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Plasma Biomarkers in Alzheimer's Disease

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<http://dx.doi.org/10.5772/64512>

Abstract

Biomarker study on dementia has developed widely. In applying biomarkers, there seems to be several utilizations such as presymptomatic- and early-stage detection, differential diagnosis, and evaluation of treatment effect. Currently, most reliable fluid markers are amyloid peptide ($A\beta$) with microtubule-associated protein tau (TAU) and phosphorylated TAU (P-TAU) detected in cerebrospinal fluid (CSF). $A\beta_{42}$ correlates with plaque pathology, TAU reflects the intensity of neuroaxonal degeneration, and P-TAU may correlate with neurofibrillary tangle (NFT) pathology. An attenuation of the level of $A\beta_{42}$ and elevation in the ratio of $A\beta_{42}$ relative to the shorter major species of $A\beta_{42}$ peptide with 40 amino acid residues ($A\beta_{40}$) has been identified as significant events in the early stage of Alzheimer's disease (AD) pathology. In addition, there is great interest in blood-based markers of AD since blood extraction is much less invasive. Moreover, plasma biomarkers can be measured at relatively low expense once a standard system of measurement is established. Although there is not yet an established or validated diagnostic test for plasma biomarkers, there is great interest in blood-based markers. We will summarize reported biomarkers, describe our novel potential plasma biomarker for AD (annexin A5), offering a strategy for selecting candidates, and show our results and evaluation.

Keywords: plasma biomarker, Alzheimer's disease, annexin A5, $A\beta_{42}$, Ca^{2+} -stress

1. Introduction

The augmented number of dementia patients has been dramatic due to the aging of society in advanced countries. Alzheimer's disease (AD) is the most common type of dementia and accounts for more than half (50–70% depending on the reports) of all dementia. AD is characterized by a gradual onset by developing neuronal damage and continuing cognitive decline related to stress-induced cell damage in the patient's brain [1], which ultimately causes significant

impairment to social and occupational functions. The mean duration from the onset of clinical symptoms to the death of the patient has been reported to be approximately 8.5 years [2].

AD is defined by the presence of plaques and tangles in the brain, thus the gold standard for the diagnosis of Alzheimer's disease (AD) is set by means of the histological examination of brain tissue at autopsy, which is usually done after a patient has died, or, rarely, following brain biopsy. On the other hand, clinical diagnosis of AD during life has been performed with a sensitivity ranging from 70.9% to 87.3% and specificity from 44.3% to 70.8% [3]. It was also reported that dementia is often overlooked in community care settings [4].

For objective diagnostic analysis, several biomarkers are available. The reliable biomarker candidates for AD include brain imaging studies using magnetic resonance imaging (MRI) or positron emission tomography (PET), and proteins in cerebrospinal fluid (CSF). MRI is utilized for structural imaging, PET for molecular imaging of amyloid deposition and fluoro-deoxy-D-glucose (FDG)-PET for metabolic imaging, while measurements of amyloid peptide ($A\beta$) and TAU protein in cerebrospinal fluid (CSF) are used for quantitative analysis. However, structural changes measured by MRI only become apparent in the late stage of AD. Moreover, structural MRI and FDG-PET images are not direct measures of the core pathological hallmarks of AD. PET imaging is relatively expensive and limited in availability. CSF $A\beta$ and TAU might be nonspecific for AD depending on each case [5]. At present, it can be stated that the most well-characterized and validated biomarkers are $A\beta$ and TAU in CSF: the decrease in $A\beta$ with 42 amino acid residues ($A\beta_{42}$) and increase in TAU and phosphorylated TAU (P-TAU) has been observed in AD patients in several of studies [6].

An alternative method to the invasive CSF collection and expensive specialized facilities for diagnostic imaging is most desirable. Thus, plasma biomarkers have raised expectations because blood sampling is a much less invasive procedure. Blood-based biomarkers have the potential to overcome access and cost barriers and greatly facilitate advanced neuroimaging and cerebrospinal fluid biomarker approaches. Due to the fact that preanalytical processing shows the largest variation in laboratory testing, there are currently no available standardized preanalytical guidelines. In this review, the primary focus is on the fluid biomarkers, especially blood plasma protein biomarkers, as indicators of AD development together with our study on results of a specific blood plasma candidate.

2. CSF markers

Diagnostic markers are anticipated to be present in secreted proteins followed by a result of cell damage in pathological states. Although CSF sampling by lumbar puncture definitely is known to be an invasive procedure, at present CSF is probably the most informative fluid in biomarker detection for neurodegenerative disease prognosis [7]. CSF has direct contact with the brain, and it does not easily escape from the brain owing to the fact that the blood-brain barrier (BBB) is tightly regulated. In general, if a biomarker candidate is identified in CSF, its possibility as a true biomarker for brain-specific activities, as well as disease pathology, would be considered to be more promising compared with any other body fluid biomarker candidate.

It has been demonstrated that senile plaque formation and neuronal loss precede clinical onset of Alzheimer's disease [8]. Senile plaques are polymorphous and comprise Amyloid β peptide ($A\beta$), a proteolytic product of amyloid precursor protein (APP) that accumulates in the brains of AD patients. Several species of $A\beta$ peptide depending on the cleavage sites on APP have been identified in the body fluid. APP processing consists of initially proteolysis by β -secretase and then by γ -secretase, which leads to the formation of $A\beta$ peptides with 38–43 residues [9]. Of these, $A\beta_{42}$ with 42 amino acid residues is the most remarkably focused due to its toxic effect. $A\beta_{42}$ is highly hydrophobic and forms oligomers and fibrils that accumulate as extracellular plaques, which correlates inversely with plaque pathology [1, 10]. Attenuated activity of $A\beta$ -degrading catabolic enzymes including neprilysin and insulin-degrading enzymes with age or abnormal production of $A\beta$ due to gene mutation(s) of related proteins such as on APP have been identified, which in turn leads to the accumulation of $A\beta_{42}$ in the brain tissue [11].

On the other hand, TAU that is an intracellular protein, is believed to be involved in filament stabilization, and has been shown to aggregate to form filaments in neurons. In normal individuals, only a low concentration of TAU is present in CSF. The function of TAU is tightly regulated by a number of posttranslational modifications possibly due to phosphorylation at serine and threonine residues. Several studies have suggested that hyperphosphorylation and formation of neurofibrillary tangles (NFTs) is the pathophysiological phenomenon of the development of AD [12]. It is remarkable that functional loss of TAU following hyperphosphorylation, the dissociation of TAU from microtubule and subsequent polymerization into insoluble paired helical filaments (PHF) could result in the loss of axonal integrity in the neuronal cells [13, 14]. NFT formation and neuronal degradation is an essential part of AD pathology.

Due to significant disruption of the neuronal architecture, the TAU and its hyperphosphorylated form (P-TAU) could appear in CSF [15]. Therefore, the phenomenon of increased levels of TAU and P-TAU in CSF represents well with the onset of neurodegeneration in AD. The total TAU (t-TAU) concentration in CSF has been measured by the method of ELISA using monoclonal antibodies against all TAU isoforms. Several groups have indicated that t-TAU concentration in CSF of AD patients is significantly higher than control [15, 16]. On the other hand, the attenuation of the amount of $A\beta_{42}$ in CSF has been noted due to accumulation in the brain [17]. Thus, decrease in level of $A\beta_{42}$, increase in t-TAU and P-TAU have been utilized as CSF biomarkers contributing to the diagnosis of AD [18]. In addition, the development of imaging biomarkers has provided evidence of an ongoing AD pathophysiological process.

The $A\beta$ ratio ($A\beta_{40}$ to $A\beta_{42}$) in the AD group was significantly increased compared with that in the normal control group, the non-AD type dementia group, and the other neuronal disease group [19]. For the enhancement of the diagnostic relevance of AD, AD index that is calculated by multiplying TAU level by the $A\beta$ ratio was shown to be useful for discrimination of AD patients from healthy controls with good sensitivity and specificity [19].

It was reported that low CSF level of $A\beta_{42}$ appeared to predict conversion of mild cognitive impairment (MCI) to AD, while a decrease in $A\beta_{42}$ level has also been observed in other neurodegenerative disorders [20]. Furthermore, it was shown that levels of TAU and P-TAU at Ser181 (P-TAU181) in CSF, but not $A\beta_{42}$, correlated oppositely with whole brain volume in

the early stage of AD, whereas levels of CSF $A\beta_{42}$, but not TAU or P-TAU181, was positively correlated with whole brain volume in nondemented controls [17].

It is thought that the production and accumulation of unfavorable $A\beta$ species proceeds over time as the disease progresses. Abnormal activity by the $A\beta$ species is initiated before pathological change and reaches a plateau before the clinical symptoms appear. Thereafter, elevation of TAU and P-TAU that are the biomarkers for neuronal injury, dysfunction, and degeneration, become apparent in the later stage of the disease and correlate with clinical symptom severity [8]. On the other hand, MRI imaging is valuable as it is the last biomarker to show abnormality. As such, MRI retains a closer relationship with cognitive performance later on in the disease compared with other biomarkers. Moreover, none of the biomarkers is stable; that is, the rate of change for each biomarker is not linear over time [8].

The revised guideline for AD diagnosis was released by a working group from the National Institute of Aging in 2011, in which both CSF and imaging biomarkers have been implemented. The new guideline provides evidence of an ongoing AD pathophysiological process, and it is also possible to make a preclinical diagnosis of MCI due to AD [21–23]. AD is classified into three separate stages: preclinical AD, MCI due to AD, and AD with dementia.

Since fluid biomarkers of either CSF or blood plasma can serve as objective criteria for dementia diagnosis, this guideline is aiming at early and reliable diagnosis. However, it is clear that at present no single biomarker plays a sufficient discriminatory role in screening for future development of late-onset AD or dementia.

3. Plasma markers

Compared with CSF, blood sampling is a less invasive procedure, more easily accessible, and cost reductive, thus the finding of reliable blood biomarkers for AD is being given the highest priority. There has been an increasing research effort to examine the potential biomarkers of AD in blood plasma. However, for blood-based biomarkers, it has to be noted that blood plasma contains several tens of thousands of different proteins. In addition, the range of protein concentrations are extremely varied (attaining to 12 orders of magnitude), and the lower the concentration, the greater the diversity of proteins [24, 25]. Moreover, none of the current methods allows us to directly detect components in the low concentration region [25]. These conditions make it extremely challenging or almost impossible to directly analyze blood, even though possible biomarker candidates are more likely to be present in the areas of low concentration. The change in concentration of the blood components may often be on a very small scale and cover a wide range of both peripheral and central processes. Additionally, the less abundant proteins may be masked by highly abundant plasma proteins such as albumin and immunoglobulin. Therefore, focusing on concentration change of a particular AD-specific marker, which may be in low concentration, can be the most challenging to discover [26, 27].

It was reported that the BBB is disrupted resulting in increased permeability with aging and in AD [28, 29]. It is also thought that this event occurs in the relatively early stage of the aging

brain, which is related to increased cognitive impairment. Although the relationship between an analyte found as a biomarker candidate in blood plasma and the behavioral changes in the brain is not easily demonstrated, there is the possibility of a connection due to BBB disruption during the early stage. This might lead one to expect the possible appearance of a brain component in the peripheral blood stream.

The widely accepted CSF biomarker, A β peptides, have also been examined in blood, but its concentration in blood plasma is considerably lower than reported in CSF by about 100-fold [30]. Elevated plasma level of either A β_{40} [31, 32] or A β_{42} [33, 34] levels was used as an indicator for the development of AD, while the opposite results [35] or no association at all between plasma A β level and AD development [36, 37] were reported. Thus, results based on plasma A β as a biomarker have been inconsistent. A low plasma A β_{42} to A β_{40} ratio was utilized for the prediction of future AD [32, 38, 39], while contrary results, reporting a higher ratio [31, 33] in the nascent AD stage patients than the subjects who did not develop AD, and no significant differences were also shown [36].

Another promising candidate of a plasma protein biomarker was identified by means of the proteomic approach. The protein clusterin/apolipoprotein J, which is involved in the clearance of cellular debris and apoptosis, was associated with both hippocampal atrophy and clinical progression [40]. Increased plasma concentration of clusterin indicated the prediction of greater fibrillary amyloid- β burden in the medial temporal lobe and AD patients had increased clusterin messenger RNA in blood. Moreover, in the transgenic AD mouse model (APP/PS1), increased plasma clusterin level, age-dependent increase in brain clusterin, as well as amyloid and clusterin colocalization in plaques were shown [40, 41]. The recent finding is that increased plasma clusterin levels have been associated with increased risk of conversion to AD and the rate of cognitive decline [42]. Clusterin may have a role in A β aggregation and clearance [43, 44], and at high concentrations, clusterin may prevent A β aggregation through its binding to A β . Furthermore, clusterin possesses neurotoxic properties by involvement in noncanonical wnt signaling, which mediates A β toxicity [45]. Therefore, clusterin might fulfill different roles. Other plasma biomarker candidates have been reported, such as desmosterol [46], transthyretin [47], chitinase 3-like 1 protein [48], and matrix metalloproteinase 2 [49], which may be associated with AD. Using protein array technology, Ray et al. found 18 signaling proteins in blood plasma that can discriminate AD samples from control subjects with approximately 90% accuracy [50].

4. Another plasma biomarker study

The brain-derived proteins present in blood plasma are limited compared with those in CSF due to the presence of the BBB. It is also likely that if potential brain-derived proteins are present in blood plasma, it is conceivable they are considerably diluted in the large volume of plasma and underwent proteolysis and excretion. These possible events make the study more challenging. As mentioned previously, in plasma, there are several tens of thousands of different proteins present at concentrations in the millimolar to femtomolar or lower range.

This extremely varied range of protein concentrations in plasma makes it almost impossible to directly analyze low concentration components.

Therefore, in our study, instead of direct examination of plasma, we initially utilized a cell culture model, mouse primary culture neuron. After A β -treatment, we identified proteins present outside of the cells (culture supernatant), in which A β -dependent secreted proteins are expected to be present, using a proteomic approach, and focused on the proteins that were increased by A β -treatment, and discovered a biomarker candidate. Ultimately, we verified the potential candidate with animal model (transgenic mice) and human plasma samples (Figure 1).

Figure 1. Process of biomarker identification (our study).

The cytotoxicity due to A β_{42} is thought to be directly linked to neural cell death [1]. Amyloid-dependent neurotoxicity is known to perturb Ca²⁺ homeostasis in neuronal cells [51]. Possibly, A β impairs membrane Ca²⁺ pumps and enhances Ca²⁺ influx through voltage-dependent channels and ionotropic glutamate receptors (Figure 2).

By focusing on this mechanism, we identified the Ca²⁺-related protein as a potential biomarker for AD using primary neurons as a cell culture model [52]. Since phosphatidylserine (PS) is flip-flopped and appears in the outer layer of the plasma membrane during the apoptotic process, we focused on PS-binding proteins in the culture supernatant and used a unique method to identify a potential biomarker candidate.

Thermoresponsive magnetic nanoparticles disperse well in an aqueous solution at a temperature below 10°C and are aggregated and become responsive to magnets at 20°C or higher. In this study, we coated magnetic beads with thermoresponsive polymers (polyethyleneimine) together with myristate and then coated them with PS [52]. We mixed these particles with a culture supernatant in the presence of Ca²⁺ and collected the PS binding fraction with ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). After running SDS-PAGE, we performed in-gel digestion with trypsin and analyzed the tryptic peptides by reverse-phase

liquid chromatography coupled with MALDI TOF/TOF MS spectrometry and performed database analysis for peptide sequencing. From this proteomic approach, about 240 types of proteins were indicated to be increased in the $A\beta_{42}$ -treated sample, compared with the control, suggesting that they were upregulated by $A\beta_{42}$. From among these proteins, we focused on annexin A5, one of the annexin family proteins that commonly bind Ca^{2+} and phospholipid. It was shown that annexin A5 was augmented in both the brain and blood plasma in an AD-model mouse (Tg2576 transgenic mice), overexpressing mutant human APP [52]. Technetium-labeled annexin A5 was detectable in the brain after intravenous injection in humans, showing that annexin A5 crosses the BBB [53].

Figure 2. $A\beta$ -dependent perturbation of calcium homeostasis in AD.

4.1. Methods

To quantify plasma annexin A5, we previously established the chemiluminescent enzyme immunoassay (CLEIA) system with two clones of monoclonal antibodies against human annexin A5: one clone was conjugated to a glass bead and used for trapping annexin A5 present in the blood plasma; the other clone was labeled with horseradish peroxidase (HRP) and used for quantification of the trapped annexin A5 [52]. The HRP catalyzes the oxidation of a luminol solution that includes a phenol-derivative acting as an enhancer, and produces light. This system was useful to quantify plasma annexin A5 in the range from 0.16 to 20.0 ng/ml [52]. We obtained blood samples from 150 AD, 50 DLB, 14 mild cognitive impairment (MCI), and six depression patients, and 298 healthy elderly individuals from the senior citizen's clubs. AD patients met NINCDS-ADRDA [54] and DLB patients diagnosed as probable DLB according

to the latest consensus diagnostic criteria [55]. Statistical analysis was done using JMP version 9.0.0 (SAS Institute Inc., Cary, NC, USA). The mean response of each experimental group was compared with its simultaneous control by unpaired Student's *t*-test, setting a significant difference at $P < 0.05$. To examine the plasma annexin A5 levels in diagnoses of AD, DLB, and MCI, logistic regression modeling was employed to construct receiver operator (ROC) curves.

The plasma level of annexin A5 was significantly increased in AD patients compared to that of a control group (P -value < 0.0001 in the logistic regression analysis), suggesting that annexin A5 is a potentially positive biomarker for AD (Figure 3) [52].

Figure 3. Comparison of plasma levels of annexin A5 in AD, DLB, MCI, depression, and age-matched healthy control.

For quantification of plasma annexin A5, we used a previously established chemiluminescent enzyme immunoassay system with monoclonal antibodies against human annexin A5 [52] (see Section 4.1). Individual plasma annexin A5 concentration is plotted in (A). The probability of either AD, DLB, or MCI can be predicted by a logistic regression model with the plasma level of annexin A5. Receiver operating characteristic (ROC) curves are shown in (B)–(D). The areas under the curve are 86.3%, 83.8%, and 91.6% for AD (B), DLB (C), and MCI (D), respectively. AD, Alzheimer's disease; DLB, dementia with Lewy bodies; MCI, mild cognitive impairment.

As annexin A5 binds not only phospholipids but also Ca^{2+} , it might have a role in protecting against Ca^{2+} -induced damage by chelating elevated intracellular Ca^{2+} . A defensive role against

apoptosis induced by the participation of annexin A5 was also reported, in that annexin A5 plays a role in reducing the toxicity of the amyloidogenic proteins through interaction with them, such as amyloid polypeptides and α -synuclein [56].

On the other hand, dementia with Lewy bodies (DLB) shares clinical and pathological features with other dementia subtypes such as AD, vascular dementia, and Parkinson's disease (PD), which makes it difficult to distinguish in clinical practice. Lewy bodies are often found in the brains of AD patients. Also, the lack of valid and reliable methods for assessing the core clinical symptoms of both AD and DLB makes its identification even more difficult. We analyzed plasma level of annexin A5 in DLB. When average concentrations of plasma annexin A5 are compared among AD, DLB, and control groups, the values of AD and DLB were significantly higher than healthy control subjects (**Figure 3A**). Moreover, the ROC analyses showed good separation of patients with either AD or DLB from the control group (healthy volunteers) (**Figure 3B and C**) [57]. These suggest that annexin A5 is a potential biomarker for both AD and DLB. There is a similarity between AD and DLB. Lewy bodies are often found in the brains of AD patients. The therapeutic agent, acetylcholinesterase inhibitor, is effective not only in AD but also in DLB. From these results, annexin A5 reflects the above-mentioned similarity of AD and DLB.

To examine when annexin A5 becomes elevated during the course of disease development, we analyzed plasma samples from MCI patients (early stage of dementia). Average concentration was significantly higher than for the control group and the level was comparable with that of AD (**Figure 3A**). The areas under the ROC curve was 91.6% ($P < 0.001$) (**Figure 3D**), suggesting that annexin A5 is also a potential biomarker for MCI. Therefore, it is presumable that elevation of annexin A5 is likely to take place from the early developmental stage of AD. Although a sample number is very limited, plasma annexin A5 level in depression was comparable with the control (**Figure 3A**).

We next tracked plasma level of annexin A5 over a 3-year period in late stage AD patients. The plasma level of annexin A5 tended to be unchanged or slightly decreased, which indicates that biosynthesis of annexin A5 might be downregulated during the late stage, due to the progression of neuronal cell damage (data not shown).

5. Issues on blood sample

Since annexin A5 is also expressed in peripheral blood lymphocytes [58, 59], the effect of physical stress (such as osmotic pressure and temperature changes) upon blood cells may induce leakage of annexin A5. In fact, if a prolonged period of time passes (such as 12 h) after collecting blood, prior to centrifugation, the amount of plasma annexin A5 increases compared with a shorter period (such as within 6 h) (data not shown). Therefore, blood samples should be centrifuged within a specified period of time after collection. In our study, we did this within 6 h after blood collection. However, the lack of consistent technical standard for blood sampling in plasma biomarker studies may induce complicated and inconsistent observations depending on the study groups [60]. With respect to some conditions, such as anticoagulant

reagent (EDTA or others), needle gauge, and 6-h fasting, standards should be proposed. For plasma preparation, a time limit until plasma separation after blood sampling may be critical to avoid induction of unwanted component leakage. Centrifugation speed (gravity force), duration, temperature, and number of spins, sample storage conditions may also require specification, though most common plasma samples are stored immediately at a temperature of -80°C for long-term storage. There will also be a number of factors that apply to subjects (patients and other participants involved): such as demographics (age, sex, and race/ethnicity), life style, overall health conditions (chronic drug administration, dietary supplements), smoking, and alcohol consumption.

6. AD risk factors

Several risk factors for AD have been indicated. Genetic factors are increasingly recognized as major risk factors for dementia. The most remarkable factor for AD from numerous studies is the ApoE gene on chromosome 19. ApoE, which is a major component of lipoproteins with 299 amino acid residues, plays a role in the metabolism and redistribution of cholesterol [61]. ApoE constitutes three major common isoforms, designated ApoE2, ApoE3, and ApoE4. ApoE isoforms interact differently with A β isoform-specific effects on A β -clearance. In ApoE4, domain interaction occurs as a result of a putative salt bridge, leading to tight structural formation. This interaction is unlikely to take place with ApoE2 and ApoE3 [62, 63]. ApoE4 is associated with an increased risk for AD along with early onset of the disease [64]. It was reported that ApoE4 carrier frequency was the highest in AD among AD, DLB, and control groups, and it was also higher in DLB than in the control groups [65]. Other findings have shown that ApoE4 carrier and allelic frequencies were comparable for those with AD and DLB with respect to Japanese subjects [57, 66].

Recently, a single nucleotide polymorphism in triggering receptor expressed on myeloid cells 2 (TREM2), an innate immune receptor expressed on the surface of microglia, were associated with both reduced hippocampal volume in healthy older adults and MCI [67, 68]. It was also shown that increased CSF sTREM2 levels were associated with higher CSF total TAU and phospho-TAU181P [69].

7. Future prospect

Biomarkers are usually employed as an indicator of processes related to the onset of a disease, specific disease conditions or response to therapeutic interventions [70]. However, it is clear that at present no single biomarker plays a sufficient discrimination role in screening for future development of late-onset AD or dementia. During development of the disease, the time when each unique biomarker becomes elevated will vary. Therefore, it is imperative to be able to determine when specific biomarkers need to be measured in order to provide timely therapeutic intervention.

Blood testing for measuring biomarkers will be easy and widely accepted due to the ease of collection and low cost. Moreover, the increasing availability of large sample sets obtained from a variety of technologies might contribute to diagnosis, prediction, and monitoring the progression of AD [71]. If a standardization of sample collection, standard operating procedures, comprehensive data management, and exchange of scientific findings is established, and if collaborative studies continue to progress, these should lead to a reduction in the variability and fragmentation of data. It is very likely that we may see plasma biomarkers become a reliable indicator for diagnosing AD.

Biomarkers of disease presence, subtypes (i.e., endophenotypes), treatment response, and progression are needed to advance therapeutic and preventative opportunities for this rapidly growing health care crisis.

8. Conclusions

In spite of the fact that reliable biomarkers have been established in CSF, no blood-based biomarker has been fully validated or qualified, even though an increasing number of plasma biomarker candidates have been reported. However, promising candidates have been emerging due to the progress in the field. Longitudinal studies from collaborative research and from the use of a variety of technologies and study designs are expected.

Acknowledgements

We are indebted to Kenneth E. Hartmann for critical reading of the manuscript. This study was supported in part by grant from the Ministry of Health, Labour and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant number 24591686). All authors are free of any conflicts of interest.

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