patients. As such, intervention methods that are not reliant on drug therapy are also in equal demand. In Japan, the Japan-multimodal intervention trial for prevention of dementia (J-MINT) is underway and assessing the use of blood-based biomarkers.⁴⁰⁾ As noted above, the A and T biomarkers of the ATN system for classifying AD pathology are now starting to see practical use. As for N, total-Tau (t-Tau) and neurofilament light chain (NfL) have been correlated with neurodegeneration and are biomarker candidates, though other reports have shown they also increase in some neurodegenerative diseases, dementias other than AD, and with increasing age, hence further studies are needed to characterize their behavior before practical application.⁴¹⁾⁻⁴⁵⁾ **Table 2** lists those biomarkers shown to exhibit correlation with stages of the ATN classification system by a high number of reports.

8. Conclusions

Biomarkers that reflect AD pathology are essential to AD drug discovery, and blood-based biomarkers that provide a less invasive means of measuring large numbers of specimens will likely become increasingly useful. Although not covered by this article, implementing the use of biomarkers in society is another important topic of study. Japan has approved drugs for treating AD patients with dementia, and biomarkers are used in a case that these drugs will provide a benefit by determining if a patient with dementia has AD.^{46), 47)} By contrast, the use of biomarkers is not recommended at the MCI stage before clinical onset, as the therapeutic benefit of these drugs has yet to be established in people with MCI. However, with the emergence of disease-modifying drugs, treatments and biomarkers are becoming inextricably linked, and "Guidelines for the Appropriate Use of Cerebrospinal Fluid and Blood-Based Biomarkers in Dementia" states that when disease-modifying drugs are brought to market, it is important to perform biomarker tests to identify which persons with MCI are likely to benefit from the drug based on determinations of background pathology.⁴⁷⁾ In the future, we will probably see a growing discussion on the utility of biomarkers in clinical practice, including their benefit for patients, assessments of health care economics, and ethical issues. In the field of biomarker research, high-performance analytical technology and the ability to differentiate between diseases are of obvious importance, but biomarker development must also take into account the benefits that biomarkers bring to drug discovery and the

Table 2 Biomarkers reported to correlate with ATN staging

Classification	Image	CSF	Blood
A	Amyloid PET	A β 42 A β 42/A β 40	A β 1-40/A β 1-42 (A β 42/A β 40) APP669-711/A β 1-42
T	Tau PET	p-Tau181 p-Tau217	p-Tau181 p-Tau217
N	MRI FDG-PET	t-Tau NfL Neurogranin SNAP-25	t-Tau NfL

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significance and application of biomarkers in health care. Development of disease modifying drugs has been greatly progressed by the advance of biomarker measurement technology. The field of neuroscience research is advancing toward the realization of therapeutic methods long awaited not only by researchers who participate in AD studies but also many patients and caregivers.

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