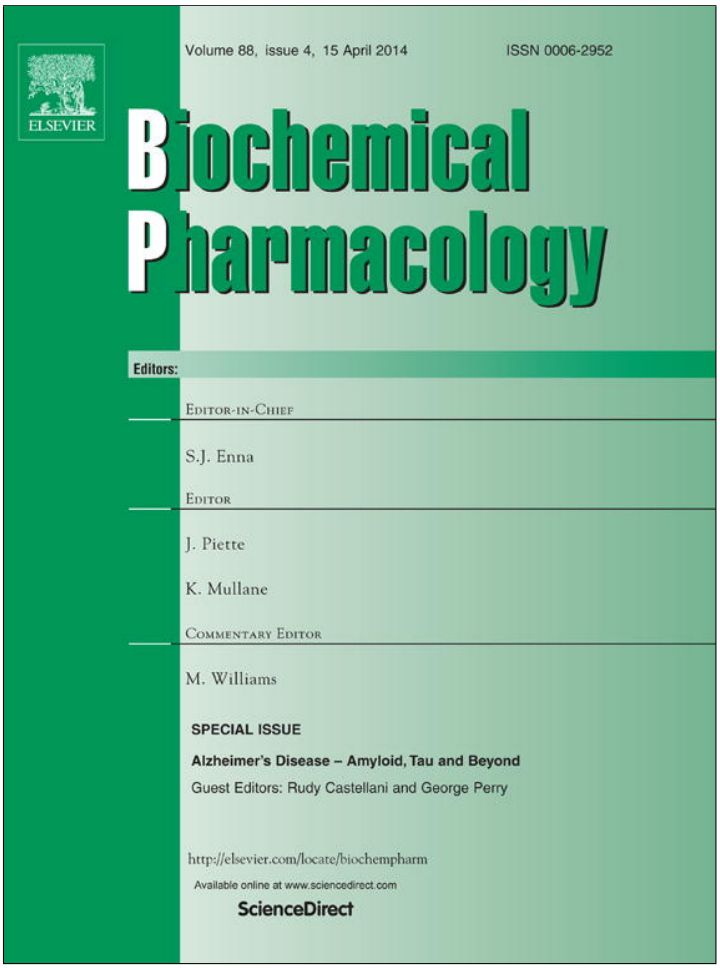

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Review - Part of the Special Issue: Alzheimer's Disease – Amyloid, Tau and Beyond

Perspective on future role of biological markers in clinical therapy trials of Alzheimer's disease: A long-range point of view beyond 2020

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ABSTRACT

Recent advances in understanding the molecular mechanisms underlying various paths toward the pathogenesis of Alzheimer's disease (AD) has begun to provide new insight for interventions to modify disease progression. The evolving knowledge gained from multidisciplinary basic research has begun to identify new concepts for treatments and distinct classes of therapeutic targets; as well as putative disease-modifying compounds that are now being tested in clinical trials.

There is a mounting consensus that such disease modifying compounds and/or interventions are more likely to be effectively administered as early as possible in the cascade of pathogenic processes preceding and underlying the clinical expression of AD. The budding sentiment is that "treatments" need to be applied before various molecular mechanisms converge into an irreversible pathway leading to morphological, metabolic and functional alterations that characterize the pathophysiology of AD. In light

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of this, biological indicators of pathophysiological mechanisms are desired to chart and detect AD throughout the asymptomatic early molecular stages into the prodromal and early dementia phase.

A major conceptual development in the clinical AD research field was the recent proposal of new diagnostic criteria, which specifically incorporate the use of biomarkers as defining criteria for preclinical stages of AD. This paradigm shift in AD definition, conceptualization, operationalization, detection and diagnosis represents novel fundamental opportunities for the modification of interventional trial designs.

This perspective summarizes not only present knowledge regarding biological markers but also unresolved questions on the status of surrogate indicators for detection of the disease in asymptomatic people and diagnosis of AD.

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1. Introduction

Sporadic Alzheimer's disease (AD) is currently conceptualized as a multifactorial neurodegenerative disease transitioning later through a prodromal cognitive stage into a late-stage dementia syndrome. This initially clinically "silent" multi-dimensional disease cascade chronically, non-linear progressively unfolds through the emergence and probably at some point convergence of a yet not fully understood and characterized parallelized and/or interrelated array of molecular mechanisms and signaling pathways. For many decades, the definite diagnosis of AD has relied on the *postmortem* detection of senile plaques (SPs) and neurofibrillary tangles (NFTs). There, these historic hallmark neuropathological lesions have been extensively studied. Their molecular constituents have been isolated (intracellular aggregation of tau protein and extracellular accumulation of amyloid beta (Aβ) peptide). The neuropathology is now better understood in terms of amyloid and tau pathology – as a consequence Aβ and tau assays having secondarily been developed and validated during the last two decades to provide first "core feasible" cerebrospinal fluid (CSF) biomarkers. The stereotyped progression of tau [1] and Aβ pathology [2] in the brain has been described and is the basis of the new National Institute on Aging and the Alzheimer's Association neuropathological criteria [3]. The amyloid cascade hypothesis, relying on the observation that all the mutations causing early-onset AD involve genes that alter Aβ production, has generated a theory emphasizing the central relevance of the amyloidogenic cascade and the Aβ peptide. As a consequence, many treatment trials in AD have been aimed at altering the abnormal production, accumulation and deposition Aβ. The optimism that reducing Aβ accumulation and/or

deposition would directly result into an improved clinical and functional patient status, however, has not yet been fulfilled. Recent evidence indicating that misfolding of Aβ and tau could be transmitted to normal proteins of the host through brain injections of affected samples is hypothesis generating and opens new translational research perspectives. A mono-linear amyloid cascade perspective, would seem reductionistic, since it fails to recognize the role of the many conformations that the proteins may adopt, explaining the progression of the disease through the connections and the transmissibility of the pathology in some experimental conditions. Therefore, in the advent of the worldwide AD epidemic, critical reassessment of the evidence-based significance and limitation of prevailing as well as of emerging fundamental concepts of AD pathophysiology seems to be necessary to foster breakthrough advances to effectively detect, treat or even prevent AD [4].

The search for biomarkers of preclinical AD is becoming increasingly important because pathogenesis-targeted neuroprotective strategies are being developed for future use in "at risk" populations. Advances in new neuroimaging probes and technologies, identification of new biochemical markers of AD in plasma, blood and CSF, and breakthroughs in molecular genetics and basic neuroscience are gradually translating into better understanding of predisposing and preclinical factors that lead to progressive neurodegeneration and finally cognitive and behavioral symptoms and dementia.

At present, the combination and integration of multimodal imaging methods, neurochemical markers, and genetic strategies are still in their infancy. However, significant indications on the existing state of the biomedicine on candidate markers of AD resulting from multiple analytical platforms – encompassing (I) structural/functional/metabolic neuroimaging modalities, (II) neurochemistry methods based on CSF and blood (plasma/serum) examination, (III) neurogenetic analyses, and (IV) procedures for cognitive and functional assessment – have been supplied [5–13].

The next-generation of studies is required to use multicenter data sets that exploit the large variety of affected systems to appraise the stability of multimodal diagnostic algorithms in a multinational multicenter setting. A growing number of national and international platforms are following this central line of research, among them the US Alzheimer's Disease Neuroimaging Initiative (US-ADNI) [14] and the European ADNI (E-ADNI) [15] that, in conjunction with other parallel projects around the globe, are collectively known as Worldwide ADNI (WW-ADNI) [16]. The ADNI has been designed to validate neuroimaging, CSF, and blood-plasma biomarker candidates for AD treatment trials, and therefore aid and speed drug development [16]. As a result, the approach of combining different source markers might be of help in the identification of those subjects who will develop AD and who are consequently potential targets for prevention as well as symptomatic pharmacological interventions.

When employed in AD clinical trials, biomarkers can be utilized: (I) to improve the diagnostic accuracy in trial participants, enabling patient cohorts to be enriched with characteristic molecular mechanisms of AD; (II) for stratification of AD patients; (III) for safety monitoring, *i.e.* to assess and predict tolerability and adverse side effects; (IV) as theragnostic markers, *i.e.* to identify and monitor the biochemical effects of drugs [5,6,17]. Notably, biomarkers provide the potential for characterization and validation of drug mechanisms of action, monitoring AD course and progression, and evaluating therapeutic response/outcome [18]. Furthermore, since biomarker profiles reflect different stages of the pathogenic process, they can be utilized to recruit optimal individuals for trials of different drugs and different clinical phenotypes at different stages of AD pathophysiology [19].

By using multimodal strategies, AD has been categorized into different stages according to the presence of biomarkers and the patterns of cognitive impairment. Following a pre-pathology stage characterized by normal biomarkers and absence of cognitive impairment, AD dimensionally (not categorically) emerges exhibiting through an asymptomatic stage (biomarkers abnormal, no cognitive impairment) subsequently to a symptomatic stage (biomarkers abnormal, cognitive impairment) that can be further differentiated into a subjective cognitive impairment (SCI) stage (AD-SCI), a prodromal, often categorized as a "mild cognitive impairment" (MCI) stage (AD-MCI), and finally a syndromal dementia stage (AD-dementia) [20]. Notably, these categories are mere restrictive research or practical clinical constructs and should not mask the true continuous dimensional character of AD.

The present review will summarize the current knowledge on the employment of biological markers in AD and provide perspectives as well as future directives on major areas of AD biomarker discovery and development emphasizing the role of such markers for use in clinical trials. Notably, since this manuscript is intended to raise evolving debate on the effective discovery, development, validation, and qualification process of biological markers resulting from all available technical modalities, it represents a major complement and extension to the antecedent perspective by Hampel et al. [7]. Current knowledge and perspectives/future directives on the employment of biological markers in AD are summarized in Tables 1 and 2, respectively.

2. International Work Group criteria

In 2007, an International Work Group (IWG) led by Dubois and colleagues has provided a novel description of AD as a clinico-biological syndrome that can be documented *in vivo*, prior to the onset of dementia, by a "core" clinical phenotype that includes an amnestic syndrome of the hippocampal type and indication from biomarkers reflecting the existence of Alzheimer-type pathology [21]. Such criteria may be used throughout any phase of the AD spectrum after the beginning of clinical signs [22]. Moreover, a specific terminology has been developed to resolve issues related to AD reconceptualization [23].

The IWG proposed two new sets of diagnostic criteria requiring the assessment of AD biomarkers. The first, covering asymptomatic AD individuals, is defined "preclinical AD". Preclinical AD has been then partitioned into the "asymptomatic at risk for AD" and the "presymptomatic AD" categories [23], the latter applying to asymptomatic individuals who carry familial autosomal dominant AD mutations. The second group, applying to symptomatic AD individuals, is designated as "AD". Individuals reflecting these criteria can be, in turn, categorized into "prodromal AD" (or "predementia AD") and "AD dementia" [23].

The most important progress inherent in the IWG criteria is the integration of biomarkers into a diagnostic scheme that allows a biology aided assessment of AD which is integrated with the clinical signs and symptoms, independent of disease severity. The use of biomarkers is integral to the diagnosis of AD in the IWG criteria; consequently, the presence of pathophysiologic or topographic aberrations representative of AD is strictly required. The pathophysiologic markers encompass the molecular signatures of AD in the CSF (low levels of the 42 amino acid-long form of the Aβ peptide ($A\beta_{1-42}$) plus increased concentrations of total-tau (t-tau) and/or hyperphosphorylated tau (phospho tau, p-tau) proteins) or significant binding of amyloid ligands using positron emission tomography (PET). The topographic markers consist of medial temporal/hippocampal atrophy on magnetic resonance imaging (MRI) or bilateral parieto-temporal hypometabolism on PET [22].

Importantly, the IWG criteria have abandoned the categorical concept of "MCI", which is heterogeneous in terms of AD progression and has many different underlying causes, in favor

Table 1
Current knowledge on the employment of biological markers in AD.

Area of markers	Key points
Genetics	
Familial AD	• "Featured genes" (causal genes): *APP*, *PSEN1*, *PSEN2* • Currently known mutations in *APP*, *PSEN1*, *PSEN2* genes do not account for all Mendelian AD cases, suggesting the existence of AD-causing mutations in other genes
Sporadic AD	• "Featured genes" (proposed susceptibility genes): *APOE*, *BIN1*, *CLU*, *ABCA7*, *CR1*, *PICALM*, *MS4A6A*, *MS4A4E*, *CD33*, *CD2AP*, *EPHA1*, *TREM2 and counting* • The advent of GWAS have led to the identification of novel loci linked to mostly LOAD risk • These genes appear to be mostly linked with three molecular pathways: (I) the amyloidogenic cascade, (II) cholesterol-lipid metabolism, and (III) immune-inflammatory mechanisms
Cerebrospinal fluid	• CSF biomarkers $A\beta_{1-42}$, t-tau, p-tau_{181}, and p-tau_{231} have a high diagnostic accuracy for AD, and for prodromal AD in patients with MCI • CSF levels of $A\beta_{1-42}$ start declining in the preclinical phase of sporadic AD, prior to any evident increase in t-tau or p-tau • CSF biomarkers, especially $A\beta_{1-42}$, convert to pathologic values several years before the first appearance of clinical signs, also in the familial form of AD • The diagnostic accuracy for the combination of CSF $A\beta_{1-42}$, t-tau, and p-tau has been reported to be higher than for any biomarker alone • CSF biomarkers are increasingly used in clinical trials, both for enrichment of patient populations with pure AD cases at the inclusion and to evaluate the biochemical effects of treatment (theragnostic markers) • CSF biomarker $A\beta_{1-42}$ is the central CSF biomarker for Aβ metabolism and deposition in clinical treatment trials. • CSF biomarkers t-tau and p-tau are the central CSF biomarkers to monitor the intensity of cortical axonal degeneration and tau phosphorylation state, respectively, in clinical treatment trials
Blood	• Definite data regarding the association of plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ concentrations with incipient AD are presently lacking • The development of mass spectrometry-based technologies has elected proteomics as the chief platform to inspect the plasma/serum proteome for the discovery of next-generation biomarkers showing diagnostic, prognostic, or therapeutic efficacy • Blood-based profiles/signatures including panels of molecules related to immune regulation and inflammatory pathways have been discovered • Issues in plasma/serum proteomics, including pre-analytical variables, requiring standardization for specimen collection/processing, quantitation, and setting strategies for managing biomarkers after their detection, currently exist. These markers do not seem to be ready for clinical applications
Structural neuroimaging	• Reduction in hippocampus volumetry, derived from structural MRI, has been consistently found in AD and MCI across a wide range of mono- and multicenter studies • Hippocampus volumetry has also been used as a secondary endpoint in clinical trials on potential disease modifiers in AD or MCI • The EMA regulatory authorities have endorsed a qualification process for the use of low hippocampus volume for enrichment of study samples • Few automated protocols have already been cleared for marketing as a medical device by the FDA • The attractiveness of MRI as endpoint in clinical trials is related to the assumption that regional brain volume can serve as *in vivo* surrogate of neuronal density • Neuropathological evidence suggests a selective involvement of specific subcortical areas, most notably the cholinergic nuclei of the basal forebrain and noradrenergic nuclei in the *locus coeruleus* in AD • Diffusion Tensor Imaging has become a leading method in investigating white matter microarchitecture and integrity and has been widely employed in AD and MCI
Functional neuroimaging	• Functional MRI, studying the neuronal activity through non-invasive means during specific cognitive states, has been able to detect functional alterations prior to onset of cognitive impairment or AD-related structural neurodegeneration • Functional MRI studies are focused on the "default mode network", *i.e.* the interplay between a set cortical areas and the hippocampal memory system
In vivo molecular neuroimaging	• FDG-PET has demonstrated to be of great value because it allows the detection of different patterns of neurodegeneration; it is also highly useful in differentiating within amyloid-positive subtypes of disease which cannot be distinguished on the basis of their amyloid PET-scan • PiB is the current gold-standard tracer for PET amyloid-imaging. Recently, ^{18}F-labeled compounds have been evaluated to enable allow a more widespread application of this method. [^{18}F]Florbetapir/Amyvid has already been approved by the FDA and the EMA • Concerning early diagnosis, several studies demonstrated a high predictive value of a positive amyloid-scan in the stage of MCI with regard to conversion to AD
Neurodynamics	• Time-resolved EEG and MEG measures have been increasingly explored to identify predementia AD (MCI)
Resting state	• Brainwave band power estimates in the delta, theta, alpha, beta and gamma frequency bands, as well as their ratios, have been used as a major tool to demonstrate RSN changes in AD and predementia AD patients as compared to healthy controls • Studied have shown a connection between clinical (MMSE) measures and frequency band power (alpha)
Functional	• ERP/ERF markers (peak latency, amplitude, brain sources) measure task-related functional changes which are not available in resting state. Deterioration of cognitive/episodic memory measures (P300, P600, *etc.*) has been demonstrated in AD and predementia AD subjects by multiple studies

Abbreviations: AD, Alzheimer's disease; $A\beta_{1-40}$, 40 amino acid-long form of the amyloid beta peptide; $A\beta_{1-42}$, 42 amino acid-long form of the amyloid beta peptide; CSF, cerebrospinal fluid; EEG, electroencephalography; EMA, European Medicine Agency; ERP, event-related potentials; ERF, event-related fields; FDA, Food and Drug Administration; FDG, [^{18}F]Fluorodeoxyglucose; GWAS, genome-wide association studies; LOAD, late-onset AD; MCI, mild cognitive impairment; MEG, magnetoencephalography; MMSE, Mini Mental State Examination; MRI, magnetic resonance imaging; p-tau_{181}, hyperphosphorylated tau protein at threonine 181; p-tau_{231} hyperphosphorylated tau protein at threonine 231; PET, positron emission tomography; PiB, [^{11}C]Pittsburgh-Compound-B; RSN, resting state network; t-tau, total-tau.

of prodromal AD in subjects showing symptomatic predementia AD. Therefore, these criteria rely on the implementation of biomarkers to detect a specific subset of MCI individuals who are in the predementia phase [22,24].

From a conceptual perspective, the IWG criteria foster the perception of AD as a dimensional clinico-biological entity and have been positively applied in clinical therapy trials approved by the US Food and Drug Administration (FDA) [22]. They have been

Table 2
Short- to mid-term perspectives and future directives of biological markers in AD.

Area of markers	Key points
Genetics	• Additional AD susceptibility variants are expected to be identified in upcoming GWAS based on larger sample sizes and/or higher resolution genetic maps • Resequencing part (*e.g.* by targeting specific functional regions such as the exome) or all of the human genome using "next-generation sequencing" technologies • "Next- and third generation sequencing" technologies will allow efficiently extending the knowledge of AD genetics to the lower allele frequency spectrum, down to low-frequency variants
Cerebrospinal fluid	• The use of multiple longitudinal CSF specimens is necessary to detect the time point at which CSF biomarkers convert from physiologic to pathologic values • Substantial progresses in the exploratory "omics" disciplines, especially proteomics/metabolomics, will enhance the detection of novel candidate CSF biomarkers • Many candidate biomarkers have the potential to increase the diagnostic accuracy of the "core" biomarkers $A\beta_{1-42}$, t-tau, and p-tau (*e.g.* BACE1)
Blood	• Progress in blood-based biomarker discovery relies on the establishment of Standard Operating Procedures (SOPs) for the appropriate selection of patients and specimens • The Human Plasma Proteome Project (HPPP) is an initiative launched that will face matters related to pre-analytical variability and to make attempts to establish SOPs • The Blood-Based Biomarker Interest Group (BBBIG), an international working group of leading AD scientists from academia and industry, will inspect the present scenario of biomarker discovery in blood in order to identify current needs that will enable the field to progress • It seems to be doubtful that a blood marker alone will be in itself adequate for the diagnosis of AD • In contrast, it seems most likely to have combinations of markers: several proteins coupled with other blood-based or non-blood-based markers, such as imaging
Structural neuroimaging	• The EADC-ADNI hippocampal harmonization project is providing an internationally consented protocol for manual hippocampus segmentation that will serve to validate automated hippocampus volumetry methods • Future studies are needed to address more specifically associations between regional brain atrophy pattern and regional markers of neuronal degeneration • The next years will see increasing use of automated volumetry of hippocampus or regional brain atrophy pattern as secondary endpoints in clinical trials in prodromal AD and AD dementia stages • Structural imaging markers are being used to enrich the risk for AD in clinical samples of MCI subjects for clinical trials. In addition, structural MRI will help to enrich study samples of asymptomatic subjects with positive molecular biomarkers of AD • The presence of hippocampus atrophy together with amyloid positivity will help to select subjects with a high risk of conversion to AD or MCI within a timeframe that is relevant for a clinical trial • The effect of a novel structural imaging marker of predementia AD on care systems worldwide, that have difficulties to provide adequate care even to patients in clinically manifest stages of disease, needs to be assessed in future studies • Novel methods, including high-field MRI at 3 Tesla and ultra-high field MRI at 7 Tesla, will gain increasing importance to understand the morphological/neuroanatomical basis of cognitive decline in AD • Based on mappings of subcortical nuclei from *postmortem* analyses, MRI scans *in cranio* will help to identify early changes in cholinergic and noradrenergic projecting nuclei in predementia and dementia stages of AD • MRI-based detection of amyloid plaques in humans will become a major topic of research in coming years. The use of 7 Tesla MRI in human studies may allow *in vivo* detection of cortical amyloid deposition in the future
Functional neuroimaging	• Functional MRI will be increasingly applied in the area of novel pharmaceutical strategies, in AD and MCI. Although drug-induced modulation of memory-related networks have been detected by functional MRI, few studies have demonstrated abnormal activation following pharmacological treatment in MCI and AD
In vivo molecular neuroimaging	• Novel imaging instrumentation such as hybrid PET/MRI scanners may offer the opportunity to merge the complementary information from different imaging modalities into new integrated *in vivo* biomarkers of neurodegeneration
Neurodynamics	• Design of enhanced EEG/MEG-based AD biomarkers: - Neurodynamic measures (such as brain connectivity, global synchronization, synchronization likelihood, detrended fluctuation analysis, approximate entropy, mutual information, source localization, and other non-linear signal features) will be used within the framework of both the resting-state and functional biomarker paradigms to adapt better to the complex characteristics and dynamics of progressive neurodegeneration and aging - Future functional EEG/MEG biomarkers will rely on multidimensional (spatio-spectro-temporal characteristics) in order to handle efficiently single-trial EEG/MEG data and increase sensitivity/specificity - Efficient biomarker selection with the final goal to evaluate the current state of AD-related functional brain networks for each individual subject • Standardization and validation of selected EEG/MEG-based AD biomarkers: - A selected battery promising neurodynamic biomarkers will pass through a rigorous multi-step and multi-center standardization/validation process before they can be used as diagnostic aids - Modular approach will be required for new biomarker standards. A robust review procedure will be put in place to facilitate fast and efficient biomarker upgrades

Abbreviations: AD, Alzheimer's disease; $A\beta_{1-42}$, 42 amino acid-long form of the amyloid beta peptide; CSF, cerebrospinal fluid; EADC-ADNI, European Alzheimer's Disease Centers-Alzheimer's Disease Neuroimaging Initiative; EEG, electroencephalography; GWAS, genome-wide association studies; MCI, mild cognitive impairment; MEG, magnetoencephalography; MRI, magnetic resonance imaging; p-tau, hyperphosphorylated tau protein; PET, positron emission tomography; t-tau, total-tau.

recognized by the European Medicine Agency (EMA) [25] for the employment in clinical drug trials as well.

3. National Institute on Aging/Alzheimer's Association criteria

Following the emerging development of the IWG/Dubois criteria, the National Institute on Aging (NIA) and the Alzheimer's Association (AA) summoned three working parties aimed at establishing criteria for the staging of AD [26–28]. Differently from the IWG that use an integrated clinico-biological approach covering all of the AD symptomatic phases, the NIA-AA employs three different categories of criteria for cases in which biomarkers have been measured: one for the asymptomatic phase ("preclinical AD"), one for the AD-MCI phase ("MCI due to AD"), and one for the

AD-dementia phase ("dementia due to AD") [20]. Notably, the NIA-AA criteria distinguish between amyloid and neuronal injury markers. This distinction is based on the hypothesis that Aβ generation drives other pathophysiological changes, an idea strongly supported by genetic evidence from familial autosomal dominant AD, Down's syndrome, and the recent demonstration of a protective mutation in the amyloid precursor protein (*APP*) gene [29]. The biomarkers of Aβ accumulation are represented by significant amyloid tracer retention using PET imaging and/or low CSF concentrations of $A\beta_{1-42}$. The biomarkers of neuronal degeneration or injury consist of increased levels of CSF tau (t-tau or p-tau), reduced fluorodeoxyglucose uptake on PET in specific areas encompassing temporoparietal cortex, and atrophy on structural MRI primarily including medial temporal lobes and parietal cortices [30,31].

Subjects with preclinical AD can be categorized into three stages using cognitive markers and biomarkers. In particular, individuals showing only anomalous amyloid markers are classified in stage 1; those with both atypical amyloid and injury markers are considered in stage 2; those showing both unusual amyloid and injury markers accompanied by minimal cognitive impairments, such as SCI, are classified in stage 3. Individuals with MCI due to AD or dementia due to AD are categorized in a risk staging model according to amyloid and neuronal injury markers, as follows: (I) high likelihood for AD if both amyloid and neuronal injury markers are aberrant, (II) intermediate likelihood for AD if only one of the two markers has been assessed and is anomalous, (III) uninformative if one marker is atypical and the other normal, or *vice versa* [20].

The IWG group considers the presence of brain amyloid accumulation in the absence of clinical features in the sporadic population to be indicative of an "at risk" group. In contrast, the NIA-AA group considers such individuals to indeed already have preclinical AD, suggesting that in time they would develop cognitive decline and the clinical dementia syndrome. This presents a fundamental hypothetical and conceptual difference of the two approaches with practical consequences for trials which needs to be further elucidated.

4. The genetics of Alzheimer's disease

AD has been designated as a multifaceted pathology characterized by a high-degree of genetic heterogeneity. This implies both that the same phenotype can be generated or modified by a number of different genetic loci and alleles, and that mutations or polymorphisms at different positions in the same gene lead to the same clinical syndrome [32]. This situation is aggravated by the fact that, in some instances, different mutations in the same gene can lead to clinically distinct syndromes. Hence, AD is considered to belong to the growing fraction of "genetically complex" diseases.

A peculiar feature observed in AD is the dichotomy of (I) familial *versus* (II) "apparently" non-familial forms of disease. The former, referred to as familial AD, accounts for less than 5% of all AD cases and is often conferred by individual disease-causing mutations transmitted in classic Mendelian fashion, mostly typically by autosomal dominant transmission. Since age of onset in these forms of AD is usually early (<65 years) or very early (≤50 years), it is often also called early-onset familial AD (EOFAD). The latter, commonly defined as non-Mendelian, "polygenic", or "sporadic" AD, accounts for about 95% of all AD cases. It is typically characterized by an onset age well beyond 65 years of age, and it is also designated as late-onset AD (LOAD) [32,33]. However, it should be highlighted that this dichotomization scheme is over simplistic, as there are cases of EOAD without evidence for familial clustering or Mendelian transmission while, on the other hand, these clustering and transmission patterns are frequently observed in LOAD [33]. In addition to these genetic causes, non-genetic (*e.g.* environmental or epigenetic) factors are likely significantly affecting an individual's risk to develop AD. However, the exact mechanisms underlying the possible pathogenic effects of these non-genetic factors are still mostly elusive which is, at least in part, owing to the fact that it is still relatively difficult to detect and evaluate them experimentally [34].

The introduction of high-throughput DNA genotyping and sequencing technologies, allowing to systematically screen the genomes of a large number of individuals simultaneously, has led to the completion of a high number of genome-wide association studies (GWAS) in AD. These studies allow simultaneously investigating literally millions of genetic markers (mostly so-called single-nucleotide polymorphisms, SNPs) in one experiment to assess their effect on disease risk, or quantitative phenotypes. Not unexpectedly, these GWAS have led to more reproducible and more consistent findings than three decades of candidate-gene-driven research before [35].

4.1. EOFAD with Mendelian transmission

EOFAD is caused by rare and highly penetrant mutations in three genes, namely: amyloid precursor protein (*APP*, located at chromosome region 21q21.2), presenilin 1 (*PSEN1*, located at 14q24.3), and presenilin 2 (*PSEN2*, located at 1q42.13) [33]. Presently, more than 220 distinct disease-causing mutations have been discovered across these genes (for an up-to-date summary, see the Alzheimer Disease & Frontotemporal Dementia Mutation Database (AD&FTDMDB) at http://www.molgen.vib-ua.be/ADMutations/ [36]). Currently, over 30 AD-causing mutations have been reported in *APP*, encoding for the precursor protein for Aβ. Interestingly, most of the *APP* mutations occur near the putative γ-secretase site between amino acidic residues 714 and 717, suggesting that the γ-cleavage event of APP or its (dys)regulation are crucial for the development of AD [32]. The vast majority of EOFAD mutations are observed in *PSEN1* located on chromosome 14. *PSEN1* encodes for a highly conserved polytopic membrane protein, presenilin 1, which is involved in mediating intramembranous, γ-secretase processing of APP to generate Aβ peptides [37]. At present, the overall number of known AD-causing mutations in *PSEN1* exceeds 180. Lastly, EOFAD is rarely caused by mutations in *PSEN2* which encodes for presenilin 2, which represents another member of the presenilin family of proteins, displaying substantial homology to presenilin 1, both at the genomic and protein level [38,39]. In summary, the currently known AD-causing mutations occur in three different genes located on three different chromosomes. Functionally, the proteins encoded by all three genes share a common biochemical pathway, *i.e.* the altered production of the Aβ peptide. Together, these findings provide strong support for the "amyloid hypothesis" indicating that an abnormal production and/or regulation of Aβ is one of the main factors underlying AD pathogenesis [40]. While the currently known mutations in these three EOFAD genes account for a large fraction of Mendelian AD, they do not account for all cases, suggesting that AD-causing mutations in other genes exist. The successful identification of these hitherto unknown Mendelian AD genes could, thus, provide entirely new insights into AD pathogenesis [33].

Recently, a study has detected mutations in the *SORL1* gene in EOFAD patients [41]. *SORL1* encodes for the protein SorLA that belongs to a set of protein-trafficking molecules in the endocytic and retromer pathways and is implicated in modulating the production of Aβ peptide [41]. These findings suggest that *SORL1* may represent a genetic risk factor for AD, although these data need independent replication.

4.2. Sporadic AD/LOAD

In contrast to EOFAD, LOAD exhibits a significantly more complex and intricate pattern of interplay between genetic and non-genetic factors. This situation, combined with the fact that each factor only exhibits exceedingly small effect sizes, has been proven to make the identification of these factors a complicated issue.

The earliest and by far best established genetic risk factor for LOAD is the presence of one or two copies of the *ε4* allele in the apolipoprotein E gene (*APOE*), located on chromosome 19q13.2 [42]. The risk effect of *APOE ε4* has been replicated in many studies across various ethnic groups. Besides the increase in AD risk conferred by the *ε4* allele, a less pronounced protective effect has been reported, albeit somewhat less consistently, for the least common *ε2* allele [43]. Despite its comparatively large effect size, it is important to note that the presence of the *APOE ε4* allele is neither necessary nor sufficient to actually cause AD. Instead, it works as a *bona fide* genetic risk modifier, likely by diminishing the age of onset in a dose-dependent manner. In spite of the accomplishments of over two dozen published GWAS in AD, *APOE ε4* remains to be the single most important genetic risk factor for AD, both in terms of effect size and statistical significance [32].

Despite its well-known genetic association, the biochemical aspects of *APOE ε4* in AD pathogenesis are still only incompletely understood. The encoded protein, apolipoprotein E (apoE), is synthesized in a large number of tissues, primarily in the liver. Hepatic apoE accounts for roughly three-quarters of circulating plasma levels of the protein [44,45]. The human brain is the second most prominent site of synthesis, chiefly occurring in the astrocytes [46] and microglia [47]. There is experimental evidence from transgenic mice that the expression of the human *ε4* allele and mutant APP promotes Aβ accumulation during the course of the disease, suggesting that amyloid may accumulate progressively with time [48]. Moreover, apoE participates in cholesterol transport and lipid metabolism and, in addition to AD, the *ε4* allele also represents a confirmed risk factor in vascular disease, likely owing to its link to augmented plasma cholesterol levels [49]. Amyloid angiopathy involving capillaries is much more prevalent in *APOE ε4* carriers [50].

After the original report suggesting *APOE ε4* to be a genetic risk factor in AD, literally hundreds of genes have been investigated for evidence of genetic association and disease risk, mostly to no avail (for an up-to-date overview of the accumulated evidence, see the AlzGene database at http://www.alzgene.org/ [51]). As outlined above, this situation changed substantially with the advent of GWAS which have led to the identification of at least ten novel loci linked to mostly LOAD risk: *BIN1*, *CLU*, *ABCA7*, *CR1*, *PICALM*, *MS4A6A*, *MS4A4E*, *CD33*, *CD2AP*, and *EPHA1* [52–56]. Functionally, these genes appear to be mostly linked with three (interdependent) molecular pathways: (I) the amyloidogenic cascade, (II) cholesterol-lipid metabolism, and (III) immune-inflammatory mechanisms [57]. Extending these leads, Jones et al. (2010) have assessed the functional role of SNPs not quite reaching genome-wide significance in AD and arrived at a very similar conclusion, *i.e.* that especially pathways related to immune system response and lipid metabolism appear to be particularly overrepresented [58]. More recently, rare amino-acid changing variants in *TREM2* (encoding for the triggering receptor located on myeloid cells 2) have been implicated as additional risk factors for LOAD [59,60]. Intriguingly, the protein encoded by *TREM2* is an immune receptor participating in the clearance of neural debris from the central nervous system (CNS), *via* processes including phagocytosis and reactive oxygen species production [61]. In all likelihood, additional AD susceptibility variants will be identified in upcoming GWAS based on larger sample sizes and/or higher resolution genetic maps. Equally, efforts are already under way to resequence part (*e.g.* by targeting specific functional regions such as the exome) or all of the human genome using "next-generation sequencing". Other than GWAS – which are based on microarray technology primarily targeting common genetic variations – these methods will allow efficiently extending our knowledge of AD genetics to the lower allele frequency spectrum, down to low-frequency variants such as the ones already observed in *TREM2*. However, even the increasingly widespread application of these powerful new technologies will not abolish the need for extensive subsequent functional genetic experiments to elucidate the pathogenic mechanisms underlying the observed genetic effects [32].

5. Cerebrospinal fluid biomarkers

Owing to its contiguity to the brain parenchyma and the free exchange with the brain extracellular space, the biochemical composition of CSF is able to provide information on the brain chemistry. The distinctive features of CSF, together with the low incidence of complications after lumbar puncture [62] have supported the introduction of lumbar puncture and analyses of CSF biomarkers into routine clinical practice in some centers [63,64]. CSF biomarkers are also increasingly used in clinical drug trials, both for enrichment of the target population at inclusion and to evaluate the biochemical effects of treatment [65–67].

5.1. AD dementia

In the early' 90s, a first publication has documented elevated CSF amounts of t-tau in patients with AD dementia [68]. After that, augmented CSF concentrations of p-tau [69] and reduced levels of $A\beta_{1-42}$ [70] have been described. A large number of studies have replicated these findings. A decrease in CSF $A\beta_{1-42}$ to about 50% of the level in cognitively normal elderly subjects has been regularly reported, whereas an increase in CSF t-tau to approximately 300% of the level in cognitively normal elderly subjects and a less evident growth in CSF p-tau to about 200% have been repeatedly detected [71]. Such biomarkers show 80–95% of sensitivity and specificity in the dementia phase of the pathology [71,72].

The CSF concentration of these markers is within the normal range in several differential diagnoses, including depression and Parkinson's disease [5,69,72]. Additionally, measurement of p-tau in CSF is of help to distinguish AD from other dementing pathologies, such as frontotemporal dementia and Lewy-body dementia. Only minimal differences among immunoassays specific for various epitopes of p-tau, including p-tau_{181}, p-tau_{231}, and p-tau_{199}, have been found [73]. The diagnostic accuracy of these CSF biomarkers has also been substantiated in analyses in which the diagnosis was then proven by autopsy [74,75] with comparable or superior discriminatory power than in studies utilizing patients with clinical diagnoses only.

5.2. Prodromal AD

CSF biomarkers exhibit a high predictive value in detecting prodromal AD in MCI subjects [72]. A study with a protracted clinical follow-up period has revealed that the combination of all three core CSF biomarkers shows a sensitivity of 95% to recognize prodromal AD in MCI [76]. Moreover, these markers are able to predict the rate of cognitive decline in patients with MCI/very mild AD dementia [77].

A high diagnostic accuracy of CSF biomarkers for prodromal AD has also been corroborated in large multicenter studies, such as the US-ADNI [75], the European Development of Screening guidelines and Criteria for Predementia Alzheimer's disease (DESCRIPA) study

[78], and the Swedish Brain Power (SBP) project [79]. These findings emphasize the role of CSF biomarkers as clinical diagnostic tools to detect enhanced risk in MCI subjects to have prodromal AD.

5.3. Preclinical AD

The notion of preclinical AD designates cognitively normal subjects harboring early AD pathology, not severe enough to induce cognitive signs. The efficacy of CSF biomarkers in the preclinical stage to recognize patients who will progress to AD dementia has been assessed. Skoog et al. (2003) have found a reduction in CSF $A\beta_{1-42}$, but normal t-tau and p-tau levels in cognitively normal 85-year-olds who later developed dementia [80]. These results are corroborated in a population-based cohort of healthy elderly subjects aged 70–78 years with 8 years follow-up [81] and in a clinical study on asymptomatic elderly subjects aged 60–94 years [82]. According to these data, CSF levels of $A\beta_{1-42}$ start declining in the preclinical phase of sporadic AD, prior to any manifest increase in t-tau or p-tau.

With reference to familial AD, Moonis et al. (2005) have uncovered that asymptomatic subjects carrying familial AD mutations exhibit both low CSF $A\beta_{1-42}$ and high t-tau concentrations [83]. This finding is confirmed in an analysis by Ringman et al. (2008) showing that mutation carriers have the full AD pattern of CSF biomarker changes long before symptom onset [84]. Bateman et al. (2012) have also suggested that CSF $A\beta_{1-42}$ may start to decrease already 25 years before the estimated clinical onset in familial AD mutation carriers, whereas increased CSF tau may be observed 15 years before predicted symptom onset [85]. Altogether, these results suggest that CSF biomarkers, especially $A\beta_{1-42}$, convert to positive several years before the first appearance of clinical signs, also in the familial form of the disease. Notably, familial AD mutation carriers – in their early 20s – may commence at higher CSF $A\beta_{1-42}$ concentrations than non-mutation carriers [84,86]. It should be noted that most of the studies published to date are "pseudo-longitudinal" in their design; they relate cross-sectional biomarker data to longitudinal clinical or neuroimaging markers or time before expected disease onset. Longitudinal examinations with repeated CSF samplings are required to define when and how fast the shift to lower CSF $A\beta_{1-42}$ and higher tau levels occurs, indicating onset of amyloid deposition and neurodegeneration.

5.4. Combined analyses of Aβ and tau biomarkers

Combining $A\beta_{1-42}$ with tau offers good discriminative value for AD patients compared to age-matched healthy controls, with a sensitivity of 85% and a specificity of 86%. Nevertheless, when these ratios are employed to discriminate AD from other dementias, a lower degree of specificity is achieved [87]. Other examinations have used the tau x $A\beta_{1-40}/A\beta_{1-42}$ ratio – referred to as the AD index – showing sensitivity 69% and specificity 88% [88] or the combination among $A\beta_{1-42}$, $A\beta_{1-38}$, and tau to make a diagnosis of AD [89]. In the latter analysis, increased p-tau and the ratio $A\beta_{1-42}/A\beta_{1-38}$ account for accuracies higher than 80 and 85%, respectively, to differentiate AD *versus* non-Alzheimer dementias (NAD). The combination of p-tau with $A\beta_{1-42}/A\beta_{1-38}$ leads to a sensitivity of 94% to identify AD and 85% specificity to exclude NAD. The ratio $A\beta_{1-42}/A\beta_{1-38}$/p-tau, robustly distinguishing AD *versus* NAD, is believed to satisfy the accuracy requirements for an appropriate screening and differential diagnostic AD biomarker [89]. When reviewing this type of sensitivity and specificity figures for the AD CSF biomarkers, it should be noted that these figures come from studies based on clinically diagnosed patients, which means that a biomarker can never show a better performance than the clinical diagnosis in such studies.

5.4.1. Progression from cognitively normal subjects to MCI

The increased ratio of tau/$A\beta_{1-42}$ and p-tau/$A\beta_{1-42}$ in normal subjects has been related to an amplified risk of conversion to MCI. A study has demonstrated that about 70% of those with a high ratio *versus* only 10% of those with a normal ratio change to MCI over a 3-year period [90]. Later, it has been observed that all subjects who have converted to MCI display increased tau/$A\beta_{1-42}$ ratios (over a follow-up of 42 months), while no conversions take place in the normal ratio group [91]. In light of this, the subset of normal elderly with high ratios seems to have already developed amyloid deposition and neurodegeneration. This might denote a subgroup with a diagnosis of preclinical AD.

5.4.2. Progression from MCI to AD

Numerous studies assessing the efficacy of CSF markers in predicting the risk of progression from MCI to AD indicate that diminished $A\beta_{1-42}$ and elevated t-tau and p-tau show in MCI a sensitivity equivalent to that observed in more advanced AD [92]. Lower CSF $A\beta_{1-42}/A\beta_{1-40}$ ratios suggest risk of progression to AD in subjects with very mild dementia [92]. A large longitudinal study of MCI subjects (18 months follow-up) has allowed the detection of a grown tau/$A\beta_{1-42}$ ratio in 90% of MCI subjects who have later converted to AD compared to 10% of those who have not converted [93]. Combining tau with the $A\beta_{1-42}$/p-tau_{181} ratio has significantly predicted progression of MCI into more advanced AD in another longitudinal study (average follow-up: 4–6 years) [76].

As emphasized by Blennow et al. (2012) [94], given that the diagnostic accuracy for the combination of CSF $A\beta_{1-42}$, t-tau, and p-tau has been reported to be higher than for any biomarker alone [76,93,95,96], a multiparameter assay, utilizing the Luminex™ xMAP technology (Luminex Corporation, Austin, TX, USA) to enable simultaneous quantification of these CSF biomarkers, has been developed [97]. The employment of this assay in multicenter studies on CSF biomarkers has yielded a good diagnostic performance [75,76,79,98].

5.5. Time course of AD biomarkers

Great consideration has been given to the hypothetical model for the sequence of pathologic events in AD suggested by Jack et al. (2010) according to which biomarkers reflecting Aβ pathology become positive before those reproducing neuronal degeneration and tangle development [99]. Two recent examinations have addressed this issue in detail. Both studies, after scrutinizing MCI cohorts with long clinical follow-up, have identified an evident reduction in CSF $A\beta_{1-42}$ along with grown levels of t-tau and p-tau. In particular, one study has demonstrated that MCI subjects with prodromal AD present with low CSF $A\beta_{1-42}$, regardless of time to dementia, whereas t-tau and p-tau are highest in patients with shorter time to conversion, thus indicating that $A\beta_{1-42}$ is completely altered before t-tau or p-tau [100]. These data support the hypothesis that modified Aβ metabolism precedes tau-related disease and neuronal degeneration. The other study has disclosed that MCI subjects with elevated concentrations of injury markers – namely, t-tau and p-tau – may develop faster, therefore presenting shorter time to conversion [101]. Since both analyses are cross-sectional in regards to the biomarker data, the use of multiple longitudinal CSF specimens is necessary to detect the time point at which CSF biomarkers convert from physiologic to pathologic values.

5.6. CSF biomarkers variability

Substantial interlaboratory discrepancies, with reference to CSF biomarker levels, make assessments and comparisons of data from different laboratories problematic. As a result, globally recognized

reference and cut-off values have not been established. For this reason, standardization efforts have been introduced to harmonize laboratory practices [102], define procedures on CSF collection and handling [103], create reference materials for assay calibration [104], and delineate reference measurement protocols [105]. In particular, the establishment of certified reference materials is presently executed as a concerted effort among the Alzheimer's Association, the International Federation of Clinical Chemistry and Laboratory Medicine, and the Institute for Reference Materials & Measurements [106].

A universal quality-control program to evaluate total analytical variability of the best-established CSF biomarkers – $A\beta_{1-42}$, t-tau, and p-tau – has been recently initiated by the Alzheimer's Association. The aim is the standardization of CSF biomarker measurements between research and clinical laboratories to increase the analytical precision and improve the longitudinal stability of biomarker measurements [79].

To date, the major cause of the experimental variability for CSF biomarkers is due to between-laboratory factors [107]. Since global biomarker cut-off levels cannot be defined owing to the high extent of variability, each laboratory should employ internally validated cut-off values and guarantee longitudinal stability in its measurements. Progresses in standardization of laboratory protocols in conjunction with the enhancement of kit performance and the use of fully automated tools are expected to improve the effectiveness of CSF AD biomarkers for both researchers and clinicians [107].

5.7. Upcoming candidate biomarkers

The composition of CSF is subject to fluctuations that mirror the complexity of AD pathophysiology, involving SPs deposition, NFTs formation, gliosis/neuroinflammation, and synaptic and neuronal loss. Accordingly, lots of molecules have been proposed as potential AD biomarkers in the CSF [108]. As the power and the complexity of the "omics" disciplines such as proteomics and metabolomics – that promise to revolutionize biomedicine – has greatly advanced over the last decade, proteins encompass the majority of viable candidates. In this context, both hypothesis-driven strategies – allowing the study of definite molecules participating to Aβ metabolism, neurodegeneration, neuroinflammation, and oxidative stress – and unbiased as well as targeted multianalyte profiling approaches – for instance, proteomic screening and molecular arrays – have been employed (see Fagan and Perrin (2012) for an exhaustive review on novel candidate CSF biomarkers [108]). Individually, various candidates are useful to evaluate statistical differences between cohorts of AD and control samples, and many of these also have the potential to increase the diagnostic accuracy of the "core" biomarkers $A\beta_{1-42}$, t-tau, and p-tau. Certain promising molecules seem to be of help for diagnosis/differential diagnosis, prognosis, and therapeutic monitoring ("theragnosis") [108].

Notably, in the framework of the "omics" revolution, mounting evidence is emphasizing the role of metabolomics to determine diagnostic biomarkers for AD. The metabolome designates a set of small-molecule metabolites discovered within a biological sample in a specific physiologic or developmental condition. Thus, different disease states disturbing biochemical networks will lead to dissimilar metabolic signatures [109]. This groundbreaking approach recognizes metabolic disturbances by assessing the activity of various metabolites at the same time. The discovery of uncommon disruptions in the metabolic network could serve to better elucidate the pathological mechanisms [110]. Notably, two analyses have reported alterations in CSF metabolome of AD. One study has showed the increase of the concentrations of eight amino acids in AD *versus* MCI [111]. Another larger examination, after measuring 343 analytes has also led to detect eight molecules with statistical significance; interestingly, one of these markers, cortisol, correlated with the advancement of the disease [112]. A disadvantage with metabolomics as compared with proteomics and peptidomics may be that, in contrast to several proteins, there is no data showing an established role for small (non-protein) molecules in AD pathogenesis.

6. Blood prospective candidate biomarkers

The attention on blood-based biomarkers for the diagnosis of AD has rapidly developed during the past decade. Although conventional AD biomarkers from CSF are highly accurate, barriers to their clinical application are still present. Since blood is a biofluid much more easily reached and manageable than CSF, searching for consistent blood-borne biomarkers is needed. In this connection, the Blood-Based Biomarker Interest Group (BBBIG), an international working group of leading AD scientists from academia and industry, has been established to scrutinize the present scenario and to support the progress in the field (see Henriksen et al. (2013) for a critical perspective on the status of blood-based biomarkers for AD [13]).

Although the association of plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ concentrations with incipient AD has been repeatedly investigated, definite data are still lacking. Increased $A\beta_{1-40}$ or $A\beta_{1-42}$ levels have been shown to predict the development of AD [113,114]; however, other analyses have revealed no associations [115,116] or opposite [117] results. A low $A\beta_{1-42}/A\beta_{1-40}$ ratio is assumed to predict future AD [113,118,119]. On the other side, an increased ratio [114,120] or no major difference [115] in subjects with incipient AD, compared with those that have not developed AD, have been described. A recent meta-analysis has suggested that a low $A\beta_{1-42}/A\beta_{1-40}$ ratio could predict the progression of AD, but no such association has been observed for the single peptides [121].

With reference to tau, some studies have highlighted differences in the modulation of CSF tau levels as compared to blood. In case of hypoxic brain damage subsequent to cardiac arrest, tau is promptly released into the bloodstream but efficiently cleared, within 24 h, in patients showing positive neurological outcome [122]. In contrast, CSF tau levels remain elevated for several weeks after an acute neurological insult [123]. In addition, tau concentrations are significantly increased in CSF of AD patients, but less in the equivalent plasma samples. Actually, measurements of tau in CSF and plasma compartments are not associated [124]. More recently, the association of plasma tau concentrations with AD has been appraised in a cross-sectional study including AD patients, MCI subjects, and cognitively healthy controls, using a newly developed ultra-sensitive immunoassay for the quantification of tau protein [125]. Plasma concentrations of tau appear increased in AD in relation to MCI and healthy controls. MCI-AD subjects (*i.e.* MCI converters to AD) display tau levels comparable to those detected in MCI-stable (*i.e.* MCI non-converters to AD) and healthy subjects. This overlap among ranges is believed to diminish the efficacy of plasma tau as diagnostic test [125].

During the last decade, the development of mass spectrometry-based technologies has elected proteomics as the chief platform to inspect the plasma/serum proteome for the discovery of next generation biomarkers showing diagnostic, prognostic, or therapeutic efficacy [126]. Mass spectrometry-based methods, together with innovative tools in progress, are welcomed because they will significantly improve the ability to detect blood markers [127,128]. By simultaneously quantifying the levels of many plasma analytes, biomarker patterns successfully distinguishing AD patients from controls [129] or associated with MCI or AD have been disclosed [130]. Since the activities of most molecules are connected to immune regulation and inflammatory pathways, the existence of

an inflammatory process in AD has been firmly proposed [131,132]. Nevertheless, such protein panels have been hard to reproduce in independent studies [133].

However, two analyses, utilizing large and well-characterized clinical cohorts, have discovered that a set of inflammatory molecules display modified expression as a function of AD [130,134]. Moreover, both Doecke et al. (2012) [134] and O'Bryant et al. (2011) [135] have found diagnostic accuracy across cohorts employing biomarker algorithms/profiles. These encouraging results provide additional support for the blood-based profiles/signatures.

The discovery of plasma/serum biomarkers for a CNS disease as AD meets both conceptual and practical challenges [136]. No findings of transcripts/proteins/metabolites in blood have been successfully replicated to be definitively approved as AD biomarkers. Moreover, based on the data from the literature, it seems to be doubtful that a blood biomarker alone will be in itself adequate for the diagnosis of AD. In contrast, it seems most likely to have a combination of markers: several proteins coupled with other blood-based or non-blood-based markers, as imaging [127]. It is also uncertain the existence of only one set of biomarkers for all conceivable uses in AD. It is probable that there will be a group of biomarkers to support AD diagnosis, a different set of molecular markers to predict outcome in AD patients or conversion in MCI, and, probably, another cluster to allow monitoring the evolution of the disease [128].

It should be also emphasized the presence of many issues in plasma/serum proteomics, including the existence of pre-analytical and analytical variables. Consequently, there is an urgent need for standardization of specimen collection/processing, quantitation, and setting strategies for managing biomarkers after their detection [137]. Progress also relies on the establishment of Standard Operating Procedures (SOPs) for the appropriate selection of patients and specimens, thus decreasing the complexity of samples to be analyzed [138]. In this regard, the Human Plasma Proteome Project (HPPP) (see http://www.hupo.org/initiatives/plasma-proteome-project/) is an initiative conceived and launched by the Human Proteome Organization (HUPO) (available at http://www.hupo.org/) to solve matters related to pre-analytical variability and to make attempts to establish SOPs [139]. In addition, the development of informatic tools for data management and collaborations with other disease-related initiatives of the HUPO to extend the area of plasma/serum biomarker discovery should be encouraged [140].

7. Neuroimaging markers

7.1. Structural MRI markers

Reduction of hippocampus volume, derived from structural MRI, is one of the key biomarkers of AD in the IWG [23] and NIA-AA criteria [30]. This reflects the consistent findings of reduced hippocampus volumes in AD and MCI subjects across a wide range of mono- and multicenter studies (for meta-analysis, see Clerx et al. (2012) [141]). Hippocampus volume has also been used as a secondary endpoint in several clinical trials on potential disease modifiers in AD or MCI, including vaccination [142], muscarinic receptor agonists [143], and glutamate modulators [144]. Although widely used since more than 20 years, standardization of manual hippocampus volumetry has only begun in 2011 with the European Alzheimer's Disease Centers-Alzheimer's Disease Neuroimaging Initiative (EADC-ADNI) hippocampal harmonization project [145] that now provides an internationally consented protocol for manual hippocampus segmentation (available at http://www.hippocampal-protocol.net/SOPs/index.php). This protocol will serve to validate automated hippocampus volumetry methods [146]. The EMA regulatory authorities have endorsed a qualification process for the use of low hippocampus volume to help enrichment of study samples (available at http://www.ema.europa.eu/docs/en_GB/document_library/Regulatory_and_-procedural_guideline/2011/10/WC500116264.pdf). Few automated protocols have already been cleared for marketing as a medical device by the US FDA. However, hippocampal atrophy is not specific to AD and is found in other conditions, including fronto-temporal dementia [147] vascular dementia [148], Lewy-body dementia [149], and depression [150].

Another structural marker beyond hippocampus volumetry is whole brain volume as longitudinal marker of disease progression and treatment effects. Automated algorithms such as voxel-based compression mapping [151] allow stable estimates of whole brain volume over time and across centers [152]. Whole brain volume has been used as secondary endpoint in several clinical trials [142,153], but has become less attractive with the advent of regionally more specific protocols. These are based on local measures of gray matter concentration or cortical thickness at each point of the space and on high dimensional warping of brain scans into a common standard space [154] to estimate regional pattern of atrophy in data driven automated analyses. Longitudinal evaluation of these pattern of atrophy has begun to be used in phase IIa type clinical trials [155]. In addition, multivariate approaches such as machine learning with support vector machines have successfully been employed to derive patterns of brain atrophy that discriminate AD patients from healthy controls and MCI converters from MCI-stable subjects [156–158]. By highlighting specific topographical patterns of atrophy, these approaches have the potential to be useful to discriminate between different types of dementia [158]. Presently, scanner manufacturers are developing radiological expert systems based on these algorithms to help the radiologist to rate the presence or absence of AD from the pattern of atrophy derived from a single brain scan. It is important to note that these technical devices need to be employed in a multidisciplinary clinical setting where the diagnostic relevance of an imaging finding is put in the context of all other relevant clinical information by a clinical dementia specialist.

The attractiveness of MRI as endpoint in clinical trials is related to the assumption that regional brain volume can serve as *in vivo* surrogate of neuronal number. Clinico-pathological comparison studies have shown that hippocampus volume obtained *antemortem* accounted for at least 50% of variability in neuron numbers determined during autopsy [159]. The amount of variation explained by MRI-based hippocampus volumetry was above 90% when MRI scans was obtained *postmortem* [160]. Thus, hippocampus volumetry can be considered as an *in vivo* surrogate measure of hippocampal neuronal number. However, one should be careful to simply interpolate these findings to *in vivo* measures of cortical atrophy. In 27 *antemortem* cognitively intact subjects, cortical thinning determined *postmortem* across age cohorts was not associated with regional neuron numbers and density, but was suggested to reflect changes in neuronal and dendritic architecture [161]. Therefore, future studies need to address more specifically associations between regional brain atrophy and regional markers of neuronal degeneration. A platform implementing *postmortem* MRI *in cranio* can help such an approach through access to *postmortem* MRI data whose signal distribution is close to *in vivo* MRI scans [162]. Moreover, hippocampal sclerosis may occur in the absence of AD pathology and hippocampal atrophy is common in fronto-temporal lobar degeneration related to mutation of the progranulin (*GRN*) or chromosome 9 open reading frame 72 (*C9ORF72*) genes [163].

An explicit framework has been proposed for a specific temporal sequence of biomarker changes during progression from

asymptomatic AD to AD dementia [99]. A study of the Dominantly Inherited AD Network has provided evidence supporting such a sequence in asymptomatic mutation carriers of familial AD [85]. In these subjects, hippocampus atrophy was estimated to follow amyloid accumulation by 10 years and to precede onset of dementia by up to 15 years. Findings from familial forms cannot simply be transferred to sporadic AD. A recent study in sporadic AD interpolated 6-year follow-up data onto a timeline from asymptomatic to clinical manifest disease covering several decades [164]. This study estimated an onset of hippocampus atrophy 4 years before onset of clinical dementia, much later than in the familial cases. It is necessary to keep in mind that these data represent interpolations from cross-sectional [85] or maximum 6 years follow-up [164] data that are projected onto a timeline of disease progression. This projection, however, relies on assumptions of disease stages that shall actually be tested in the specific study. Therefore, there is some circularity in testing these models that need to be validated in further studies.

7.1.1. Future directions: application of existing methods in a new context

The next years will see increasing use of automated volumetry of hippocampus or regional brain atrophy pattern as secondary endpoints in clinical trials in prodromal AD and AD dementia stages. Regulatory authorities are seeking for biological surrogate markers for disease modification in a situation where neuropsychological endpoints require large cohorts and complex study designs to differentiate symptomatic from disease modifying effects [6]. Structural imaging markers will play a key role in this respect, because they provide stable measures over time and across scanners and are closely associated with underlying changes of neuronal integrity [159]; moreover, the functional relevance of regional atrophy for some specific cognitive impairments has been established [165]. Therefore, the use of structural imaging endpoints will help to reduce sample size in future clinical trials. Due to the wide availability of structural imaging markers, they are also being used to enrich the risk for AD in clinical samples of MCI subjects for clinical trials. In addition, structural MRI will help to enrich study samples of asymptomatic subjects with positive molecular biomarkers of AD. The presence of amyloid alone does not predict progression to cognitive decline with sufficient accuracy, as only 25% of amyloid positive cognitively healthy subjects progress to MCI or AD within 3 years [166]. Therefore, the presence of hippocampus atrophy together with amyloid positivity will help to select subjects with a high risk of conversion to AD or MCI within a timeframe that is relevant for a clinical trial.

Use of such markers in the well-controlled setting of a clinical trial will be in the interest of probands participating in such trials. However, these protocols will also be increasingly used for diagnostic purposes outside of clinical trials. If embedded into a multidisciplinary diagnostic setting and applied to symptomatic patients, the use of such protocols will probably help make full use of the anatomical information in a structural MRI scan to the benefit of the patient. The situation is different, when such measures are employed as screening instruments. Even today, private companies offer an analysis based on regional brain and hippocampus volumetry to people who pay to get a confirmation that their brain is still structurally intact. The problem with this business model is that we are far from knowing what an atrophic hippocampus or regional brain atrophy means in terms of risk for AD and dementia in an asymptomatic person without further clinical information. Moreover, there is no point in identifying a hypothetical risk of AD without offering an intervention and support scheme to an individual. There are still many issues to be resolved on how to adequately communicate the negative aspects of a screening to a client, such as the risk of false-positive findings, the lack of a treatment option, and the probable lack of clinical relevance of a true positive finding. The "litmus test" for the usefulness of an imaging marker is its application in the "intent to diagnose" population, *i.e.* in those patients that will be confronted with this diagnostic test in primary care. There is almost no evidence available on the usefulness of imaging markers, including MRI, to support an early diagnosis of AD outside of clinically highly selected samples. Future studies are needed to determine the efficacy of MRI to detect AD type pattern of atrophy in the presence of comorbidities that had usually been excluded in studies so far. In addition, the effect of a novel structural imaging marker of predementia AD on care systems worldwide that have difficulties to provide adequate care even to patients in clinically manifest stages of disease needs to be assessed in future studies [4].

7.1.2. Future directions: novel methods

Novel methods will gain increasing importance to understand the neurobiological basis of cognitive decline in AD. The wide availability of high-field MRI at 3 Tesla and the increasing availability of ultra-high field MRI at 7 Tesla render subfield measurements of the hippocampus a feasible diagnostic approach in selected samples. Pathological evidence suggests a selective vulnerability of hippocampal subfields in AD [167]. Manual methods to determine hippocampal subfields are based on the direct identification of anatomical boundaries and serve as gold-standard to assess the performance of automated methods. Using hippocampus subfields can significantly decrease the rate of false positive findings in the prediction of future conversion from MCI to AD using manual [168] or automated [169] measurement. Sequences at 7 Tesla provide higher spatial resolution, new contrasts and access to even finer substructures of the hippocampus [170,171], but the clinical relevance of these measures needs to be explored in future studies [172].

Neuropathological evidence suggests a selective involvement of specific subcortical areas, most notably the cholinergic nuclei of the basal forebrain [173,174] and noradrenergic nuclei, in the *locus coeruleus* in AD. Based on mappings of subcortical nuclei from *postmortem* analyses, MRI scans *in cranio* will help to identify early changes in cholinergic and noradrenergic projecting nuclei in predementia and dementia stages of AD [175,176].

MRI-based detection of amyloid plaques has been successfully implemented in transgenic animals [177–180]. Further, a recent study indicates that detection is also possible in non-transgenic mouse lemur primates, in which plaques are formed naturally and are more similar to those found in humans [181]. MRI detection of plaques in humans will thus become a major topic of research in coming years. Using 7 Tesla MRI in human studies may allow *in vivo* detection of cortical amyloid deposition in the future, based on susceptibility related imaging [182] or direct visualization of amyloid plaques using intrinsic or extrinsic contrast agents. The validity of first findings and their relevance for early diagnosis will be explored in the coming years.

7.2. Diffusion tensor imaging

Diffusion Tensor Imaging (DTI) is a magnetic resonance (MR) technique that measures the random thermal motion of water molecules, *i.e.* Brownian motion, within tissue [183]. This modality does not require the injection of contrast material or radiation exposure and provide, non-invasively, unique information of the axonal organization of the brain, which is not feasible with standard MRI techniques. During the last decade, this technique has become a leading method in investigating white matter (WM) microarchitecture and integrity and has been widely employed in AD and MCI [184–187].

In a clinical context, modern MR high-field scanners (between 1.5 and 3.0 Tesla) allow rapid whole-brain assessment (4–10 min) of the apparent water diffusion tensor (DT) field using echo-planar imaging sequences. Images generated from DTI data may be qualitatively interpreted by using directionally encoded color (DEC) maps in which each color represents the axonal orientation of WM tracts. By contrast, using quantitative scalar metrics, most commonly the mean diffusivity (MD) and fractional anisotropy (FA), tissue integrity may be inferred [188]. DT-derived rotational invariants such as single eigen-values may be exploited in quantifying WM tracts integrity through region of interest (ROI), voxel- or tract-based spatial statistics approaches [185]. Furthermore, information regarding WM architecture may be quantified through deterministic and/or probabilistic tractography algorithm [189].

Normal human brain exhibits higher hindrance to water motion (diffusion) perpendicular to the long axis of WM bundles than parallel. This restriction is mostly attributed to macromolecules and cellular barriers (cell membrane) [190]. Neuronal damage, because of loss of the barriers, causes an increase in MD and a decrease FA.

Increased MD and decreased FA values have been reported in AD and MCI in parietal and temporal areas, including the hippocampal region, suggesting unspecific bundle degeneration [191,192]. Abnormal DT derived indices have also been demonstrated in frontal region, and specifically in the *cingulum posterior*, *corpus callosum*, *fasciculus longitudinalis superior*, and *fasciculus uncinatus* [193–195].

A recent study including both AD and MCI subjects [196] demonstrated a circumscribed increase in FA. These findings were aided by examining variations of a third tensor invariant, tensor mode [197] allowing to differentiate the type of anisotropy (planar, *e.g.* in regions of crossing fibers *versus* linear, in regions with one predominant orientation). Using this method, authors postulated a selective degeneration of only one of two crossing fibers suggesting a relative sparing of motor-related projection fibers crossing the association tracts of the *fasciculus longitudinalis superior*. In addition, DTI has been able to track the age-related WM degeneration in AD and, in agreement with the retrogenesis model (regions that mature late are more vulnerable to age- and disease-related degeneration), WM changes have been shown to appear earlier in specific areas such as temporo-parietal regions, the *fasciculus longitudinalis inferior*, and prefrontal regions [186,198,199].

Importantly, the reproducibility and robustness play a major role in DT data acquisition; this is a delicate point as tensor techniques employ extremely noisy echo-planar sequences, requiring strict quality control and quality assurance routines [200]. A recent meta-analysis highlights the high variability in both the anatomy of regions studied and DTI metrics [201]. Also, a recent European multicenter study, the European DTI Study in Dementia (EDSD) [202], revealed significant center-related effect in DT-derived measures.

One shortcoming of conventional DTI methods is related to the use of the simplistic model of a Gaussian propagator, which is not sufficiently accurate in regions where mixed tissue types can give rise to significant partial volume effects and/or where two or more WM fiber cross [203]. To this aim, more advanced methods such as Kurtosis Imaging [204–207], Diffusion Spectrum Imaging [208], higher-order tensor models [209], compartment models [210,211], and anomalous diffusion [212,213] have been introduced in order to augment their suitability in a clinical setting [214]. These upcoming techniques have been succesfully used in some pathologies, including AD, to enhance information of earlier microstructural tissue alterations linked to disease progression [215,216]. Among these, Kurtosis Imaging seems to be the most promising developing modality in relation of its easy setup/optimization and relatively short time acquisition in clinical MR scanners.

7.3. Functional MRI markers

Functional MRI (fMRI) represents an extraordinary technique that can study the neuronal activity through non-invasive means during specific cognitive states. This technique exploits the blood-oxygen-level-dependent (BOLD) contrasts in the vascular capillary network around the cerebral cortex. The regional metabolic demand, due to cortical activity (specific tasks/paradigms), determines an increase in local capillary hemodynamic and in the oxygenated/deoxygenated blood ratio. The increase of local deoxyhemoglobin concentration, because of its paramagnetic properties, generates an increase in local signal intensity. This technique has a relatively high spatial and temporal resolution and can be acquired along with structural MR images during the same scan session.

Several fMRI studies have been able to detect functional alterations prior to onset of cognitive impairment or AD-related structural neurodegeneration [217–219]. Task-based fMRI has been employed to study memory-related activation in the hippocampus and medial temporal lobe, typically reporting a decrease in hippocampal or parahippocampal activity during information encoding [220–224]. Also, several other studies have reported a decreased activation in the medial temporal lobe in MCI subjects [225–227].

A growing body of fMRI studies have focused on the "default mode network" (DMN), *i.e.* the interplay between a set cortical areas and the hippocampal memory system [228], the activity of which is thought to be reduced during memory intensive tasks to favor encoding and to be increased during retrieval [229]. Several studies have found dysfunctional modulation of encoding-related network activity in AD [227,230–233] or abnormal default mode pattern activity in AD and MCI patients using resting-state fMRI [234–238]. Interestingly, these results in MCI subjects have been correlated with a higher risk of progressing to AD-related dementia [239].

A bright future of fMRI or resting-state fMRI in AD and MCI might come in the area of novel pharmaceutical strategies, to date underexploited. Although drug-induced modulation of memory-related networks have been detected by fMRI [240], only few studies have demonstrated abnormal activation following, for example, long-term treatment with cholinesterase inhibitors in MCI and AD [241–244]. Therfore, additional studies are needed to test the potential role of fMRI as biomarker in clinical trials [245].

The speed of the innovation and the optimization of all these emerging modalites will be strictly related to stronger and faster MR gradients. Also, the integration of complementary information through a multimodal approach will be very useful to overcome the shortcomings of each single protocol, requiring advanced analysis tools which are able to integrate information from different protocols into the same processing pipeline. Similar approaches are likely to aid in better discrimination and staging of AD [8,246–248]. In this context, information from different modalities may be simultaneously combined using the support of machine learning algorithms enabling the classification of a single subject into a predefined group while dealing with any type of input features (*e.g.* genetic, clinical, and neuropsychological imaging data). Importantly, the classification performance is not significantly degraded if same-modality data are collected in different centers [249]. Recent results based on multimodal approaches have achieved encouraging results in discriminating AD and MCI subjects [250,251]. In the coming years, machine learning algorithms will be incorporated into scanner software to

enhance the semi-automated detection of prodromal AD stages based on high-dimensional pattern recognition.

7.4. Amyloid PET and fluorodeoxyglucose-PET markers

7.4.1. Fluorodeoxyglucose-PET

PET imaging biomarkers represent highly valuable tools for non-invasive assessment of molecular and functional pathologies which are considered to be early phenomena in the development of AD. [^{18}F]Fluorodeoxyglucose (FDG) is a well-established tracer, which allows the imaging of cerebral glucose metabolism, known to be tightly associated with neuronal function. Synaptic activity leads to an increased energy demand, which is covered by glial cells surrounding the synapse by increased glucose uptake from the blood [252]. Inversely, synaptic/neuronal dysfunction results in a decreased energy demand which is mirrored in regional metabolic decline.

Typical patterns of hypometabolism have been described in AD, including posterior parietal regions, precuneus, and also frontal cortical regions, sparing sensorimotor and visual cortex. These characteristic findings have been demonstrated to be superior to neuropsychological testing, regarding early and differential diagnosis of AD, even when *postmortem* histopathological analysis of brain tissue served as a gold-standard [253–255].

Numerous studies were able to demonstrate that early abnormalities particularly in posterior cingulate/precuneus cortical regions have a high positive and negative predictive value with regard to prediction of conversion to AD in the stage of MCI [256,257]. Even in some subjects with subjective memory impairment changes in metabolism have been observed, potentially reflecting early AD-typical pathological changes in the brain [258]. Interestingly, Reiman et al. (1996) were able to demonstrate abnormalities even in *APOE ε4*-positive subjects in younger age without any cognitive symptoms, underlining the high sensitivity of this method [259].

Regarding differential diagnosis, FDG-PET has demonstrated to be of great value because it allows the detection of different patterns of neurodegeneration, which are specific for various non-AD (amyloid-negative) forms of neurodegeneration. This includes the subtypes of frontotemporal lobar degeneration (frontotemporal dementia, progressive aphasia, semantic dementia) as well as subtypes of Parkinson-plus syndromes such as multiple system atrophy, corticobasal degeneration, and progressive supranuclear palsy [260]. Most importantly, FDG-PET is also highly useful in differentiating within amyloid-positive subtypes of disease which cannot be distinguished on the basis of their amyloid PET-scan. This includes Lewy-body dementia, posterior cortical atrophy, and the logopenic variant of progressive aphasia [255,261].

A tight correlation between the level of metabolic decline with the degree of cognitive impairment has been demonstrated consistently [262], which qualifies this method for follow-up and therapy control studies [263]. This correlation can, however, be somewhat influenced by cognitive reserve effects, expressed in variable magnitude [264]. It has also been demonstrated that FDG-PET is capable to capture therapy effects of cognitive as well as pharmacological intervention trials [265,266].

Regarding the plethora of data underlining the clinical value of FDG-PET for early and differential diagnosis of neurodegenerative disorders, as well as its complementary features as compared to amyloid-imaging, it can be expected that this method will remain an important biomarker in the coming years. Suitable MR-procedures such as resting-state fMRI or arterial spin labeling may generally bear the potential to provide information on neuronal dysfunction relatively similar to FDG-PET findings. However, the individual clinical value of these methods remains to be established in the future.

7.4.2. Amyloid-PET imaging

Today, several tracers for PET amyloid-imaging have been evaluated successfully, including clinical phase I–III studies in humans. The greatest overall number of studies has been performed with the tracer [^{11}C]Pittsburgh-Compound-B (PiB), which can be considered as the current gold-standard [267]. More recently, several ^{18}F-labeled compounds have been evaluated which would allow more widespread application of this method. One of these compounds ([^{18}F]Florbetapir/Amyvid^{TM}) has already been approved by the FDA and the EMA for commercial distribution and several others will follow in the near future. For a comprehensive review, see Rowe and Villemagne (2011) [268].

Consistently, in the great majority of all studies, a distinct uptake of the amyloid tracers has been observed in AD-patients throughout the cerebral cortex, including frontal, temporoparietal regions, and the precuneus. Whereas the basal ganglia are also regularly affected, sensorimotor and visual cortical regions show less uptake and the cerebellum is free of any relevant gray matter tracer accumulation. In young healthy control subjects, no gray matter binding of the amyloid tracers is observed but only non-specific tracer uptake in the white matter has been demonstrated. In general, this white matter uptake has been described to be less pronounced for [^{11}C]PiB as compared to the ^{18}F labeled compounds, which may somewhat decrease the sensitivity of the ^{18}F-labeled versions of amyloid tracers. The tracer ^{18}F-AZD4694 may form an exception, because it has been demonstrated to show comparably lower white matter retention [269]. The apparent differences in tracer distribution between different types of amyloid tracers have raised concerns about the comparability/standardization of amyloid-PET results. In this context, different initiatives are underway, trying to define a common standard for quantification of different amyloid-imaging results [270]. This may be particularly important with regard to clinical studies.

In vivo versus postmortem histopathological cross-evaluation studies have been performed, in general confirming that increased cortical tracer-uptake corresponds to amyloid aggregation in the brain [271,272]. The tracers are also considered to be specific for amyloid deposition with the exception of [^{18}F]FFDNP, which has been demonstrated to bind also to tau aggregates [273]. Although the tracers are specific for the protein aggregation (*i.e.* amyloid-plaques), the protein aggregation is not specific for AD. For example, it is known from histopathological studies that in Lewy-body dementia, amyloid plaques aggregation will be found in the brain in addition to the pathognonomic synuclein deposits, in most cases [274]. Thus, amyloid-imaging may not be able to differentiate between Lewy-body dementia and AD. Furthermore, amyloid-imaging alone may not be helpful with regard to distinguishing between amyloid-positive subtypes of AD (typical AD, logopenic variant of progressive aphasia, and posterior cortical atrophy) [261].

With regard to early diagnosis, a number of studies demonstrated a high predictive value of a positive amyloid-scan in the stage of MCI with regard to conversion to AD [275,276]. Even in subjects with subjective memory impairment, increased levels of amyloid deposition have been described [277] and Reiman et al. (2009) were able to demonstrate elevated amyloid-levels in asymptomatic carriers of the *APOE ε4* allele [278]. Furthermore, in a relevant proportion of elderly subjects without any cognitive complaints elevated cortical tracer-uptake was observed consistently. The meaning of these findings is not definitely clear so far, but a number of findings indicate that these subjects may indeed suffer from early AD-pathology, potentially leading to dementia later in life. This includes relatively worse performance in cognitive tests [279,166] as well as abnormal findings in other imaging tests such as resting-state connectivity [280]. In addition, recent trials in

autosomal dominantly inherited forms of AD were able to demonstrate cerebral amyloid deposition decades ahead of the expected onset of disease, using amyloid-PET. However, currently the expected time to a potential conversion to AD cannot be estimated on the basis of a positive amyloid-scan alone. Furthermore, it has to be taken into account that amyloid-imaging is not suitable to detect soluble amyloid oligomers, which have been discussed to potentially represent the most toxic species [281].

Only a limited correlation has been observed between *in vivo* measured amyloid burden and cognitive decline. This may particularly depend on the stage of disease: (I) in cognitively healthy elderly subjects amyloid pathology may not yet have induced neurotoxic effects downstream from amyloid aggregation sufficient enough to have an impact on cognition; (II) in patients with manifest Alzheimer's dementia, a plateau of amyloid deposition has been observed, indicating that amyloid deposition reaches saturation, whereas subsequent neurodegeneration (and cognitive decline) continues [282]. As for FDG-PET, in the presence of cerebral compensation mechanisms, expressed to different degree in different subjects, it may also lead to a discrepancy between cortical amyloid load and symptomatic appearance. These factors do not necessarily limit the value of amyloid-imaging for therapy trials. First, the value of amyloid-imaging with regard to patient selection is undoubted. Second, amyloid-imaging may allow the measurement of the increase in amyloid deposition over time particularly in early stages, *i.e.* ahead of a plateau phase. Finally, it has been demonstrated that the response to anti-amyloid therapy may be quantified at least in a group based evaluation [283].

Regarding the commercial availability of amyloid-imaging tools, appropriateness of use criteria have recently been published in the *Journal of Nuclear Medicine and Molecular Imaging* [284]. These criteria suggest a useful application of amyloid-imaging in patients with MCI, in AD with atypical presentation (*e.g.* early-onset) and when the diagnosis is uncertain after evaluation by a dementia expert. Without doubt, amyloid-imaging may represent one of the most important biomarkers for scientific and clinical assessment of AD in the future. The establishment of this sophisticated method for *in vivo* assessment and quantification of a molecular neuropathology will certainly also depend on reimbursement issues and on the question if anti-amyloid therapy trials will be followed further and yield in first promising pharmacotherapeutic approaches.

7.4.3. Complementary value of FDG-PET and amyloid-PET and order of abnormalities

As mentioned above, recently introduced guidelines recommend the integration of biomarkers into the classification/estimation of likelihood of preclinical, prodromal, and manifest stages of AD. According to these guidelines, both FDG-PET and amyloid-PET are suited to play an important role as diagnostic biomarkers in all stages of disease. In short, the proof of amyloid pathology (as possible with amyloid-PET) accompanied by proof of neuronal injury (as possible with FDG-PET) and finally proof of cognitive impairment sum up to an increasing probability for AD [25–27]. All these guidelines are based on the assumption that amyloid pathology is the first biomarker to become positive, followed by neuronal injury/tau-pathology and finally cognitive decline. Some recent data from a study in subjects with inherited AD in presymptomatic stages seems to confirm this notion in principle [85]. On the basis of the currently available information, it seems that amyloid-PET and $A\beta_{1-42}$-changes in the CSF behave relatively similar with regard to early detection of ongoing AD-pathology. Bateman et al. (2012) were able to demonstrate a very early decline in CSF $A\beta_{1-42}$-levels in mutation carriers but coming from a higher preexisting overall $A\beta_{1-42}$-level [85]. Thus, an early detection of ongoing disease on the basis of single time-point absolute $A\beta_{1-42}$ CSF levels would not be possible. A significant difference in $A\beta_{1-42}$-levels between carriers and non-carriers was detected relatively later as compared to the onset of significant abnormalities detected with amyloid-PET.

The mentioned guidelines and recent models of biomarker time courses treat CSF tau, FDG-PET, and structural MRI as equivalent markers of neuronal injury, appearing subsequently to amyloid pathology [285]. However, this assumption may represent an oversimplification for several reasons. First, it is known that FDG mirrors neuronal dysfunction and, from a pathophysiological point of view, it appears obvious that functional changes should be detectable ahead of neuronal loss/brain atrophy. In fact, studies were able to demonstrate higher sensitivity of FDG-PET as compared to structural MRI with regard to prediction of AD in the stage of MCI. FDG-PET may also be able to monitor changes in neuronal function in response to therapy, which may not be detectable with MRI or CSF tau measurements.

Furthermore, several recent findings are challenging the classic amyloid-hypothesis. This includes imaging studies demonstrating the presence of neuronal injury in absence of any proof of amyloid pathology [286,287]. Thus, further studies are required to gain deeper insights into the actual order of appearance of the pathologies and the threshold of their detectability. In this context, novel PET-tracers for tau-imaging may be of extraordinary importance. Fortunately, first successful experiments to establish such novel imaging biomarkers are currently on their way [288].

As mentioned above, the advantage of imaging biomarkers as compared to CSF biomarkers can be found in the provision of information not only on the presence of a certain pathology but also about the topography and the actual extent in the brain. This may be an important advantage with regard to disease staging, follow-up/therapy control, and differential diagnosis. Novel imaging instrumentation such as hybrid PET/MR scanners may offer an additional opportunity to merge the complementary information from different imaging modalities into new integrated *in vivo* biomarkers of neurodegeneration.

8. Neuroelectrical and neuromagnetic markers

The full potential of neurodynamic time-sensitive biomarkers using electroencephalography (EEG) [289] and magnetoencephalography (MEG) [290] for quantification of degenerative brain changes during various stages of AD has yet to be realized. Subtle but consistent deviations in the electromagnetic neuronal dynamics have been shown to precede explicit cognitive manifestations in AD [291] which could enable a future role of EEG/MEG biomarkers not only as a clinical diagnosis and treatment option, but also as a new mode for AD stage discovery. Dramatic progresses in dense-array active-EEG and MEG sensor technology, as well as in advanced signal processing techniques [292] have generated a recent surge of interest to use these promising capabilities in the context of improved clinical AD diagnosis. The added value of the EEG/MEG markers as an inexpensive, fast, and time-resolved tool is set to be explored rigorously both as a standalone approach and as a complementary measure together with other biomarker modalities.

8.1. Resting-state neuroelectrical/neuromagnetic markers

The spontaneous activity of the brain's resting-state networks (RSN), while the subject is idle with eyes closed or open, can be characterized by quantitative EEG/MEG measures (qEEG/qMEG), often using frequency band power or time-frequency estimates. Brainwave components of the resting EEG could be altered in the early stages of AD. There is evidence that EEG power in the alpha band declines with AD-related cognitive impairment [293]. Other

studies have shown enhanced low-frequency brain oscillation in the theta [294] and delta bands in temporal and occipital areas as well as reduction of beta power in temporal and occipital areas in MCI [295]. However, frequency band-power methods need to address some current limitations, notably regarding the necessity to adjust band limits depending on task and individual, as well as to study more completely each band's significance in relation to neuronal phenomena.

A next generation of more sophisticated resting-state signal analysis approaches [292] is set to improve upon and to replace band-power markers in the next decade by capturing better the complex characteristics and dynamics of progressive neurodegeneration and aging. Promising methods involve brain connectivity [296], global synchronization, synchronization likelihood [291], detrended fluctuation analysis, approximate entropy, mutual information, source localization, and a host of further non-linear signal features. This will open new possibilities and raise new questions such as a recent study showing in AD not only that an EEG synchronization marker was suppressed in the 10–30 Hz range (upper-alpha and beta bands) but also that the temporal fluctuations of this synchronization measure carry additional diagnostic value in the lower alpha and beta bands [297].

8.2. Functional neuroelectrical/neuromagnetic markers

Functional neuroelectrical/neuromagnetic biomarkers represent an emerging candidate for a diagnostic tool in AD clinical practice, created to evaluate specific functional activities of the brain, as opposed to resting-state. Their main purpose is the dynamic detection of cognitive-task-related deviations in brain function following the onset of AD due to impairment of neuronal connections or neuronal components participating in the functional response. Such deviations are not always manifested during the resting-state due to the targeted activation of task-related pathways and areas of the brain. Although existing topographical and pathophysiological biomarkers have shown substantial capabilities for identification or follow-up of AD [298], functional neurodynamic measures provide differential information that is advantageous and complementary in relation to cognitive impairment and progression of the disease [299]. AD biomarkers of pathophysiological type using amyloid-imaging can expose early changes in cognitively normal individuals leading to dementia, yet the subsequent structural brain changes during the various stages of the disease are more optimally followed using topographical biomarkers such as MRI and FDG-PET [300]. There is a clear need to bridge the drawbacks of these biomarker approaches in view of the challenging tasks of detection and follow-up of subjects on the way to convert to clinical AD [301].

Currently, most functional EEG biomarkers [302] are based on spatio-temporal features such as the peak amplitude or latency of event-related potentials (ERPs) [303] (*e.g.* the N400/P600 ERPs which are cognitive indicators of episodic memory encoding [304]). Yet, the event-related potential/event-related field (ERP/ERF) approach is in need to address further some well-known usability issues [305]. Similarly to resting-state measures, in the future a wider application of new biomarkers based on evoked spatio-spectro-temporal measures and task-related dynamic synchrony methods will be needed to bring in additional capability for handling single-trial EEG/MEG data more reliably, and to reflect the state of the functional brain networks for each individual subject.

8.3. Future steps toward establishing the neuroelectrical/neuromagnetic markers

The main challenge for establishing the EEG/MEG biomarkers as an AD diagnostic instrument is the diversity of approaches in existing studies. While this richness of possibilities is quite promising, a first practical step would be to select a first battery of neurodynamic biomarkers based on existing results and to initiate proposals for full standardization and implementation in practice. A modular approach would ensure that future advances can be efficiently integrated. Possible standardization modules could include data recording procedures, specific guidelines on suppression of signal noise interferences, as well as recommendations on feature extraction and diagnostic decision-making. Special attention is necessary to ensure an adaptive approach as a prerequisite for success, including the integration of individual biomarker baselines for the subjects. The goal to recognize reliably each AD stage using EEG/MEG biomarkers is particularly challenging since it is necessary to overcome known brain plasticity effects due to compensatory mechanisms in the preclinical and prodromal stages of AD as neurodegenerative and cerebrovascular lesions impose progressive impairment. The final steps would involve an extensive multi-step, multi-center validation of the biomarker standards, as well as a modality integration with other measures (a compatibility study).

The existing record of neuroelectrical/neuromagnetic biomarker performance in the scientific literature suggests a promising potential in enhancing the reliability and specificity of AD prognosis while circumventing technical, experimental, financial and logistic limitations of other biomarker measurements [306].

9. Regulatory perspectives

Despite remarkable progress in understanding the molecular underpinnings of AD during the last three decades, there are no effective interventions for altering the progression of the disease. Even those few medicinal products approved for symptomatic treatment of mild to moderate stages the disease are inadequate for long term amelioration of symptoms in more severe cases. The positive results of pre-clinical studies aimed at rescuing synaptic dysfunction or preventing behavioral impairment in animal models [307] have yet to be translated into disease-modifying compounds in humans. The latest clinical trial failure of bapineuzumab and the very modest results from two major phase III studies for solanezumab raises several questions regarding: (I) prevailing ideas-theories about the pathogenesis of the disease, (II) the appropriates of the therapeutic targets, (III) selection or inclusion criteria of subjects into clinical trials, *e.g.* pre-clinical subjects *versus* mild-moderate, and (IV) study design. US and EU regulatory agencies are facing these questions as well recent recommendations of various task forces for clinical trials in AD.

Recently, the FDA has proposed a draft guideline for Industries (available at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM338287.pdf) allowing alternative targeting of intervention at the early stages of AD. According to this new guidance FDA suggests potential approaches to clinical trial design and execution that allow for regulatory flexibility and innovation [308]. There they cover the selection of patients for trials at early stages of AD and for this there is a consensus within the AD research community that clinical diagnosis of early cognitive impairment might be coupled with specific appropriate biomarkers of disease. Diagnostic criteria have been established and are under validation by various working groups [6]. Such biomarkers include brain Aβ load, as measured by PET and CSF levels of Aβ and tau proteins [309] as outlined earlier in this article.

However, adequate validation of these biomarkers is still lacking despite over 19,000 published papers. Approximately 150 longitudinal studies related to the biomarkers of interest were identified which included subjects who had objective cognitive impairment but no dementia at baseline. The authors report that

the body of evidence for these imaging and CSF biomarkers is still limited and variable across the different types of biomarkers [310]. As far as the CSF biomarkers are concerned, it was recently reported that the overall variability of data coming from a total of 84 laboratories remains too high to allow the validation of universal biomarker cut-off values for the specific intended use [107], which underscores the urgent need for better harmonization and standardization of these methods.

The use of biomarkers as endpoints in earlier stages of drug development is well established for regulators, and there are examples to approve medicinal products on the basis of their effects on validated surrogate markers, *e.g.* antihypertensives, or cholesterol-lowering products. However, these examples have been considered as validated surrogate markers as they allow substitution for a clinically relevant end point. In their validation a link between a treatment-induced change in the biomarker and long-term outcome of the relevant clinical measure was undoubtedly established. Therefore, the regulatory requirements on biomarkers used as endpoints in clinical trials are high as outlined earlier [309]. In consequence EU regulators help applicants in their research and development by issuing opinions on the acceptability of using such biomarkers or a distinct methodology in clinical trials. Since 2011, EMA's Committee for Medicinal Products for Human Use (CHMP) has adopted and published four of these qualification opinions for use in the development of medicines for AD. Three of these qualification opinions are for biomarkers to help identify and select patients at the pre-dementia stage of the disease. The fourth one is for a biomarker to be used to select patients for clinical trials in mild and moderate AD. In August 2013, a public consultation ended on a qualification opinion for a novel model of disease progression and trial evaluation in mild and moderate AD. The simulation tool is intended to provide a quantitative rationale for the selection of study design and inclusion criteria for the recruitment of patients.

In the diagnostic area, the approval of the first radiopharmaceutical for PET imaging of Aβ neuritic plaques in the brain by the European Commission, in January 2013, on the recommendation of the CHMP has been another step forward. This diagnostic agent can be used in patients who are being evaluated for AD and other causes of cognitive decline. Two other diagnostic radiopharmaceuticals for AD ([^{18}F]Florbetaben and [^{18}F]Flutemetamol) are currently under evaluation by the CHMP. However, interpretation of amyloid scans is not without hurdles: amyloid positivity does not reliably distinguish between clinical diagnoses, so that neuropsychiatric normal people as well as those with MCI, AD, and other neurodegenerative diseases can all be "amyloid positive". Therefore, a positive amyloid scan must be considered in the full clinical picture of a patient, on the other hand a negative amyloid scan indicates that the likelihood of cognitive impairment due to AD is low [311,312].

Another issue in future clinical trials is the appropriate choice of clinical endpoints. In established AD the CHMP guidance requires co-primary endpoints in cognition (mandatory) together with functional or global outcome measures; moving now to earlier asymptomatic or prodromal stages of AD might change this requirement. Thus, the FDA suggests for clinical trials focusing on patients in whom overt dementia seems imminent the use of a single scale that combines assessment of both cognition and function such as the score on the Clinical Dementia Rating Sum of Boxes (CDR-SB) [308]. For patients whose disease is at an even earlier clinical stage, it might be possible to approve a drug through an accelerated procedure pathway on the basis of assessment of only cognitive symptoms in the US. The accelerated approval mechanisms will allow drugs that address an unmet medical need to be approved on the basis of a surrogate endpoint or an intermediate clinical endpoint (*i.e.* a sensitive cognitive measure). In the EU, a similar approach is possible *via* a "conditional" approval, which implies that the applicant accepts after such an preliminary approval the obligation to carry out further long-term clinical studies to confirm clinical efficacy and safety. Only after the approval and long-term treatment, it would be possible to properly follow the amelioration of cognitive and behavioral disorders as well as the slowing of the progression of neurodegenerative lesions as shown by neuroimaging techniques [309]. Pharmaceutical industry is encouraged to seek scientific advice on their development program as soon as possible with the regulators, if they intend to use new methods to define the patient population or specific study designs and assessment tools. For instance, Richard et al. (2013) have proposed a new memory test for improving the diagnostic accuracy in patients with mild cognitive impairment recently. In particular, the Net Reclassification Improvement (NRI), followed by MRI and CSF analysis, might be an attractive and easy way to interpret certain measures for clinicians [313]. The development and validation of such new assessment tools is encouraged by regulators.

By the end of 2013, CHMP will decide whether or not there is a need to revise the guideline on the clinical investigation of medicines for the treatment of AD on the basis of new knowledge obtained from the use of biomarkers in clinical evaluation and new trends in research and development. It has already been acknowledged that AD is more a "continuum" of different stages and that the focus of new drug development has shifted to earlier stages. It is desirable that regulators and all involved stakeholders work together to decide the best design at the various stages of disease of the new clinical trials for AD prevention and treatment.

10. Conclusions

According to the new diagnostic criteria of AD recommended by the IWG [21,23] and the revised NIA-AA [26–28] initiatives, biomarkers are expected to play a prominent role in future development-validation of technologies-algorithms for: (I) accurate detection of people in the early stage of the disease, (II) more reliable diagnosis, and (III) accurate prognosis or prediction of asymptomatic people at elevated risk. This will be also possible in equivocal cases with unusually presenting clinical symptoms and problematic classification/differential diagnosis [314]. As argued by Visser et al. (2012), the IWG and the NIA-AA criteria display both commonalities and important differences [20]. Notably, they concur in recognizing the onset of AD prior to dementia [24] and highlight the employment of biomarkers as critical and supportive data for the early diagnosis of prodromal AD. In clinical trials, biomarkers can be utilized to enrich early or asymptomatic AD, thus decreasing both the extent of heterogeneity within diagnostic groups and the number of individuals necessary to detect statistically significant group differences. As a result, the statistical power will be increased [315].

Besides their diagnostic significance, biomarkers may contribute to the progress in the development of novel drugs for the treatment of AD related molecular mechanisms. They may be employed for the *in vitro* monitoring of drug discovery plans intended to identify new molecules inhibiting amyloidogenic mechanisms and to provide surrogate measures assessing treatment efficacy of novel Aβ-targeting drugs, which would decrease the time and cut the costs of clinical trials [94]. In addition, biomarkers may help demonstrate the usefulness of a certain therapy in a specific patient, thus assisting the physician to find the proper medication. Intriguingly, Lu et al. (2013), employing solid-state nuclear magnetic resonance (NMR) approach, have reported the existence of an original structural model of Aβ fibrils

from AD brain, characterized by significant differences from *in vitro* fibrils [316]. These novel structural data can be utilized to construct novel structure-specific PET radioligands for *in vivo* amyloid-imaging and conceptualize more selective small molecule inhibitors, and therapeutic antibodies [317]. These unique structure-specific PET radioligands, once validated by future follow-up studies, might be used in cooperation with CSF and blood biomarkers to help refining patient stratification [317].

Controlled and observational longitudinal studies utilizing combinations of biofluid markers in conjunction with other types of diagnostic and therapeutic approaches are required. In the absence of such studies, it is challenging to recommend exhaustive diagnostic algorithms that integrate fluid biomarkers. Moreover, the paucity of standardized procedures to quantify the existing biomarkers impedes the use of validated biomarker cut-off values to guide and monitor clinical decision-making. Attaining the validation of these cut-off points is one of the key objectives of present research performed into biomarker discovery both for AD and for other neurological disorders [318].

Finally, the standardization of the methodologies and the development of external control assays/tools/methodologies are compulsory requirements to enable the successful use of biomarkers in the diagnosis and management of AD [6,319].

At present, trials aiming at exploring early AD have been developed. In this regard, an umbrella group–the Collaboration for Alzheimer's Prevention (CAP), sponsored by Fidelity Biosciences Research, Inc., and the Alzheimer's Association – has been established which incorporates three separate, but interconnected, long-term prevention initiatives [18]: the Dominantly Inherited Alzheimer's Network (DIAN) [85], the Alzheimer's Prevention Initiative (API) [320], and the Anti-amyloid Treatment of Asymptomatic Alzheimer's (A4) trial [321]. CAP has been promoted to harmonize the studies and encourage data sharing: it exists as a setting for DIAN, API, and A4 to keep a systematic discussion among them as they plan and execute their preclinical treatment trials [18]. All of the three trials will focus on the concept that AD pathological mechanisms initiate long before the onset and progress of dementia and that amyloid is critically involved in the disease pathogenesis [322].

The paradigm shift toward early AD detection/characterization/diagnosis is essential to redefine and launch successful interventional trials. Such a paradigm embraces both secondary prevention (*i.e.* preventing the progression of pathological mechanisms and subsequent symptoms) and primary prevention (*i.e.* preventing the beginning of molecular and cellular mechanisms/signaling pathways). This objective may be attained by integrating the clinical trial approach to disease into a public health model, using long-term longitudinal databases that include large populations [323]. In this connection, significant initiatives showing a worldwide perspective are: the Organization for Economic Co-operation and Development (OECD) Task Force on AD (available at http://www.oecd.org/), the EU/US Task Force on Clinical Trial Development in AD [18,324], and the non-profit corporation Prevent Alzheimer's Disease 2020 (PAD2020) (available at http://www.pad2020.org) [17], all stressing that a world-wide database should be established by integrating/expanding existing cohorts and registries [323].

Given the vibrant and as of yet relatively unexploited future potential of the multimodal biomarker development, the current status of the integration of biomarkers in clinical trials seems only the beginning of the evolving paradigmatic "systems biology and neural network" era of AD [7,12]. This seems to be the most promising road ahead to breakthrough advances in this highly complex scientific arena. It is recognized that we can learn much from existing research in early asymptomatic populations as well as in familial autosomal dominant AD. However, it will be necessary to chart the full spectrum biomarker map in complex, non-linear sporadic AD [7,12] to progress and improve effective treatment perspectives.

Systems biology is an emerging interdisciplinary approach to AD research [12] that allows the integrated examination and assessment of interrelated biological pathways where structurally/functionally different biomolecules are simultaneously measured over time in cells, networks of cells, organs, or whole organisms [325]. Systems biology, embracing a large set of divergent methodological approaches, has become realistic owing to multiple high-throughput "omics" technologies, namely genomics/epigenomics, transcriptomics, proteomics, and metabolomics/lipidomics. These platforms, in association with accurate bioinformatic analyses using powerful computational and statistical modeling tools, will permit the investigation of various types of molecular interactions [325], such as transcriptional modules [326], gene-interaction networks [326], protein–protein interaction networks [327], and signaling networks [327]. Studying these network models will help unveil previously unknown molecular network properties of AD as well as identify genes, proteins, and cellular pathways critically involved in AD mechanisms. This, in turn, will be of support for the detection of the most appropriate gene and protein targets for AD treatment.

Conflict of interest

H.H. declares no competing financial interests related to the present article. During the last two years he has received lecture honoraria and/or research grants and/or travel funding and/or participated in scientific advisory boards and/or as a consultant to diagnostic, biotechnology, and pharmaceutical companies involved in the manufacture and marketing of biomarkers and/or diagnostics and/or drugs or medicinal products for cognitive impairment and Alzheimer's disease including Boehringer-Ingelheim, Bristol-Myers Squibb, Elan Corporation, Wyeth, Novartis, Eisai Inc., Pfizer, Schwabe, Sanofi-Aventis, Roche Pharmaceuticals and Diagnostics, GE Healthcare, Astra-Zeneca, Avid, Eli Lilly and Company, Janssen-Cilag, Merz Pharmaceuticals, GlaxoSmithKline-Biologicals, Jung-Diagnostics, Thermo Fisher Scientific Clinical Diagnostics, Cytox. He is co-inventor in pending patent submissions relating to biological markers and/or diagnostics and has not received any royalties.

K.B. has served at Advisory Boards for Innogenetics, Kyowa Hakko Kirin Pharma, Pfizer, and Roche.

H.B. declares no conflicts of interest. He does not have any financial support from commercial companies regarding this study.

A.D. declares the following potential conflicts of interest: Piramal, AVID Pharmaceuticals/Lilly, GE Healthcare, and Siemens Healthcare.

O.C. declares no financial activities related to the present article. He reports having received lecture fees from Lundbeck, consulting fees from Guerbet and no other biomedical financial interests or any potential conflicts of interest.

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P.S.A. declare no competing financial interests related to the present article. He serves on a scientific advisory board for NeuroPhage; he has served as a consultant to Elan Corporation, Wyeth, Eisai Inc., Schering-Plough Corp., Bristol-Myers Squibb, Eli Lilly and Company, NeuroPhage, Merck & Co., Roche, Amgen, Genentech, Inc., Abbott, Pfizer Inc, Novartis, Bayer, Astellas, Dainippon, Biomarin, Solvay, Otsuka, Daiichi, AstraZeneca, Janssen,

Medivation, Ichor, Toyama, Lundbeck, Biogen Idec, iPerian, Probiodrug, Somaxon, Biotie, Cardeus, Anavex, Kyowa Hakko Kirin Pharma, and Medtronic.

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