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Biomarkers in Amyloid-β Immunotherapy Trials in Alzheimer's Disease

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Drug candidates directed against amyloid-β (Aβ) are mainstream in Alzheimer's disease (AD) drug development. Active and passive Aβ immunotherapy is the principle that has come furthest, both in number and in stage of clinical trials. However, an increasing number of reports on major difficulties in identifying any clinical benefit in phase II–III clinical trials on this type of anti-Aβ drug candidates have caused concern among researchers, pharmaceutical companies, and other stakeholders. This has provided critics of the amyloid cascade hypothesis with fire for their arguments that Aβ deposition may merely be a bystander, and not the cause, of the disease or that the amyloid hypothesis may only be valid for the familial form of AD. On the other hand, most researchers argue that it is the trial design that will need refinement to allow for identifying a positive clinical effect of anti-Aβ drugs. A consensus in the field is that future trials need to be performed in an earlier stage of the disease and that biomarkers are essential to guide and facilitate drug development. In this context, it is reassuring that, in contrast to most brain disorders, research advances in the AD field have led to both imaging (magnetic resonance imaging (MRI) and PET) and cerebrospinal fluid (CSF) biomarkers for the central pathogenic processes of the disease. AD biomarkers will have a central role in future clinical trials to enable early diagnosis, and Aβ biomarkers (CSF Aβ42 and amyloid PET) may be essential to allow for testing a drug on patients with evidence of brain Aβ pathology. Pharmacodynamic Aβ and amyloid precursor protein biomarkers will be of use to verify target engagement of a drug candidate in humans, thereby bridging the gap between mechanistic data from transgenic AD models (that may not be relevant to the neuropathology of human AD) and large and expensive phase III trials. Last, downstream biomarker evidence (CSF tau proteins and MRI volumetry) that the drug ameliorates neurodegeneration will, together with beneficial clinical effects on cognition and functioning, be essential for labeling an anti-Aβ drug as disease modifying.

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INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder and a huge socioeconomic and humanistic problem. There are several symptomatic treatments registered for AD, including the cholinesterase inhibitors donepezil, galantamine, and rivastigmine and the noncompetitive *N*-methyl-D-aspartate receptor antagonist memantine (for review, see Blennow *et al*, 2006). As these drugs only provide a temporary effect on cognition and functioning, and do not affect the underlying neurodegenerative process, there is an urgent need for novel drugs that may slow down the neurodegenerative process and exert a disease-modifying effect.

The identification in the mid-1980s of amyloid-β (Aβ) as the main component of plaques (Masters *et al*, 1985); the cloning of its precursor, amyloid precursor protein (APP), on chromosome 21 (Kang *et al*, 1987); and the finding that mutations in the *APP* gene can cause familial forms of AD (Goate *et al*, 1991), together with the knowledge that plaque counts correlate with dementia severity (Roth *et al*, 1966) and that persons with Down's syndrome (who have an extra copy of chromosome 21) develop AD pathology with plaques early in life (Mann *et al*, 1984), highlighted the role of Aβ and plaque formation as the potentially central mechanism in AD. The leading hypothesis for AD

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pathogenesis is the amyloid cascade hypothesis, which posits that Aβ, especially the 42 amino-acid form of the protein (Aβ42), is the initiating event and driving force in the disease process (Hardy and Selkoe, 2002). An imbalance between Aβ production and clearance would result in a conformational change in Aβ, with aggregation and formation of toxic oligomers and larger plaques, which ultimately leads to neuronal degeneration and cognitive symptoms (Hardy, 2009).

The amyloid cascade hypothesis has served as the backbone for the overwhelming part of AD drug development, and today there are a large number of anti-Aβ therapies in different phases of clinical trials with potential disease-modifying effects (ClinicalTrials.gov, 2013). These anti-Aβ drug candidates have three general principles for mode of action. The first is to lower Aβ production by inhibiting either of the two enzymes that cleaves APP and thereby generates Aβ, β-site APP-cleaving enzyme 1 (BACE1) or γ-secretase, or to lower the relative proportion of the Aβ42 isoform by γ-secretase modulators (May *et al*, 2011; Panza *et al*, 2011). An alternative target is to inhibit the aggregation of Aβ by small molecules such as PBT2, a metal-protein-attenuating compound that affects Aβ oligomerization (Lannfelt *et al*, 2008). The third mode of action is Aβ immunotherapy, which can be divided into active immunization using full-length Aβ or fragments of Aβ, or passive immunotherapy using monoclonal anti-Aβ antibodies or intravenous immunoglobulins (Lemere and Masliah, 2010).

However, despite very promising preclinical data showing that Aβ immunotherapy prevents, or even clears, amyloid plaques in AD transgenic mouse models, AD research in recent years has been dominated by an increasing number of reports on anti-Aβ drug trials that show no, or only marginal, positive effects on primary clinical outcome measures (Blennow, 2010; Lemere and Masliah, 2010). These negative trials have caused concern that the amyloid cascade hypothesis is wrong, that is, Aβ aggregation and plaque development is merely a by-product of the neuronal degeneration, or is valid only in familial AD (FAD). In this context, it should be noted that the bulk of data supporting the amyloid cascade hypothesis is derived from studies on cellular models and laboratory animals harboring mutations in the *APP* and presenilin (*PSEN1* and *PSEN2)* genes found in the rare FAD variants of the disease. Another plausible consequence of the disappointing results from anti-Aβ trials is that it may stimulate both research and drug development in other aspects of AD neuropathology and neurochemistry. However, there are several other possible explanations, including that the design of future trials will need refinement so that treatment can be initiated at an earlier stage of the disease, before neurodegeneration is too severe and widespread, and that the diagnostic procedure in trials needs refinement so that only patients with AD, and not dementia in general, are included.

In this review, we give an overview on the role of biomarkers in clinical trials on Aβ immunotherapy and the type of anti-Aβ drug candidates that has come furthest in development, with many ongoing, but also arrested, drug programs. We do not aim at giving a historical review covering all preclinical data and clinical trials on Aβ immunotherapy. Instead, we present clinical trials for which there are published data available, with focus on cerebrospinal fluid (CSF) biomarkers. We discuss the position of biomarkers in AD immunotherapy trials and try to hypothesize on how to interpret data from trials on different forms of Aβ immunotherapy.

BIOMARKERS IN AD CLINICAL TRIALS

The term 'biomarker' refers to an objective measure of a biological or pathogenic process that may be used in clinical medicine as diagnostic tools to predict disease risk or prognosis or to monitor the effect of therapeutic interventions. Numerous studies have shown that all of magnetic resonance imaging (MRI) volumetry of the hippocampus to gauge brain atrophy, PET measurements of (^{18}F)-fluorodeoxyglucose (FDG) to assess glucose metabolism rate in cortical neurons and glial cells in specific brain regions, and global cortical retention of amyloid ligands, such as Pittsburgh compound B (PiB), and CSF biomarkers have high diagnostic accuracy for AD (for review, see Quigley *et al*, 2011; Herholz, 2012; Reiman and Jagust, 2012). These biomarkers have also been used as secondary end points in clinical trials on anti-Aβ compounds in AD (for example, see Fox *et al*, 2005; Gilman *et al*, 2005; Rinne *et al*, 2010; Blennow *et al*, 2012; Dodel *et al*, 2013).

A hypothetical model has been presented for how to interpret the longitudinal evolution of biomarker changes in AD and how biomarkers may be used to track the ongoing pathophysiological processes (Jack *et al*, 2013). As discussed elsewhere (Blennow, 2010), interpretation of biomarker data may depend on whether the biomarkers reflect the intensity of the degenerative disease process (i.e., how fast the synaptic and neuronal degeneration progress) or the stage of the disease (i.e., the amount of brain pathology). As an example, the CSF level of total tau (T-tau) reflects the intensity of the neuronal degeneration, with very high-level disease in cases with Creutzfeldt–Jakob disease (Otto *et al*, 1997), which is characterized by very intense neuronal degeneration, and high T-tau levels also correlate with rapid cognitive decline and a high mortality rate in AD patients (Wallin *et al*, 2009; Sämgård *et al*, 2010). On the other hand, CSF Aβ42 may be regarded as a disease stage marker, as low CSF levels correlate with Aβ plaque load, both at autopsy (Strozyk *et al*, 2003) and as measured during life using amyloid PET scanning (Fagan *et al*, 2006; Degerman Gunnarsson *et al*, 2010). In addition, interpretation of biomarker results will also depend on both the sensitivity of the analytical technique and the (patho)physiology underlying the biomarker signal (Frisoni and Blennow, 2013). For these reasons, true longitudinal studies with repeated biomarker assessments will be needed to further explore the temporal evolution of AD pathophysiological events,

that is, which comes first of Aβ/plaque pathology, tau/tangle pathology, and neuronal/synaptic degeneration, during the progression of the disease.

As reviewed elsewhere (Hampel *et al*, 2010), there is a consensus among the academy, pharmaceutical industry, and regulatory authorities that biomarkers have several uses in clinical trials. One is as diagnostic tools to enrich the trial cohort with pure AD cases, another to facilitate drug development as tools to identify and monitor the biochemical effect of a drug both as pharmacodynamic markers to verify target engagement and as downstream biomarkers to provide objective data that a drug ameliorates neurodegeneration, and a third to enable early and specific detection of side effects of the drug (Hampel *et al*, 2010). Still another use is for patient stratification, with the aim to identify biochemical phenotypes that may be more responsive to therapy (Table 1).

Biomarkers for Patient Enrichment

Trials on Aβ immunotherapy that have reported data on clinical effects have so far been based on treatment of AD patients in the later stages of the disease, with mild-to-moderate dementia. However, it is unlikely that anti-Aβ disease-modifying drugs will have other than very minor effects on the cognitive symptoms or daily life functioning in this late stage of the disease, in which there is quite advanced neurodegeneration with severe neuronal degeneration and synaptic loss. An example of this may be that despite developing long-lasting anti-Aβ titers in blood and post-mortem evidence of Aβ plaque removal, treated patients deteriorated to severe dementia before death in the follow-up study of the AN1792 phase I active Aβ immunotherapy trial (Holmes *et al*, 2008).

For this reason, it is plausible that trials have to be designed to test novel drug candidates on prodromal AD cases, that is, AD patients who are in the mild cognitive impairment (MCI) stage of the disease, or even in the asymptomatic (preclinical) phase of AD, to give promising anti-Aβ drugs a fair chance of exhibiting a disease-modifying effect. However, MCI is a heterogeneous syndrome, with only approximately 50% of patients having prodromal AD, whereas the other half having benign memory problems as part of the normal aging process, stress-related symptoms, depression, or other brain disorders such as vascular dementia, Lewy body dementia, or tauopathies (DeCarli, 2003). Considering that MCI patients have vague symptoms with mild memory disturbances, whereas other symptoms characteristic of AD are absent, the diagnostic problems are obvious.

CSF biomarkers have been shown to have a value to identify prodromal AD in MCI cohorts. Andreasen *et al* (1999) showed that MCI patients who during follow-up progressed clinically to AD with dementia had the AD CSF profile with high T-tau and phosphorylated tau (P-tau) together with decreased levels of Aβ42 already in the baseline examination. Hansson *et al* (2006) presented a

TABLE 1 CSF Biomarkers in Immunotherapy Trials

Application	Method	Biomarkers	Benefit
Patient enrichment	CSF biomarkers analyzed for diagnostic purposes before enrollment into a clinical trial	High T-tau, high P-tau and low Aβ42 are indicative of AD	Improved diagnostic accuracy in mild AD and enrichment of MCI trials with prodromal AD cases may improve the possibility to identify a clinical effect of the drug candidate
Patient stratification	CSF samples taken before trial initiation, and analyses performed after the end of the clinical trial	*Post hoc* analysis	AD cases with CSF biomarker evidence (low Aβ42) of a disturbance in Aβ metabolism might be more responsive to anti-Aβ drugs than patients without clear evidence of disturbed Aβ metabolism
Safety monitoring	CSF samples taken before trial initiation for comparison and new samples taken if an adverse event occurs	CSF cell count, CSF/serum albumin ratio, IgG/IgM index, and isoelectric focusing to identify IgG/IgM oligoclonal bands to identify inflammatory processes and disturbances in the blood–brain barrier	Aβ immunotherapy might elicit adverse effects, such as meningoencephalitis or ARIA-E/vasogenic edema
Pharmacokinetics	Analysis of plasma and CSF samples after a single dose or multiple dosing	The therapeutic antibody	Antibody ratio between CSF and plasma will indicate whether the therapeutic antibody passes the blood–brain/CSF barrier to the same degree as endogenous IgG
Theragnostics	CSF biomarkers analyzed before study initiation and at time points during the trial including last week of the study	Aβ1–42 as the main biomarker for Aβ metabolism and deposition; other Aβ isoforms (e.g., AβX-42, Aβ1–40, Aβ1–16, Aβ5-X, and total Aβ) for complementary information on the Aβ metabolism, and APP isoforms (sAPPα and sAPPβ) and BACE1 activity for information on APP processing	Pharmacodynamical information on whether, and how, the drug candidate affects Aβ metabolism and deposition and APP processing
		Downstream biomarkers (e.g., T-tau, P-tau, HFABP, and VLP-1)	Biomarker information on whether the drug candidate has downstream effects on the intensity of neuronal degeneration and tau phosphorylation state/tangle formation

Abbreviations: Aβ, amyloid-β; AD, Alzheimer' disease; APP, amyloid precursor protein; ARIA-E, amyloid-related imaging abnormalities—edema; BACE1, β-site APP-cleaving enzyme 1; CSF, cerebrospinal fluid; HFABP, heart fatty acid-binding protein; P-tau, phosphorylated tau; sAPP, soluble APP extracellular domains; T-tau, total tau; VLP-1, visinin-like protein-1.

study with an extended clinical follow-up period, which is needed to be more confident that stable MCI cases will not progress, showing that the AD CSF profile has a very high sensitivity to identify prodromal AD cases, at a specificity exceeding 90% against controls and 80% against stable MCI cases and MCI patients with other dementias. A predictive value for prodromal AD for the AD CSF profile has thereafter been verified in large multicenter MCI studies, including the Alzheimer's Disease Neuroimaging Initiative (ADNI) study (Shaw *et al*, 2009), the European Descripa study (Visser *et al*, 2009), and the US/European MCI CSF study run by the Swedish Brain Power project (Mattsson *et al*, 2009). When using highly standardized clinical diagnostic and laboratory procedures, the diagnostic accuracy for prodromal AD compared with stable MCI and MCI patients having other dementias may be as high as 97% (Johansson *et al*, 2011).

A large body of literature shows that biomarkers have diagnostic value to identify AD in the MCI stage of the disease. For this reason, new research criteria for the diagnosis of prodromal AD were presented by an international work group in 2007 (Dubois *et al*, 2007), based on combining the core symptom of episodic memory impairment with at least one or more abnormal AD biomarkers (volumetric MRI, amyloid PET, and/or CSF analyses of Aβ and tau protein) (Dubois *et al*, 2007). In 2011, similar criteria for MCI due to AD were also published by the National Institute on Aging—Alzheimer's Association workgroups (Albert *et al*, 2011).

Thus, it is plausible that the use of CSF biomarkers as positive inclusion criteria in clinical MCI trials will enrich the trial with prodromal AD cases or, in other words, increase the proportion of patients with underlying Alzheimer's pathology, thereby increasing the chance of identifying a positive clinical effect of the drug. The European Medicines Agency (EMA) has also presented a qualification opinion stating that the CSF biomarker signature of low Aβ1–42 and high T-tau is useful for the enrichment of clinical trial populations with prodromal AD cases (Isaac *et al*, 2011).

It may be argued that diagnostic biomarkers have no value in trials on AD patients with mild-to-moderate dementia, as the diagnosis can be made on pure clinical grounds. However, identifying subjects with pure AD may be difficult also in this stage of the disease due to the heterogeneity of the disease with variable symptomatology also in the dementia stage, making it difficult to differentiate from other complex neurodegenerative disorders, with variable and overlapping phenotypes, such as Lewy body dementia, vascular dementia, and tauopathies such as frontotemporal dementia and argyrophilic grain disease (Kotzbauer *et al*, 2001; Jellinger, 2008; Nelson *et al*, 2010). An aggravating example of this problem is that approximately 20% of clinically diagnosed AD patients enrolled in clinical trials have negative PiB–PET scans (Rinne *et al*, 2010), that is, they do not have AD. Unpublished data confirm this figure in later trials, with even higher percentages in *APOE* ε4 noncarriers (Fagan, 2012). It might be logical to assume that enrolling such a large percentage of patients with other disorders than AD, for which the anti-Aβ drug is intended, will minimize the chance of identifying a positive effect on clinical outcome measures. Thus, the use of CSF or imaging biomarkers as positive inclusion criteria also in future clinical trials on AD patients with mild dementia may be a wise strategy.

As CSF samples must be obtained by lumbar puncture, which introduces a risk of post-lumbar puncture headache in a percentage of cases (Zetterberg *et al*, 2010) and may warrant some training to perform and implement as a routine procedure in the memory clinic, AD biomarkers that can be assayed in blood samples would be valuable. A large number of serum and plasma proteins have been examined as potential AD blood biomarkers, but the original findings have been notoriously difficult to verify in independent studies (for review, see Blennow *et al*, 2010; Noelker *et al*, 2011; Bazenet and Lovestone, 2012; Henriksen *et al*, 2013). The reason for this failure to find blood biomarkers for AD (as well as for other brain disorders) is probably multifactorial. First, dilution of a brain-specific protein in the large volume of plasma, and in the extracellular fluid of peripheral organs, will result in very low concentrations. Second, the protein may be degraded by blood proteases or cleared by hepatic metabolism or renal excretion. Third, analysis of brain proteins in blood will be confounded by release of the same protein from peripheral tissues. Fourth, analyses may be complicated by interference from the million times more abundant plasma proteins.

One example is plasma Aβ, which has been examined in numerous studies with conflicting results, ranging from an increase over no change to a decrease (for review, see Blennow *et al*, 2010; Koyama *et al*, 2012). However, the studies have in common a close to complete overlap in plasma levels of both Aβ1–42 and Aβ1–40 levels between individuals with preclinical and prodromal AD as well as AD with dementia compared with matched control groups. These discouraging results are probably due to the fact that Aβ in plasma is derived from peripheral tissues and does not reflect brain Aβ metabolism (Mehta *et al*, 2000) and that the hydrophobic nature of Aβ makes analyses difficult due to binding to plasma proteins with epitope masking and other analytical interferences (Kuo *et al*, 1999).

For this reason, highly sensitive analytical techniques are needed to measure the very low amounts of brain-specific proteins in plasma or serum samples. A novel highly sensitive technique to measure tau protein in plasma or serum samples, called single-molecule digital ELISA, has recently been published (Randall *et al*, 2013). This assay has a limit of detection of 0.02 pg/ml, which is 1000-fold more sensitive than conventional immunoassays, and has a broad linear range (Randall *et al*, 2013). A first study also showed a marked increase in plasma tau levels in AD (Zetterberg *et al*, 2013), suggesting that this biomarker might develop into a quick and sensitive screening tool for AD.

Biomarkers for Patient Stratification

The use of biomarkers for patient stratification relies on the hypothesis that the effect of anti-Aβ disease-modifying drugs may vary between AD patients depending on the degree of Aβ plaque pathology and the finding that the relative amount of plaques and tangles shows a marked difference between AD patients (Nelson *et al*, 2010). Stratification of patients based on biomarker data in *post hoc* analyses of clinical trial results may be a way to identify subgroups of patients more prone to respond to therapy (Table 1). Such subgroups may constitute, for example, clinically diagnosed AD patients with clear biomarker evidence (high binding of amyloid tracers on PET or low CSF Aβ42) of Aβ pathology, indicating more pronounced and homogeneous pathology with higher chance of being responsive to anti-Aβ immunotherapy.

CSF Biomarkers as Safety Measures

Anti-Aβ immunotherapy trials have been associated with serious adverse events, such as meningoencephalitis in the AN1792 active immunotherapy trial (Orgogozo *et al*, 2003) and vasogenic edema, or amyloid-related imaging abnormalities—edema (ARIA-E), in the passive immunotherapy trials with the bapineuzumab antibody (Salloway *et al*, 2009; Sperling *et al*, 2012).

Except for that CSF analyses are the standard way in the clinic to diagnose meningoencephalitis and conditions associated with impairment of the blood–brain barrier (Tibbling *et al*, 1977; Blennow *et al*, 1993; Andersson *et al*, 1994), CSF samples taken at baseline, before enrollment in the trial, are useful to identify and exclude patients with chronic infectious or inflammatory brain disorders that can mimic AD, such as Borrelia encephalitis (Andreasen *et al*, 2010). If such infectious or inflammatory disorders are not ruled out before enrolling patients in an immunotherapy trial, any unrelated clinical deterioration may erroneously be taken as evidence of adverse effect of the therapy, such as meningoencephalitis, if a CSF sample is taken at this time point. By comparing CSF samples during the study period with samples taken at baseline, even minor immune activation in the brain due to adverse effects of the drug can be identified. This type of biochemical safety monitoring may be valuable to exclude that an immunotherapy drug candidate induces any harmful immune activation (Figure 1).

Theragnostic CSF Biomarkers

The term 'theragnostic' biomarker was suggested for the use of biomarkers to identify and monitor the effect of a drug candidate on biochemical pathways or pathogenic processes (Blennow *et al*, 2010). Conceptually, theragnostic biomarkers may be divided into primary and downstream biomarkers (Blennow, 2005). Primary biomarkers refer to the use to identify and monitor the pharmacodynamic effect of the drug, thereby providing evidence for target engagement, that is, that the drug candidate indeed has the proposed mechanism also in humans or patients with the disease. As an example of primary biomarkers, CSF acetylcholine esterase (AChE) activity shows a marked and dose-dependent change following treatment with AChE inhibitors such as donepezil and galantamine, which also correlates with clinical benefit (Davidsson *et al*, 2001). In an anti-Aβ clinical trial, primary biomarkers may include, for example, CSF (and plasma) Aβ42 and Aβ40 together with sAPPβ and sAPPα (Table 1). A downstream biomarker is a biomarker used to monitor effects downstream of the primary target of the drug (Blennow, 2005). In an anti-Aβ clinical trial, downstream biomarkers may be neuronal proteins reflecting the intensity of the neuronal degeneration, for example, T-tau but also other neuronal proteins such as heart-type fatty acid-binding protein (H-FABP) (Steinacker *et al*, 2004) and visinin-like protein 1 (VLP-1) (Lee *et al*, 2008). The findings that the intraindividual variability of CSF biomarkers (Aβ42, T-tau, P-tau, and H-FABP) is very low over time, with coefficients of variation for repeated CSF samples over 6–24 months of 4–9%, suggest that even minor changes in biomarker levels can be identified (Blennow *et al*, 2007; Zetterberg *et al*, 2007; Olsson *et al*, 2012).

Theragnostic biomarkers may have a central position throughout the different stages in AD drug development, from early clinical to late registration trials (Figure 1). First, one explanation for some of the failures of anti-Aβ drugs is that poor drug candidates have gone all the way to late-stage clinical trials, based on promising, but misleading, results from preclinical studies performed in AD transgenic mice (Blennow *et al*, 2010). In 2006, we argued that the very large number, at that time 46, of compounds shown to reduce Aβ pathology in AD transgenic mice makes these models poor predictors of treatment success in sporadic AD patients (Blennow *et al*, 2006), a figure that some years later exceeded 100 anti-Aβ drugs (Zahs and Ashe, 2010). Increasing attention is drawn to the low predictivity of disease models for success in late-stage clinical trials and is by no means unique to AD drug development (Prinz *et al*, 2011). For this reason, it may be wise not to rely only on preclinical findings of a plaque-lowering effect in AD transgenic mice for the decision to move into large and expensive phase II or III clinical trials, without any data in humans speaking for appropriate target engagement. We believe that it may be valuable to perform early biomarker studies in humans to bridge the gap between preclinical studies and large and expensive clinical trials. Such trials could be short-term proof-of-principle studies on a limited number of healthy volunteers in phase I, as well as proof-of-concept studies on AD patients in phase IIa; the outline will vary depending on the type of anti-Aβ drug. An encouraging example that this type of studies provides valuable data that the drug candidate does engage the proposed target was obtained in a recent single-dose study on 30 healthy volunteers for a novel BACE1 inhibitor (May *et al*, 2011). In this study, marked and sustained reductions in Aβ40 and

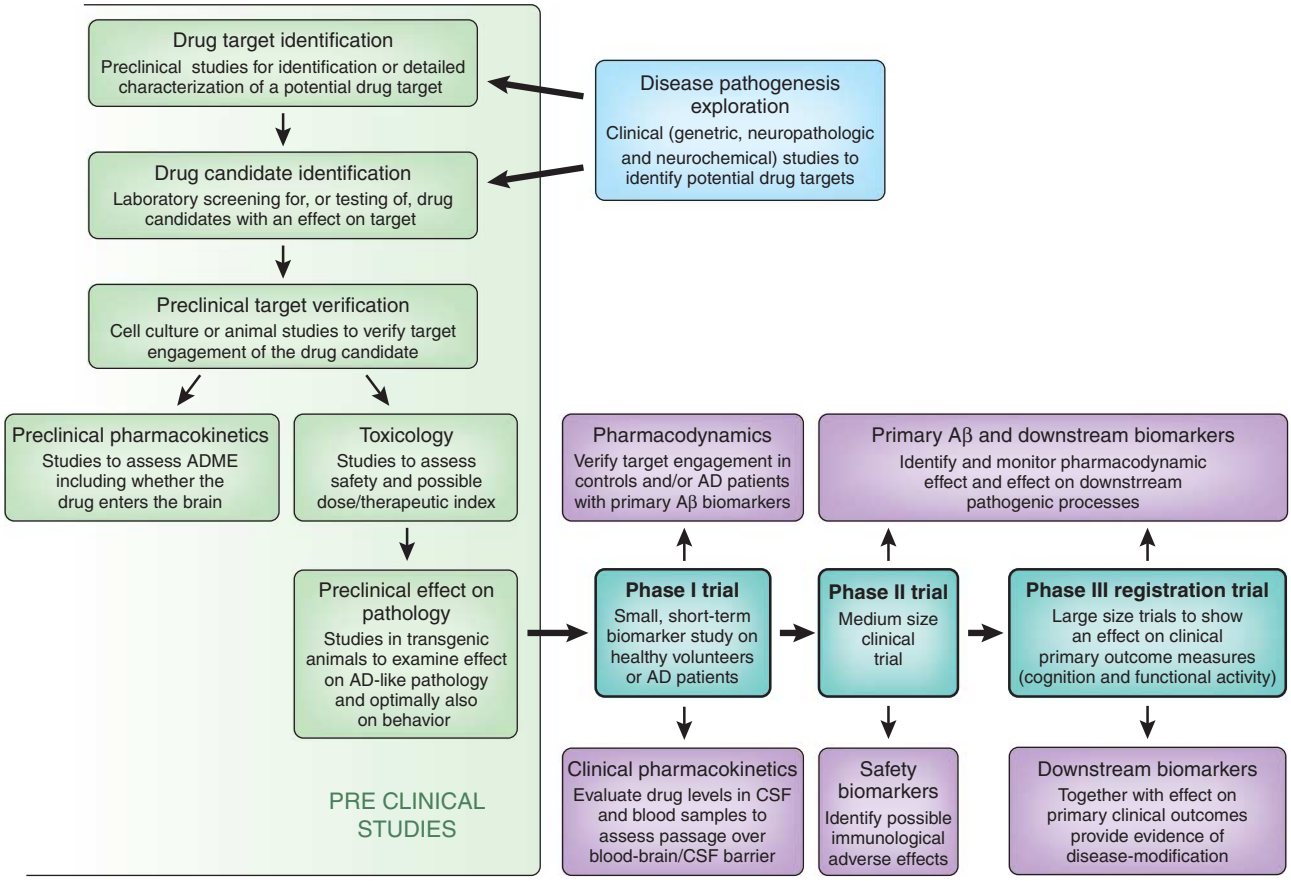

Figure 1. Position of biomarkers in Alzheimer's disease drug development. Flowchart showing how biomarkers may be implemented in Alzheimer's disease drug development. To the left the preclinical stages of development and to the right the clinical phases are shown. In an anti-Aβ treatment trial, primary biomarkers may include measures of brain amyloid load (amyloid PET or CSF Aβ42 levels) and amyloid metabolism (e.g., CSF Aβ40, sAPPα, and sAPPβ), whereas downstream biomarkers may include measures of the intensity of the neuronal degeneration (CSF tau protein or VLP-1), the rate of brain atrophy (longitudinal volumetric MRI), glucose metabolism rate in cortical neurons and glial cells (FDG-PET), and tau phosphorylation state (CSF P-tau). NB: There is no intention that this flowchart should provide a complete or exact overview of all steps in drug development. AD, Alzheimer's disease; ADME, absorption, distribution, metabolism, and excretion; CSF, cerebrospinal fluid.

Aβ42 levels in CSF accompanied by a decrease also in sAPPβ, and a compensatory increase in sAPPα, were found, verifying preclinical data that the drug inhibits BACE1 in brain, thereby reducing Aβ production. Data from this type of early clinical biomarker studies may be valuable to select drug candidates with a proven effect on Aβ metabolism or clearance also in humans, which would be of value in the decision making whether to embark on expensive phase II and III trials.

The design of an increasing number of phase II and III trials also includes different modalities of theragnostic biomarkers (Figure 1). Biomarker data from this type of trails may provide data on target engagement as well as evidence of downstream effects on the primary drug target.

Last, theragnostic biomarkers may be important in phase III registration trials to provide evidence of disease modification (Figure 1). In 2010, we suggested that even if there are no validated surrogate biomarkers to predict clinical outcome in an AD clinical trial, it is logical that biomarker evidence that a drug candidate affects the central disease processes in AD will be required to label the drug as disease modifying (Hampel *et al*, 2010). In this context, it may be important to ask the question whether an effect on primary biomarkers denotes disease modification, or merely target engagement. The finding that BACE1 inhibitors have a clear effect on CSF Aβ40, Aβ42, and sAPPβ speaks for the latter. In a recent draft document by the US Food and Drug Administration (FDA, 2013), it was also stated as a possibility that a positive biomarker result, evaluated as a secondary outcome measure in a trial, in combination with a positive finding on a primary clinical outcome measure, may support a claim of disease modification in AD, given that there is widespread evidence-based agreement in the research community that the chosen biomarker reflects a fundamental entity in AD pathophysiology (FDA, 2013).

IMPLEMENTING CSF BIOMARKERS IN TRIALS AND IN THE CLINIC

As discussed above, the AD CSF biomarkers have a high diagnostic accuracy both for AD dementia and for

prodromal AD. However, the relatively large variability in absolute concentrations between laboratories has been highlighted as a problem (Mattsson *et al*, 2009) for a general introduction of CSF biomarkers in clinical routine. A number of standardization initiatives have been initiated to control this type of between-laboratory variability, which is a general problem for all novel fluid (CSF, plasma, serum, and urine) biomarkers in clinical medicine. A proficiency program for the AD CSF biomarkers, called the Alzheimer's Association Quality Control (QC) Program for CSF Biomarkers, is ongoing, with more than 90 laboratories worldwide participating (Mattsson *et al*, 2011). Other initiatives will develop certified reference materials and methods to serve as 'gold standards' for CSF biomarker measurements (Mattsson *et al*, 2012a, 2012b). These initiatives will, together with novel assays produced under rigorous QC measures and run on fully automated analytical instruments, improve the quality and reduce between-lab variability, thereby allowing uniform cutoff levels for diagnosis and a more widespread use of CSF biomarkers in the routine clinical diagnostic setting. The situation is different for the implementation of CSF biomarkers as diagnostic aids in clinical trials, as assay variability can be minimized by the use of internal control samples and the use of a central laboratory will preclude between-laboratory variability (Andreasen *et al*, 2001; Bjerke *et al*, 2010).

Aβ IMMUNOTHERAPY

The first preclinical study on Aβ vaccination was published by Schenk *et al* (1999) and showed that immunization with Aβ in AD transgenic mice basically prevented development of plaques, neuritic dystrophy, and astrogliosis in young animals and markedly reduced this type of pathology in older mice (Schenk *et al*, 1999). One year later, Bard *et al* (2000) showed that infusion of anti-Aβ antibodies reduced plaque load by entering the brain, binding to plaques, and inducing microglia to Fc receptor-mediated phagocytosis and subsequent peptide degradation. These studies, showing clearance of Aβ and plaques in the brains of AD transgenic mice, served as the basis for the two main principles for Aβ immunotherapy that are now being evaluated in clinical trials: active immunotherapy (or Aβ vaccination) that involves activation of the patients' immune system to produce endogenous anti-Aβ antibodies and passive immunotherapy that involves infusion of humanized or human anti-Aβ antibodies produced in the laboratory.

Active Aβ Immunotherapy

The first clinical trial on Aβ immunotherapy was on active immunization with AN1792, which was composed of full-length aggregated Aβ1–42 together with the QS-21 adjuvant (Bayer *et al*, 2005). Treatment with AN1792 resulted in long-lasting endogenous antibody response and an apparent reduction in Aβ plaque load in patients followed until autopsy (Holmes *et al*, 2008), but further development had to be stopped due to a percentage of patients developing meningoencephalitis (Orgogozo *et al*, 2003). These side effects were due to the induction of a strong T-helper cell-mediated immune response (for review, see Lemere and Masliah, 2010).

With the aim to minimize potentially harmful T-cell activation, and optimize antibody production, an alternative approach for active Aβ immunotherapy has been developed, which involves synthetic fragments of Aβ coupled with a carrier protein. CAD106 is a novel immunotherapy designed to stimulate the generation of antibodies against a small Aβ peptide fragment (Aβ1–6) coupled with the virus-like particle Qβ acting as a B-cell epitope and avoiding an Aβ-specific T-cell response (Wiessner *et al*, 2011). Preclinical studies show that the anti-Aβ antibodies produced by immunization with CAD106 react with all of Aβ monomers, oligomers, and Aβ present in amyloid plaques (Wiessner *et al*, 2011). Further, CAD106 treatment has been found to reduce brain amyloid accumulation in two APP transgenic mouse lines (Wiessner *et al*, 2011).

There are also several other ongoing active Aβ immunotherapy trials based on different strategies to elicit an anti-Aβ immune response and avoid adverse effects (for review, see Lemere and Masliah, 2010), but no biomarker data are available from these trials.

Passive Immunotherapy with Anti-Aβ Antibodies

Passive Aβ immunotherapy refers to humanized or fully human monoclonal antibodies directed against Aβ. There are several different such anti-Aβ antibodies that have come to evaluation in clinical trials in different phases, which all differ depending on which domain (N- or C-terminal or mid-portion) on Aβ they are directed against and whether they bind soluble or aggregated Aβ, or both (for review, see Lemere and Masliah, 2010; Liu *et al*, 2012).

Bapineuzumab is a humanized monoclonal antibody directed against the N-terminal part of Aβ, which is a humanized version of the murine antibody 3D6 (Black *et al*, 2011). Bapineuzumab (and 3D6) recognizes both soluble and oligomeric Aβ and binds to Aβ plaques in the brain (Bard *et al*, 2000; Zago *et al*, 2012). Treatment of PDAPP mice with 3D6 results in a very marked reduction of Aβ plaques, probably by Fc-mediated microglial activation and phagocytosis (Bard *et al*, 2000).

Gantenerumab is a fully human IgG1 anti-Aβ antibody that was derived from a human phage display library and optimized for high-affinity binding with fibrillar Aβ (Ostrowitzki *et al*, 2012). Gantenerumab binds Aβ present in plaques in human and AD transgenic mice brain tissue, and long-term gantenerumab treatment also reduces plaque load in PS2APP mice (Bohrmann *et al*, 2012). Incubation of AD brain tissue slices with gantenerumab and human primary microglia cells showed a dose-dependent binding of the antibody to plaques and active intracellular uptake by

migrating microglia (Ostrowitzki *et al*, 2012). Taken together, these results suggest that gantenerumab treatment results in a dose-dependent reduction in brain Aβ load by microglial uptake and degradation.

Solanezumab is the humanized version of the anti-Aβ monoclonal antibody m266 and is directed against the mid-domain of the Aβ protein (DeMattos *et al*, 2001). Solanezumab selectively binds to soluble Aβ and has little or no affinity for fibrillar Aβ or plaque Aβ (DeMattos *et al*, 2001). Preclinical studies have shown that treatment of AD transgenic mice with the murine variant of the antibody (m266.2) results in reduced plaque burden (DeMattos *et al*, 2001).

Ponezumab (PF-04360365) is a humanized monoclonal anti-Aβ antibody with an epitope on the C terminus (AβX-40) of the Aβ protein (La Porte *et al*, 2012; Burstein *et al*, 2013). Ponezumab binds only to soluble Aβ, but not to fibrillar Aβ or plaques, probably as the C terminus is not as exposed as the N terminus in Aβ fibrils and plaques (Landen *et al*, 2013). There are two amino-acid substitutions in the Fc region of ponezumab, leading to a reduced capacity for complement activation and cell-mediated cytotoxicity (Landen *et al*, 2013).

Several other passive Aβ immunotherapy programs based on antibodies directed against epitopes on monomeric or oligomeric/fibrillar Aβ antibodies are in different stages of development (Delrieu *et al*, 2012), but as no biomarker data are published, it is beyond the scope of this review to go into detail on these drug candidates.

Intravenous Immunoglobulins

A third principle for AD anti-Aβ immunotherapy is intravenous immunoglobulins (IVIGs). IVIG is purified human immunoglobulin (IgG) from healthy blood donors and is a therapy that since long is approved for treatment of immune-deficiency disorders and several other diseases. The rationale for IVIG in AD is that the antibody preparation contains low titers of naturally occurring polyclonal anti-Aβ antibodies together with the finding in some studies of lower blood levels of anti-Aβ antibodies in AD patients than in healthy elderly (Dodel *et al*, 2002; Weksler *et al*, 2002). Later studies have shown that human plasma contains immunoglobulins that react with both N-terminal and central epitopes on Aβ monomers as well as with conformational epitopes on Aβ oligomers and fibrils (O'Nuallain *et al*, 2006; Szabo *et al*, 2008). Two IVIG preparations are under development as treatments for AD including Octagam (Octapharma) and Gammagard (Baxter).

FLUID BIOMARKERS IN AD IMMUNOTHERAPY TRIALS

There are a number of published papers presenting data on plasma and CSF biomarkers in AD immunotherapy trials. In the following section, these data are reviewed considering both the type of immunotherapy used and the type of biomarkers examined.

Active Vaccination

Results on CSF biomarkers were reported in a small subgroup of patients in the interrupted phase IIa AN1792 trial on active immunization with full-length Aβ1–42. In 11 patients that were antibody responders, there was no change in CSF Aβ42 compared with 10 placebo patients (Gilman *et al*, 2005). In contrast, there was a significant decrease in CSF T-tau towards normal levels, which may be interpreted as indicating that the treatment had an effect on the intensity of the neuronal degeneration.

In a double-blind, placebo-controlled, 52-week phase I clinical trial on CAD106, an endogenous antibody response was found in 70–80% of treated patients, and serum samples from treated patients specifically labeled Aβ plaque cores in both APP23 transgenic mouse and AD brain sections, with intensity of plaque staining correlating with measured anti-Aβ titers in blood (Winblad *et al*, 2012). Although there was no significant change in the CSF levels of either Aβ42 or Aβ40 or in the downstream biomarkers T-tau and P-tau, total plasma Aβ levels increased and free Aβ levels decreased in parallel, suggesting that the produced antibodies bound to Aβ *in vivo* (Winblad *et al*, 2012).

Passive Immunotherapy

Pharmacokinetic data. Comparisons of levels of the therapeutic antibody in plasma and CSF samples may provide useful information on to which degree the antibody passes the blood–brain barrier and enters the brain. Pharmacokinetic data on the anti-Aβ antibodies show that the CSF level of bapineuzumab is approximately 0.3% of the corresponding plasma concentration (Blennow *et al*, 2012) and 0.5% for ponezumab (Landen *et al*, 2013), both of which are within the same range as the normal CSF to serum ratio for endogenous IgG (Blennow *et al*, 1993), suggesting that these antibodies pass the blood–brain barrier at the expected ratio and enter the brain.

Bapineuzumab. The effect of bapineuzumab treatment on cortical fibrillar Aβ load as measured by PiB–PET was evaluated in a double-blind, placebo-controlled clinical trial over 78 weeks of treatment. This study showed a reduction in cortical PiB retention with treatment in several cortical brain regions, both compared with baseline levels and compared with placebo (Rinne *et al*, 2010), suggesting that bapineuzumab treatment reduces amyloid burden in AD patients. Changes in AD biomarkers following bapineuzumab treatment were evaluated in a study combining two phase II randomized, double-blind, placebo-controlled 12-month clinical trials (Blennow *et al*, 2012). In total, 27 AD patients receiving bapineuzumab and 19 placebo patients were included in the combined analysis. There was no clear change in either CSF Aβ1–42 or AβX-40 with treatment,

either compared with baseline levels or compared with placebo groups, whereas a mild increase in CSF AβX-42 was found with treatment compared with baseline levels, but this difference was not significant when comparing the change between the bapineuzumab and placebo groups (Blennow *et al*, 2012).

This study also reported bapineuzumab effects on downstream CSF biomarkers. There was a decrease in both CSF T-tau and P-tau with treatment, both approximately 10% lower at the end of the study compared with baseline levels, and for CSF P-tau this decrease was significant also when comparing the treatment and placebo groups (Blennow *et al*, 2012). Unpublished data from the phase III clinical bapineuzumab trials also suggest significant treatment effects for both P-tau and T-tau between bapineuzumab and placebo groups (Fagan, 2012; Streffer *et al*, 2013).

Solanezumab and ponezumab. Already the preclinical studies of the solanezumab mouse version m266 showed a very marked (1000-fold) and rapid increase in plasma Aβ levels in the PDAPP AD transgenic mouse model (DeMattos *et al*, 2001). Pronounced increases in both Aβ42 and Aβ40 were also found in a phase II randomized, double-blind, placebo-controlled clinical trial on solanezumab in mild-to-moderate AD (Farlow *et al*, 2012). Solanezumab treatment also increased the total (antibody-bound and free Aβ) levels of both Aβ40 and Aβ42 in CSF. Also the CSF level of free Aβ42 increased with treatment, whereas no significant change was seen for Aβ40 compared with placebo (Farlow *et al*, 2012).

A short single intravenous infusion of ponezumab results in a very marked increase in plasma levels of both Aβ42 and Aβ40 (Burstein *et al*, 2013). This was verified in a phase I randomized, double-blind, placebo-controlled clinical trial in mild-to-moderate AD patients, which also showed that ponezumab, to a much lesser extent, increased the CSF Aβ level (Landen *et al*, 2013). The increase in plasma Aβ is dose dependent and closely follows the plasma pharmacokinetic profile of ponezumab, and the terminal half-life ($t_{1/2}$) is similar for plasma Aβ (approximately 50–60 days) and ponezumab (approximately 35–52 days) (Burstein *et al*, 2013). This half-life is in a similar range as for IVIG preparations and endogenous IgG (Mankarious *et al*, 1988).

Both solanezumab and ponezumab preferentially bind soluble Aβ, with little affinity for fibrillar Aβ in plaques (DeMattos *et al*, 2001; Landen *et al*, 2013). Based on the very marked increase in plasma Aβ after administration, and that m266 reduces Aβ deposition in PDAPP mice, the peripheral sink hypothesis was suggested as the mechanism for m266/solanezumab, positing that the antibody acts by changing an equilibrium in Aβ between the CNS and plasma, which results in increased clearance of Aβ from the brain (DeMattos *et al*, 2001). A change in the systemic Aβ equilibrium between the circulation and the brain following treatment, resulting in that Aβ diffuses out from the brain to the circulation, was also suggested as a possible mechanism for ponezumab (Landen *et al*, 2013).

To test whether a dynamic equilibrium exists between brain and blood Aβ, Walker *et al* (2013) administered the Aβ-degrading enzyme neprilysin intravenously to AD transgenic and wild-type mice. Neprilysin infusion resulted in a very marked reduction in plasma Aβ without any corresponding change in brain levels of either soluble or fibrillar Aβ or any compensatory increase in APP expression (Walker *et al*, 2013). These data do not support the existence of a brain-to-blood equilibrium of Aβ or sink mechanism for Aβ clearance. However, other studies suggest that peripheral expression of a soluble form of neprilysin that is secreted into plasma decreases both plasma and brain Aβ levels (Liu *et al*, 2010). A possible alternative mechanism for the increase in plasma Aβ is that binding of anti-Aβ antibodies to circulating soluble Aβ in peripheral blood may protect Aβ from degradation by proteases. Clearance of Aβ in peripheral blood is mediated by a number of Aβ-degrading proteases, such as insulin-degrading enzyme and angiotensin-converting enzyme (Liu *et al*, 2012). A further alternative mechanism might be that immunotherapy with anti-Aβ antibodies that preferentially bind soluble Aβ, such as solanezumab and ponezumab, sequesters soluble Aβ monomers in the brain, which may prevent from aggregation into oligomers and higher MW aggregates (Yamada *et al*, 2009).

Gantenerumab. The effect of gantenerumab treatment on brain Aβ load was evaluated in a study with PET scans to measure retention of the amyloid ligand PiB as primary outcome (Ostrowitzki *et al*, 2012). The mean percent change in PiB retention during the 2- to 7-week treatment period relative to placebo was −16% for the 60 mg and −36% for the 200 mg gantenerumab groups, suggesting that treatment resulted in a dose-dependent reduction in fibrillar brain Aβ levels (Ostrowitzki *et al*, 2012). There are no published results on CSF biomarker levels in relation to gantenerumab treatment.

Intravenous Immunoglobulins

A small (five patients) open 6-month IVIG (Octagam) pilot clinical trial found that the CSF levels of total Aβ decreased with treatment in all patients (approximately 20–40% decrease during 6 months of treatment), whereas CSF Aβ42 did not change (Dodel *et al*, 2002). In the same study, blood levels of total Aβ increased with treatment, whereas no change was found in Aβ42 levels (Dodel *et al*, 2002). A later phase I trial of IVIG (Gammagard) in mild AD included a 6-month period of active treatment followed by a washout period of 3 months and an open label extension of 9-month additional treatment (Relkin *et al*, 2009). Also, this study was small, with a total of eight patients enrolled, and divided into several dose regimes, and repeated blood and CSF sampling was performed throughout the study (Relkin *et al*, 2009). Plasma Aβ42 and Aβ40 levels showed transient increases after each infusion. Compared with baseline levels, a decrease with treatment was found for both CSF

Aβ42 (approximately 40% reduction) and Aβ40 (approximately 20% reduction) at 6 months, with both isoforms returning to baseline levels after the washout period and decreasing again after the IVIG extension period (Relkin *et al*, 2009).

Results from a large phase II randomized, double-blind, placebo-controlled, dose-finding trial on IVIG treatment with Octagam were recently published (Dodel *et al*, 2013). In this 6-month trial, 55 patients received three different doses at different time intervals, leaving 5–7 patients in each treatment group eligible for the final analyses. During the treatment period, there was no significant change in either the primary CSF biomarkers Aβ42 and Aβ40 or the downstream biomarkers T-tau and P-tau. At the same time, plasma levels of Aβ42 and Aβ40 did not change in five of the six treatment groups, although a reduction was found in the highest dose given every 2 weeks (Dodel *et al*, 2013). Therefore, CSF and blood biomarker data from IVIG trials are inconclusive, and it is clear that longer trials with higher number of patients and, thus, larger power are needed to evaluate the effect of IVIG on AD pathogenesis.

FUTURE RESEARCH DIRECTIONS

The amyloid cascade hypothesis has (for good reasons) dominated thoughts about the pathogenesis of AD for several decades and, hence, directed research efforts towards finding means to clear Aβ from the brain, for example, by active or passive immunization. However, in the last few years, several clinical trials of anti-Aβ immunotherapy have, despite of amyloid PET results suggesting plaque clearance, failed to reach their primary clinical end points. Some promising effects on downstream tau biomarkers have been reported, suggesting an effect of therapy on the neurodegenerative process.

These results should in our view encourage further research on patients earlier in the disease process. To that end, biomarkers of Alzheimer's pathology will be essential to allow for including subjects on route to AD but at a much earlier stage than in previous studies and with limited manifest neuronal damage. These studies will not be easy to conduct, as they need to be large and will require extensive follow-up periods, but are in our view essential to assess not only anti-Aβ drugs but also the role of Aβ in AD pathogenesis in humans.

One option is treatment trials in FAD mutation carriers in the preclinical disease stage, cognitively normal *APOE* ε4 carriers in their 60s, or patients with mild cognitive symptoms and a positive biomarker profile indicating prodromal AD. In fact, three complementary initiatives for presymptomatic treatment of AD have been initiated. The Alzheimer's Prevention Initiative (API) is a project led by the Banner Alzheimer's Institute (BAI), in collaboration with the National Institutes of Health (NIH), the University of Antioquia in Colombia and Genentech (Alzheimer's Prevention Initiative, 2013). The API will examine whether treatment with crenezumab, an anti-Aβ monoclonal antibody, in the preclinical phase of FAD will reduce the risk of developing the clinical disease. Similarly, the Dominantly Inherited Alzheimer Network (DIAN), an international research partnership, are performing a clinical FAD prevention trial on the anti-Aβ monoclonal antibodies gantenerumab and solanezumab (National Institute of Aging, 2013; Strobel, 2013). Last, the Anti-Amyloid Treatment in Asymptomatic AD Trial (or A4 Trial) is aiming for clinically normal elderly with evidence of amyloid pathology by PET imaging, and the anti-Aβ monoclonal antibody solanezumab will be the first drug candidate to be tested (Fierce Biotech, 2012; Strobel, 2011).

Apart from changes in study design regarding already existing drug candidates, it may also be important to develop new ones. Perhaps immunization should be directed against a specific form of Aβ that is particularly neurotoxic. It may also be important to explore possible mediators of Aβ toxicity, such as microglial overactivation. Recent genetic data suggest that induction of the pro-inflammatory M1 phenotype of microglia by aggregated Aβ may be detrimental to neurons and may be an important pathway in AD pathogenesis (Guerreiro *et al*, 2013; Jonsson *et al*, 2013). Last, other aspects of AD pathophysiology may also be suitable targets for disease-modifying treatments, including tau immunization strategies and drugs to reduce tau hyperphosphorylation and aggregation as well as compounds that control oxidative stress and inflammation (Götz *et al*, 2012; Panza *et al*, 2012; Singh *et al*, 2012).

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