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Evolving relevance of neuroproteomics in Alzheimer's disease

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ABSTRACT

Substantial progresses in unraveling the biological fundamentals of Alzheimer disease (AD) have occurred over the past decades. However, the early diagnosis of AD, as well as that of many other neurodegenerative diseases, remains still challenging. Therefore, the discovery and validation of clinically appropriate biomarkers addressing the criteria of specificity, sensitivity, and repeatability is eagerly needed.

Remarkable innovations in the area of high-throughput technologies, such as sequencing, microarrays, and mass spectrometry-based analyses of proteins/peptides, have led to the generation of large global molecular datasets from a multiplicity of biological systems, such as biological fluids, cells, tissues, and organs. Such a methodological progress has shifted the attention to the execution of comprehensive analyses, with the aim of fully understanding the biological systems as a whole. The systems biology paradigm integrates experimental biology with accurate and rigorous computational modelling to describe and foresee the dynamic features of biological systems. The use of dynamically evolving technological platforms, including mass spectrometry, in the area of proteomics has enabled to rush the process of biomarker discovery and validation for refining significantly the diagnosis of AD. Currently, proteomics – which is part of the systems biology paradigm – is designated as one of the dominant mature sciences needed for the effective discovery of prospective biomarker candidates expected to be of major clinical value in the early diagnosis and prognosis of AD.

KEY WORDS: Alzheimer's disease; systems biology; omics sciences; proteomics; neuroproteomics; biomarkers; mass spectrometry; cerebrospinal fluid; blood; plasma/serum.

INTRODUCTION

The most recent years have been characterised by a growing understanding of the molecular bases of Alzheimer disease (AD). The pathogenesis of this complex neurodegenerative disorder implicates sequentially interacting pathological cascades, including both central events – the accumulation of the 42 amino acid-long amyloid β ($A\beta_{1–42}$) peptide into amyloid plaques and the formation of intraneuronal neurofibrillary tangles – and downstream processes – for instance, widespread neuroinflammatory reactions. These cellular/molecular events, in the end, cause a disintegration of the synaptic structures and induce perturbations of the anatomical and functional neural connectivities ***(1)***. According to the traditional "amyloid cascade hypothesis", the clearance and degradation of extracellular Aβ play a role of primary importance in the modulation of Aβ deposition; therefore, alterations of these processes are considered critical events in the pathogenesis of AD ***(2, 3)***.

A plethora of molecular alterations have been observed in the AD brain including, but not restricted to, changes in amyloid precursor protein (APP) metabolism ***(4)***, tau phosphorylation ***(5)***, lipid alterations ***(6)***, membrane lipid dysregulation ***(7)***, mitochondrial dysfunction, amplified oxidative stress, activation of neuroinflammatory pathways ***(8)***, and anomalous interplay of neurotransmitters ***(9)***. Given that these perturbations are reciprocally interrelated, a *systemic approach* is necessary in order to shed more light on the pathogenesis of AD at a complex network level ***(10, 11)***. The aim of the current manuscript is to provide a concise outline of the impact of proteomics – as part of the *systems biology paradigm* – in the context of AD pathophysiology.

NEUROPROTEOMICS AND THE ROLE OF SYSTEMS BIOLOGY TO UNDERSTAND ALZHEIMER'S DISEASE MECHANISMS

Like most neurodegenerative disorders, AD affects not only the neurological system. The central nervous system (CNS), encompassing the brain and the spinal cord, plays a role of paramount importance in all aspects of life, including some levels of modulation of the activity of all other systems in the human body ***(12)***. It is recognized that AD patients show physical decline; therefore, AD has been evidently associated with systemic manifestations that spread beyond the CNS ***(13)***. The physical decay is certainly driven to some extent by the progressive functional and behavioural failures linked to CNS degeneration ***(14)***. Thus, changes in CNS function unavoidably result in systemic dysfunction that affects multiple outside organs ***(12)***. The high extent of heterogeneity in the biological as well as behavioural-clinical genotypes of AD is reflected in the extensive variations at the level of neuropathological lesions, age of onset, patterns and types of

behavioural-clinical manifestations. These well-validated observations regarding AD emphasize the complexity of the multigenic nature of this disorder ***(15)***. Conventional biomedical strategies for exploring the speculated molecular mechanisms responsible for the pathogenesis of AD have commonly been focused on only a few significant genes and their associated products. The final outcome of such a line of research has been an insufficient and somewhat reductionistic understanding and knowledge of the intricate etiopathogenesis of AD. In contrast, the evolving hypothesis-free paradigm of *systems biology* – also referred to as *network biology* or *integrative biology* ***(16, 17)*** – is an integrative interdisciplinary approach using advances in multimodal high-throughput technological platforms that enable the examination of networks of biological pathways where elevated amounts of structurally/functionally different molecules are simultaneously explored over time at a system level (i.e., at the level of cells, tissues, organs, apparatuses, or even whole organism) ***(11)***. One of the prerequisites of systems biology is the "all-inclusive" enumeration and quantification of biological processes, followed by rigorous data inspection and integration, in order to allow the generation of hypotheses that need to be confirmed at a system level ***(11)***. Technologies utilized in systems biology have become relevant and applicable thanks to the development of various high-throughput screening strategies applied to the *omics sciences*. Omics disciplines cover genomics/epigenomics, transcriptomics, proteomics, and metabolomics/lipidomics. The concrete application of such omics sciences, in conjunction with dedicated computational tools, makes unquestionably realistic the full depiction of various biomolecules including DNA sequences, transcripts, proteins, metabolites/lipids ***(18)***. It should be highlighted that, among the rapidly emerging omics disciplines necessary to scrutinize the neurodegenerative disorders, with AD being investigated most comprehensively, *proteomics* has undeniably gathered substantial consideration. Proteomics is a rather newly developed area of study encompassing the large-scale inspection of protein structures, functions, interactions, and dynamics ***(19)***. Over the last years, the interest in using proteomics for clinical diagnosis purposes has grown exponentially. Actually, clinical proteomics is employed to reveal and/or corroborate novel diagnostic and prognostic *biomarkers*, drug targets, as well as to elucidate novel molecular mechanisms ***(20)***. *Neuroproteomics* represents one of the most relevant subcategories of proteomics ***(21)***. The general aim of clinical neuroproteomics is to explicate the protein-driven biological processes and the associated activities at the level of the CNS. As a result, neuroproteomics supports the analysis of protein expression and the detection of new original protein/peptide biomarkers in order to actively support the diagnosis, treatment monitoring, and prognosis of CNS pathologies ***(19)***. The depiction of the CNS proteome under both healthy and pathological

conditions is fully supported by the Human Proteome Organization (HUPO, available at http://www.hupo.org/), the largest global consortium that is committed to facilitate proteomic exploration bodily fluids and human tissues. Interestingly, the HUPO Brain Proteome Project (HUPO BPP, available at http://www.hbpp.org/), an open international project under the patronage of the HUPO, aims at explicating the CNS proteome in both aging and neurodegenerative disorders, including AD ***(22)***.

BIOMARKER EVIDENCE OF ALZHEIMER'S DISEASE PATHOLOGY

Remarkable methodological progress has led to a multimodal framework of AD biomarkers, including both biochemical and imaging markers ***(15, 23-26)***. Existing biomarkers of AD result from neurogenetics ***(27-29)***, structural/functional/metabolic neuroimaging and neurophysiology ***(30, 31)***, neurobiochemistry on biological fluids ***(32, 33)***, including both cerebrospinal fluid (CSF) ***(34-37)*** and blood (plasma/serum) ***(38-42)***. However, the diagnostic power of such a multimodal approach to AD diagnosis is yet to be established and needs to be additionally validated in terms of sensitivity, specificity, and predictive power ***(43-46)***. To this aim, substantial efforts among biotechnology, pharmaceutical and regulatory stakeholders, clinicians, researchers, and health care decision makers are eagerly required ***(47, 48)***. For a biomarker to mature to a validated and standardized clinical test, it should be feasible, reproducible, and fully available with quality control. Currently, the best established fluid biomarkers for AD include three *core* CSF molecules: $A\beta_{1-42}$ (that reflects Aβ plaque formation in the brain), phospho-tau (P-tau; that mirrors neurofibrillary tangle pathology in AD), and total-tau (T-tau; a marker of axonal degeneration) markers. All of these biomarkers have been validated against pathology and have 85-95% sensitivity and specificity for AD in both dementia and prodromal mild cognitive impairment (MCI) stages ***(35)***. At present, the amyloid cascade hypothesis, where aggregated forms of Aβ – especially soluble and diffusible Aβ oligomers – have been assumed to have a key role in AD pathogenesis, is highly relevant ***(2)***. However, the hypothesis has been challenged by failures of some recent phase III clinical trials aimed at blocking Aβ production, by using secretase inhibitors ***(49)***, or enhancing its clearance from the brain, by employing anti-Aβ immunotherapy ***(50, 51)***. As a result, such ineffective clinical trials of Aβ-targeting drug candidates have raised the idea that additional pathological pathways may be involved in the clinical expression of AD ***(52)***. In order to acquire more knowledge about the pathophysiology of AD, searching for unique and original biomarkers for potential disease-related molecular alterations is of great significance. A significant effective system for detecting these *biological markers* is via *proteomic* investigations ***(53)***.

OVERVIEW OF TECHNOLOGIES FOR NEUROPROTEOMIC ANALYSIS

Over the last 30 years, numerous technological platforms have been employed in neuroproteomics to scrutinize different biological samples collected from patients with different neurodegenerative disorders. Several steps characterize the workflow of a typical proteomic investigation, including: sample preparation; protein separation, identification and quantification; protein biomarker candidates validation (see Schevchenko and colleagues (2015) for a detailed methodological overview) ***(19)***. In particular, currently acknowledged proteomic methods include a sequence of separation, enzymatic digestion (often in conjunction with some kind of isotopic labelling) and mass spectrometric analysis. The step of protein *separation* is commonly performed by using two-dimensional polyacrylamide gel electrophoresis (2-DE), liquid chromatography (LC), or protein-chip arrays. The protein content of the sample is then subjected to tryptic (or another enzyme) digestion to generate peptides that are amenable to analysis by mass spectrometry (MS) or tandem mass spectrometry (MS/MS) ***(54)***. Notably, MS is considered a key technology for the systemic examination of proteins, i.e., from identification and quantification to characterization of post-translational modifications and protein-protein interactions. All mass spectrometers, regardless of type, ionization mode, or performance details, generate mass spectra, which plot the mass-to-charge ratio (m/z) of the detected ions *versus* ion abundance. The *identification* step is performed by correlating the experimentally discovered molecular masses with known protein/peptide masses via database search algorithms ***(19)***. However, whether a protein/peptide has been identified, what confers it the status of a candidate marker is its consistent variation in some characteristic such as abundance between two states, for instance, presence or absence of a given disease ***(15)***.

In recent times, there has been a remarkable development of methodologies and instrumentation for protein/peptide quantification including label-based and label-free technologies. A number of quantitative MS-based methods have been applied to neuroproteomics studies, such as traditional approaches – 2-DE and differential two-dimensional gel electrophoresis (2D-DIGE) – as well as more innovative quantitative systems, for instance: isotopic chemical labelling, label-free quantification methodologies, and selected reaction monitoring (SRM) ***(19)***. The labelled approaches – for instance, isobaric tags for relative and absolute quantitation (iTRAQ) ***(55)***, stable isotope labelling by amino acids in cell culture (SILAC) ***(56)*** and tandem mass tag (TMT) multiplex isobaric labelling (ref: Hölttä M et al., J Proteome Res. 2015 Feb 6;14(2):654-63) – display a substantial decrease in operation-related sample-to-sample variations since all samples are mixed and processed simultaneously after being labelled with different tags. The quantity of specimens

utilized in a single experiment is determined by the availability of tags ***(12)***. Differently from the labelled approaches, the label-free methods have the advantage of using protein quantification without needing any isotope labelling. Consequently, these methods are not laborious and can be applied to all biological samples ***(19)***. Label-free methods offer more flexibility regarding the amount of samples to be used in an experiment and need less hands-on operation. However, these approaches necessitate more computation time for data analysis and higher consistency from sample preparation. Furthermore, a stable mass spectrometer is needed because they are reliant on data comparison from independent MS runs ***(12)***. Notably, SRM is an MS-based, targeted protein measurement to identify relative and absolute peptide levels ***(57)***. In contrast to global protein profiling approaches, SRM enables the detection of well-defined target peptides with both high selectivity and sensitivity to measure low abundant proteins in complex specimens in relation to labelled standard peptides ***(19)***. The major benefits of the SRM compared to the conventional enzyme-linked immunosorbent assay (ELISA) are the substantially shorter lead-time and decreased costs and that the method does not rely on the optimal interaction of the target analyte with antibodies. Whereas the optimization of a single ELISA assay may take over 1 year, the development of a SRM assay may occur within weeks and allows multiplexing of assays for numerous biomarkers at the same time ***(58)***. As a result, SRM is considered a novel ground-breaking method to complement the traditional immunoassays, like ELISA, as a diagnostic tool in clinical practice ***(19)***. Definitively, *quantitative neuroproteomics* is a discipline commonly utilized to measure the relative and absolute protein abundance between two or more conditions (for example, healthy *versus* diseased or treated *versus* placebo) with the aim of investigating significant alterations in the proteome and disclosing novel protein biomarkers.

These neuroproteomic tools are now being combined to establish robust platforms for further improving quantitative, high-throughput proteomics. This effort is supported by the constant introduction of novel high-performance mass spectrometers. Actually, specialized laboratories with MS facilities can identify and quantify hundreds of proteins per day on a single MS system; thus, rapid advances in sample throughput, sensitivity, and accuracy are anticipated to occur ***(16, 17)***. In summary, the evolving area of neuroproteomics needs to address the issues of the heterogeneity and complexity of the CNS, both at cellular and subcellular level. However, if suitable sample preparation and separation procedures are executed, neuroproteomics tools utilizing MS are expected to provide clinically relevant data on biological fluids. These include proteins/peptides identification, characterization, and significant alterations in their relative/absolute amount according to the pathological conditions ***(19, 21)***.

One of the most important benefits of employing proteomics consists of the simultaneous characterization and quantification of hundreds or even thousands of proteins ***(59)***. Both *explorative* and *targeted* proteomic strategies have been performed to reveal major quantitative discrepancies in terms of protein expression among AD patients, patients with other non-AD associated disorders, and healthy individuals. *Explorative proteomic* studies aim at exploiting technological platforms on clinical samples – body fluids, tissues, definite groups of cells – in order to recognize distinctive biomarkers and, accordingly, characteristic pathological signatures that can facilitate the comprehension of the mechanisms responsible for the advancement of the disease. Therefore, the knowledge about the pathology will be also enriched. Differently, *targeted proteomics* studies are focused on the examination of one protein (and its altered forms), or a precise group of proteins/peptides that are exposed to proteomic investigation. In this setting, proteomic methods in cooperation with immunopurification and immunoprecipitation protocols are frequently employed. According to Brinkmalm and colleagues (2015), such a methodological strategy preserves the capacity and specificity of proteomic approaches and often results in higher analytical sensitivity ***(53)***.

LIMITATIONS OF NEUROPROTEOMICS

The variations of the proteome in AD have been scrutinized at different stages of the disease using a plethora of high-throughput systems. Different technological platforms have been utilized both in CSF ***(60–81)*** and blood (i.e., plasma/serum) ***(82–97)***. The most recent data in terms of applications of proteomics in the field of AD – both in CSF and blood specimens – are comprehensively reported by Brinkmalm and colleagues (2015) ***(53)***, Lista and colleagues (2015) ***(23)***, and Rosén and colleagues (2013) ***(32)***.

CSF is produced by filtration of blood in the choroid plexus and by diffusion from the extracellular matrix of the brain into the ventricles ***(98)***. CSF generation occurs at a rate of 500 mL/day and turns over about 4 times per day by drainage into the blood ***(99)***. It surrounds both the brain and the spinal cord and constantly receives a stream of proteins from the brain; indeed, nearly 20% of the amount of proteins in the CSF are known to originate from the brain ***(100)***. Given its contiguity to the diseased brain tissue and its anatomical interaction with the brain interstitial fluid, where neurochemical alterations associated to CNS pathologies are reflected, CSF is an invaluable source of protein/peptide biomarkers that supports the diagnosis of neurodegenerative disorders and can aid in monitoring their progression ***(101)***. In addition to intact proteins, CSF presents a large number of endogenous peptides ***(102)*** which are created by specific enzymatic reactions, while

others are generated by common degradation pathways. These peptides mirror a multitude of mechanisms in the brain, such as processes of secretion and aggregation as well as enzymatic activities and tissue re- and degeneration. For this reason, the investigation of the CSF is of high relevance to get more in detail into the pathology of CNS disorders. Nevertheless, the main drawback limiting the clinical application of the CSF is the invasive nature of the procedure for its collection: lumbar puncture, colloquially known as a spinal tap, is still considered in many countries a quite intrusive practice that may cause patient discomfort and displays side effects as the post-lumbar puncture headache ***(103)***. Even though there are studies demonstrating the low incidence of lumbar puncture-associated headaches and almost no additional clinical complications in a memory clinic setting ***(104)***, CSF sampling still suffers from a negative public reputation together with high rates of reservation among patients ***(105)***. Furthermore, the collection of CSF from healthy individuals is still an ethical concern.

Blood is considered a complex liquid tissue that includes cells and extracellular fluid. It can be divided into two compartments: plasma, i.e. the cell-free content of the blood, and the blood cells. Serum is blood that has been allowed to clot, removing cells and coagulation factors (i.e., plasma without coagulation factors). Potentially, blood is a rich source of biomarkers since proteins, nucleic acids, lipids, as well as other metabolic products can be detected in plasma, serum, and cellular compartments. The cellular fraction of blood includes erythrocytes, leukocytes, and platelets, which can be separated either crude, for instance via buffy coat after density gradient centrifugation, or isolated by flow cytometry into distinct cell clusters. The diversity of potential candidate biomarkers in blood is substantial and may embrace: proteins (in terms of their concentration, isoforms, and post-translational modifications); metabolic products subject to considerable alterations; nucleic acids (DNA and RNA). All these aspects emphasize that, unlike CSF and other body fluids, blood is quite a multifaceted tissue ***(106)***.

It should be highlighted that the search for protein biomarkers in blood present some challenges ***(107)***. Even though the high complexity of blood – as element reproducing the condition of the whole organism – may be considered a benefit, it may be also seen as a restriction. A first limitation, from a methodological viewpoint, is given by the high diversity of proteins and peptides. Alternative splicing mechanisms, conformational changes, and post-translational modifications allow proteins to appear in various forms, thus impressively increasing the heterogeneity of the plasma/serum proteome ***(108)***. A second restriction is represented by the large concentration range of plasma/serum proteins encompassing 10^{11} - 10^{12} orders of magnitude, from mg/mL to pg/mL ***(108)***. Third, around 90% of the content of total plasma/serum proteins belongs to one of the ten

major groups of the high-abundance proteins (HAPs): albumin (covering alone approximately 50%), immunoglobulins, transferrin, fibrinogen, α-2 macroglobulin, α-1 antitrypsin, C3-complement factor, and haptoglobin ***(107)***. These proteins are supposed not to be adequately "informative"; therefore, they cannot be exploited as disease biomarkers ***(109)***. The residual 10% is a complex mixture of middle- and low-abundance proteins (MAPs and LAPs); the tens of thousands of LAPs, assumed to be clinically significant and, thus, to denote potential biomarkers, are "masked" under few non-informative HAPs and referred to as the *"hidden" proteome* ***(110)***. The content of LAPs is many orders of magnitude inferior to that of HAPs. Usually, biological markers for malignant or non-malignant pathologies cover a range of concentrations from ng/mL to pg/mL ***(111)***. As a result, methods for separating and/or removing HAPs seem to have a key role to limit such a dynamic range and identify/quantify the majority of protein species in a sample. Overall, because of the *wide concentration range* and the well-known *complexity* of proteins (compared to other types of biological molecules), all proteomic technologies presently implemented are scrutinizing only a minor fraction of potential biomarker-relevant changes that can be detected in pathological conditions ***(106)***.

Similar to omics projects performed on other body fluid, plasma/serum proteomics displays various methodological issues, such as preanalytical variables, the need for standardizing specimen collection/processing, quantitation, and strategies on how to deal with biomarkers once they have been detected ***(107)***. Advancement in blood biomarker discovery is also based on the development of standard operating procedures (SOPs) for the suitable selection of patients and specimens to diminish the complexity of samples intended to be examined ***(112)***. Global initiatives such as the Human Plasma Proteome Project (HPPP) (available at http://www.hupo.org/initiatives/plasma-proteome-project/), as part of the Human Proteome Organization (HUPO) (available at http://www.hupo.org/), have been established to address matters associated with preanalytical variability and to initiate the process of drafting SOPs ***(113)***. Furthermore, there is ongoing progress in the development of informatic tools for data management as well as collaborations with other disease-related initiatives of the HUPO to expand the area of plasma/serum biomarker discovery ***(114)***. Additionally, an international collaboration for the initiation of preanalytical guidelines for AD blood-based biomarkers is underway as part of the Alzheimer's Association Professional Interest Area (PIA) on Blood Based Biomarkers (BBB-PIA) ***(39)***. The BBB-PIA is a part of the Alzheimer's Association's International Society to Advance Alzheimer's Research and Treatment (ISTAART), that has been established in order to develop a field-wide consensus on the harmonization of both preanalytical and analytical protocols and to address the requirement of a

biorepository of clinical reference samples, thus enabling not only assay harmonization but also clinical performance assessment ***(40)***. Finally, it should be also highlighted that preanalytical procedures are also provided by the Clinical and Laboratory Standards Institute (CLSI, available at http://clsi.org/) that represents a guiding source for individuals seeking to take research-derived techniques to clinic.

CONCLUSIONS

The working hypothesis of AD is that all forms of this pathology advance through the emergence and, at some time, convergence of failures in several systems, networks, signalling pathways, and the appearance of pathophysiological processes such as neuroinflammation, perturbed lipid homeostasis, apoptosis, oxidative stress, tau hyperphosphorylation with subsequent neurofibrillary tangles formation, and the amyloidogenic cascade leading to the production and release of different Aβ peptides. In the post-genomic era, the understanding of biological systems is dynamically evolving and progressing. Genomic research advanced to proteomics and led to a deeper knowledge of the structure and function of proteins. Proteomics is indeed considered one of the fastest developing disciplines enabling the fully elucidation of the crucial processes in growing, differentiation, and regulation occurring at various stages at cellular and intercellular levels. Proteomics has then given birth to the discipline of neuroproteomics as the aspects of neurological disorders – which appeared to be yet unclear less than a generation ago – commenced to be unveiled. Because of the highly degree of heterogeneity and inaccessibility of the human brain, neuroproteomics studies in humans have addressed biological fluids, mainly CSF and blood. CSF, in particular, is a highly relevant biological fluid for biomarker discovery as it is in close contact with the brain. From a basic research perspective, the development of proteomics – as well as the interrelated omics sciences, namely genomics/epigenomics, transcriptomics, metabolomics/lipidomics – has revealed the approach for the identification of novel molecular biomarkers from biofluids (besides cells, group of cells, and tissues). High-throughput molecular profiling approaches have the ability to accumulate large amounts of data concerning a given disease status or a specific phenotype, in an unbiased way ***(15)***. The close relationships among the various omics platforms are crucial to developing a clinically operative AD biomarker panel: such a *multi-omic* interdisciplinary system is anticipated to considerably advance the biomarker discovery area ***(41)***. Omics strategies, in combination with bioinformatics, including computational and statistical modelling, support and simplify the identification/characterization of DNA/RNA sequences, transcripts, proteins/peptides, metabolites/lipids, and other biomolecules. As a result,

large amount of composite information is collected via multiple high-throughput and high-content technological platforms for molecular profiling in AD which impacts various cellular/molecular pathways ***(15, 27)***. Since these heterogeneous data need to be integrated in an effective way, *systems biology* strategies are triggering the combined exploration of multiple interacting biochemical and genetic pathways and are providing the full depiction of the complex molecular pathogenesis of all forms of AD. This will represent the basis of providing effective *targeted* drugs and therapeutic strategies for AD treatment. Upcoming developments in the study of AD heterogeneity will possibly allow clinicians to provide more efficacious and helpful pharmacological treatments designated as *customized* (or *"tailored"*) – that is to say adapted – to the definite profiles of their AD patients. Proteomic information, integrated with data obtained from the other omics sciences, can inform a more accurate prediction of the risk of developing the disease, its progression as well as severity of symptoms, in a specific individual. This information needs to be utilized to "tailor" prevention and therapy to that subject as well as to make informed choices regarding lifestyle, screening, and preventative treatments. In order to develop the conception of *targeted therapeutic strategies* in the field of AD, it is necessary to integrate cutting-edge biomarker technologies and *transfertilization* from *more mature translational research fields* – such as the areas of *oncology* and *cardiovascular diseases* – which satisfy regulatory requirements for an accurate, sensitive, and well-validated surrogate marker of specific pathophysiological processes and/or clinical outcomes ***(15)***.

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