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REVIEW

Proximity extension assay-based proteomic studies in Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is the most common neurodegenerative disease in older age. Pathophysiological changes begin in the brains of affected individuals many years before any clinical signs are observed. Although brain imaging and neurophysiological analyzes are useful to reveal anatomical and functional changes in patients whose diagnosis of AD is considered based on clinical examination, their contribution to the diagnosis is quite limited, particularly in the early stages of the disease. Some biological markers are important as laboratory support in the early diagnosis of AD. Biomarkers are objectively measurable and evaluable indicators that serve to identify normal biological processes, pathological processes, and therapeutic response rates. Biomarkers have the potential to predict the likelihood of disease, assist in early diagnosis, and contribute to monitoring treatment effectiveness. This article aimed to provide information about the use of proximity extension assay technology in biomarker studies in AD.

Keywords: Alzheimer's disease, proximity extension assay, proteomic.

Proximity extension assay

Proteomics, an expanding field, has the potential to examine the protein alterations in cells, biological fluids, or tissues that could elucidate the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD). The identification of protein changes might play a crucial role in discovering effective markers for early disease detection.

Proximity extension assay (PEA), an extraordinary proteomic technique developed by Olink Proteomics (Uppsala, Sweden), is a method that allows for the high-specificity and high-sensitivity identification of multiple proteins in just a 1-µL sample.[1] This method can be used to detect proteins secreted in biological fluids, such as the cerebrospinal fluid (CSF) and blood.[2] Biomarker studies can also contribute to drug development efforts in drug efficacy testing.

The principle of PEA relies on the specific binding of oligonucleotide-labeled antibodies and target proteins. Each antibody is labeled with specific oligonucleotide sequences. Two antibodies targeting different epitopes of the target protein are used to detect a specific protein in the sample. When the antibodies bind to the target protein, they bring the oligonucleotide sequences closer, facilitating the hybridization of these oligonucleotides. Hybridization results in the formation of a double-stranded DNA (dsDNA) that acts as a barcode for the identification of the target protein (Figure 1).[3] In the detection phase of PEA, the dsDNA is amplified using microfluidic quantitative polymerase chain reaction to determine the initial amount of protein.[2] Additionally, instead of polymerase chain reaction, protein changes can be identified using next generation sequencing.[4] This review focused on the use of PEA technology in biomarker studies for AD.

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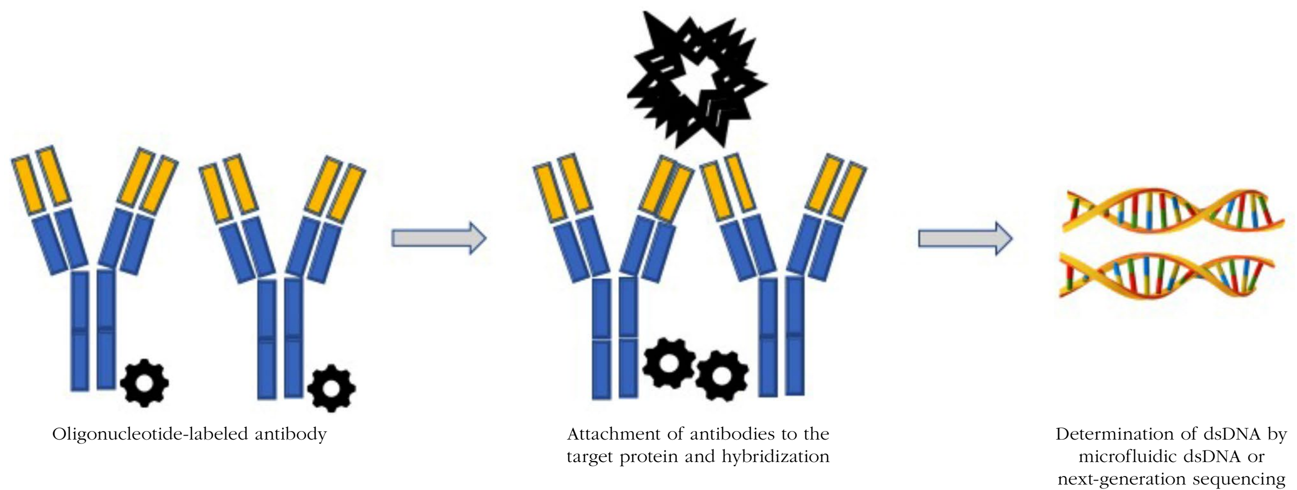

Figure 1. The principle of PEA.
PEA: Proximity extension assay; dsDNA: Anti-double stranded deoxyribonucleic acid.

Biomarkers in Alzheimer's disease

Alzheimer's disease is the most common neurodegenerative disease in old age. Clinically, it is a slowly progressive type of dementia characterized by progressive cognitive decline with a predominance of episodic memory impairment. Alzheimer's disease is characterized by the accumulation of amyloid beta (Aβ) protein in senile plaques in the brain parenchyma and phosphorylated tau accumulation in neurofibrillary tangles in cerebral neurons. Pathophysiological changes begin in the brains of affected individuals many years before any clinical signs are observed.[5] Although commonly used brain imaging and neurophysiological analyses are quite useful in revealing anatomical and functional changes, their contribution to the diagnosis in the early stages of the disease is limited in sensitivity.[6-10]

The primary aim of using biomarkers is to enhance the accuracy of differential diagnosis and strengthen clinical diagnosis. Biomarkers are objectively measurable and assessable indicators that serve to identify normal biological processes, pathological processes, and therapeutic response rates. Biomarkers have the potential to predict disease likelihood, assist in early diagnosis, and contribute to monitoring treatment effectiveness. Recent developments in molecular biomarkers with results obtained from analyses of blood and CSF samples have fostered hope that these biomarkers may be introduced into clinical practice in the near future.[11-15] Cerebrospinal fluid is considered the most suitable source of biomarkers due to its direct connection with the extracellular space of brain tissue. It is assumed that all biochemical changes in the brain are reflected in the CSF.[16]

The primary biomarkers in AD are Aβ, particularly the $A\beta_{42}$ isoform, total tau protein (t-tau), and phosphorylated tau protein (p-tau), more specifically the phosphorylated form at position 181 ($p\text{-}tau_{181}$).[17] Alzheimer's disease is characterized by a decrease in $A\beta_{42}$ levels and an increase in the levels of t-tau and p-tau in the CSF. This combination serves as a prodromal biomarker in the early stages and in mild cognitive impairment (MCI), as well as in the dementia stage.

$A\beta_{42}$ and $A\beta_{40}$ are the most extensively studied blood markers for the diagnosis of symptomatic and prodromal AD. Determining the levels of Aβ peptides in blood plasma as potential biomarkers for AD is challenging due to the very low concentrations of Aβ and the presence of matrix components that can interfere with measurements. Studies utilizing new techniques such as mass spectrometry and ultrasensitive immunological tests have shown that measurements of Aβ in blood samples could potentially serve as a biomarker.[18-20]

Neurofilaments are the main structural components of long myelinated axons. Neurofilament is a protein that provides structural support to axons as part of the cellular cytoskeleton and regulates axon diameter.[21] It has been

reported that neurofilaments increase in both CSF and serum in various neurological conditions and indicate neuronal and axonal injury.[22] The neurofilament concentrations in the CSF and serum are highly correlated. In addition to studies that identify CSF neurofilament as a reliable marker of neurodegeneration,[23,24] studies have found that serum neurofilament levels are elevated in AD,[25] vascular dementia,[26] Parkinson's disease dementia,[27] frontotemporal dementia (FTD),[25] and traumatic brain injury[28] compared to healthy controls. High concentrations in CSF generally reflect rapidly progressing neurodegenerative processes. Therefore, they can be used in differential diagnosis to distinguish AD from other diseases accompanied by dementia.[29,30]

Studies on PEA in AD

In addition to standardized fundamental biomarker assessment methods, the use of innovative large-scale omics technologies in AD has led to the discovery of new biomarkers for disease diagnosis and monitoring. In recent years, advancements in unique and highly sensitive protein measurement methods such as PEA have accelerated quantitative proteomic studies in both CSF and plasma samples in AD (Table 1).[31] Various proteomic studies in AD have been conducted using PEA technology with plasma, CSF, and extracellular vesicles as samples. In two of the studies that used only CSF samples, an inflammation panel was preferred. Gaetani et al.[32] included 34 patients with MCI due to AD and 25 patients with other neurological disorders and demonstrated that changes in the levels of SIRT2, HGF, MMP-10, and CXCL5 proteins in CSF samples could distinguish these two groups using machine learning (Table 1). The most significant finding of the second study, which included patients with stable MCI, MCI due to AD, and FTD, was the increased level of MMP-10 in the CSF samples of patients with AD and MCI due to AD (Table 1).[33] The increase in MMP-10 and -11 additional proteins observed in patients with AD and MCI due to AD, which was not observed in stable MCI or FTD, suggests that the analysis of inflammatory proteins in CSF can assist clinicians in predicting the progression of MCI to AD and in differentiating between FTD and AD. Previous studies have demonstrated that MMP-10 levels increase in patients with AD.[34,35] Another study utilizing an inflammation and neurology panel in CSF samples reported that the protein changes observed in infectious delirium were similar to those in AD (Table 1).[36] A recent study involving 979 participants showed that changes in eight proteins in the CSF could accurately distinguish patients with AD and MCI (Aβ positive) from controls and that changes in MMP-10, TGB2, and TREM1 were specific to AD.[37]

The accessibility of peripheral samples such as plasma has led to the preference for using plasma samples in biomarker studies employing PEA. Among studies focusing on AD, four studies used only plasma,[38-41] two studies used both plasma and CSF,[31,42] and one study used extracellular vesicles[43] as the source of samples. The patient groups exhibited a wide variability in studies using plasma samples. Two studies included a control group along with an AD group,[38,40] whereas another study incorporated groups with MCI and non-AD dementia.[41] Another study collected samples from two different cohorts in Greece.[39] The heterogeneity of participants complicates the ability to merge and analyze results across studies and identify biomarkers. Although it appears that validating the results of the discovery group in another independent patient group increases the reliability of the findings, studies using plasma samples have not been able to identify proteins that exhibit significant changes across multiple studies.[41]

Validating the protein changes in plasma with CSF samples is not only crucial for understanding the pathogenesis of the disease but also strengthens the biomarker potential of these proteins. The ability of these biomarkers to predict the disease in its presymptomatic stages can be clinically beneficial. In the Rotterdam study using PEA with a neurology panel in plasma samples from 316 participants, CDH6 and HAGH levels were elevated in AD patients carrying the apolipoprotein E epsilon 4 allele (Table 1).[42] These results were replicated in the BioFINDER study group, which included 186 AD patients and 485 controls, and a replication study was conducted with CSF samples from 242 AD patients and 199 cognitively normal control participants from the Amsterdam dementia cohort.[42] In apolipoprotein E epsilon 4 allele carriers, CSF levels of CDH6 were correlated with t-tau and p-tau; however, a similar correlation was not detected with $A\beta_{42}$ levels.[42] The increase in CDH6 levels in the cortices of APP/PS1 mice suggests that the CDH6 protein may play a potential role in AD.[44] In another study, levels of 1,196 proteins in both plasma and CSF samples were comparatively analyzed using PEA, SomaLogic SomaScan, and tandem mass tag mass spectrometry (Table 1).[31] The findings revealed an increase in the SMOC1 protein in both plasma and CSF samples in AD. The SMOC1 protein is closely associated

TABLE 1
Proximity extension assay-based proteomic studies utilizing CSF and plasma samples in AD

Study name and year	Sample type	The number of patients and controls	The panel type	Results
Ahmad et al.,[42] 2020	Plasma	Discovery cohort: 161 AD patients, 155 controls Validation cohort: 186 AD patients, 485 controls	Neurology	Increase in CDH6 and HAGH levels in AD patients carrying APOE ε4
	CSF	242 AD patients, 199 controls		The correlation of CDH6 levels with t-tau and p-tau in APOE ε4 carriers
Peters van Ton et al.,[36] 2020	CSF	Five patients with infectious delirium, 29 patients with AD, 30 infected patients without delirium, 15 controls with no infection	Neurology and inflammation	Similar expression of 34 proteins in patients with infectious delirium and in those with infections without delirium; a decrease in levels of 23 proteins, including CD200R1, and an increase in levels of eight proteins, including CX3CL1 (fractalkine), in patients with delirium and AD
Boström et al.,[33] 2021	CSF	AD (n=42), MCI due to AD (n=29), stable MCI (n=22), FTD (n=42), and 49 controls	Inflammation	Increase in MMP-10 in AD, MCI due to AD, and FTD; Increase in MMP-10, beta-NGF, CDCP1, CSF-1, FGF-5, HGF, LIF-R, PD-L1, SCF, SIRT2, TWEAK, and VEGFA proteins in MCI due to AD compared to stable MCI; decrease in 36 protein levels in FTD (no change in AD and MCI)
Dammer et al.,[31] 2022	Plasma and CSF	18 AD patients, 18 controls	13 human Olink Target 96	Increase of SMOC1 in CSF and plasma
Gaetani et al.,[32] 2021	CSF	34 patients with MCI due to AD, 25 patients with other neurological disorders	Inflammation	Increase in SIRT2, HGF, MMP-10, and CXCL5 in patients with MCI due to AD
Ellegaard Nielsen et al.,[43] 2020	Plasma and ECV	10 AD patients, 10 MCI patients, 10 controls	Neurology, inflammation	Increase of TGF-α in plasma and CCL20 in ECV
Jiang Y et al.,[40] 2022	Plasma	Discovery cohort: 106 AD patients, 74 controls Validation cohort: 36 AD patients, 61 controls	Olink biomarker	Change in the levels of 429 proteins, particularly 19 hub proteins, in AD (increase in KLK4, CD8A, LIF-R, and hK14; decrease in AOC3, GSAP, NELL1, GAMT, CD164, LGMN, VPS37A, VAMP5, NFKBIE, TMSB10, PRKCQ, PRDX1, CASP-3, CETN2, and LYN)
Kivisakk et al.,[41] 2022	Plasma	Discovery cohort: 30 stable MCI patients, 30 progressive MCI patients Validation cohort: 21 stable MCI patients, 21 progressive MCI patients Additional cohort: 20 AD patients, 30 non-AD dementia patients, 34 controls	Immunooncological, neurological, cardiovascular, inflammation, and cardiometabolic	In the comparison of stable and progressive MCI, change in progressive MCI in the levels of 44 proteins related to inflammation (CCL23, CX3CL1, CSF-1, CXCL9, IL-8, and TNFRSF12A), extracellular matrix (MMP-3, PTN, and TIMP-4), neurodegeneration (NF-L), and vasculature (PGF, MB, and VEGFA)

AD: Alzheimer's disease; MCI: Mild cognitive impairment; FTD: Frontotemporal dementia; CSF: Cerebrospinal fluid; ECV: Extracellular vesicle; APOE ε4: Apolipoprotein E epsilon 4 allele.

with amyloid accumulation, and changes in CSF and plasma samples of AD patients have been also reported in previous studies.[45,46]

DISCUSSION

The use of PEA technology in biomarker studies and protein analysis of neurodegenerative disease has proven to be a robust and promising approach. It has facilitated the identification and validation of new biomarkers, enabled early and accurate diagnosis, allowed monitoring of disease progression, and assisted in the evaluation of therapeutic interventions.

Proximity extension assay possesses several strong features compared to traditional proteomic methods. Primarily, PEA offers higher sensitivity and specificity, enabling the precise detection and measurement of low-abundance proteins. The dual recognition principle, which combines antibody-based recognition and DNA amplification, enhances the signal-to-noise ratio and minimizes background noise, yielding highly reliable and reproducible results. This sensitivity is crucial when working on neurodegenerative diseases in which biomarkers may be present in extremely low concentrations. Furthermore, PEA allows for the simultaneous measurement of multiple proteins in a single sample, providing a highly efficient analysis that significantly reduces time and cost compared to traditional methods. This capability is invaluable in neurodegenerative disease research, where the identification of biomarker panels and protein signatures can revolutionize diagnostic and therapeutic interventions. Moreover, the compatibility of PEA with various sample types, including the CSF, blood, and tissues, makes it a versatile and adaptable tool for biomarker discovery and validation. This versatility allows researchers to analyze clinical samples in a noninvasive manner and monitor protein changes over time, potentially facilitating the development of personalized diagnostic and treatment strategies.

Overall, PEA technology significantly enhances biomarker studies and protein analysis in neurodegenerative diseases. Its sensitivity, dynamic range, and compatibility with different sample types make PEA a potent and promising approach in resolving the complexity of these diseases. As research continues to advance, PEA has a high potential to accelerate the development of diagnostic tools and therapeutic interventions, ultimately improving patient outcomes and advancing our understanding of neurodegenerative diseases.

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