Fluid Candidate Biomarkers for Alzheimer’s Disease: A Precision Medicine Approach

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Abstract: A plethora of dynamic pathophysiological mechanisms underpins highly heterogeneous phenotypes in the field of dementia, particularly in Alzheimer’s disease (AD). In such a faceted scenario, a biomarker-guided approach, through the implementation of specific fluid biomarkers individually reflecting distinct molecular pathways in the brain, may help establish a proper clinical diagnosis, even in its preclinical stages. Recently, ultrasensitive assays may detect different neurodegenerative mechanisms in blood earlier. ß-amyloid (Aß) peptides, phosphorylated-tau (p-tau), and neurofilament light chain (NFL) measured in blood are gaining momentum as candidate biomarkers for AD. P-tau is currently the more convincing plasma biomarker for the diagnostic workup of AD. The clinical role of plasma Aß peptides should be better elucidated with further studies that also compare the accuracy of the different ultrasensitive techniques. Blood NFL is promising as a proxy of neurodegeneration process tout court. Protein misfolding amplification assays can accurately detect α-synuclein in cerebrospinal fluid (CSF), thus representing advancement in the pathologic stratification of AD. In CSF, neurogranin and YKL-40 are further candidate biomarkers tracking synaptic disruption and neuroinflammation, which are additional key pathophysiological pathways related to AD genesis. Advanced statistical analysis using clinical scores and biomarker data to bring together individuals with AD from large heterogeneous cohorts into consistent clusters may promote the discovery of pathophysiological causes and detection of tailored treatments.

Keywords: biomarkers; Alzheimer’s disease; neurodegeneration; cerebrospinal fluid; mild cognitive impairment; synaptic biomarkers; neuroinflammation; neurofilament light chain

1. Introduction

Alzheimer’s disease (AD) is the most common neurodegenerative disease (NDD), with 5.8 million Americans aged 65 years and older living with AD in 2020 [1]. Since Alois Alzheimer’s first description of the typical histological alterations of neuritic plaques (NP) and neurofibrillary tangles (NFT) in 1906 [2], more than eighty years have passed before amyloid beta (Aß) and phosphorylated-tau (p-tau) were identified as the main component of NP and NFT, respectively [3]. In 1984, the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) [4] set postmortem examination as the reference standard of AD diagnosis. Since then, the broad phenotypical variability of neurodegenerative diseases (NDDs) has pushed the efforts toward developing a classification based on the main misfolded protein deposition [5,6]. Nevertheless, the occurrence of these aggregates in multiple combinations is frequent, and NDDs are rather emerging as a spectrum of disorders characterized by the loss of proteostasis [7].
Due to the failure of numerous trials against amyloid pathology, the idea of “one drug fits all” treatment as an ultimate solution for an AD cure is fading [8]. In brief, the current framework on AD is more complex than previously thought because AD is not a mere plaque and tangle disorder. The following pathophysiological pathways leading to neurodegeneration have been recognized as clearly implicated in AD pathogenesis: (1) accumulation of misfolded proteins in the brain (Aβ peptides, tau and p-tau proteins, other co-pathologies), (2) vascular dysfunction, (3) synaptic disruption, and (4) neuroinflammation. The discovery of biomarkers indicating the modification of these processes at different levels in space and time is gaining momentum, especially in design tailored disease-modifying trials (Figure 1).

Our aim is to review the development of novel candidate fluid biomarkers tracking these key pathophysiological mechanisms in different matrices, especially cerebrospinal fluid (CSF) and blood. In relation to AD, we mainly focused on the diagnostic and prognostic value of these biomarkers, with particular attention to the novel ultrasensitive techniques.

2. Literature Search Methods

We performed a narrative review of literature focusing on novel candidate fluid biomarkers for AD. A systematic review of literature focused on plasma biomarkers detected by means of novel ultrasensitive techniques was performed in PubMed. We used the combination of the keywords “plasma”, “serum”, “amyloid-β”, “NFL” (neurofilament light chain), “p-tau”, “p-tau181”, “phospho-tau181”, “phosphorylated tau181”, “t-tau”, “α-syn”, “Simoa”, “immunoassay”, “immunomagnetic reduction”, “fully automated”, “immuno-infrared sensor”, “mass spectrometry”, and “multimer detection system”. Only papers in English published between 2014 and July 2020 and focused on AD were included in the final analysis. Overall, we identified 21 studies that provided relevant diagnostic and/or prognostic information (Figure 2). Among them, 10 were focused on amyloid-β
peptides, 7 were focused on p-tau or tau or both, and 4 were focused on NFL. For each paper, the study population, the study design (cross-sectional, perspective, retrospective), and the diagnostic and/or prognostic value of the investigated biomarker were analyzed. We classified the diagnostic value of each biomarker according to previously published recommendations as follows: “excellent” (area under ROC curve [AuROC] 0.90–1.00), “good” (AuROC 0.80–0.89), “fair or moderate” (AuROC 0.70–0.79), “poor” (AuROC 0.60–0.69), or “fail or insufficient” (i.e., no discriminatory capacity) (AuROC 0.50–0.59).

Figure 2. Flowchart displaying the article selection process.

3. Toward a Pathophysiological Definition of Alzheimer’s Disease

With the 1984 NINCDS-ADRDA criteria, the accuracy for probable AD diagnosis was suboptimal, with sensitivity between 70.9% and 87.3% and specificity between 44.3% and 70.8% [9]. The definite diagnosis relied on postmortem examination, with obvious limitations, since it is not applicable in vivo. For this reason, the International Working Group (IWG) [10] and later the National Institute on Aging and Alzheimer’s Association (NIA-AA) [11] published novel criteria for the diagnosis of AD incorporating in vivo biomarkers. According to the 2007 IWG criteria, AD can be identified in vivo by the presence of amnestic syndrome of the hippocampal type, which is characterized by low free recall that does not improve with cueing. Moreover, biomarkers must be consistent with AD pathology. These biomarkers are pathophysiological and topographical. The pathophysiological ones are low CSF Aβ1-42 peptide concentration, high CSF total tau (t-tau) or p-tau levels, and an increased cerebral uptake of amyloid tracers (e.g., Pittsburgh compound) with PET. The formers are hippocampal atrophy on volumetric Magnetic Resonance Imaging (MRI) and cortical regional hypometabolism on fluorodeoxyglucose FDG-PET, involving bilateral temporal parietal regions and posterior cingulate. IWG criteria managed to move from the static and binary/dichotomic vision of AD as a clinicopathological entity to its current dynamic clinical-radio-biological description [10]. The subsequent 2010 revision of IWG criteria overtook the amnestic-centered concept of AD and broadened the spectrum, adding the
rarer atypical forms of AD, such as primary progressive non-fluent aphasia, in particular logopenic aphasia, posterior cortical atrophy, and frontal variant AD. The so-called “asymptomatic at risk of AD” condition without clinical symptoms but with positive biomarkers of AD pathology was stated out, as well as the concept of “mixed AD”, implying the co-occurrence of clinical and biological features of other disease, such as parkinsonism (e.g., Lewy body pathology) or cerebrovascular disease [12]. Later on, these concepts were implemented in the IWG-2 criteria (2014) [13], where clinical diagnosis required specifying whether typical or atypical AD phenotypes occurred. Furthermore, the condition of a preclinical AD stage (for asymptomatic at risk and presymptomatic subjects) was defined in the presence of cognitive normal individuals with biomarkers indicative of AD pathophysiological process. Topographical biomarkers were used only for disease staging and monitoring. In parallel with IWG criteria, the NIA-AA diagnostic guidelines developed in 2011 [14–16] moved forward, defining the concept of mild cognitive impairment (MCI) due to AD (clinical MCI individuals with biomarkers indicating AD pathology). In fact, MCI due to AD had a high likelihood of developing AD over time. Subsequently, in 2016, the joint IWG-Alzheimer’s Association (IWG-AA) formalized a purely biological definition of AD, based on the positivity of biomarkers of both amyloidosis and tauopathy [17]. In the same years, the “A/T/N” classification system for AD was published. In this classification, the validated AD biomarkers were reported into three binary categories (presence or absence) based on the nature of the pathophysiology. “A” refers to the β-amyloid pathology (cerebral amyloid PET or CSF Aβ42); “T,” refers to taupathology (CSF p-tau, or cerebral tau PET); and “N” refers to neurodegeneration or neuronal injury tout court ([18F]-fluorodeoxyglucose-PET, structural MRI, or CSF total tau [18]). This unbiased biomarker-based scheme was recently incorporated in the current NIA-AA criteria published in 2018, with the addition of C for clinical change, to integrate the biomarkers condition with clinical cognitive status [19]. All A+ individuals are considered part of the “Alzheimer’s continuum”, while only A+ and T+ define AD. Non AD-specific parameters, namely neurodegenerative/neuronal injury biomarkers (N) and cognitive symptoms (C), define staging [19]. A- individuals fall either in the “normal AD biomarker” category with A-T-(N-), or “Suspected non-Alzheimer’s pathophysiology” (SNAP) with A-T+(N)-, A-T-(N)+, or A-T+(N)+.

Among the mimics of typical AD-type dementia, Primary Age-Related Tauopathy (PART) should be mentioned [20,21]. PART identifies individuals with cerebral NFT indistinguishable from those of AD, in the absence of Aβ plaques; notably, NFT are restricted to the medial temporal lobe, basal forebrain, brainstem, and olfactory areas [21]. At a clinical level, associated manifestations range from normal cognition to amnesic cognitive impairment, but they are rarely a frank dementia. Similarly, a recently described entity is the limbic-predominant age-related TDP-43 encephalopathy (LATE) [22]. LATE is a common TDP-43 proteinopathy that generally affects older adults, and it is frequently associated with hippocampal sclerosis. Aβ plaques or tauopathy may also coexist. Generally, co-pathologies in AD subjects are common with approximately 30% of AD patients showing a cerebrovascular disease [23]. The concomitant deposition of Aβ and α-syn proteins is also described in postmortem examination in about 30% of AD individuals [24,25] but also in up to 40% of patients with Parkinson’s disease (PD), Parkinson disease dementia (PDD), and dementia with Lewy bodies (DLB) clinical diagnoses [26–28].

Thus, in this scenario, it is likely that no single biomarker could reach a 100% diagnostic accuracy, being AD biologically multifaceted with a clinical picture reflecting pathology only in terms of probabilistic association. Despite these intrinsic limitations, the use of core biomarkers in the AD diagnostic workup improves accuracy (up to 90%) with a relevant impact on AD stratification and selection for disease-modifying trials tailored against Aβ and tau pathologies [29].

To date, the neuropathological hallmarks of AD remain extracellular Aβ plaques and NFTs [30,31]. First proposed in 1992, the “amyloid cascade hypothesis” [32] has been later corroborated by genetic and biochemical data and currently represents the dominant pathogenetic model of AD. According to this hypothesis, the deposition of fibrillar Aβ plaques within the brain promotes the accumulation of NFTs, synaptic disintegration and neuronal death by inflammatory mechanisms, modification of ions homeostasis, kinase/phosphatase activity, and oxidative stress [33]. In particular, Aβ plaques create an
unique environment that facilitates tau aggregation, initially as dystrophic neurites surrounding Aβ plaques, followed by the formation and spread throughout the brain in a prion-like manner of NFTs and neuropil threads [34]. NFTs are characteristic of AD and are composed of hyperphosphorylated tau [35–37]. The hyperphosphorylation of tau protein reduces its affinity for microtubules and promotes its capacity to aggregate and fibrillize [38]. Therefore, microtubules are destabilized, and axonal transport is impaired [39].

The hyperphosphorylated tau could also migrate in the somatodendritic compartments where it interacts with Aβ and enhances synaptotoxicity [40], finally causing cell death due to a toxic gain of function mechanism [41,42].

At the same time, much interest is growing around the role of inflammation in the pathogenesis of AD. The contribution of inflammation to the pathophysiology of AD has been already hypothesized more than 20 years ago [43–45]. The attention has been focused especially on microglia activation, which seems to occur decades before AD onset [46–48]. Furthermore, a correlation between neuroinflammation and amyloid or tau accumulation in the human brain has been reported in several investigations [46,49–51]. The microglial activation produces two different phenotypes. The microglial “pro-inflammatory” phenotype (M1) displays pro-inflammatory cytokines (IL-1β, IL-6, IL-12, tumor necrosis factor (TNF)-α, CCL2), nitric oxide, reactive oxygen and nitrogen species. The “anti-inflammatory” one (M2) sustains the production of IL-10 and TGF-β, and it increases the expression of neurotrophic factors (nerve-derived growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins, glial cell–derived neurotrophic factor (GDNF)), and several other signals involved in downregulation, protection, or repair processes [52]. The chronic stimulus on microglia by Aβ peptides accumulation is likely to lead to a protracted inflammation, and, in turn, increase Aβ deposition, in a vicious circle [53].

The inflammatory state would promote the production and release of pro-inflammatory cytokines, which could themselves have a detrimental effect by inducing neuronal cell death.

Another relevant key pathophysiological mechanism that contributes to AD is vascular dysregulation [54]. Several pieces of evidence support the role of chronic cerebral hypoperfusion as the primum movens of AD pathology [55,56]. Hypoxia can activate β-secretase-1 and γ-secretase as well as increase Aβ peptides accumulation [57]. Furthermore, the reduced supply of oxygen and nutrients affects neurons per se, and, in turn, it promotes blood–brain barrier dysfunction, increasing oxidative stress and inflammation [58]. Since Aβ deposition derives essentially from an imbalance between production and removal, clearing system impairment is emerging as a further key pathophysiological mechanism leading to AD. In particular, this mechanism involves the alteration of astroglial-mediated interstitial fluid (ISF) bulk flow or glymphatic system [59,60]. This pathway is mainly modulated by the sleep–wake cycle and seems to be important for the sleep-driven clearance of Aβ [61]. Vascular pathology seems to be additive or even synergic to AD pathology as a cause of cognitive impairment [62,63]. This cross-talk is most evident for cerebral amyloid angiopathy (CAA), which shares Aβ deposition with AD typical neurotic plaques that are localized within leptomeningeal and intracortical arteries, arterioles, and capillaries. CAA is commonly found in AD brains: up to approximately 50% of subjects with severe NP load [64]. CAA can affect perivascular drainage impairing glymphatic circulation, thus reducing a major route of Aβ clearance from the brain [59]. Intracranial atherosclerosis was found to be an additional, although not strictly neurodegenerative, strong risk factor for AD dementia [65].

4. Fluid Biomarkers: Ultrasensitive Measurement Techniques

Due to several advantages over invasive (e.g., CSF Aβ peptides), expensive and scarcely available (e.g., cerebral amyloid-PET) diagnostic tools, technologies aiming at quantifying NDDs biomarkers in blood are gathering momentum.

However, the discovery of CNS-related biomarkers in blood presents challenging issues: (a) the concentration of a biomarker released in CNS is lower than in CSF, considering that it has to cross the blood–brain barrier and that the blood volume is consistently larger than the CSF one, (b) biomarkers
could also be directly expressed peripherally, and the contribution of CNS might be difficult to quantify, (c) proteolytic degradation of the analytes by plasma proteases and confounding blood proteins may interfere with biomarker measurement [66].

The traditional enzyme-linked immunosorbent assay (ELISA) was extensively used in the last few decades. It showed a substantial intrinsic variability in the quantification of plasma/serum biomarkers and provided overlapping results in the discrimination between NDDs and cognitively healthy subjects [67,68]. The large sample volume required in the analysis combined with a sensitivity limited to the picomolar range could be addressed as the main weakness of this method. Therefore, ultrasensitive techniques often representing ELISA-based evolutions have been developed for blood biomarker discovery.

The automated xMAP (multi-analyte profiling) Luminex technology, a flow cytometric method, allows the adaptation of several immunoassay formats to simultaneously detect multiple analytes on different sets of microspheres in a single well [69]. Through pre-made calibrators, it reduces measurement variability, partially overcoming some limitations of conventional ELISA methods (Table 1).

Another emerging technique is the single-molecule array (Simoa), which is essentially a digital ELISA. This fully automated method is based on capturing antibody-coated paramagnetic beads loaded in arrays of femtomolar-sized reaction chambers with a volume 2 billion times smaller than conventional ELISA. Ultimately, by acquiring fluorescence images, an increase in signal will reflect the presence of single enzyme-associated immunocomplexes [70] (Table 1). This method is a candidate prescreening tool but may be potentially useful throughout the whole AD spectrum [71]. Large-scale longitudinal multicenter studies are anyway needed for the standardization and harmonization of preanalytical and analytical variables [72].

Combining the unique advantages of highly specific immunoreactions and electrochemiluminescence (ECL) biosensors, ECL immunoassays (ECLIA) have been implemented in several automated platforms. A wide dynamic detection range, low background noise, and simple optical set-ups are the strengths of this technique [73,74] (Table 1).

For protein analysis, immunoprecipitation has also been coupled with mass-spectrometry (IP-MS) providing a robust quantitative tool to identify antigens based on their intrinsic chemico-physical properties [75]. A significant advantage of this method is the possibility to analyze complex mixtures of Aβ peptides at very low concentrations in a single assay. However, multi-step structured analysis strategies are required in order to reduce the influence of non-specific binders and improve the signal quality (Table 1) [76]. A tailored approach for the identification of oligomers has been adopted in the Multimer Detection System (MDS), which is a modified sandwich ELISA originally designed to detect prion proteins. As opposed to the conventional method, this strategy relies on two epitope-overlapping antibodies for capturing and detecting an epitope, so that only multimers will bind to both antibodies, allowing their selective detection over monomers, which conversely will only bind to one of them (Table 1) [77,78].

| METHOD | PROS | CONS |
|--------|------|------|
| xMAP   | It is a flexible technology with a workflow ranging from semi- to fully automated options. It enables the concomitant evaluation of multiple analytes in a single sample representing a time-, cost-, and labor-saving method. It enables a shift from a hypothesis-based analysis of known targets to a data-driven approach [83,84]. | The simultaneous measurement of multiple ligands may favor cross-reactivities (“matrix effect”). A rigorous adherence to the manufacturer’s protocols is required to minimize any artifacts when using these kits [85]. |

Table 1. Key points of ultrasensitive techniques for the detection of putative blood biomarkers for AD.
### Table 1. Cont.

| METHOD | PROS | CONS |
|--------|------|------|
| **Simoa** | It is a fully automated technology based on antibody-coated paramagnetic microbeads. It has a great sensitivity (x1000 greater compared to conventional immunoassays), being able to detect single proteins at subfemtomolar concentrations. It is capable of multiplexing with short turnaround times and a remarkable throughput (up to 66 samples/h). It represents the most established ultrasensitive technology for blood biomarkers of AD to date (kits to measure Aβ1-42, p-tau181, t-tau, and NFL are available). A higher sensitivity compared to both ELISA and ECLIA-based methods was shown for the detection of NFL in serum [86–88]. | Wide longitudinal multicenter studies are warranted for the standardization of preanalytical and analytical protocols parameters [72,88]. |
| **ECLIA (MSD, Elecsys)** | ECLIA-based methods are adopted in semi- to fully automated (MSD) and fully automated (Elecsys) platforms. MSD is a flexible multi-array technology enabling the detection of biomarkers in single and high throughput multiplex formats. It provides a high inter-laboratory reproducibility, low matrix effects, reliability and cost-effectiveness [75,74]. Aβ peptides measured with Elecsys showed among the best accuracies in predicting the Aβ status assessed by either amyloid-PET or CSF Aβ1-42/Aβ1-40 ratio when compared to other techniques [89]. MSD provides good to optimal accuracy regarding the discriminative role of plasma p-tau181 to detect AD [90,91]. | Antibodies and solid matrices also isolate many non-specific “contaminants”. To reduce the interferences with the signals and increase specificity in the detection of the antigens, targeted precautions are recommended (e.g., two rounds of repeated processing during the immunoprecipitation) [75,76]. Compared to automated ELISA-based techniques, IP-MS is a labor-intensive, low-throughput and time-consuming method not easily implementable on a wide scale [92]. |
| **IP-MS** | It is able to characterize and quantify peptides by introducing them into the mass spectrometer after isolation through antibody-driven immunoprecipitation. Using this technique, optimal discriminative accuracies in detecting AD were reached by the Aβ1-40/Aβ1-42 ratio measured in plasma [75]. | Its sensitivity in detecting Aβ oligomers failed to reach the cut-off of >80% that is needed for the validation of a biomarker [94]. |
| **MDS** | It is an ELISA-based sandwich assay aiming at measuring oligomerization tendency in blood. It uses capture antibodies and epitope-overlapping detection antibodies to identify oligomers or multimers [93]. | Its sensitivity in detecting Aβ oligomers failed to reach the cut-off of >80% that is needed for the validation of a biomarker [94]. |
| **Immuno-infrared sensor** | It is an antibody-based method to extract all the Aβ peptides from blood samples, allowing the identification of β-sheet enriched conformations [79]. Compared to established ELISA-based tests, it does not measure the absolute biomarker concentration but the relative frequency shift in the infrared, reducing the influence of concentration fluctuations caused by biological variances [80]. Unique features of this assay are the absence of labels (enzymes, fluorescent or radioactive molecules) with potentially confounding effects, being the analytes detected based on their intrinsic physical properties, a simple and low-cost procedure and the low sample volume needed. It is able to identify the initial Aβ misfolding, occurring several years before clinical manifestation of AD [80]. | Further tests in different clinical set-ups are needed to investigate the potential effects of sample handling and to evaluate their potential as screening-assays [79–81]. |
| **IMR** | It measures the change in magnetic susceptibility caused by the association of antigens with antibody-coated paramagnetic nanobeads [82]. In contrast to ultrasensitive digital ELISA methodologies, IMR is a single-antibody immunomassay. Less stereocistical interferences and a better ability to detect Aβ42 oligomers failed to measure in plasma [82]. It is a fully automated technology based on antibody-coated paramagnetic microbeads. It has a great sensitivity (x1000 greater compared to conventional immunoassays), being able to detect single proteins at subfemtomolar concentrations. It is capable of multiplexing with short turnaround times and a remarkable throughput (up to 66 samples/h). It represents the most established ultrasensitive technology for blood biomarkers of AD to date (kits to measure Aβ1-42, p-tau181, t-tau, and NFL are available). A higher sensitivity compared to both ELISA and ECLIA-based methods was shown for the detection of NFL in serum [86–88]. | In regard to Aβ peptides, it provides results that are not consistent with those of the ELISA- and MS-based methods. The unspecific detection of Aβ aggregates or Aβ binding proteins likely caused by the single-antibody nature of the technique may explain the increase of plasma Aβ1-42 levels in AD patients compared to healthy controls [96]. |

**Abbreviations:** Aβ: amyloid β; Aβ1-40: amyloid β-peptide 1-40; Aβ1-42: amyloid β-peptide 1-42; AD: Alzheimer’s disease; CSF: cerebrospinal fluid; ECLIA: electrochemiluminescence immunoassay; ELISA: enzyme-linked immunosorbent assay; IMR: immunomagnetic reduction; IP-MS: immunoprecipitation coupled with mass spectrometry; MDS: multimer detection system; MSD: meso scale discovery; NFL: neurofilament light chain; p-tau: phosphorylated-tau; Simoa: single molecule array; t-tau: total tau; xMAP: multi-analyte profiling.
Among the recently developed biosensors in AD research, the immuno-infrared sensor represents a promising label-free technique not aiming at discriminating particular Aβ species, but rather aiming at identifying the secondary structure distribution of all misfolded peptides. Thus, it is potentially exploitable in a preclinical setting (Table 1) [79–81].

A further virtuous application of the immunoassay principles is part of the immunomagnetic reduction (IMR) technique, in which magnetic antibody-coated nanoparticles dispersed in aqueous solution oscillate under external multiple alternating current (AC) magnetic fields. The association of target molecules determines a reduction in the AC magnetic susceptibility of capturing nanoparticles that will be as high as the concentration of the analytes (Table 1) [82]. Compared to ELISA-based techniques (e.g., Simoa), this method does not make use of beads to purify or concentrate antigens, and it is virtually able to quantify smaller proteins in higher number. Whether this could represent an advantage to detect Aβ peptides in plasma is still to be elucidated (Table 1).

Huge efforts have been made to develop and refine these technologies. Nevertheless, there is an urgent need to promote unbiased cross-platform evaluations for an effective method standardization.

In view of a targeted-oriented approach to AD, the adoption of guidelines to systemize preanalytical and postanalytical procedures across laboratories, aiming at finding consensus on a high-performance scalable platform for the discovery and approval of blood biomarkers, would be strongly recommended.

5. Biomarkers Tracking Amyloid Pathology

Accumulating evidence from the clinical research consistently supports that CSF Aβ1–42 peptide shows an inverse correlation with plaque load in the brain [97–101] and provides important diagnostic information throughout the continuum of the AD spectrum. Therefore, this biomarker is currently integrated in the diagnostic criteria of AD; it is used for subject selection in clinical trials and approved in medical practice as well [13,14,19]. On the contrary, CSF Aβ1–40 peptide alone, albeit prevailing over the other Aβ species in both CNS and periphery, showed no relevant correlation with AD dementia [76,87–89]. Notably, the ratio of CSF Aβ1–42/Aβ1–40 has been found to predict cortical amyloid-PET positivity more accurately than CSF Aβ1–42 alone [90–92], improving the discrimination of AD vs. non-AD demented patients (Table 2) [93,94].

Table 2. Overview on the possible context of use of fluid biomarkers in AD.

| Diagnostic Value | Prognostic Value | Monitoring Treatment |
|------------------|------------------|----------------------|
| Preclinical Phase | Prodromal Phase  | Full-Blown Picture   |
| Amyloid pathology|                  |                      |
| Aβ peptides      | Blood            | +                    |
| Aβ peptides      | CSF              | +                    |
| p-tau            | Blood            | +                    |
| p-tau            | CSF              | +                    |
| Neuroinflammation| YKL-40           | +                    |
| Synthetic dysfunction| Ng             | +                    |
| NFL              | CSF              | +                    |
| NFL              | Blood            | +                    |

Legend: plus sign (+): potential use, supportive data available. Abbreviations: Aβ: amyloid beta; t-tau: total tau; p-tau: phosphorylated-tau; YKL-40; Ng: neurogranin; NFL: neurofilaments; CSF: cerebrospinal fluid.

Some investigations suggest that also the CSF Aβ1–42/Aβ1–38 ratio turned out to improve cerebral amyloid deposition compared with CSF Aβ1–42 alone [94–96]. In addition, in pharmacological trials, short Aβ peptides detection in CSF may help monitor patients receiving drugs that modulate γ-secretase (Table 2) [97,98]. Among Aβ species, Aβ oligomers that are likely to play a key role in AD
pathogenesis could be potentially used as preclinical biomarkers in CSF. Unfortunately, the detection of Aβ species and Aβ oligomers is challenging due to their polymorphous and unstable nature. Moreover, their concentration in biofluid is low, and they compete with other proteins and Aβ monomers. For the aforementioned reasons, most of the existing techniques are not satisfactory and reporting conflicting results so far [99]. As it also emerged from the Olsson and colleagues meta-analysis in 2016, Aβ1–42 and Aβ1–40 peptides measured in blood with traditional ELISA methods did not discriminate AD from healthy controls [95].

Undoubtedly, the validation of relatively new technologies in the last few years—e.g., Simoa [100], immunoprecipitation-mass spectrometry (IP-MS) assays [74], stable labeling kinetics protocols [101], multimer detection system (MDS) [102], xMAP technology [103], immuno-infrared sensor [104], electrochemiluminescence immunoassays (ECLIA) [105–112]—led to a significant increased sensitivity in amyloid peptides detection in periphery when compared to the conventional ELISA technique, with drastically lower concentrations (up to the femtolitre) in blood than in CSF [65]. Particularly, in a 2017 study based on an IP-MS method, the plasma Aβ1-42/Aβ1-40 ratio was significantly lower in amyloid-PET positive compared with amyloid-PET negative participants. This ratio reported a good accuracy in distinguishing the two populations [101] (Table 3) [113–131].

In 2018, Nakamura and colleagues used the same technique to measure Aβ peptides in plasma of cognitive normal individuals, MCI and AD with dementia subjects, finding higher levels of plasma Aβ1–40/Aβ1–42 ratio in amyloid-PET positive compared with amyloid-PET negative individuals [75]. Regardless of the clinical diagnosis, the ratio of Aβ1–40 and Aβ1–42 peptides (Aβ1–40/Aβ1–42), and that of Aβ precursor protein fragment (APP69–711) and Aβ1–42 (APP69–711/Aβ1–42), had an excellent diagnostic accuracy in discriminating cerebral amyloid-PET positive and amyloid-PET negative subjects (Table 3). Similarly, in another study including a cohort of subjective memory complainers, a condition at risk for AD, the plasma Aβ1–40/Aβ1–42 ratio, turned out to be the best predictor of cerebral amyloidosis among a series of tested candidate biomarkers (e.g., β-site amyloid precursor protein cleaving enzyme 1 or BACE1, t-tau, NFL [72] (Table 3). Additional investigations performed using the Simoa technique reported a moderate accuracy of Aβ1-42/Aβ1-40 ratio in identifying the amyloid status. The Aβ1-42/Aβ1-40 ratio was lower in amyloid-PET positive compared with amyloid-PET negative participants [122,132] (Table 3). A further study in which Aβ peptides concentrations were assessed with a fully automated ECLIA technique confirmed the good discriminative role of Aβ1-42/Aβ1-40 ratio, in both validation and discovery cohorts [92] (Table 3). Similarly, Hanon and colleagues in a large investigation including 1040 MCI or AD participants reported that plasma Aβ1–42 and Aβ1–40 concentrations assessed by means of a kit based on a multiplex xMAP technique were lower in AD than in both MCI and non amnestic MCI (naMCI), suggesting a gradual decrease of these peripheral biomarkers with the course of the disease, in accordance to previous findings [109,133,134]. Conversely, in some studies where IMR was used, plasma Aβ1–42 concentrations are higher in AD patients than in healthy subjects [95,121]. Lue and colleagues reported a moderate diagnostic accuracy of this plasma biomarker in one cohort and excellent in the other [121] (Table 3). Moreover, in a 2018 study carried out by Nabers and colleagues using an immuno-infrared sensor, when compared with controls, not only were the concentrations of β-sheet-enriched Aβ peptides higher in severe AD dementia patients, as previously demonstrated [80], but they were also higher in prodromal AD patients, reaching a good diagnostic accuracy in identifying the amyloid status assessed by PET (Table 3) [80]. A recently developed ELISA method detecting Aβ multimers from monomers (MDS) showed a good accuracy of plasma Aβ oligomers in distinguishing AD patients from healthy controls [118] (Table 3). In parallel, in a subsequent study in which traditional ELISA was applied, plasma BACE-1 increase has emerged as another surrogate hallmark of AD progression [135]. In an effort to assess the plasma concentrations of Aβ1–38, Aβ1–40 and Aβ1–42 simultaneously, Shahpasand-Kroner and colleagues found that the Aβ1–42/Aβ1–40 and Aβ1–42/Aβ1–38 ratios are significantly lower in patients with AD dementia than in patients with dementia due to other causes and have a good accuracy in differentiating the two groups [120] (Table 3).
Table 3. Diagnostic and prognostic role of blood Aβ peptides, p-tau, t-tau, and NFL proteins measured with ultrasensitive techniques in AD.

| Reference            | Population Description                     | Study Design   | Technique                        | Diagnostic Value                                                                                           | Prognostic Value |
|----------------------|--------------------------------------------|----------------|----------------------------------|------------------------------------------------------------------------------------------------------------|-----------------|
| Ovod V. et al., 2017 | N = 41 (CU, AD dementia)                   | Longitudinal   | IP-MS and stable labeling kinetics protocols | Aβ₁₋₄₂/Aβ₁₋₄₀ in differentiating amyloid positive participants vs. negative: AuROC = 0.89 with amyloid-PET and CSF Aβ₁₋₄₂ as reference standards | NA |
| Wang M. et al., 2017 | N = 61 (CU, AD dementia)                   | Cross-sectional| MDS                              | Aβ oligomers in differentiating AD patients vs. CU subjects: AuROC = 0.84 with clinical diagnosis (AD) as reference standard | NA |
| Lue L. et al., 2017  | N = 124 (CU, AD dementia); U.S. cohort: N = 32; Taiwan cohort: N = 92 | Cross-sectional| IMR                              | Aβ₁₋₄₂ in differentiating AD patients vs. CU subjects: AuROC = 0.69 (U.S. cohort); AuROC = 0.96 (Taiwan cohort) with clinical diagnosis (AD) as reference standard | NA |
| Nakamura A et al., 2018 | N = 484 (CU, MCI, AD)                  | Cross-sectional (retrospective) | IP-MS                            | APP/Aβ₁₋₄₂ and Aβ₁₋₄₀/Aβ₁₋₄₂ in differentiating amyloid positive participants vs. negative: AuROC ≥0.90 compared with amyloid-PET as reference standard | NA |
| Nabers A. et al., 2018 | N = 385 (CU, prodromal AD, AD); Sweden cohort: N = 73; Germany cohort: N = 312 | Cross-sectional and nested case control | Immuno-infrared sensor       | β-sheet-enriched Aβ peptides in differentiating: - amyloid positive participants vs. negative: AuROC = 0.78 (Sweden cohort) compared with amyloid-PET as reference standard; - AD vs. CU subjects: AuROC = 0.80 (Germany cohort) | NA |
| Shahpasand-Kroner H. et al., 2018 | N = 40 (AD dementia, dementia due to other reasons) | Cross-sectional | ECLIA                            | Aβ₁₋₄₂/Aβ₁₋₄₀ in differentiating AD dementia vs. dementia due to other reasons: AuROC = 0.87 with clinical diagnosis as reference standard | NA |
| Verberk I. et al., 2018 | N = 248 (SMC)                              | Longitudinal   | Simoa                            | Aβ₁₋₄₂/Aβ₁₋₄₀ in differentiating amyloid negative SMC vs. positive: AuROC = 0.77 with CSF Aβ₁₋₄₂ and amyloid PET as reference standards | Low Aβ₁₋₄₀/Aβ₁₋₄₂ is associated to MCI or dementia conversion (HR = 2.0) also after correcting for age and sex (HR=1.67) |
| Palmqvist S. et al., 2019 | N = 1079 (CU, MCI, AD); Sweden cohort: N = 842; Germany cohort: N = 237 | Multicenter and longitudinal | ECLIA                            | Aβ₁₋₄₂ + Aβ₁₋₄₀ (used as separate predictors in a logistic regression) in differentiating amyloid negative participants vs. positive: AuROC = 0.80 (Sweden cohort) and AuROC = 0.86 (Germany cohort) compared with CSF Aβ₁₋₄₂/Aβ₁₋₄₀ ratio as reference standard | NA |
| Vergallo A. et al., 2019 | N = 276 (SMC)                              | Longitudinal   | Simoa                            | Aβ₁₋₄₀/Aβ₁₋₄₂ in differentiating amyloid positive SMC vs. negative: AuROC = 0.77 compared with amyloid-PET as reference standard | NA |
| Chatterjee P. et al., 2019 | N = 95 (CU)                                | Cross-sectional | Simoa                            | Aβ₁₋₄₀/Aβ₁₋₄₂ along with age and APOE ε₄ status in differentiating amyloid positive participants vs. negative: AuROC = 0.78 compared with amyloid-PET as reference standard | NA |
| Reference                        | Population                              | Study Design              | Technique     | Diagnostic Value                                                                 | Prognostic Value                                                                 |
|---------------------------------|-----------------------------------------|---------------------------|---------------|----------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| Mielke MM. et al., 2017 [123]   | $N = 458$ (CU, MCI)                     | Longitudinal              | Simoa         | Both the middle (HR = 2.43) and the highest (HR = 2.02) tertiles of plasma t-tau levels are associated with increased risk of MCI in CU participants |
| Mielke MM. et al., 2018 [124]   | $N = 269$ (CU, MCI, AD)                 | Cross-sectional           | Simoa         | In the discrimination between amyloid negative participants vs. positive: - plasma p-tau181: AuROC = 0.80; - plasma t-tau: AuROC = 0.60 compared with amyloid-PET as reference standard |
| Yang C. et al., 2018 [125]      | $N = 73$ (CU, MCI, very mild AD)        | Cross-sectional           | IMR           | Plasma p-tau181 discriminating: - CU vs. MCI due to AD: AuROC = 0.85; - MCI due to AD vs. mild AD: AuROC = 0.78 with clinical diagnosis as reference standard |
| Park JC. et al., 2019 [126]     | $N = 76$ (CU, MCI, AD)                  | Both cross-sectional and longitudinal designs | Simoa (tau protein)/xMAP(Alβ1-42) | In the discrimination between tau positive participants vs. negative: - plasma t-tau/Alβ1-42 ratio: AuROC = 0.89; - plasma t-tau: AuROC = 0.80 with tau-PET as reference standard |
| Janelidze S. et al., 2020 [90]  | $N = 589$ (CU, MCI, AD dementia, non-AD dementia) cohort 1: $N = 182$ cohort 2: $N = 344$ cohort 3 (neuropathology cohort): $N = 63$ | Both cross-sectional and longitudinal designs | ECLIA         | Plasma p-tau181 in differentiating: - tau positive vs. negative participants: AuROC = 0.87–0.91 depending on different brain regions with tau-PET as reference standard (cohort 1); - AD dementia vs. non-AD dementia: AuROC = 0.94 with clinical diagnosis as reference standard (cohort 1); - Alβ positive vs. negative participants: AuROC = 0.80 (cohort 1 and cohort 2) with Aβ PET as reference standard; - AD dementia vs. non-AD dementia group: AuROC = 0.85 with neuropathology autopsy as reference standard (cohort 3) |
| Thijssen E. et al., 2020 [91]   | $N = 404$ (CU, MCI, AD, CBS, PSP, FTLD, nvfPPA, svPPA) 3 independent cohorts | Both cross-sectional (retrospective) and longitudinal designs | ECLIA         | Plasma p-tau181 in differentiating: - AD (56) vs. FTLD (190) participants: AuROC = 0.89 with clinical diagnosis as reference standard; - Aβ-PET positive CU (11) vs. negative (29): AuROC = 0.86 with amyloid-PET as reference standard; - AD (15) vs. FTLD-tau participants (52): AuROC = 0.86 with neuropathology autopsy as reference standard |

**Table 3. Cont.**
Table 3. Cont.

| Reference               | Population                                                                 | Study Design  | Technique | Diagnostic Value                                                                                           | Prognostic Value |
|-------------------------|---------------------------------------------------------------------------|---------------|-----------|-----------------------------------------------------------------------------------------------------------|------------------|
| Karikari T. et al., 2020 [127] | kari                                                                     | Longitudinal  | Simoa     | Plasma p-tau181 in differentiating AD participants vs:                                                   |                   |
|                         |                                                                           |               |           | - amyloid β negative young adults: AuROC = 0.99;                                                         |                   |
|                         |                                                                           |               |           | - CU older adults: AuROC = 0.90–0.98 across cohorts;                                                     |                   |
|                         |                                                                           |               |           | - vascular dementia participants: AuROC = 0.92;                                                          |                   |
|                         |                                                                           |               |           | - PSP or CBS participants: AuROC = 0.89;                                                                 |                   |
|                         |                                                                           |               |           | - PD or MSA participants: AuROC = 0.82 with clinical diagnosis as reference standard                     |                   |
|                         |                                                                           |               |           | - tau-PET positive vs. tau-PET negative individuals AuROC = 0.83–0.93 across cohorts with tau-PET as reference standard | NA                |
| Mattsson N. et al., 2017 [128] | N = 570 (CU, MCI, AD)                                                   | Case-control  | Simoa     | Plasma NFL in differentiating CU vs. AD participants:                                                   | NA                |
|                         |                                                                           |               |           | - AuROC = 0.87 with clinical diagnosis as reference standard                                             |                   |
| Lewczuk P. et al., 2018 [129] | N = 99 (CU, MCI, AD)                                                    | Cross-sectional | Simoa     | Plasma NFL in differentiating CU vs. diseased participants:                                             | NA                |
|                         |                                                                           |               |           | - AuROC = 0.85 with clinical diagnosis as reference standard                                             |                   |
| Steinacker P. et al., 2018 [130] | N = 132 (CU, MCL, AD, bvFTD)                                            | Longitudinal  | Simoa     | Serum NFL in differentiating bvFTD vs:                                                                  |                   |
|                         |                                                                           |               |           | - AD: AuROC = 0.67                                                                                       |                   |
|                         |                                                                           |               |           | - MCI: AuROC = 0.90                                                                                      |                   |
|                         |                                                                           |               |           | - CU: AuROC = 0.85 with clinical diagnosis as reference standard                                         |                   |
|                         |                                                                           |               |           | Serum NFL in differentiating:                                                                           |                   |
|                         |                                                                           |               |           | - bvFTD vs. AD groups selected on the basis of CSF Aβ_{1–42} levels: AuROC = 0.79                    |                   |
|                         |                                                                           |               |           | - bvFTD vs. AD groups selected on the basis of both CSF Aβ_{1–42} and tau/p-tau levels: AuROC = 0.78   |                   |
| Preische O. et al., 2019 [131] | N = 405 (controls - AD mutation non-carriers, AD mutation carriers subdivided into presymptomatic mutation carriers, converters and symptomatic mutation carriers) | Longitudinal  | Simoa     | Rate of change of serum NFL in differentiating:                                                          |                   |
|                         |                                                                           |               |           | - non-mutation carriers vs. presymptomatic mutation carriers: AuROC = 0.70                              |                   |
|                         |                                                                           |               |           | - non-mutation carriers vs. symptomatic non-mutation carriers: AuROC = 0.89                             |                   |
|                         |                                                                           |               |           | Baseline serum NFL levels in differentiating:                                                             |                   |
|                         |                                                                           |               |           | - non-mutation carriers vs. presymptomatic mutation carriers: AuROC = 0.49                              |                   |
|                         |                                                                           |               |           | - non-mutation carriers vs. symptomatic non-mutation carriers: AuROC = 0.85                             |                   |

Abbreviations: AD: Alzheimer’s disease; AuROC: area under the receiver operating curve; Aβ: amyloid β; Aβ_{1–40}: amyloid β-peptide 1–40; Aβ_{1–42}: amyloid β-peptide 1–42; bvFTD: behavioral variant frontotemporal dementia; CBS: corticobasal syndrome; CSF: cerebrospinal fluid; CU: cognitively unimpaired; ECLIA: electrochemiluminescence immunoassay; FTD: frontotemporal dementia; FTLD: frontotemporal lobar degeneration; HR: hazard ratio; IMR: immunomagnetic reduction; IP: immunoprecipitation; IP MS: immunoprecipitation coupled to mass spectrometry; MCI: mild cognitive impairment; MDS: multimer detection system; MS: mass spectrometry; MSA: multiple system atrophy; NA: not assessed; NFL: neurofilament light chain; nfvPPA: non-fluent variant primary progressive aphasia; PD: Parkinson’s disease; PPA: primary progressive aphasia; PSP: progressive supranuclear palsy; p-tau181: phospho-tau181; Simoa: single molecule array; SMC: subjective memory complainers; svPPA: semantic variant primary progressive aphasia; t-tau: total-tau; xMAP: multi-analyte profiling.
In summary, quite concordant results regarding low plasmatic concentrations of Aβ1–42 and low Aβ1–42/Aβ1–40 ratio in AD are reported in most of the studies in which an ultrasensitive technique has been applied. Overall, recent data suggest a low plasma Aβ1–42 and Aβ1–42/Aβ1–40 ratio as being a specific feature of AD patients with a weak to moderate and a moderate to high concordance with amyloid-PET outcomes, respectively [136] (Table 2). Further investigations comparing the different ultrasensitive techniques in the same populations will provide more accurate information regarding advantages and drawbacks.

6. Biomarkers for Tau Pathology

Together with CSF Aβ1–42, CSF t-tau and p-tau are considered as core biomarkers for AD diagnosis [11,137], and they are currently used for subject selection in clinical trials. Both CSF tau species are higher in patients compared to non-demented individuals. P-tau is more specific than t-tau for AD pathology and plays a main role in differential diagnosis being substantially normal in non-AD dementias [138]. Recent studies have shown that CSF tau can predict disease progression in both cognitively unimpaired and MCI subjects (Table 2) [139–141]. In 1999, Hulstaert and colleagues reported that the combined measurements of CSF Aβ1–42 and tau had a better outcome than the individual biomarker in differentiating AD patients from controls and other dementias [142]. In an effort to establish the utility of both CSF t-tau/Aβ1–42 and p-tau/Aβ1–42 ratios, several authors ended up confirming those preliminary findings [98,143,144]. Moreover, the ability of both ratios to predict disease onset and progression was proven in other studies, including normal individuals and MCI subjects, respectively [140,145,146].

Since 2013, it has been reported that plasma t-tau levels, measured through an assay based on digital array technology, were higher in AD participants compared with both MCI and healthy subjects, but they did not show significant modifications in subsequent longitudinal evaluations (Table 2) [116,147]. Shortly thereafter, a large meta-analysis demonstrated the increase of plasma t-tau levels to be strongly associated with AD (Table 2) [115].

In the last four years, highly sensitive immunoassay techniques significantly implemented tau levels detection in peripheral blood. Indeed, Mattsson and colleagues to assess plasma t-tau concentration in two separate cohorts applied the Simoa technique. In AD patients compared with both MCI and healthy controls, an increase of plasma tau was demonstrated but with overlapping results. More interestingly, longitudinal evaluations revealed that high baseline levels of this biomarker were predictive of cognitive decline, higher atrophy rates at MRI, and hypometabolism at 18F-FDG-PET as well [148]. A longitudinal study carried out using the same technique found increased plasma tau levels associated with a higher risk of MCI and cognitive decline in MCI subjects, irrespective of the total Aβ-burden in the brain [123] (Table 3). In 2019, Park and colleagues highlighted that both plasma t-tau measured by the Simoa technique as well and the plasma t-tau/Aβ42 ratio positively correlated with cerebral tau-PET uptake. Moreover, plasma tau-related biomarkers concentrations were significantly higher in Tau-PET+ subjects compared with Tau-PET- subjects and could differentiate the two groups with good accuracy (Table 3). It is noteworthy that the plasma t-tau and t-tau/Aβ42 ratio could predict the cerebral accumulation of this misfolded protein after a 2-year follow-up [149]. A clear association between plasma and CSF levels of p-tau was also found in Aβ+ patients, including the presymptomatic stage (Aβ+ cognitively unimpaired), but not in Aβ− individuals [90]. Overall, these data suggest to some extent that plasma t-tau concentration is high in AD patients, but the substantial overlap with normal controls hinders its diagnostic utility (Table 2). Interestingly, an additional study found out that plasma p-tau concentrations improve diagnostic accuracy significantly compared to plasma t-tau by reaching a good capability in the discrimination of amyloid-positive and amyloid-negative subjects (Table 3) [124]. Moreover, plasma p-tau levels assessed by IMR have shown a good accuracy in differentiating unimpaired and MCI subjects [125] (Table 3).

The potential diagnostic role of plasma p-tau has been outlined in three recent investigations. In a first study performed with a novel ECLIA technique, plasma p-tau concentrations not only could
discriminate AD and frontotemporal lobar degeneration (FTLD) participants with good accuracy, but they also identified amyloid-PET positive participants among elderly and MCI, and they predicted the rate of cognitive decline in AD and MCI over a 2-year follow-up (Table 3) [91]. The second study including three separate cohorts with 589 individuals (controls, MCI, AD, and non-AD NDDs) revealed that plasma p-tau levels likewise assessed by means of the MSD platform increase with disease progression (from preclinical to frank dementia phases encompassing prodromal/MCI stage) and can distinguish AD dementia from non-AD dementia with excellent accuracy (Table 3). Furthermore, plasma p-tau concentration was more elevated in Aβ+ cognitively unimpaired individuals than in Aβ− ones and in Aβ+ MCI who progressed to AD dementia compared to those who did not convert [90]. These results were confirmed in another study involving four independent cohorts (1131 total subjects) in different clinical contexts. Actually, plasma p-tau concentration measured by the Simoa technique discriminated AD dementia patients from both cognitively unimpaired subjects and other NDDs (including tauopathies such as Progressive Supranuclear Palsy and Corticobasal Syndrome) with optimal diagnostic accuracy (Table 3). Moreover, plasma p-tau predicted future cognitive decline over time. Interestingly, plasma p-tau concentration strongly correlates with cerebral amyloid-PET burden, even in amyloid-PET positive but tau-PET negative subjects, suggesting its crucial role in detecting the earliest disease phases. Thus, this biomarker might represent a screening tool implementable in different clinical settings and contexts of use [127].

7. Biomarkers for Neuroinflammation

The role of inflammation in AD pathogenesis was first suggested more than 20 years ago. Microglia, astrocytes, cytokines, and chemokines play a central role in disease pathogenesis since early phases [45,150]. Furthermore, amyloid and tau accumulation is linked to neuroinflammation [46,49,50,151], and Aβ accumulation evokes an exaggerated or heightened microglial response inducing and amplifying inflammatory reactions [152]. Therefore, biomarkers of neuroinflammation are gaining momentum in preclinical stages of AD and are useful to establish the eligibility of patients into new clinical trials [153–156].

A potential biomarker of neuroinflammation is the microglia/astrocyte-expressed protein YKL-40. YKL-40 is a glycoprotein belonging to the family of 18 glycosyl hydrolases, and it is alternatively named human cartilage glycoprotein-39 (HC gp-39) or chitinase-3-like-1 protein (CHI3L1) [157]. CSF YKL-40 concentration is able to differentiate patients with typical AD dementia from cognitively normal controls with fair diagnostic accuracy [158,159]. Limited data regarding the ability of CSF YKL-40 to discriminate different NDDs are available so far. Actually, CSF YKL-40 differentiated AD from DLB, PD [160], FTLD [161], and non-AD MCI [162] with only a moderate diagnostic accuracy. Furthermore, CSF YKL-40 concentration is higher in AD versus Aβ-positive MCI subjects [163], and it significantly increases over time in the former (Table 2) [163]. CSF YKL-40 showed no ability in differentiating stable and progressing MCI [164,165], although it may predict progression to overt dementia in MCI [165] (Table 2). CSF YKL-40 levels negatively correlated with cortical thickness in specific AD-vulnerable areas, such as middle and inferior temporal areas in Aβ42-positive subjects [166] and grey matter volume in APOE ε4 carriers [167]. Interestingly, a positive association between CSF YKL-40 and t-tau has been reported in asymptomatic preclinical stages of AD and other NDDs [110,168], thus suggesting a link of YKL-40 with an underlying tau-driven neurodegenerative mechanisms [169].

YKL-40 has also been investigated in plasma, and elevated levels have been reported in patients with mild AD [170] and early AD [171] compared with controls. Unfortunately, plasma YKL-40 did not, so far, demonstrate utility as a diagnostic biomarker and for predicting cognitive decline (Table 2) [170,172]. To sum up, YKL-40 is an unspecific pathophysiological biomarker tracking immune/inflammatory response in NDDs, and it could be helpful as a monitoring biomarker for targeted anti-inflammatory therapies [169].

Another emerging biomarker of inflammation is “Triggering Receptor Expressed on Myeloid cells 2” (TREM2). TREM2 receptors play an important role in the pathogenesis of AD [173]. In the
early stages of AD, TREM2 seems to be upregulated, probably in a protective intent [174]. However, due to the activation of inflammatory response, a detrimental role may prevail in later stages [175]. Some TREM2 genetic variants are related to AD possibly impairing microglia Aβ phagocytic ability and reducing, as a consequence, the cerebral Aβ peptides clearance [167]. TREM2 has been proposed as AD biomarker in CSF, but with conflicting results so far. Some studies found higher CSF levels of TREM2 in AD [176–179] and MCI [176] compared to controls, and in subjects with MCI due to AD (or prodromal AD) compared with preclinical AD and AD dementia patients (Table 2) [179]. However, another study showed no difference between AD or MCI patients and cognitively normal controls [180]. A link between high CSF TREM2 value and neurodegeneration was proposed in MCI, considering that it positively correlated with gray matter volume and a negative correlation with mean diffusivity was detected [181]. Higher levels of TREM2 mRNA and TREM2 protein expressed in peripheral blood mononuclear cells were identified in AD patients compared to controls, with an inverse correlation with MMSE [182]. Moreover, TREM2 gene expression was found to be higher in MCI than AD patients [183]. Finally, a possible role of TREM2 as CSF and blood biomarker for AD has been suggested, but few data are currently available, and additional research is needed.

Another interesting candidate inflammatory biomarker is the monocyte chemoattractant protein-1 (MCP-1), which is a member of the C-C chemokine family and a potent chemotactic factor for monocytes [184]. Elevated CSF MCP-1 levels were found in AD patients compared to controls [185]. Noteworthy, also in blood, MCP-1 could be higher in MCI subjects than in controls [185].

Several other inflammatory biomarkers in CSF and blood have been investigated for their potential use as biomarkers for AD. A large metaanalysis exploring inflammatory biomarkers in CSF demonstrated that AD patients could express higher levels of TGF-β compared with controls [350 Molievo]. TGF-β1 is a neurotrophic, anti-inflammatory factor, and it enhances Aβ clearance by microglia activation. Since the early phases of disease, a reduced expression of TGF-β has been described both in postmortem AD studies [186,187] and in animal models [188–190]. Two recently published meta-analyses investigating inflammatory biomarkers in blood reported an elevated tumor necrosis factor (TNF)-α, IL-12 [191], IL-1β, IL-2, IL-6, IL-18 [191,192], and reduced IL-1 receptor antagonist concentration in AD patients compared with controls. Mounting data about IL-6 as an AD biomarker are available. Blood IL-6 levels are associated with severity of cognitive decline in AD [193] and positively correlated with the cerebral ventricular volumes [194] and with matched CSF samples [195]. Blood IL-6 concentration was even higher in MCI individuals [135], suggesting that this biomarker is altered also in prodromal AD stages.

8. Biomarkers for Synaptic Dysfunction

Synaptic dysfunction is a core feature of AD, occurring early in the disease course. Synaptic strict correlation with cognitive impairment and with Aβ and tau accumulation in AD, thus suggesting a central role in the underlying neurodegenerative process [196]. Based on these observations, several synaptic proteins have been investigated as potential diagnostic and prognostic biomarkers in AD. These include the quite well-characterized Neurogranin (Ng) and other emerging biomarkers such as Synaptosomal-Associated Protein 25 (SNAP-25), Synaptotagmin 1 and 2 (SYT-1 and SYT-2), Neurpentraxin 2 (NPTX-2), and Growth Associated Protein 43 (GAP-43) [197,198].

Ng is a post-synaptic protein largely expressed in the excitatory neurons of the hippocampus and cerebral cortex that acts as a calcium-sensitive modulator of post-synaptic signaling pathways and of long-term potentiation (LTP) [199]. Two recently published meta-analyses reported higher CSF Ng levels in AD compared to MCI and normal controls, thus supporting a role of CSF Ng as a useful diagnostic tool (Table 2) [200,201]. In particular, Ng reported good or even optimal diagnostic accuracy in differentiating AD patients with a full-blown clinical picture from cognitively normal subjects [202]. However, CSF Ng concentration discriminates between stable and converting MCI with poor diagnostic accuracy [200,201,203]. As regards its prognostic value, higher baseline CSF Ng levels are detected in controls and in MCI subjects who will convert to AD compared to non-converters, indicating a role of Ng in predicting progression to AD dementia in both cognitively normal [204]
and MCI individuals (Table 2) [205,206]. CSF Ng could be also a reliable biomarker in the diagnostic workup of dementia being specifically more elevated in AD than non-AD dementias (FTD, DLB, but also VaD) [168,207–210]. Intriguingly, CSF Ng levels are high in AD patients with a typical amnesic phenotype, suggesting its role in the stratification and identification of AD subtypes, as a selective indicator of hippocampal degeneration [207]. In addition to Ng, other synaptic proteins have been explored as candidate biomarkers for AD. In particular, SNAP-25, a pre-synaptic protein involved in vesicle docking and neurotransmitter release, showed good accuracy in differentiating AD and MCI from normal controls (Table 2) [211–213]. Furthermore, high baseline CSF SNAP-25 levels predict future conversion to AD in MCI individuals [212]. Other pre-synaptic proteins such as SYT-1, SYT-2, NPTX-2, and GAP-43 are candidate as biomarkers to differentiate AD, MCI, and cognitive normal controls [197,213]. Importantly, an inverse correlation between CSF and neuron-derived plasma exosomes (NDE) levels of Ng, SYT-2, and GAP-43 has been observed [200,214]. Even if these results would deserve further supporting evidence, NDE may represent a window on the early synaptic dysfunction in AD and pave the way to a minimally invasive assessment (blood sample) of synaptic biomarkers in cognitively impaired and unimpaired subjects.

9. Biomarkers of Neuronal Injury

NFL is a subunit of neurofilaments that are neural cytoplasmic proteins designated to the structural stability of neurons; they are present in dendrites, soma, and also in axons. Axons physiologically release a low amount of NFL proteins that increase with aging [215]. The concentration of NFL significantly increases in both CSF and in blood as a result of axonal injury or neurodegeneration [216–219]. NFL in CSF is usually measured by sandwich ELISA technology. On the contrary, blood NFL concentration is 40-fold lower than in CSF, and it is below the sensitivity of ELISA or electrochemiluminescence assay technology [215]. Promising results came from recently developed ultrasensitive techniques capable of detecting even low concentrations of NFL in blood (Simoa) [220]. Despite being a sensitive biomarker of axonal injury, NFL is unspecific and did not discriminate between neurological diseases with a similar rate of axonal loss. However, growing data showed that CSF and, above all, blood NFL identifies neurodegeneration from early stages [215]. Indeed, NFL (CSF and blood) showed a good diagnostic accuracy in differentiating AD and FTD from healthy controls (Table 3) [128,129,131,208,221,222]. According to these results, a possible context-of-use of this biomarker is to rule out neurodegeneration in mimics such as psychiatric disturbances, or to early detect, within screening programs, the neurodegenerative process in a specific population at high risk (e.g., diabetes, elderly, genetic mutation carriers). Increased blood NFL concentration could also help clinicians to proceed or not with more invasive and expensive examinations in individuals with subjective memory complaints [215]. Moreover, CSF NFL but not t-tau, p-tau, and Ng might be a reliable risk biomarker being associated with a threefold higher risk to develop MCI over a median follow-up of 3.8 years in a population of cognitively healthy individuals [223]. CSF [224,225] and blood [128,226,227] NFL tightly correlated each other and with disease severity. In this regard, in a prospective case-control study including normal controls, MCI, and AD dementia patients, plasma NFL correlated with CSF NFL, poor cognition, cerebral atrophy, and brain hypometabolism [128].

Serum NFL concentration correlated with the estimated years to symptom onset and disease severity in autosomal dominant AD mutation carriers, suggesting its possible role as a risk biomarker in subjects with autosomal genetic mutations for AD (Table 3) [227]. Promising data concern the role of NFL in the differential diagnosis between FTD and AD. Actually, CSF NFL is higher in FTD patients compared to early onset AD, and the addition of NFL analysis improves the diagnostic accuracy of the traditional core biomarkers (p-tau181 and Aß42) up to a sensitivity of 86% and a specificity of 100% [228]. Similar findings were also reported in an autopsy-confirmed AD and FTD study (Table 3) [229]. Moreover, serum NFL could help in the differentiation of Primary Progressive Aphasia (PPA) variants. Indeed, serum NFL is increased in PPA compared to controls and discriminates...
between nfvPPA/svPPA (with a more likely FTD pathology) and lvPPA (where an AD pathology is expected in more than 50% of cases) with 81% and 67% of sensitivity and specificity, respectively [230].

Visinin-like protein 1 (VILIP-1) is emerging as a surrogate of signaling disruption and neuronal injury. VILIP-1 is a neuronal calcium sensor protein involved in signaling pathways related to synaptic plasticity [231]. A high intracellular concentration of Ca2+ induces the reversible translocation of VILIP-1 to the membrane components of the cell modulating signaling cascade in the neurons via the activation of specific membrane-bound targets [232]. The dysregulation of Ca2+ homeostasis is involved in AD neurodegeneration, bringing to a reduced intracellular expression of VILIP-1 and a quite selective damage of VILIP-1-containing neurons (cortical pyramidal cells, interneurons, septal, subthalamic, and hippocampal neurons) [232]. Therefore, this biomarker significantly increases in CSF [231]. Since VILIP-1 contributes to an altered Ca2+ homeostasis leading to neuronal loss [232], it is mainly considered a biomarker of neuronal injury. CSF VILIP-1 is higher in AD than in controls, but its diagnostic accuracy remains limited, especially in the prodromal stage (Table 2) [115,233,234]. Although VILIP-1 tightly correlated with p-tau and t-tau in CSF, conflicting results concern its relationship with Aβ peptides [235,236]. CSF VILIP-1 and the VILIP-1/Aβ-42 ratio negatively correlate with MMSE and with the cerebral amyloid load, and they may predict a cognitive decline over time [233,234,236–240].

10. Toward Alternative Pathophysiological Pathways and Novel Matrices

The research on novel putative biomarkers in AD recently focused on two main directions: the exploration of new still under-characterized pathophysiological pathways, including mixed neuropathology models, and the identification of alternative easily accessible matrices.

TAR DNA-binding protein 43 (TDP43) is a DNA and RNA binding protein involved in transcription and splicing. TDP-43 contributes to neuroinflammation and may play a role in mitochondrial and neural dysfunction. In ALS and FTLD, its hyperphosphorylated and/or ubiquitinated cytoplasmic inclusions are detected [241,242] but also 20–50% of AD patients may show concomitant TDP-43 pathology [243–245]. Interestingly, TDP-43 pathology can be triggered by Aβ peptides [244]. In AD, increased plasma TDP-43 levels have been found compared to normal controls [246]; furthermore, plasma levels were increased also in the preclinical stage of subjects who subsequently progressed to AD dementia [247]. However, the evidence of a diagnostic and prognostic role of TDP-43 in AD is currently quite limited as well as its role in differentiating AD from other dementia mainly involving the hippocampus and memory (e.g., LATE) [22].

Lewy-related pathology (LRP), primarily consisting of α-synuclein (α-syn) aggregates, has been detected in more than half of autopsied AD brains, and higher levels of α-syn in the CSF of patients with MCI and AD have been associated with AD pathology and cognitive decline [248,249]. Moreover, CSF total α-syn (t-α-syn) and oligomeric α-synuclein (α-α-syn) levels were higher in AD [250] compared to PD, PD dementia and DLB individuals [251,252]. The use of standard ELISA methods to assess CSF α-syn levels does not ensure good diagnostic accuracy in discriminating AD from synucleinopathies [250]. Nevertheless, RT-QuIC [253] and protein misfolding cyclic amplification (PMCA) [254] are promising tools to identify AD individuals with α-syn co-pathology. Furthermore, growing interest toward the evaluation of α-syn heterocomplexes with Aβ1–42 (α-syn/Aβ) or tau (α-syn/tau) measured in red blood cells (RBCs) as peripheral pathophysiological markers of NDDs has been displayed [254]. Despite both α-syn alone, α-syn/Aβ and α-syn/tau heteroaggregates being found lower in AD compared to cognitive normal controls when isolated from red blood cells (RBC), only RBC α-syn/Aβ and α-syn/tau heterodimers discriminated AD from controls with fair accuracy [254].

Exploring alternative easily accessible matrices as a source of putative biomarkers is another key point of the search for novel fluid biomarkers. In this frame, exosomes represent an innovative and promising non-invasive tool to track early neurodegenerative changes occurring within the central nervous system. Exosomes are vesicles containing potential biomarkers for NDDs released into the extracellular space (that can be isolated from several body fluids) [243,254]. Proteins reflecting key
events of the neurodegenerative process have been isolated in exosomes extracted from CSF and blood by using proteomic analysis [244–246]; in particular, p-tau was isolated in CSF exosomes from patients with mild AD (Braak stage 3) [247], and increased levels of exosomes-associated tau and Aβ were found in AD patients compared to controls [248]. Finally, other easily accessible matrices such as the retina may represent an open window on early neurodegenerative events in AD [249]. Amyloid pathology was demonstrated in the retina, and high-resolution non-invasive retinal imaging [47–51] represents an in vivo approach for visualizing Aβ deposits [250–252]. Indeed, retinal Aβ accumulation positively correlated with cerebral amyloid plaques [8]. Furthermore, decreased flow velocities in the retinal central veins were found in both MCI and AD compared to controls, thus suggesting a strict correlation with the underlying early neurodegenerative changes [253]. However, this field of research remains in its pathfinding stages, and a consensus on retinal imaging modalities, methodologies, and measures is still missing [253].

11. Conclusions

Recent research efforts are expanding the array of biomarkers on detecting and stratifying NDDs. Since 2007, fluid biomarkers have been reported within the diagnostic criteria of AD. In particular, Aβ42 peptide, p-tau, and t-tau proteins measured in CSF became essential for a “modern” AD definition. The conceptual shift from a phenotype to a biomarker-based (or a precision medicine) diagnostic approach allowed the inclusion of the atypical subtypes within the AD spectrum and the exclusion of AD-mimics. For instance, patients with early and predominant behavioral impairment but positive for core pathophysiological biomarkers are categorized as AD and not as FTD. By contrast, individuals showing cognitive impairment of the hippocampal type but negative for core biomarkers are not considered AD. Definitely, the identification of a specific pathophysiological process in vivo by one or more biomarkers prevails on clinical phenotype. Unfortunately, validated fluid biomarkers used for AD diagnosis are invasive, time-consuming, expensive, not easily repeatable and, most importantly, not applicable as screening tools in large asymptomatic populations. On the other hand, the preclinical or prodromal identification of AD is urgent for patient recruitment in future disease-modifying treatments. This is an “expert” opinion based on the current literature, reporting the diagnostic and prognostic value of fluid biomarkers in AD. Five candidate molecules—three in plasma measured using ultrasensitive techniques (Aβ peptides, p-tau, and NFL proteins) and two in CSF (Ng and YKL-40)—with different potential context-of-use (Table 2) may be proposed. These molecules may enrich the current array of fluid biomarkers—CSF Aβ42, t-tau, and p-tau—for a more precise management of AD, and, broadly speaking, NDDs. These biomarkers are useful to both classify patients in different diagnostic categories and to track the pathophysiological mechanisms underlying neurodegeneration. The blood biomarkers (Aβ peptides, p-tau, NFL) are probably not more accurate than the respective molecules measured in CSF. However, they may be easily repeated over time, proposed in screening programs, and monitor treatments in disease-modifying trials. On the other hand, CSF YKL-40 and Ng are proxies of additional pathophysiological mechanisms related to AD, namely neuroinflammation and synaptic disruption, that cannot be efficiently evaluated with peripheral blood biomarkers. Therefore, CSF YKL-40 and Ng may be used in a subsequent diagnostic step to better stratify patients with prodromal or definite AD.

Plasma p-tau is increased in AD patients compared to controls and MCI individuals, discriminating AD demented patients from both cognitively unimpaired subjects and other NDDs with optimal diagnostic accuracy [91]. In several recent studies from different research groups, its classificatory accuracy surprisingly overlaps with cerebral amyloid-PET [90,124]. Moreover, plasma p-tau predicts a future cognitive decline over time [91]. Therefore, plasma p-tau, being easily repeatable, could be proposed in screening, diagnostic, prognostic, and monitoring context-of-uses. Simoa is the ultrasensitive technique used with more successful results across the studies on plasma p-tau so far. Of course, additional studies are needed. In addition, plasma t-tau concentration correlated with future cognitive decline, increased atrophy rates measured by MRI, and cerebral hypometabolism in
FDG-PET images, but the results are less convincing and charted overlapping values among AD with dementia, prodromal, and preclinical AD groups [148].

Plasma Aβ peptides may represent a further significant improvement to facilitate the in vivo detection of amyloid pathology, substituting traditional core CSF biomarkers in next years. The combination of CSF traditional biomarkers (e.g., Aβ1-42/Aβ1-40 ratio, t-tau/Aβ1-42, and p-tau/Aβ1-42 ratios) can improve the diagnostic accuracy as well as the prediction of cognitive decline in AD patients. Similarly, the combination of plasma and serum biomarkers into ratios may increase the diagnostic power, although further evidence is needed [109,133]. Mounting data revealed that a low plasma Aβ1-42 and Aβ1-42/Aβ1-40 ratio are quite specific of AD pathology, although the concordance with cerebral amyloid-PET examination is variable and should be carefully evaluated in future studies [75,92,120]. In brief, further investigations should clarify in larger prospective studies: (1) the more accurate method to detect Aβ peptides and related by-products in plasma, (2) the pathophysiological role of plasma Aβ peptides and Aβ oligomers, and (3) their diagnostic and prognostic value as biomarker of AD.

NFL is mainly a marker of axonal degeneration and considered an unspecific indicator of neurodegeneration. Importantly, CSF and plasma NFL strictly correlated in all studies, suggesting that plasma NFL would be a reliable peripheral biomarker consistently reflecting modifications within the CNS. Indeed, many studies on NDDs demonstrated that diagnostic and prognostic accuracies of plasma and CSF NFL overlap [254]. NFL is a good example as a versatile biomarker for multiple context-of-use. It is useful to differentiate NDDs from mimics such as psychiatric disturbances or to early detect neurodegenerative processes in particular populations at risk (e.g., diabetes, elderly, genetic mutation carriers). NFL values were associated with a threefold higher risk to develop MCI, demonstrating a potential prognostic value [128,226,227]. Finally, the negative predictive value of plasma NFL might be used as a first step in screening programs for neurodegeneration, involving individuals with subjective memory complaints and late-onset psychiatric disorders. Concerning AD, plasma NFL showed a promising role in differentiating AD from bvFTD patients. It is likely that bvFTD individuals with an underlying TDP-43 pathology (related to amyotrophic lateral sclerosis) reported significantly higher plasma NFL value than AD subjects. Plasma NFL could early discriminate AD from more aggressive neurodegenerative dementia such as CJD [244]. Simoa was the only ultrasensitive technique used to measure plasma NFL in AD studies.

An increasing number of studies are focused on the development of precise biomarkers tracking additional key pathophysiological pathways leading to neurodegeneration, such as synaptic disruption and neuroinflammation. The pre-synaptic protein Ng measured in CSF is the most promising indicator of a synaptic dysfunction and hippocampal damage [202]. It could help stratify patients suffering from NDDs involving the hippocampus, including AD but also hippocampal sclerosis, LATE, and PART. LATE and PART have been recently defined in postmortem examinations, but in vivo diagnostic biomarkers are needed. CSF Ng could be also used as predictive indicator of an anticholinesterasic treatment response in patients showing a prevalent hippocampal impairment (typical AD phenotype, etc.) [200,201,205,206]. CSF Ng demonstrated from good to optimal diagnostic accuracy in discriminating AD dementia patients from the control group and a reliable prognostic value for AD conversion in MCI individuals.

Growing data reported an abnormal neuroinflammatory response in AD. Currently, CSF YKL-40 is the most promising fluid biomarker of glia activation, and it has been extensively investigated in other NDDs as well. Neuroinflammation is a common pathway of several NDDs, and not surprisingly, YKL-40 is an unspecific biomarker. This biomarker could be helpful in monitoring tailored anti-inflammatory trials in AD. Studies exploring a possible correlation between CSF YKL-40 concentration and cerebral inflammatory tracer as the translocator protein (TSPO)-PET uptake could clarify the role of this fluid biomarker as an indicator of neuroinflammation. CSF YKL-40 showed a fair diagnostic accuracy to discriminate AD patients from the control group and other neurodegenerative dementias. YKL-40 also reported a certain predictive value for MCI-AD progression.
In summary, we found that reliable fluid biomarkers might track three out of four of the main pathophysiological pathways of AD (Figure 1). The concentration in different biofluids of Aβ peptides and p-tau proteins reflect the cerebral misfolded protein deposition in AD. Moreover, plasma NFL might help the early identification of a general neurodegenerative process independently from the specific pathology. The diffusion of ultrasensitive techniques in the last few years is radically revolutionizing the context-of-use of these biomarkers in AD. The possibility to measure biomarkers in blood opens a completely novel scenario for the detection of multiple neurodegenerative mechanisms with a low cost and minimally invasive examination. This should encourage the development of screening tools in selected populations and improve the monitoring of disease-modifying trials. Of note, validated surrogates of co-pathologies such as α-syn and TDP-43 protein accumulation are currently not available as well as of cerebrovascular impairment. Finally, YKL-40 and Ng measured in CSF are promising proxies of neuroinflammation and synaptic disruption, respectively.

The studies described have several shortcomings. Many investigations reported different inclusion criteria and sometimes, they were not biomarker-based. Moreover, there is frequently a lack of data about comorbidities, especially cerebrovascular burden, contemporary pharmacological treatments, or stratification for age, gender, and genetic profiles [53]. Hepatic and kidney dysfunctions may impact biomarker levels as well as modifications of blood cell counts and plasma protein composition [67]. Nonetheless, these variables were not systematically considered, thus constituting a possible methodological bias, since individuals with AD present relevant vascular comorbidities.

From the prospective of a precision medicine approach, increasing attention is paid to find biomarkers associated to pathogenic pathways leading to neurodegeneration. The contemporary use of multiple biomarkers can help dissect the pathological mechanisms dynamically acting in space and time providing an accurate stratification of AD population.

AD is more a spectrum of different pathological mechanisms that brings a loss of proteostasis, which is the accumulation of several misfolded and aggregated proteins in multiple combinations rather than a single entity. Advanced statistical analysis, including unsupervised clustering strategies, combining clinical, biomarkers, and genetic data to collect subjects from large diversified cohorts into consistent clusters might be an innovative representation of AD [6] and other NDDs [254] and significantly contribute to the discovery of causes and tailored treatments. Novel data-driven classifications based on quantitative measurements of biomarkers and clinical information (e.g., standardized clinical scores) could improve the identification of effective and personalized therapies [253,254]. In conclusion, we assume that the identification and inclusion of AD patients in disease-modifying trials will be soon changed, mainly based on the demonstration of specific pathophysiological mechanisms and minimally influenced by phenotypes.

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