Alzheimer's disease at the cross-roads

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ABSTRACT

Alzheimers Disease (AD) research is at a cross-road. So many drugs have failed in clinical trials in that a serious rethink of the pathological hypotheses is required. The amyloid β and tau pathologies are still the major focus but there are now so many theoretical potential points of intervention, such as the neurotrophic/inflammatory interface, that perhaps the definition of the disease should be reconfigured. There is a new momentum for changes in diagnosis supported by the EMA, and an FDA-supported initiative for rapid registration of drugs active in cognitive testing. Above all it is the possible transmission of the pathology by the seeding of polypeptides that may change the paradigm.

Introduction

Up until now, AD drug development has been mainly driven by the amyloid hypothesis, which predicts that amyloid β (Aβ) peptides are precipitating factors in the neurodegenerative process leading to abnormal phosphorylation of tau, which go on to produce neurotoxic neurofibrillary tangles[1]. But other research groups have placed greater emphasis on the role of tau and a lesser role of Aβ in the pathological process, giving rise to two opposing camps, the baptists versus tauists. While this debate has been going on for over twenty years, both camps now consider that the pathology cannot be accounted for by just one of these and that both Aβ and tau concomitantly, even synergistically, contribute to the etiology and progression of the disease. The debate is now focusing on the question which amyloid or tau species is/are the most important toxic trigger(s) and what other factors contribute. More than ever it is clear that AD is multifactorial and heterogeneous disease. The role of other physiological systems (loss of neurotrophic support, inflammation, mitochondria and/or vascular changes) and regulatory mechanisms (epigenetics and micro-RNAs (miRNAs)) may be major. The difficulty is how to test so many potential approaches in clinic trials, particularly as animal models are not fully predictive. This article resulted from a meeting of experts and puts together the main crucial points – it is not a systematic review but focuses on key issues.

Previously, the diagnosis of AD was a clinical diagnosis of dementia with a post-mortem histopathological validation, but recent advances in the development of biomarkers of AD allow a diagnosis at a prodromal stage of AD that was previously incorporated in the non-specific concept of Mild Cognitive Impairment (MCI) (box 1).

Thus, the recommended diagnostic procedure for prodromal AD includes a specific clinical phenotype with an amnestic syndrome of the hippocampal type and the presence of a pathophysiological biomarker that can be either specific changes of CSF with low Aβ and high tau or phospho-tau or positron-emission tomography (PET) amyloid ligand retention[2].

Genetic background of Alzheimer's disease.

Familial autosomal dominant AD is rare (<3%) with amyloid precursor protein (APP), presenilin 1 (PS1), presenilin 2 (PS2) mutations, causing alterations in APP cleavage resulting in increased Aβ production[3], supporting the "amyloid cascade hypothesis"[4]. However for sporadic AD, the most frequent form of the disease, Apolipoprotein E (APOE) is the strongest susceptibility gene[5].

In less than 3 years, nine new genes associated to AD have been identified using genome-wide association studies (GWAS) (table 1). More are expected to be discovered thanks to the IGAP consortium (International Genomic Alzheimer Project), which will coordinate 4 consortia (EADI, GERAD, CHARGE, ADGC)in their analysis of 17,008 cases and 37,646 controls, followed by a confirmatory phase in more than 8,572 cases and 11,312 controls.

The biological evidence suggests that CLU and CR1, along with APOE, PICALM are involved in Aβ peptide clearance. These data indicate that familial early-onset forms of AD are mainly linked to genes that are implicated in Aβ overproduction. Patients having genetic variants at the APOE locus and other late onset forms of the disease appear to have a pathology more closely linked to Aβ clearance[6]. Pathway analyses from these GWAS strongly reinforce the implication of the immune system and inflammation or lipid metabolism [7]. However the predictive value of the new susceptibility genes identified by GWAS, in the general population is weak compared to the known risk factor of increasing age[8]. But the identification of a panel of specific alleles of genes associated to AD may help to classify patients and identify both endophenotypes and individuals with (presymptomatic) prodromal AD, in whom different therapeutic approaches may be needed.

It still seems likely that there are hereditable factors that remain to be discovered. This "missing heritability" is now being searched using mitochondrial DNA studies, deep sequencing hunting for rare variants, epigenetics and haplotype wide association studies which identifying genome blocks for further screening.

The longitudinal clinical and biomarker changes in familial AD have just been published[9] in 128 participants from the DIAN (Dominantly Inherited Alzheimer's Network) cohort, which allow the precise timing of the amyloid and tau changes before symptom onset (Figure 1). The results allow a new perspective on the use of biomarkers, even if the application to sporadic AD can be debated.

The results showed that, for an average age of onset of parental symptoms of 46 years, in comparison with controls, the changes prior to expected symptom onset were:

- 25 years prior: Aβ42 levels in the cerebrospinal fluid (CSF) started to decline,
- 15 years prior: both tau protein levels in the CSF increased and brain atrophy began to be detected,
- 10 years prior: hypometabolism in precuneus area of the cortex is detectable together with impaired episodic memory ,
- 5 years prior: global cognitive impairment was detected,
- 3 years after: patients met diagnostic criteria for AD.

These data show in a very precise way how the disease starts early, develops insidiously, and how episodic memory is affected much earlier than the global measures usually used for drug evaluation. The deposition of amyloid over a 30 year disease progression is elegantly, and worryingly, shown in a film[9].

Table 1. Penetration of genetic mutations in Alzheimer's disease (odd ratio, OR).

Chromosome	Gene	Transmission	OR[95%CI]	Putative function	Possible pathways
19	Amyloid Precursor Protein, APP[10]	autosomal dominant / recessive	Complete penetrance	Precursor of Aβ peptide, Tau phosphorylation, GSK-3β activation	Amyloid cascade, Aß pathway Tau pathway
14	Presenilin 1, PS1[11]	autosomal dominant	Complete penetrance	γ-secretase activity, intracellular signaling, transmembrane protein processing	Aß pathway, synaptic plasticity, neuronal survival
2	Presenilin 2, PS2[12]	autosomal dominant	Complete penetrance	γ-secretase activity, intracellular signaling, transmembrane protein processing	Aß pathway, synaptic plasticity, neuronal survival
19	Apolipoprotein E, APOE[5]	semi-dominant	e4e4 : 14.9 [10.8-20.6] e3e4 : 3.2 [2.8-3.8]	Aβ aggregation, Aβ clearance, Aβ metabolism, Aβ accumulation,Tau phosphorylation,Tau accumulation,Tau aggregation, lipid metabolism, inflammation, neuronal repair, synaptic plasticity	Lipid pathway, Aβ pathway, synaptic plasticity, neuroinflammation, oxydation
8	Clusterin, CLU (APOJ)[13, 14]	susceptibility gene	0.86 [0.81-0.90]	molecular chaperone, synapse turnover, Aβ clearance, Aβ metabolism, Aβ accumulation, Aβ toxicity	Lipid pathway, Aβ pathway, neuroinflammation, oxydation, apoptosis, immune pathway
1	Complement Receptor 1, CR1[13]	susceptibility gene	1.21 [1.14-1.29]	activator of complement system, Aß clearance, Aß metabolism	immune system, Aβ pathway
11	Phosphatidylinositol binding clathrin assembly protein, PICALM[14]	susceptibility gene	0.88 [0.81-0.96]	clathrin mediated endocytosis	protein trafficking, synaptic cell functioning, Aß toxicity

2	Bridging Integrator 1, BIN1[8]	susceptibility gene	1.13 [1.06-1.21]	synaptic vesicle endocytosis, formation tubular membrane structure	synaptic cell functioning, Tau pathway, caspase independent apoptosis
7	Ephrin receptor A1, EPHA1[14, 15, 15, 16]	susceptibility gene	0.90 [0.85-0.95]	synaptic development and plasticity	immune system
19	ATP-binding cassette, subfamily A, member 7, ABCA7[15, 16]	susceptibility gene	1.23 [1.17-1.28]	substrate transporter across cell membrane	Cholesterol pathway, APP processing, immune pathway
11	Membrane spanning 4-domains, subfamily 4, MS4A6A/MS4A4E[15, 16]	susceptibility gene	0.91 [0.88-0.93]	No known function	Cell surface signaling (?)
19	CD33 molecule, CD33[15, 16]	susceptibility gene	0.89 [0.84-0.95]	clathrin mediated endocytosis	immune system, synaptic cell functioning
6	CD2-associated protein, CD2AP[15, 16]	susceptibility gene	1.11 [1.04-1.18]	receptor mediated endocytosis	synaptic cell functioning, actin skeleton
6	Triggerin receptor expressed on myeloid cells 2 TREM2[17]	susceptibility gene	4.97 [2.42-10.21]	Innate immune receptor	Immune system, phagocytosis, neuroinflammation

1. AMYLOID PATHOLOGY

A brief overview of the amyloid hypothesis.

The « classical » amyloid cascade hypothesis predicts that accumulation of 40 or 42 amino acid-long amyloid β peptides are driving the neurodegenerative process[18]. Therapeutic strategies were designed to inhibit Aβ production by targeting secretases, the enzymes that generate Aβ or by neutralizing Aβ-associated toxicity by a vaccinal approach, respectively (see below)[19]. So far these approaches have not been successful. Thus, either the postulate of secreted Aβ as the trigger of AD pathology is an oversimplification of the etiological hypothesis or the drugs were not developed against the genuine Aβ-related toxic trigger or were not tested in an appropriate (early) population. It may be that full length peptides (Aβfl) are not the *primum movens* of AD etiology but that additional Aβ-related species: βAPP-derived catabolites, Aβ bio-transformed shorter species, could contribute to the degenerative process.

The subcellular localization[20] may have been underestimated as Aβ-like species can be found intracellularly, adding another difficulty for design of bioavailable drugs.

Soluble oligomers may be the toxic species[21] (see box 2) and a potential receptor, the cellular prion protein, are important current targets in drug discovery and the evidence for this is listed in box 2. These toxic species may act as "seeds" to transmit the disease and allow cellular spreading. This hypothesis has enormous therapeutic implications (box 3),

The definition of toxic species reinforces the therapeutic strategy aimed at developing active or passive immunization (box 4). Past experience of immunization is reviewed in box 4). The passive antibody clinical trials represent an enormous therapeutic investment and their success or failure will impact the field for decades to come.

However, other toxic amyloid species exist. N-terminal truncation and cyclisation of Aβfl by aminopeptidase and glutaminyl cyclase, respectively[22-24] correspond to early proteolytic events yielding highly toxic Aβ species[25]. Interestingly, the proteolysis of βAPP by α- or β-secretases combined with subsequent cleavage by γ-secretase yields N-terminally truncated Aβ11-40/42 or Aβ17-40/42. Finally, Aβ itself can undergo degradation that produces a complex set of catabolites; N- and C-terminally truncated Aβ-related species[26], with as yet unknown toxicity .

From the intracellular domain βAPP another catabolite is also derived (from an additional ε-cleavage). Known as AICD (APP Intracellular Domain), this is a transcription factor able to modulate the expression of a variety of genes that are implicate in Aβ generation, degradation and in the development of the Aβ-associated toxic phenotype[27]. The precursor for AICD is produced in the the β-secretase-mediated pathway[28] and the main β-secretase, BACE1 [29] has been proposed as a therapeutic target, but in view of its role in AICD production (and on myelin formation), the side effects may be far ranging..

Therapeutic implications:

- **Beta-secretase 1 (BACE 1)**

The interests of BACE inhibition may be highlighted by the opening up of new research directions associated with Aβ production. Locally elevated BACE1 immunoreactivity occurs in AD localized to swollen/sprouting axon terminals. These BACE1-labeled dystrophic axons are near to, or in direct contact with, blood vessels, and may be responsible for vascular or metabolic deficits, explaining why senile plaques are present preferentially near the cerebral vasculature [30].

DeCODE, studying 1,795 Icelanders, found a coding mutation (A673T) in the APP gene that was highly protective against cognitive decline in AD and in the elderly without AD. The substituted amino acid, which is adjacent to the aspartyl protease β-site in APP, results in the reduced synthesis of amyloidogenic peptides by ~40%. These data would support a re-investigation of the clinical effectiveness of BACE inhibitors. Indeed, five BACE1 inhibitors (ACI-91, LY2889721, MK-8931, E2609, RG7129) are in clinical trials; LY2889721 has progressed to phase II and MK-8931, which can reduce Aβ production by almost 90%, has just started a 1700 patient phase III study.

- **γ-secretase**

γ-Secretase is an intramembrane protease that is involved in the last step of Aβ genesis[31]. Its other substrates include some which are vital for cellular functioning hence the pharmacological blockade of γ -secretase can lead to deleterious effects*. Thus while the failure of Myriad's inhibitor, tarenflurbil, was ascribed to poor brain penetration and potency, the failure of Lilly's semagacestat (300 patients) may have in part been due the fact that γ-secretase is essential to notch signaling[32].

The design of γ-secretase "modulators" is still an active research field[33], with the objective of reducing Aβ production, but sparing the βAPP-unrelated substrates, Three γ-secretase inhibitors are in clinical trials (NIC5-5, avagacestat, CHF5074).The recently described termed γ-secretase substrate recruiting (γSSR) factors, bind to specific γ-secretase substrates and appear to favour their processing by this protease[33, 34]. These cellular factors therefore constitute novel targets for the inhibition of Aβ production, which may not affect the processing of other substrates.

The fact that many Familial Alzheimer disease (FAD)-linked mutations fail to increase toxic Aβ species[35] suggests these mutations promote neurodegeneration by mechanisms independent of Aβ[35, 36]. In this respect, it is interesting that γ-secretase catalyses, in addition to the amyloidogenic processing of APP, the ε cleavage of cell surface receptors, which produces carboxy-terminal peptides (termed CTF2s) that are in turn important in signal transduction and are modulators of gene expression. FAD mutations at the presenilin (PS) locus appear to be associated with a loss of γ-secretase activity in respect to the ε cleavage of substrates and leads to decreased levels of CTF2 peptides (Ref?). These data support the theory that FAD mutants may promote neurodegeneration by inhibiting production of peptides with important biological functions[35-37]. Furthermore, by reducing the ε cleavage, PS FAD mutations promote accumulation of membrane-bound γ-secretase substrates[37, 38] that have been shown to cause toxicity[39-41]. Thus, these mutations may promote neurotoxicity by both reduction of intracellular signaling peptides and accumulation of extracellular toxic precursors [37, 42].

Additional evidence shows that proteins such as progranulin, involved in several neurodegenerative disorders, protect neurons from excitotoxic and oxidative stresses by stimulating survival signaling. Genetic mutations may decrease the neuroprotective functions of these proteins thus contributing to neurodegeneration[43].

While the contribution of multiple forms of Aβ to the pathology of AD seems in doubt, toxic species may play a precipitating role in disease initiation. There is also the distinct possibility that amyloid seeds of Aβ oligomers play an important role in the pathogenesis (see below).

The seeding hypothesis

Progressive protein aggregation is a hallmark of most neurodegenerative disorders, including AD, Parkinson's disease and transmissible spongiform encephalopathies. In these disease states, certain structural proteins which are normally soluble, adopt the alternative β-sheet conformation forming insoluble multimeric and fibrillar structures[44]. While this

conformational shift does not occur under normal physiological conditions, several circumstances enhance the propensity of a given protein to adopt its alternative, amyloidogenic and disease-associated conformation: mutations in the protein sequence, high concentration of the protein, and, in particular, templated misfolding.

The transition from the native protein structure to its amyloid variant has been suggested to follow a nucleation-dependent polymerization process[45], i.e. a seeding hypothesis. The formation of the nucleus (a putative β-sheet rich multimeric structure) is energetically unfavorable and is the rate-limiting step (Figure 1). In vivo the formation of such a protein "seed" may or may not occur during the lifetime of a healthy individual, but if it does, it will normally be immediately removed by the proteostasis network[46]. However, if the protein seed escapes this housekeeping process, it can act as a template for the misfolding of additional soluble homologous proteins, forming a steadily growing insoluble protein aggregate.

Interest in this concept has been recently renewed by reports of experiments in transgenic mice and from the outcome of Parkinson patients receiving neural transplants. Both lines of evidence support a templated misfolding protein aggregation as the underlying cause of disease progression (for review see[47, 48]). Cerebral β-amyloidosis can be exogenously induced by inoculation of APP+ transgenic mice using brain extracts containing aggregated Aβ (Figure 2). This seeded induction of the Aβ deposits is first evident within the injected brain area. With increasing incubation times the induced pathology spreads to neighboring regions and along neuronal pathways[49] (for review see[50]). Remarkably, recent studies show that also systemic inoculations, i.e. intraperitoneal injections of brain extracts containing aggregated Aβ, can induce progressive Aβ aggregation in the brain of APP+ transgenic mice[51] (Figure 2). The Aβ aggregation-inducing seed is probably the aggregated protein (i.e. Aβ) itself, in a conformation generated directly in the brain[52]. Moreover, small and soluble components of brain extract, presumably small and soluble Aβ aggregates, are the most potent inducers of cerebral β-amyloidosis[53]. Not only the protein seeds, but also specific soluble components for incorporation into the growing aggregates determines the progression of protein aggregation n[49, 52].

Therapeutic Implications:

These findings have multiple implications regarding therapeutic targets for the treatment and the prevention of protein misfolding disorders (Figure 2). First, because disease-specific protein aggregation can be detected many years to decades before clinical symptoms manifest[54, 55], proteopathic seeds in bodily fluids (if identified) could serve as an early and disease-specific biomarker. Evidently, transmission of these seeds between individuals must be avoided, particularly as the seeds may be chemically stable and resistant to some common inactivation procedures (as is known with prion transmission in transmissible spongiform encephalopathies ref). Second, because the formation of proteopathic seeds is slow and is thought to initiate a self-sustaining disease process, the prevention of proteopathic seed formation should become the prime therapeutic target. This could potentially be achieved by reducing the levels of the (proteopathic) proteins, by increasing protein degradation or by compounds that stabilize the native structure of the proteins[44]. If proteopathic seed formation and protein aggregation has already occurred, additional therapeutic strategies include the removal of protein aggregates by immunotherapy or the inhibition of further protein aggregation by compounds that specifically cap the template part in the misfolding process[44]. A promising future strategy may be the inhibition of the spreading of proteopathic seeds from cell to cell, between brain regions, and within an organism, although at present these mechanisms are poorly understood.

Active and passive immunisation against Aβ

Immense efforts have been put into the development of therapies that use active or passive immunization as a means to reduce or eliminate cerebral Aβ (Box 4). The first clinical trials using active immunization failed, because they found that clearing amyloid plaques did not equate to therapeutic benefit. Other clinical trials of active immunization did however show benefit in phase II. Passive immunization studies (using humanized monoclonal antibodies against Aβ) has so far failed to give encouraging results. Bapineuzumab failed to reach its primary endpoint and its development has halted. The results with solanezumab have been mixed, but it did show some benefit in some patient subgroups; its development is continuing. Antibodies against tau epitopes or amyloid seeds are the next horizon. But, if amyloid or tau peptides are the consequence of a primary immune reaction, including secretion of cytokines that increased MHC class I expression overloading protein degradation capabilities, then immune interventions will provide strong benefit. If however, the accumulation of unfolded/misfolded proteins causes a secondary immune reaction, an immunointervention will have a limited effect.

2. TAU PATHOLOGY

2.1. Pathological forms of tau

Microtubule-associated proteins tau are phosphoproteins that regulate microtubule stability and microtubule-dependent processes[56]. Abnormally hyperphosphorylated tau proteins result in the generation of neurofibrillary tangles characterized by paired helical filaments (PHF)[57]

Tau aggregates are found in many neurodegenerative disorders known collectively as tauopathies[58]. These include Alzheimers disease, argyrophilic grain disease (AgD), corticobasal degeneration (CBD), frontotemporal dementia linked to chromosome 17 (FTD-17), progressive supranuclear palsy (PSP) and myotonic dystrophy type 1.

The background to tau proteins is provided in the supplementary material.

There are six tau isoforms, produced from a single gene on 17q21 by alternative splicing. They are not equally expressed amongst neuronal subgroups. In pathological conditions such as FTD-17 or myotonic dystrophy, a mis-splicing of tau is associated with neurofibrillary degeneration[59-61]. The comparative biochemistry of the tau aggregates shows that the difference isoforms may co-exist and that they may be subjected to different post-translational modifications. This could eventually lead to a molecular classification of tauopathies.

Tau proteins are subjected to multiple control mechanisms, particularly phosphorylation. There are 85 potential phosphorylation sites on the longest tau isoform. Abnormally modified tau proteins mainly result from changes in phosphorylation, but also in acetylation, glycosylation, glycation, oxidation, nitration, ubiquitination, prolyl isomerization and truncation[62].

In AD, hyperphosphorylated tau accumulates in the somatodendritic compartment of neurons. A dendritic function of the ‘‘axonal’’ protein tau has been shown, which could play a pivotal role in AD, in particular in mediating early Aβ toxicity[63].

Furthermore, although tau is primarily seen as a cytosolic protein, a nuclear localization indicates a role in DNA protection. Heat stress and oxidative stress induce a reversible accumulation of Tau in the nuclei of neurons. This nuclear tau is able to preserve neuronal DNA integrity in stress conditions[64].

2.2. Spatiotemporal brain spreading of Tau pathology.

Disease progression is well correlated for neurofibrillary tangle (NFT) pathology. At the earliest stages of AD, tau-containing NFTs are most predominant in the somatodendritic compartment of neurons located in the transentorhinal cortex (EC)[65, 66] which then spreads to mono and transynaptically connected regions of the hippocampus and eventually to neocortical regions[65], correlating with worsening cognitive decline[67]. The most vulnerable circuit in the cerebral cortex is the medial perforant path (pp) that originates in layer II of entorhinal cortex and terminates in the middle third of the outer molecular layer of the dentate gyrus. There is extensive (up to 50%) neuron loss in layer II of entorhinal cortex even in early or possible AD (Clinical Dementia Rating score of 0.5). In late stage AD, up to 90% of the neurons in layer II of entorhinal cortex are lost[68].

The difference between regional depositions of amyloid is not clear. Amyloid plaques have a more diffuse distribution than tangles[65] which reflects the fact that the Aβ peptide is secreted into the extracellular space where it can diffuse through the brain.

2.3. Trans-synaptic spreading of tau pathology

A transgenic mouse model overexpressing tau specifically in the trans-entorhinal cortex showed trans-synaptic spreading of the tau pathology to the hippocampus[69, 70]. Crucially, the observation that tau protein, generated in EC neurons where AD pathology starts, can cross a synapse to cells in the dentate gyrus (DG) granule cell (GC) layer, *in vivo*, indicates that tau is released into an extracellular space. Now, there is also good evidence that tau is secreted, as it is found in the cerebrospinal and interstitial fluid of a human tau transgenic mouse line, in the absence of neurodegeneration[71]. It has also been identified in secreted vesicles termed exosomes[72, 73]. Uptake of filamentous tau and conversion of normal tau to abnormal tau has been shown *in vivo*. Injections of brain extract containing filamentous tau into human wt-tau expressing mice induced filamentous assembly of the endogenous tau, and the spread of this pathology to connected brain regions[74], reminiscent of the seeding hypothesis for Aβ.

How an extracellular transmissible agent could reach the intracellular compartment to transform the normal protein remains an open question. Different hypotheses were raised from extracellular tau aggregates release to the formation of nanotunelling. Recent research has shown that extracellular tau aggregates can be internalized by neurons (probably by endocytosis[75]) and promote de-novo tau mis-folding and fibrillization[76].

Internalized preformed tau fibrils have also been shown to reduce microtubule-stabilization in the host neuron suggesting a loss-of-function of normal tau in infected cells. These data support the possibility of prion-like transmissibility of tau-PHF in AD and possibly in other tauopathies[58, 77].

2.4. Therapeutic approaches based on tau.

In AD, while many amyloid-based therapeutic approaches have reached human clinical trials, most of the Tau-based therapeutic approaches are still in preclinical or exploratory stages. Those that have reached clinical trial are inhibitors of tau aggregation (methylene blue), modulators of tau phosphorylation (e.g. by inhibiting the glycogen-synthase kinase 3β (GSK-3β) using lithium), and microtubule stabilizers (davunetide) [78].

Methylene blue (Rember) is now in phase III. Rember and a similar TauRx Therapeutics drug, LMTX®, are being investigated in specific Tauopathies such as PSP and FTLD-Tau. The rationale for anti-aggregating agents[79] relies on the notion that prevention or reduction in tau fibrillization would prevent the gain of toxic function(s) by tau oligomers. Nevertheless, for

some authors, protein aggregates are also considered as neuroprotective[80]. Thus, more in vivo data are needed.

The clinical trials of inhibitors of the key kinase in Tau phosphorylation, GSK-3β, are instructional. Lithium and valproate, which are non-specific GSK3 inhibitors used in bipolar disorders, failed in clinical trials of AD. Furthermore, the phase IIb trials of the specific GSK3 inhibitor, tideglusib, (Noscira) missed its primary, cognitive, and secondary end-points, although the drug appeared to be safe[81]. This trial highlights the difficulties in AD. The animal data had been encouraging and a small phase II with 30 patients had shown positive trends in MMSE and Adas-Cog. Failure may be due to redundancy in the mechanism of action because of the multiple enzymes capable of phosphorylating tau.

Abnormal hyperphosphorylation converts tau from a microtubule-stabilizing to a microtubule-disrupting protein and promotes the self-assembly into PHF. There is redundancy built in to the phosphorylation process in that the action of GSK-3β can be replaced by cdk5 and most of the phosphorylation sites on tau can be accessed by both kinases [82]. The protein phosphatase 2A (PP2A) can dephosphorylate tau and can dissociate neurofibrillary tangles/PHF and convert the abnormally hyperphosphorylated tau into a normal protein[82, 83].

PP2A is therefore the major regulator of phosphorylation of tau in human brain[84, 85] and it is clear that its activity is compromised in the AD brain[86, 87]. One of the reasons for this decreased PP2A activity appears to be the overexpression of its negative regulator inhibitor-2, I_2^{PP2A}, in AD brain[88] and preventing this pathway could provide therapeutic benefit. Other therapeutic approaches based on tau phosphylation pathways include a PP2A demethylase purified from coffee (Sig-1012; Signum Biosciences) and the phosphatase activator, sodium selenite (a well-known dietary supplement), is now being investigated in phase II AD studies.

Since Tau phosphorylation leads to microtubule depolymerization, microtubule stabilizers such as epothilones, taxanes and other natural product microtubule-stabilizing agents that bind to the taxoid site on β-tubulin are being explored[89]. Taxane derivates do not cross the blood-brain barrier but they can couple to small haptens which allow for transcytosis. Similar to taxanes are the epothilones and of these epothilone D show promise. Bristol-Myers-Squid is currently evaluating the effects of 9 weekly intravenous (IV) infusions of epithilone D (NCT01492374) on CSF tau. The taxoid site on β-tubulin is also bound by the peptide, davunetide now in development by Allon pharmaceuticals [78, 90].

Therapeutic implications: There are similarities between the effects of toxic species of tau and Aβ, yet tau appears to be responsible for the trans-synaptic propagation of the disease. This intercellular transmission allows novel therapeutic opportunities, for example, to the production of antibodies against specific tau isotopes. Different academic and pharmaceutical laboratories published some encouraging data on Tau immunotherapy approaches[91-96]. The change in emphasis between amyloid and tau also allows us to redefine disease progression and diagnosis with biomarkers.

3. BIOMARKERS FOR AD

The last ten years has seen some major advances in the development of biomarkers for AD. These can now trace abnormal Aβ processing as well as secondary events such as inflammation, neuronal cell loss and disconnectivity. There are two major types, the biomarkers in body fluids and the imaging biomarkers.

Biomarkers in body fluids

The best-validated biomarkers are Aβ peptides and tau in CSF[97]. Many Aβ isoforms are present in CSF but in particular the Aβ1-42 isoform is clinically important as it is more prone to aggregate compared to other isoforms and forms the main component of amyloid plaques in AD brains. In AD patients CSF Aβ1-42 is decreased and correlates inversely with the amyloid burden in the brain as assessed by neuropathological examination or PET imaging[98, 99]. The decrease in CSF Aβ1-42 can be present at least 10 years before dementia diagnosis[100]. Other Aβ oligomers, which may also play an important role in the pathophysiology can be measured, however the clinical value of these assays is still unclear.

In contrast to Aβ, total tau and phosphorylated tau concentrations are increased in CSF of AD patients[97]. CSF tau concentrations correlate with the presence of neocortical neurofibrillary tangles in the brain[99]. The increase in tau starts several years after the decrease of CSF Aβ[100].

The plasma levels of Aβ and tau have little diagnostic value and are not used in clinical practice.

Imaging biomarkers

The standard magnetic resonance imaging (MRI) techniques provide identification of vascular lesions in grey and white matter and help the diagnosis of mixed and vascular dementia. In addition other advanced MRI techniques such as functional (resting state), fMRI, diffusion tensor imaging (DTI), arterial spin labelling (ASL) are being explored as imaging biomarkers in dementia.

The other major imaging technology is positron emission tomography (PET)[101]. ^{18}F-fludeoxyglucose (FDG-PET) provides a sensitive indicator of impaired synaptic function and there are also commercially developed amyloid ligands (e.g., 18F-florbetapir [Amyvid®], ^{18}F-flutemetamol) and the ^{11}C-labelled Pittsburgh Compound B (PIB). Other neurotransmitter and receptor labels for PET are being explored as imaging biomarkers for the cholinergic, noradrenergic, dopaminergic systems and microglial activation in dementia.

Clinical Significance of biomarkers

The disease specific biomarkers are critically important both in clinical practice and drug development. The use of body fluid biomarkers helps the differential diagnosis of demented patients, and provides an opportunity for early diagnosis of prodromal AD in patients with mild cognitive impairment (MCI). The sensitivity of an abnormal Aβ1-42/tau ratio for the prediction of AD-type dementia in subjects with MCI is 87% and the specificity 70%[102]. The use of the ratio for the selection of subjects with MCI for AD trials was recently proposed by the European Medicine Agency[103].

Hippocampal volumetry provides good discrimination between AD and controls, can contribute to the diagnosis of prodromal AD, and is closely related to memory deficits[101]. Amyloid-PET is the most sensitive imaging technique for early diagnosis of AD and it provides a high degree of accuracy for differentiation from frontotemporal dementia (FTD) and other non-amyloid diseases[104] including pure vascular dementia[105].

Recent studies showed that biomarkers in AD may be useful to predict rate of decline. Hippocampal atrophy and CSF tau predicted time to dementia in subjects with prodromal AD[106]. In another study CSF tau and hippocampal atrophy but not CSF Aβ1-42 predicted time

to dementia[103]. Similar findings were observed in demented subjects with AD[107, 108]. Thus, markers for secondary events in AD rather than amyloid markers may be useful for prognosis.

Therapeutical implications?

Biomarkers have several applications in AD drug development and clinical trials. In trials, they can be used as diagnostic tools to enrich for patients with evidence of AD pathology and in stratification of patients in different subgroups of the disease[109]. Amyloid markers will be in particular useful for the recruitment of asymptomatic subjects in secondary prevention AD trials. In addition, they may be used for post-hoc stratification of cases with biomarker evidence of Aβ pathology, and as safety measures[110]. CSF biomarkers may also be of great value as tools to identify and monitor the biochemical effect of a drug candidate in humans. This type of biomarkers may be labelled 'theranostic' biomarkers[110]. Theranostic biomarkers can be subdivided into primary (or pharmacodynamic) and downstream biomarkers (Table 2). Primary theranostic biomarkers are used to identify and monitor the specific biochemical effect, or mode of action, of a drug. In trials with Aβ-targeting compounds, primary biomarkers include different APP and Aβ-isoforms. A change in primary biomarkers can serve as proof-of-principle for target engagement of the drug in either healthy volunteers or AD patients, but a treatment effect on a primary biomarker does not necessarily predict a clinical effect on symptoms or disease progression. Downstream biomarkers are used to identify and monitor effects on pathogenic processes downstream of the drug target, for example on the intensity of the neuronal degeneration in an anti-Aβ trial (Table 2). An effect by an Aβ-targeting compound on downstream biomarkers is more likely to predict a clinical effect on symptoms and disease progression. The coupling of a biomarker and a sensitive cognitive test has immensely aided diagnosis, and points the way forward to more powerful phase II studies.

4. TRANSLATIONAL VALUE OF ANIMAL MODELS

Many promising therapeutic approaches, which were shown to be successful in transgenic animal models of AD, failed when evaluated in human clinical trials. So the question is raised frequently regarding the utility of these models.

Genetically-engineered mouse models are principally based on our knowledge of the etiology of familial AD (and not the idiopathic variant), but only 2% of human AD cases are inherited in an autosomal dominant fashion. The biggest difference between the two forms is that Aβ accumulation is probably the result of clearance issues in sporadic cases and of overproduction in familial cases. It is also clear that in animal models, the very short time frame (1-2 years) for the development of the pathology strains the extrapolation to the human condition, but this does not satisfactorily explain the false positives in animal models.

Failures in clinical trials have at least partly been attributed to inadequate internal and external validities of preclinical studies. Use of more than one animal model, testing at multiple sites, and using adequately powered designs are necessary[48 111, 112].

Most importantly, the outcome measures in animal studies have rarely focused on relevant translational parameters and biomarkers. For example, Aβ-imaging and CSF measurement of Aβ and tau have become valuable biomarkers for the diagnosis and progression of AD, yet few mouse studies have analyzed Aβ and tau in CSF, and the endpoint of most animal studies was postmortem neuropathology, which is not very helpful for translational purposes.

A key feature lacking in many transgenic AD models is an extensive neuronal atrophy as seen in humans. The therapeutic approaches investigated in preclinical studies are aimed at the

induced pathologies (Aβ and/or tau) and do not address the consequences of extensive neuronal loss (i.e. cognition). Hence, many of these therapies may be highly effective at modulating the induced pathology, but ineffective after a significant number of neurons or synapses have been destroyed. Second, cognitive measures are used as endpoints in some transgenic models, but the most revealing deficit in AD is episodic memory.

Despite these caveats, novel approaches to reducing AD pathology have been discovered and developed in transgenic mice. For example, the studies using the 3xTg-AD mice did predict that humans receiving immunotherapy against Aβ would show reduced Aβ but no reduction in the pathological tangles (box 3) [113].

One potentially exciting but unexplored area of AD research relates to the regenerative potential of stem cells. Preliminary work in the 3xTg-AD model has shown that transplanting neural stem cells can restore cognition via a Brain-Derived Neurotrophic Factor (BDNF)-dependent pathway without altering Aβ or tau levels. Moreover, work in a model with extensive neuronal loss, showed that stem cells can restore cognitive deficits in mice with robust hippocampal neuronal loss, via BDNF[114]These findings highlight the need to consider other non-pharmacologic based approaches.

Inducible tau transgenic mouse models have been created with expression of pro- and anti-aggregant variants of either full-length human Tau (hTau40/ΔK280 and hTau40/ΔK280/PP) or the truncated Tau repeat domain (Tau_{RD} /ΔK280 and Tau_{RD} /ΔK280/PP): all cause co-aggregation of human and mouse tau. The repeat domain Tau_{RD} /ΔK280 causes hippocampal NFTs and neuronal loss. Switching-off pro-aggregant Tau leads to rescue of the effectsand synaptic recovery provided that amyloidogenic Tau is removed[115].

Furthermore, the entire APOE field has seen a revolution because of the study of humanized proteins expressed in transgenic mice, sometimes crossed with the more "traditional" AD mice. Studies using transgenic mouse models of AD have demonstrated the important role of APOE in AD neurodegenerative process and identified the differential effects of its isoforms (see below).

- **Therapeutic Implications – transgenic animals.**

In the past transgenic animals have been blamed for some failures. However, this may be that they were used as being "mini-Alzheimer patients" although they express neither the synaptic dysfunction nor the complicated memory deficits of AD patients. Now they can be constructed to reflect specific molecular lesions which allow hypothesis testing. Each hypothesis should be developed using at least two animal models, linked to clinical biomarkers for extensive testing prior to phase III.
Transgenic animals should therefore form part of the translational process depending on the drug mechanism being investigated.

5. THERAPEUTIC APPROACHES

Specific therapies dealing with Aβ and tau production were mentioned in the preceding sections. However there are many other therapeutic possibilities which need exploring:

- Synaptic dysfunction and neuronal plasticity affecting neuronal networks,
- Cholesterol, APOE and APP processing,
- Stress, depression and lifestyle changes,
- Mitochondrial damage,

- Epigenetics and non-coding RNAs
- Neurotrophic and inflammatory aspects.

5.1. Emerging novel non-conventional targets (not directly related to Aβ and tau pathology)

- **Disturbed protein recycling and synaptic dysfunction**

Amyloid and tau proteinopathies impact synapses and, while the causes of different neurodegenerative diseases vary, the lesions that initially disrupt the integrity of neuronal communication at the synaptic compartment are common and cause significant functional impairment[116]. Synapses are sites with high metabolic activity, where neuronal signaling and physical remodeling pose high-energy demands. Abnormal signaling or lack of sufficient local energy supply can rapidly inhibit neuronal communication and cause synaptic loss. Loss of synaptic spines is found in animals that show features of neurodegenerative disorders and it is also a feature of the ageing process in primates[117]. Both extra- and intracellular processes seem to be involved in these process, including protein recycling, autophagy and mitochondrial dysfunction. For example, disturbed protein recycling has been implicated in spine loss in Huntington Disease (HD) models. Remarkably, in Drosophila HD models, re-establishing synaptic vesicle recycling by the replenishment of RAB11 recovers the major pathological features[118].

Neurons utilise the lysosomal system to degrade organelles and synaptic proteins including receptors such as AMPA and NMDARs[119]. Autophagy can target proteins and organelles for lysosomal degradation by fusion of the autophagosome (containing the proteins or organelles to be degraded) with the lysosomes. Impairment of autophagy and vesicle recycling can cause spine loss in HD models [118] and Aβ can directly impair the lysosomal degradation of autophagic cargo in AD[120]. In turn, lesions of the autophagic machinery promote formation of plaques, accumulation of APP and increased secretion of Aβ. Defects in lysosomal protein processing and defective clearance of damaged mitochondria by autophagy (mitophagy) are also found in Parkinson's disease[121]. Finally, mutations in mitochondrial proteins that are involved in mitochondrial DNA synthesis and maintenance can result in neurodegeneration, and inhibition of autophagy causes a massive neurodegenerative phenotype in mice[122].

Altered synaptic signaling can trigger several of the above-mentioned pathological mechanisms. Synaptic dysfunction and loss in AD can be initiated by abnormal Aβ signaling and/or tau missorting[116, 123]. Initial hyper-excitability and subsequent depression of neuronal network activity can result in dysfunction and degeneration of the synaptic compartment, which may trigger stress responses and/or inflammatory reactions.

Several drugs in development affect synaptic signaling, and how these fare will be important for the future. However they also show that there is a blurring of differentiation between drugs that were previously thought just to be symptomatic but may be disease modifying. Positive allosteric modulators of AMPA receptors can result in an increased BDNF production which may not only affect memory but also cell fate[124]. α7-Nicotinic receptor modulators that were previously thought to improve AD symptoms are now believed to be active in the pathways of disease progression[125], since Aβ appear to activate homomeric α7 receptors at picomolar concentrations but block them at higher concentrations.

Synaptic network function is critical and their dysfunction in AD is also being addressed. Certain antiepileptic drugs have been shown to block inappropriate synaptic activity and thus

limit cell damage. Levetiracetam has been shown to suppress abnormal hippocampal spiking activity and prevent memory deficits in animal models[126]. In a clinical imaging study in patients with amnestic MCI, the hyper activation of the hippocampus and entrorhinal cortex was inhibited by leviracetam [127]. This drug binds to SV2A on synaptic vesicles and prevents transmitter spill-over from the synaptic cleft[128]. Phase II clinical trials are ongoing.

Thus, while the efforts to avert accumulation of toxic proteins continue, it seems possible to design strategies to delay synaptic dysfunction and eventually promote synaptogenesis as an alternative disease modifying approach. The processes involved in synaptic damage and regeneration may become interesting new targets and restoring synaptic function/architecture would buy time and ensure better life quality. Examples are already evident in PD where in many cases L-DOPA ameliorates disease symptoms and improves, albeit for a limited time, quality of life. Neuroprotective strategies may not reverse the course of disease, but slow their progression. Unfortunately, the attempts to use neuroprotective strategies have not been very successful in the clinic thus far. Nevertheless some exciting drugs are to be tested, and breakthroughs have been made by consortia working on APOE[129].

Can we act at APOE and cholesterol metabolism?

- **Cholesterol, APOE and APP**

Cholesterol can be synthesized de novo within the cell body or can be recycled from degenerating spines, by the glial origin APOE/LDLR pathway (the primary source). APOE2 has a protective effect in ADand has very low affinity to LDLRs, its affinity being only 1% of the normal E3 isoform. The affinity of E4 isoform is slightly higher than that of E3. The efficiency of cholesterol recycling might be a key factor in AD pathology coupled with Aβ metabolism. This has now been explained by the findings[130, 131] that APP has a flexible transmembrane domain which binds cholesterol, with the theory that cholesterol-bound-APP can then move into lipid rafts, where γ-secretase can act.

APOE is an excellent Aβ scavenger from the extracellular space. APOE4 has a much lower expression than the other forms, therefore the lack of efficient removal of Aβ (through enhanced cellular uptake and degradation and removal through the BBB) from the extracellular space might be a major contributor to the toxicity associated with this genotype[132].

The ATP binding cassette A1 transporters (ABC transporters), transport cholesterol and in gene association studies, ABCA1, A2, A7, G1 are linked to AD[133]. A crucial issue is that cholesterol cannot pass the blood brain barrier and is oxidized by cytochromes (Cyp46A1) to 24S-hydroxysterol (cerebrosterol) or 27S-cholesterol to pass the blood-brain barrier – oxysterols activate LXR receptors which increase synthesis of the transporters ABCA1, ABCG1[133] particularly on the pericytes which surround the endothelial cells to make up the blood brain barrier. Thus exposure to cerebrosterol increases elimination of oxysterols from the brain. There is a 4-fold accumulation of 27-OH oxysterol in the brain of familial AD subjects[134]. 27-OH oxysterol also activates the brain renin-angiotensin sytem[135]. If this is important then LXR agonists should be useful.

Therapeutic implications: Very impressive results were found with the LXR/RXR agonist, bexarotene, in the mouse APP/PS1 model with enhanced clearance of soluble Aβ within hours in an apoE-dependent manner. Aβ plaque area was reduced >50% within just 72 hours.

Bexarotene stimulated the rapid reversal of cognitive, social, and olfactory deficits and restored electrophysiological dysfunction[136], although whether these effects are sustained is controversial. Thus, LXR-RXR activation stimulates physiological Aβ clearance mechanisms, resulting in the very rapid reversal of a broad range of Aβ-induced deficits. However, LXR-RXR agonists have deleterious effects on liver metabolism so future progress depends on whether this may be surmounted.

- **Stress, depression and lifestyle changes in AD**

Sporadic Alzheimer's is often comorbid with treatable conditions such as cardiovascular disease, diabetes and obesity. Depressed subjects are at 2-3 times greater risk for developing AD[137, 138]. Depression can give reductions in cognitive measures as measured by the Free and Cued selective Reminding Test (FCSRT), which are reversed by antidepressants. Stress-associated psychopathology has been associated with dendritic atrophy in the hippocampus and frontal cortex and functional disconnection of these areas[139], resulting in mood, emotional and cognitive impairments similar to those found in depression and Alzheimer's acutely and chronically[140-142]. First episode depression in the very elderly (>75 years old) is often linked with AD and this may be a useful clinical entry point.

Growing evidence indicates that stress 'programs' brain function through epigenetic mechanisms and that the resulting phenotype is determined by the life stage and context in which the stress is experienced[143]. Several lifestyle interventions are being tested in AD which could modify stress and its impact on the brain areas at risk (e.g. increased physical and cognitive activity)[144]. A recent essay described how improvements in education, health and general socio-economic status can be expected to contribute substantially to improving the "mental capital" of nations[145]. If these approaches have significant benefit, association of changes in life style will introduce another variable to take into account in drug trials.

Stress is a precipitating factor in Aβ production[146] and in pathologic Tau phosphorylation, and associated cortical deficits[147]. Stress is associated with increased glucocorticoids, and glucocorticoid receptors are located in the limbic areas at risk of AD, modifying cellular metabolism and mitochondrial activity. These receptors are normally anti-inflammatory but recent studies in rats have indicated [148] they can exacerbate inflammatory signaling in the brain.

- **Mitochondrial damage and AD**

Mitochondrial dysfunction is frequently associated with AD and other aging-related neurological disease processes [149-152] . Because the brain is characterized by an unusually high metabolic rate and high oxygen consumption, the consequences of mitochondrial dysfunction are more severe in brain than in tissues characterized by lower bioenergetics demand.

There are about 50,000 DNA lesions per cell per day, and most of these are caused by reactive oxygen species primarily generated by the oxidative phosphorylation in mitochondria. Mitochondrial dysfunction is often associated with large deletions and/or an elevated number of unrepaired oxidative DNA lesions in the mitochondrial genome and several recent studies demonstrate that oxidative DNA damage is higher and DNA repair capacity is lower in brain from individuals with AD than in control cells[153].

Tau accumulates in mitochondria and may contribute significantly to mitochondrial dysfunction and altered bioenergetics associated with AD[154-156]. Deficiencies in the capacity to perform DNA repair, leads to additional oxidative stress, and might also influence Tau-related downstream events.

Furthermore, BDNF has been shown to directly increase the respiratory coupling index of brain mitochondria via the neurotrophic pathway, and this effect is opposed by inflammatory cytokines, so mitochondrial efficiency may also be modified in AD[157], and this may contribute to the metabolic disruption which is so apparent in the progression of the disease (Figure 1). Thus drugs which may increase BDNF, such as AMPA modulators, may have beneficial effects on metabolism. Diagnostic procedures based on mitochondrial DNA repair capacity or altered mitochondrial bioenergetics are being investigated to improve early detection of individuals with prodromal AD.

- **MicroRNA and Alzheimer disease: new perspectives**

MicroRNAs (miRNA) are short, non protein-coding stretches of RNA some 19-24 nucleotides in length which act as post-transcriptional regulators of gene expression by base pairing with complementary sequences of target mRNAs. Over a thousand exist in the human brain and each possesses many (generally hundreds) of targets.

Recent studies have shown that AD (and plaque formation) is accompanied by alterations in the levels of many distinct classes of miRNA, certain of which target mRNAs encoding proteins involved in its pathogenesis, and in particular the generation of Aβ [158-162]. Several miRNA species, like miR-101 and miR-106, target APP, and their down-regulation in AD favours an elevated generation of Aβ [158, 161, 163, 164]. Interestingly, several nucleotide polymorphisms associated with AD located in the miRNA-binding region of the APP mRNA which can modulate protein expression[165]. Another class of miRNAs which are reduced in AD include the neurone-specific miR-124, and this leads to the over-expression of its target mRNA: polypyrimidine-tract binding protein 1 (a pre-RNA splicing regulator) and hence to the altered splicing of APP[166]. The miR-124 also targets BACE1, so its loss promotes the transformation of APP into beta-amyloid[167]. By analogy, reduced levels of several other miRNAs in AD (and mouse models of AD), like miR-9, miR-29a, miR-29b and miR-107, may result in elevated BACE1 expression and over-production of beta-amyloid[161, 162, 168]. Studies in mutant mice suggest that miR-298 and miR-328 fulfill similar roles[169]. Lastly, and further underpinning the complexity of miRNA control of Aβ generation in AD, loss of the above mentioned miR-9, miR-29a and miR-29b in AD, together with loss of miR-137 and miR-181c will disinhibit serine palmitoyltransferase, the rate-limiting enzyme of ceramide synthesis, leading to mislocation of BACE1 in lipid rafts and further augment the excessive processing of APP into Aβ [170]. Interestingly, while the above changes are deleterious, loss of miR-107 also disinhibits the alpha-secretase ADAM10 to favouring the non-amyloidogenic pathway of APP processing with compensatory effects shunting APP cleavage away from Aβ plaque generation towards soluble APPalpha[171].

The detrimental effects of miRNA changes in AD may not be restricted to Aβ formation. Loss of miR-15a favours Tau hyperphosphorylation by disinhibiting ERK1[172]. In addition, increased levels of miR-128 lead to decreased activity of Bcl2-associated athanogene, and hence reduced (ubiquitin-independent) removal of sarkosyl-insoluble tau: this favours the formation of toxic tau inclusions[173]. Based on studies in a transgenic mouse model, putative increases in levels of miR-34a suppress expression of Bcl2 itself hence exacerbating neuronal

loss by caspase-3 recruitment and apoptosis[174]. Increases in miR-206 in the temporal cortex of AD brains leads to suppression of BNDF contributing to compromised morphological and functional synaptic plasticity[175]. Two miRNAs that are increased in AD, miR-125b and miR-146, may lead to decreases of Complement Factor H and an aggravation of neuroinflammation[176, 177], and alterations of miR-106b impact the expression of Transforming Growth Factor beta[178]. Moreover, the neuronal release (and cellular reuptake) of mRNAs like miR-146a and miR-155 may, lead to inflammatory spreading in AD (at least partially) by provoking the down-regulation of complement factor H[177, 179]. Underscoring the significance of miRNA release, Lehmann et al[180] recently suggested a detrimental role of the miRNA, Let-7b, which, following its release, activates the Toll-like receptor 7, whichresults in neuronal degeneration. Finally, loss of the above-mentioned miR-29a will disrupt the activity of another target gene, Neuronal Navigator 3, a protein controlling axonal guidance which is enriched in degenerating pyramidal neurons in AD[181].

In addition to this wealth of evidence that alterations in miRNAs impact (mostly unfavourably) pathological processes in AD, there is in vitro evidence that Aβ itself reciprocally impacts, by as yet unclear (and presumably indirect) mechanisms, the production of miRNAs including several mentioned above like miR-9, miR-106b and Let7[178, 182, 183].

Despite this wealth of evidence for a role of miRNAs in AD, it remains clear whether they play a causal and primary role in its induction. Nor is it clear what are the effects of clinically-employed and prospective drugs on miRNA levels. Thus, efforts continue to clarify the interrelationship of miRNA to pathological processes of AD, and also to exploit miRNA levels in CSF and peripheral cells as precocious biomarkers of AD and of the actions of pharmacotherapy[184, 185]. miRNAs represent potential targets for novel pharmacotherapy themselves, in view of recent progress in technologies like locked nuclear acids and antagomirs for their manipulation[159, 186].

Finally, miRNAs are just one class of a suite of non-coding RNAs of potential importance to AD like long non-coding and small nucleolar RNAs so the extension of work on miRNAs to these not so distant relatives will likely also reveal important insights into its pathogenesis, diagnosis and, ultimately, treatment of this and related neurodegenerative disorders[187].

- **Neurotrophic and inflammatory aspects**

Vacuolar protein sorting 10 (Vps10) receptors (sortilin, SorL1, SorCS1, 2 and 3) are a key link between neurotrophins and amyloid production. One of these, SORLA (also known as SorL1), is an accessory protein for APP which affects Aβ processing and is deficient in AD. In familial AD[188], SORLA is reduced by about 50% and this in turn increases Aβ by 50% [189]. SORLA production is dependent on BDNF signaling and when this is impaired increased Aβ production ensues[190]. SORLA forms a 1:1 complex with APP and prevents dimerization of two APPs. The APP dimer is metabolized much more efficiently, allosterically, by BACE[191]. However in the presence of SORLA, APP is metabolized as a monomer with competitive kinetics.

A further link is provided by sortilin which binds to the proBDNF and pro-NGF domains. These pro-neurotrophins when released may have the opposite effects to the mature neurotrophins, and sortilin has been proposed as a molecular switch acting on both p75NTR

and TrkA positive neurons whereby proNGF can cause apoptotic or antiapoptotic effects – and presumably modify Aβ production via SORLA[192].

The 3xTg mouse has helped us to define the role of inflammation and infection at the neurotrophic/inflammatory interface. Infection exacerbates both the neuropathology and tau phosphorylation, probably via a combination of GSK3β activation[193], TLR4 receptors and IL1β signaling[111, 112]. In transgenic mice, a key role of Aβ in inducing inflammasome activation (particularly caspase-1 activation; the common component of all inflammasomes), and hence augmented IL1β production, has been clearly demonstrated[111, 112, 194]. NLRP3 and caspase-1 inflammasome knockout mice were found to be largely protected from spatial memory defects and a variety of biochemical and electrophysiological abnormalities associated with murine AD when crossed into the APP/PS1 model of AD[195]. This protection might be due to reduced levels of IL1β, although the NLRP3 inflammasome also is responsible for the processing of IL18, a proinflammatory cytokine that has not yet been assessed in AD. Recently it has been shown that humans AD brain specimens exhibit activated forms of caspase-1, which is responsible for the processing of IL1b, IL18 and IL33[195].

Combined new approaches may be even more effective. Developing miRNA-based strategies to improve synaptic plasticity is achievable in animal models. Identification of epigenetic factors that are involved in neurodegenerative diseases may prove more effective than general trophic stimulation of synaptic function and plasticity. Stress and inflammation are triggers of epigenetic changes.

6. CLINICAL ASPECTS

6.1. Why have so many drugs failed?

Between 1998 and 2012, 104 drugs failed in development for only three drugs registered. Several failures were in phase III, where trials were based on outcomes from phase 2 trials which were not necessarily mechanistically determinant and also underpowered. The multitude of unresolved mechanistic hypotheses (see above) and the fact that transgenic animls can be used for hypothesis generation rather than as disease-models, which can predictively filter new compounds, means that it is therefore critical to have the capacity to put many compounds through proof of concept clinical trials which are sufficiently powered to reduce the risk of phase III failure.

In consequence we need to rethink both phase 2, with better hypothesis-testing use of biomarkers and phase 3 to improve our detection of any futility signal as soon as possible, thus limiting the risks.

Phase 2 trials should be experimental and deterministic. Biomarkers must be used to help determine if a compound meets its hypothesis-led expectations in animals and then man. This phase must assure the sponsor that a potential therapeutic has a reasonable chance of working in phase III and there are now sufficiently powerful experimental tools (table 2) to allow this. However, this does not mean that there are not drugs already available for other indications which would not be effective – a recent review [196] listed a series of drugs for repositioning in AD. However development in AD is extremely costly and adequate reimbursement is unlikely for repurposed drugs out of patent, if the development risks are taken into account. The social need for new therapies means that new directions must be found by a consensus between

discovery, development, regulatory and reimbursement centres, which can only be settled at the highest level.

6.2. Improved design of clinical trials.

The trials in the field of AD over the past few years have allowed progress to be made on methodological aspects, cognitive tests, global function, neuropsychiatric measures, biomarkers, and imaging. Advances in biomarkers (CSF amyloid and Tau protein) as well as amyloid PET allows the possibility to have much greater information on the effects of the drugs in proof of concept study for a Go / No-Go decision. Concerning clinical outcome for phase IIb and III studies various Task Forces[197-201] have recommended performing 18 months trials for mild to moderate AD and 2 years for prodromal AD, as well as use Clinical Dementia Rating Scale-Sum of the Boxes (CDR-SB) as co-primary end point[202]. Imaging MRI appears now probably more useful to exclude other dementia and treatment side effects, e.g. vasogenic edema, than an endpoint. Amyloid PET appears to be crucial to demonstrate some effects on amyloid deposition.

Major problems remain in the recruitment of patients, their retention, site-to-site variability, and other methodological issues. It is thus incumbent on the clinical community to find solutions to these problems, particularly as the field moves toward treating the disease in its prodromal stages, where these methodological issues will become even more critical. A better understanding is needed as to why participants refuse to enter drug trials, and why primary care physicians and Alzheimer's specialists refuse to involve their patients. There is a need to communicate on the simplicity of neural imaging assessments and of the banality of lumbar punctures. We have also a better knowledge of the progression of AD patients under symptomatic treatment to better design therapeutic trials in mild to moderate AD[203].

The new concept of prodromal Alzheimer's, using episodic memory testing and accompanying biomarkers, before the onset of dementia (see above) is a major breakthrough, and the EMA has, informally, issued guidelines for diagnosis of Alzheimer's and inclusion in clinical trials which embrace this concept[2]: *"The definition of prodromal AD is acceptable. The "Dubois Criteria" (in box 1) as criteria to define the population must be validated in full at the time of the submission of the dossiers. Including a positive CSF biomarker profile is considered predictive for the evaluation of the AD dementia type. However, although high CSF tau and low CSF Aβ42 are predictive of Alzheimer's disease, the criterion "positive CSF tau/Aβ42 ratio" is not well defined. The qualification of biomarkers in the pre-dementia stage of Alzheimer's disease will allow better inclusion criteria of patients in pre-dementia trials in which the benefit/risk is higher for treatment with these novel compounds".*

The FDA have also issued a draft guidance (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM338287.pdf) for industry about the development of drugs for the treatment of early stage disease, with a call for comments. This document propose the clinical dementia rating-sum of boxes (CDR-SD) as a measure of cognitive changes, which is positive would allow an accelerated approval mechanism, using this single primary efficacy measure for marketing approval: the sponsor could then demonstrate that the observed benefit persists and positively affects the patient's position[204]. The FDA guideline allows companies to design clinical trials in prodromal Alzheimer's with sensitive episodic memory tests and biomarkers which can also, to an extent, be defined according to the hypothesis to be tested. This is revolutionary and may well change the entire field.

In familial AD, a clinical revolution has taken place as the Alzheimer's Prevention Initiative (comprising NIH, the Banner Alzheimer Institute and Genentech), will test an anti-amyloid antibody treatment (crenezumab developed by AC Immune and Genentech) in 300 people with a rare genetic mutation that triggers Alzheimer's symptoms around age 45. Crenuzumab is also being studied in mild to moderate AD patients. This is the first time that familial AD will have been addressed clinically and success will further encourage genotyping.

ADAS-Cog, the gold standard in later stages of the disease, is not very sensitive to drug effects and limits the possibility of demonstrating success. In early stages, FCRT is much more sensitive but insufficiently validated and comparisons with CDR-SD are needed. It is very difficult to develop a test to be used in very mild, mild and moderate populations. However new EMA guidelines give hope that companies may be able to construct their own regulatory strategy.

Subtypes of Alzheimers (relatively specific deficits in memory, apraxia, language, visioconstruction, behavioral/dysexecutive), are underestimated Vascular dementia which is a clearly different pathology and easily screened out, is not desirable in an AD study. The exclusion of other AD subtypes from clinical trials however could have very negative effects on recruitment (which is already difficult). The question of whether peripheral models of amyloidosis (inclusion body myositis) may be relevant to testing specific agents is intriguing but not yet validated (see supplementary material).

Biomarkers are critical – but they are often not fully validated quantitatively. They also considerably increase the cost of clinical development . They can show in phase I and II whether a drug is having its biochemical effect, if disease modifying. Thus they should be used to construct development packages. Initial studies in highly selected patients with imaging, biomarkers and specific memory tests may therefore be crucial in testing whether a new drug is worth pursuing. The negative effect on recruitment of spinal fluid sampling needs to be taken into account.

In conclusion the drug discovery and development process should be reconsidered and performed differently, with a fully translational approach depending on the mechanism of action. Biomarkers should then be proposed based on mechanism and the patient population proposed. Tests must be proposed for phase I and II which validate the hypothesis before phase III. This requires a continuing interaction with regulators and both the EMA and FDA have now agreed that this is important.

Therapeutic implications: However, the failure of bapineuzumanb and the modest efficacy of solanezumab put pressure on active and passive immunization therapies. There must be a real mobilization to define which of the multiple other mechanisms are valid, using exploratory clinical research, with efficacy backed up by biomarkers in specific patient populations. However the possibility of registration based on validated cognitive benefit alone yields a new impetus. Alzheimer's disease is really at a cross roads.

Table 2A : panel of theragnostic biomarkers in anti-aß clinical trials in Alzheimer's disease

Type of biomarker	Pathogenic process	CSF biomarker	Position in clinical trials	Comment
Pharmacodynamic biomarkers (target engagement)	Brain Aβ load	Aβ42	• Proof of principle for an effect on brain Aβ load	• The CSF level of Aβ42 correlates inversely with brain Aβ load measured by PiB-PET [205, 206] • No data support that brain region specific PiB retention provides additional information than global retention.
	γ-secretase dependent APP/Aβ metabolism	Aβ38, Aβ40	• Proof of principle for reduced Aβ production	• CSF Aβ38 and Aβ40 are less prone to aggregate than Aβ42, and may be more suitable to monitor Aβ metabolism than Aβ42
		Aβ42/38 and Aβ42/40 ratio	• Proof of principle for γ-secretase modulators	• Decrease in Aβ42/38 and Aβ42/40 ratio is the expected effect of γ-secretase modulators[207]
		Aβ15, Aβ16, Aβ34	• Indirect biomarkers for γ-secretase inhibitors	• Aβ15/16 are produced by an alternative APP processing pathway [208] • Increase in Aβ15/ Aβ16 and decrease in Aβ34 may be more sensitive to γ-secretase inhibitors than Aβ42 or Aβ40 [209]
	β-secretase (BACE1) dependent APP/Aβ metabolism	Aβ38, Aβ40	• Proof of principle for reduced Aβ production	• CSF Aβ38 and Aβ40 are less prone to aggregate than Aβ42, and may be more suitable to monitor Aβ metabolism than Aβ42
		sAPP-β, BACE1 activity	• Proof of principle for inhibition of BACE1	• Decrease in sAPP-β is the expected effect of inhibition of BACE1[210]
		sAPP-α	• Indirect biomarker for inhibition of BACE1	• Increase in sAPP-α may be a compensatory response to inhibition of BACE1[210]
		Aβ 5-40	• Indirect biomarker for inhibition of BACE1	• Aβ 5-40 is produced by an unknown APP processing enzyme • Increase in Aβ 5-40 may be a sensitive marker for β-secretase inhibitors [211]
	Aggregation state of Aβ	Aβ oligomers	• Core biomarker for effect on Aβ aggregation	• Effect on CSF Aβ oligomer levels may vary between drugs with different mode of action
Downstream biomarkers	Intensity of neuronal degeneration	T-tau, H-FABP	• Change towards normalization may indicate that the drug reduces the neuronal degeneration	• Change in downstream biomarkers may be important to label a drug as disease-modifying
	Tau phosphorylation and tangle formation	P-tau	• Change towards normalization in CSF P-tau indicate a drug effect on tau phosphorylation and possibly on tangle formation	• Different phosphorylated tau epitopes may respond differently to drug treatment

Abbreviations: APP: amyloid precursor protein; CSF: cerebrospinal fluid; H-FABP: heart fatty-acid binding protein; P-tau: phosphorylated tau; SILK: stable isotope labeling kinetics: T-tau: total tau.

Table 2B : Implementation of theragnostic biomarkers in anti-Aβ clinical trials in Alzheimer's disease				
Type of trial	**Design**	**Type of biomarkers**	**Interpretation**	**Example**
Phase I	• Single-dose, placebo-controlled study • Continuous CSF sampling through lumbar catheter for 36h, including samples before and after drug administration	• Pharmacodynamic markers (e.g. Aβ and APP isoforms) • Pharmacokinetic analyses of drug	• Proof-of-principle data on target engagement of drug • Data on CNS availability of drug	Phase I study showing that the BACE1 inhibitor LY2811376 lowers CSF Aβ42, Aβ40 and sAPPβ levels [210]
	• Single-dose, placebo-controlled study • SILK technique with isotope labeled amino acids • Continuous CSF sampling through lumbar catheter36h, including samples before and after drug administration	• Pharmacodynamic data on Aβ production and clearance • Other pharmacodynamic markers (e.g. Aβ and APP isoforms) can be measured simultaneously • Pharmacokinetic analyses of drug possible	• Proof-of-principle data on target engagement of drug by SILK analyses and other pharmacodynamic markers • Data on CNS availability of drug	SILK study showing that the γ-secretase inhibitor LY450139 decreases Aβ production in CNS [212]
	• Randomized, placebo-controlled study • Multiple dosing possible • Short duration (weeks-months) • CSF sampling by lumbar puncture before trial initiation and at end of study	• Pharmacodynamic markers (e.g. Aβ and APP isoforms) • Pharmacokinetic analyses of drug	• Proof-of-principle data on target engagement of drug • Data on CNS availability of drug	Phase I study showing that proposed γ-secretase modulator R-flurbiprofen (tarenflurbil) does not affect CSF Aβ42 levels[213]
Phase II	• Randomized, placebo-controlled study • Multiple dosing possible • Medium duration (months) • CSF sampling by lumbar puncture before trial initiation, mid study (optional) and at end of study	• Pharmacodynamic markers (e.g. Aβ and APP isoforms) • Downstream biomarkers (e.g. T-tau and P-tau)	• Proof-of-principle data on target engagement of drug • Downstream biomarker data may support that the drug reduces the intensity of neuronal degeneration or tau phosphorylation	Phase II study showing that passive Aβ immunotherapy reduces brain Aβ load[214] Phase II study suggesting that active [215] and passive [216], Aβ immunotherapy may lower neuronal degeneration.
Phase IIII	• Randomized, placebo-controlled, multiple dose study • Long duration (years) • CSF sampling by lumbar puncture before trial initiation, mid study (optional) and at end of study	• Pharmacodynamic markers (e.g. Aβ and APP isoforms) • Downstream biomarkers (e.g. T-tau and P-tau)	• Proof-of-principle data on target engagement and downstream biomarker data may be necessary to label the drug as disease-modifying	None available
Abbreviations: APP: amyloid precursor protein; CSF: cerebrospinal fluid; CNS: central nervous system; P-tau: phosphorylated tau; SILK: stable isotope labeling kinetics: T-tau: total tau.				

Box 1: Mild Cognitive Impairment (MCI)

- **Figure 1 : The importance and mechanism of Aβ oligomer toxicity**

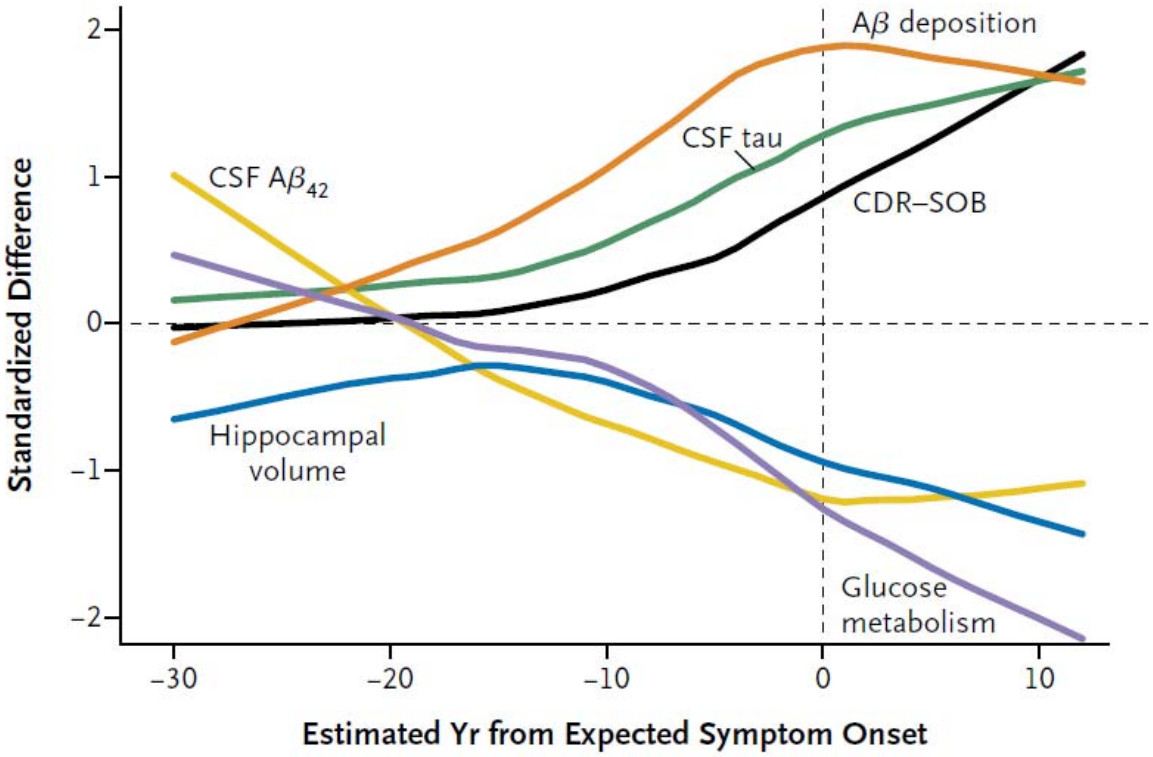


Figure 1. The insidious onset of familial Alzheimer's disease (adapted from reference 9)**.** The normalized differences between mutation carriers and noncarriers are shown with the estimated years from expected symptom onset (~49 years old). Decreasing Aβ42 levels in the CSF (CSF Aβ42) is concomitant with increasing fibrillar Aβ deposition. Slightly later, the levels of CSF tau increase and hippocampal atrophy (volume) and hypometabolism (glucose metabolism) in the precuneus start to manifest (5-10 years before symptom onset), and cognitive and clinical changes (as measured by the Clinical Dementia Rating–Sum of Boxes [CDR-SOB]) begin. Mild dementia (CDR 1) occurred ~ 3.3 years before expected symptom onset.

Box 2: Soluble oligomers as toxins

The rationale for Aβ oligomers, or Aβ-derived diffusible ligands (ADDLs), as a therapeutic target emerges from an extensive literature. Since first found to be fast-acting synaptotoxins in 1998 , oligomers have been investigated in over 1500 publications, giving rise to the conclusion that toxic oligomers play a significant role in AD pathogenesis[217, 218]. Toxic Aβ oligomers accumulate in brain tissue of humans with AD[219, 220] and in animal AD models[221]; their presence in CSF indicates value as an AD biomarker[222, 223]. Besides disrupting mechanisms of memory formation, oligomers can instigate a wide array of AD-linked neuropathology (reviewed in[222]). Crucially, they damage the structural integrity of synapses[224, 225], which is the best pathological correlate of dementia[226], and they induce tau hyperphosphorylation[227], placing their action upstream in the pathogenic process but linking to tauopathies.

However it is an issue that the Aβ vaccines that have been reported show no specificity with respect to oligomers. Bapineuzumab binds the N-terminus of Aβ and binds fibrils[228], and Solanezumab binds the C terminus and binds monomers[229], a lack of specificity that dilutes their capacity to neutralize oligomers. Whether binding of Bapineuzumab to vascular amyloid may be the cause of the observed incidence of transient vasogenic edema[230].

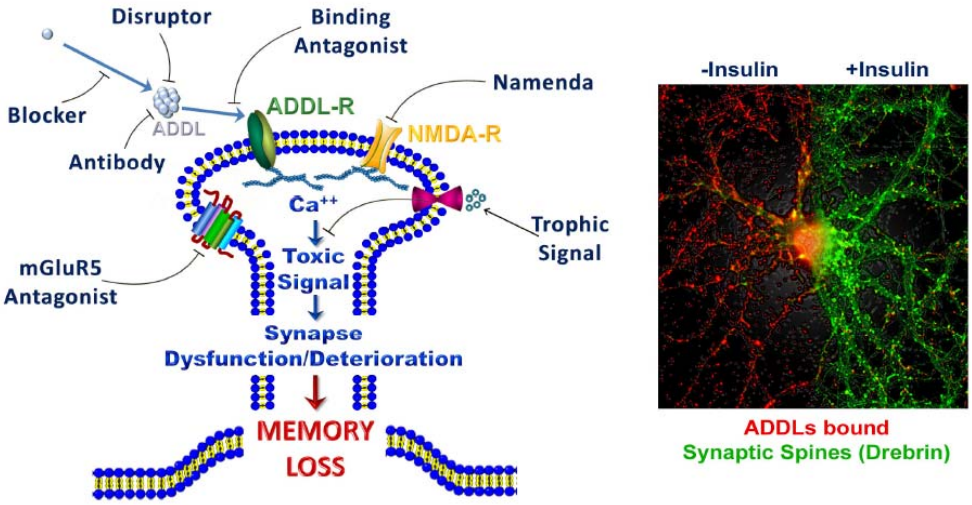


Figure 1. Targeting toxic Aβ oligomers (ADDLs) for Alzheimer's therapeutics.
(Left) Therapeutic strategies under development that target oligomers or their toxicity.
(Right) Insulin protects hippocampal synaptic spines against oligomer binding and toxicity.

Therapeutic strategies : Some antibodies are oligomer specific[231], including new scFv antibodies[232]. Oligomers occur in the CSF at lower concentrations than monomers, yielding better stoichiometry for antibodies, which penetrate the brain poorly. scFvs are especially intriguing given recent findings that these smaller molecules can reach the brain following intranasal administration[233].

A second approach exploits neuronal insulin signaling. Although oligomers cause insulin receptors to be removed from synapses, rendering neurons insulin resistant and triggering a loss of synaptic spines,the binding and synaptotoxicity of oligomers can be prevented by robust insulin signaling[234] (Fig 1-right). Age-onset deficiencies in CNS insulin signaling thus may be a potential risk for AD[235], especially given the additional role of insulin in lowering Aβ levels[236]. Insulin mimetics seem to offer therapeutic potential[237, 238]. Indeed the overlap between insulin signaling and more general neurotrophin signaling is an open research issue. Nonetheless, this may provide an explanation for the convergence between diabetes and Alzheimer.

Oligomers and prion receptors.

Oligomers may use cellular prion protein as a "toxin receptor"[239]. This could account for the specificity of oligomer accumulation at certain postsynaptic sites[224, 240, 241], although the mechanism may be more complex [242, 243]. Critically, the Aβ oligomer receptor hypothesis allows the possibility of therapeutic antagonists.

Lauren et al. identified the cellular prion protein PrP^C as an Aβ oligomer binding site by expression cloning[244]. Aβ oligomer binds to PrP^C with nM affinity and high selectivity (>30-fold by weight, and >1000-fold by molar concentration) for oligomeric Aβ over monomeric Aβ[244-248]. Aβ oligomer binding leads to downstream dysfunction of NMDA receptors[239, 249, 250] (Fig. 2A) .

Synaptic responsiveness in hippocampal slices from adult PrP^C null mice is normal, but Aβ oligomers fail to block long-term potentiation (LTP) in the absence of PrP^C [244]. The precise preparation of Aβ oligomers is critical for demonstration of PrP^C-dependence[248, 251]. Moreover, Aβ oligomers extracted from human AD brain require PrP^C to inhibit LTP in slices[248] and in vivo[252]. A physical association of Aβ with PrP^C in human AD brain has been documented[253]. In vivo, Aβ oligomer-triggered cell death also requires PrP[254], and PrP^C is essential for oligomer-induced alterations in cholinergic neuron electrophysiology[255]. The Aβ/PrP^C complex activates Fyn kinase to disrupt NMDA receptor function at post-synaptic sites[241]. Thus, PrP^C serves as a receptor component capable of mediating multiple neuronal toxicities of Aβ oligomers in vitro and in vivo.

The necessity for PrP^C in mediating those aspects of AD modeled in APPswe/PSen1ΔE9 transgenic mice has been tested[256] - absence of PrP^C has no detectable effect on APP/Aβ metabolism or plaque deposition but they are rescued from serotonin axon degeneration, loss of synaptic markers and early death, and from learning and memory deficits[256] (Fig. 2B) there was no effect on cognitive dysfunction in young APP transgenic J20 mice[257].

Can PrP^C be a therapeutic target? The Wisniewski laboratory treated aged, memory-impaired APPswe/PSen1M146L mice with an anti-PrP^C antibody, 6D11, that blocks Aβ oligomer binding[258]. Treatment completed reversed memory deficits and restored synaptic density. Because *Prnp*-/- mice have little or no phenotype, blockade of this site is not predicted to yield "on-target" side effects, other than blocking the deleterious effects of oligomers on synaptic toxicity.

• Box 4: Active and passive immunization therapies: what have we learned?

Active immunisation. Now over a decade ago, Dale Schenk and colleagues working at Elan Pharmaceuticals performed a seminal experimental study which initiated a new therapeutic concept in neurodegenerative diseases –immunotherapy[259]. They found that immunisation of PDAPP mice with full length Aβ42 peptide markedly reduced Aβ plaques. If immunized at an early age, before plaques had developed, this treatment prevented the formation of plaques; if immunised later (aged mice) this treatment resulted in a reduction of plaque load. These experiments were followed by a clinical study in 80 patients with mild-moderate Alzheimer's disease, 64 of whom received the active agent (AN1792: Aβ42 with adjuvant) and 16 received adjuvant alone. Approximately half of the actively immunised patients developed circulating antibodies to Aβ, without significant safety concerns[260].

The systematic long term clinical and neuropathological follow up studies provided evidence for plaque removal from the cerebral cortex which varied from minimal to extensive, with even a few cases in which plaque clearance was almost complete[261-265]. Histological quantification demonstrated a significantly lower cortical Aβ42 load in immunised AD patients compared to the control group (1.42% vs. 5.25%)[262]. However, despite the evidence of a substantial reduction in Aβ plaques, there was no benefit either in terms of time to the development of severe dementia or survival time[263]. Even those patients who demonstrated almost complete clearance of plaques had declined to a state of end stage dementia when assessed shortly before death: plaques are therefore not the defining aspect of AD.

Neurological side effects of Aβ immunotherapy in patients with AD were reported, which led to the termination of th eAN1792 development project[264, 266, 267]. Specific attempts have been made to alter the immunotherapy approach to avoid these complications, including modifying the immunogen to avoid a T lymphocyte response, conjugating antibodies to large molecules to prevent them crossing the blood-brain barrier, specifically targeting Aβ oligomers. Recently a new N-terminal Aβ-specific antibody (CAD106) without a specific T-cell response has proved to be safe and tolerable in a phase 1 clinical study. The majority (80%) of patients developed an Aβ antibody response without severe neurological side effects[268].

It remains to be seen whether immunization approach can be used to target the toxic species of amyloid seeds.

Passive immunisation. Passive immunotherapy consists in the use of a antibody raised in an animal which has been "humanized" so that it can circulate in the human body without being recognized by the innate immune system. It has the advantage of providing immediate protection but, it is costly, effective for only a short duration of time (monthly injections) and may lead to complications.

Unfortunately the latest results of Phase III clinical trials have not been encouraging. Bapineuzumab, a monoclonal antibody against the N-terminus of Aβ reduces brain amyloid load, as demonstrated in vivo by PiB PET scans before and after treatment, was shown to provide some protection against cognitive decline in subgroup analysis[269], however the drug failed to reach its primary endpoint. Moreover neurological side effects were observed which have led to the halt of further development. Almost at the same time it has been announced that solanezumab, another N-terminal Aβ antibody did not meet its primary endpoints in two phase III studies on mild-to moderate stage AD patients. Nevertheless, pre-specified secondary analysis of pooled data (>2000 patients enrolled into the two studies EXPEDITION1 and EXPEDITION2, with dosing over 18 months) on the subset of mild AD patients showed a statistically significant deceleration of cognitive decline (p=.001) compared with placebo, as measured by the ADAS-Cog_{14}; this finding represented a 34 percent reduction in decline. There were no CNS side effects and the sponsor, Lilly, is discussing with regulatory bodies whether to continue the project.

It may well be that a major reason of failures is that the antibodies have been tested in patients with relatively advanced phase of AD, where the role of Aβ-mediated effects already less significant. Nevertheless, the clinical trials of monoclonal antibodies in prodromal AD are ongoing and the developers decided to transform the phase II studies into phase III by doubling the number of patients in case of gantenerumab. The results are expected in 2015. The efficacy of early intervention will be tested also in familial AD patients (API trial), where crenezumab will be administered before the onset of symptoms.

Immunization against tau. Recently, some reports indicated that active and passive immunization against tau were also successful in rodent models of Tauopathies[91-94, 96, 270]; These preclinical studies support that tau immunotherapy, targeting selectively pathological tau is a promising approach against tau pathology. Different research groups conducted in at least six different animal models, using both immunization modes: active immunization with different tau antigens, administering them either subcutaneously or intraperitoneally, varying the number immunisations (1-8 injections), in differently aged animals [young (before disease onset), middle aged , or aged]; and passive immunization (with different antibodies raised against different epitopes). These various immunization studies revealed a decrease in tau pathology burden, and in some studies, also a functional improvement was reported, either in motor function, and more importantly and relevant to dementia – also an improvement in cognitive impairment.

Therapeutic implications. The failure of bapineuzumab shows that neutralizing the right epitope is criticalThe multiple clinical trials which are ongoing may demonstrate that Aβ immunisation can slow down the cognitive decline in patients with established AD. Alternatively, it may prove this approach is too simplistic once the complex dynamic interactions of AD pathology are established in the brain. Already there is considerable interest in much earlier intervention. Is it possible that younger adults could be immunised safely, before the AD process has begun? However, do we immunize against oligomers, seeds or even tau? Would this prevent accumulation of Aβ or tau in humans and if so would this prevent the development of the other features of AD pathology and the associated cognitive decline? These are crucial questions not only for Alzheimer's disease, but also for the many other neurodegenerative diseases which are characterised by abnormal protein accumulation which may be amenable to a similar therapeutic or preventative approach.

- **BOX 3. The new diagnostic framework for Alzheimer's Disease (Clinical Diagnosis Box)**

1) In 1984, the NINCDS-ADRDA criteria for Alzheimer's disease (AD) were based on the following rules:
- the diagnostic of Alzheimer's disease *cannot be certified* clinically and needs a post-mortem confirmation to be ascertained ;
- therefore, the *clinical* diagnosis of AD can only be "probable";
- and the diagnosis of AD can only be made when the disease is advanced and reaches the threshold of a *dementia*.

2) This definition has open the way to the concept of MCI (Mild Cognitive Impairment) for patients at a predementia stage of the disease.
3) The NINCDS-ADRDA criteria had two main limitations:
- a low accuracy: because they do not take into account the specific features of the disease;
- a late occurrence in the course of the disease: because they apply only when the dementia threshold is reached.

4) Since 1984, specific features of clinical AD have been demonstrated in different domains:
- an amnestic syndrome of the hippocampal type with specific memory test (FCSRT): it is characterized by a very poor free recall performance and a decreased total recall (free + cued) score because of an insufficient effect of retrieval facilitation with cueing;
- a structural atrophy of medial temporal lobe structures, mainly the hippocampus, on volumetric MRI;
- a low Aβ, high tau and phospho-tau levels in the cerebro-spinal fluid (CSF);
- a parieto-temporal hypometabolism in PET-FDG;
- a specific retention of amyloid radioligand in PET.

1) Interestingly, each of these specific patterns has been shown to be present even at the prodromal, predementia stage of AD.
2) Accordingly, new research criteria for the diagnosis of AD has been proposed in 2007 by an International Working Group, that highlighted for the first time the added value of biomarkers for the diagnosis of AD. These criteria are based on:
- *a specific clinical phenotype* consisting of an amnestic syndrome of the "hippocampal type" that can be isolated or associated with other cognitive/behavioral changes;
- *the presence of one or several biomarkers* that can be structural (atrophy of the hippocampus with MRI); biological (specific changes in CSF) and/or functional/molecular (neuroimaging pattern/amyloid ligand retention on PET).

3) These new criteria testify of a conceptual shift from a clinico-pathological entity to a clinico-biological entity that is characterized by new rules :
- it is possible to access the underlying pathology via biomarkers that can be considered as surrogate markers of the histopathological changes;
- the clinical diagnosis of AD can now be established in vivo and no more reference to dementia is needed.

4) A new lexicon was proposed in 2010 where:
- Alzheimer's disease is considered as a symptomatic entity that encompasses both the prodromal and dementia stages;
- a new concept of atypical AD is proposed which refers to the less common clinical phenotypes of the disease (primary progressive non-fluent and logopenic aphasias, frontal variant of AD and posterior cortical atrophy) that occur with Alzheimer's pathology;
- Mild Cognitive Impairment (MCI) is only applied when no disease can be diagnosed;
- preclinical states of AD refer to the long asymptomatic stage of the disease and they include 2 different conditions:
    - *asymptomatic at risk for AD* : that characterizes cognitively normal individuals with evidence of amyloidosis in the brain or in CSF;
    - *presymptomatic AD*: that characterizes cognitively normal autosomal dominant mutation carriers who will develop AD.

5) According to this new lexicon, the biomarkers of AD can be divided into :
- *pathophysiological biomarkers* including CSF changes and PET amyloid ligand retention;
- and *topographical biomarkers* including medial temporal lobe atrophy on MRI and temporal parietal hypometabolism in PET FDG.

6) Based on this proposal, the recommended diagnostic procedure for prodromal AD is:
- *a specific clinical phenotype* with an amnestic syndrome of the hippocampal type;
- with the presence of *a pathophysiological biomarker* that can be:
    - specific changes of CSF: low Aβ and high tau or phospho-tau
    - PET amyloid ligand retention.

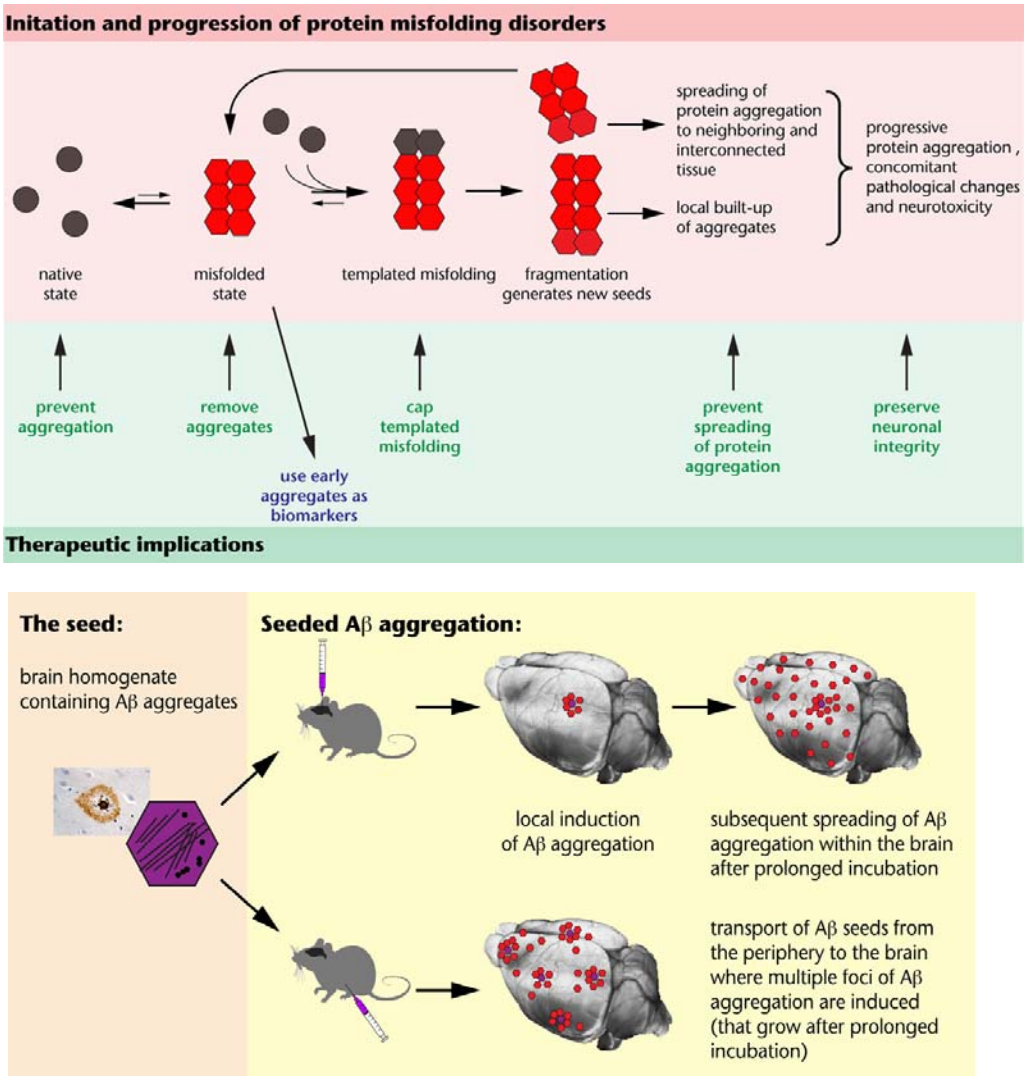
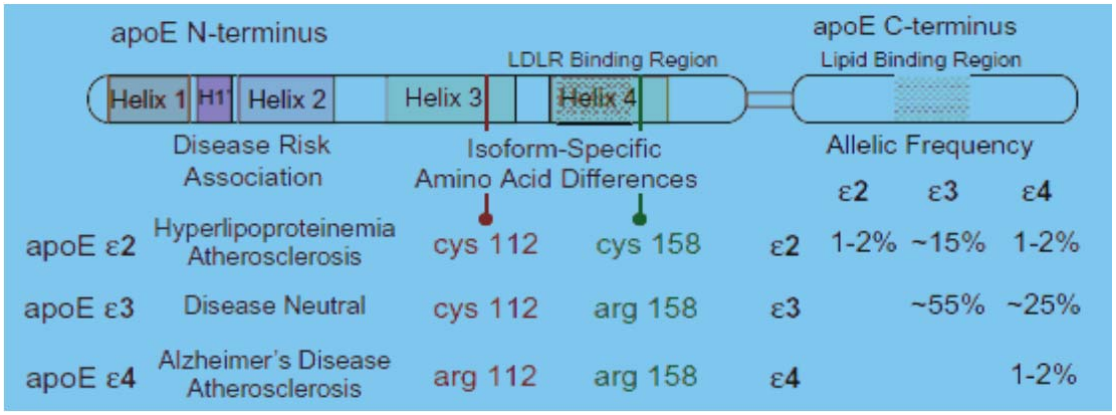


Figure 2. The seeding hypothesis

Figure


apoE N-terminus
apoE C-terminus
LDLR Binding Region
Lipid Binding Region
Helix 1
H1'
Helix 2
Helix 3
Helix 4
Disease Risk Association
Isoform-Specific Amino Acid Differences
Allelic Frequency
ε2
ε3
ε4
apoE ε2
Hyperlipoproteinemia Atherosclerosis
cys 112
cys 158
ε2
1-2%
~15%
1-2%
apoE ε3
Disease Neutral
cys 112
arg 158
ε3
~55%
~25%
apoE ε4
Alzheimer's Disease Atherosclerosis
arg 112
arg 158
ε4
1-2%

Supplementary Material

• Background to tau protein

Supplementary material. Tau proteins belong to the family of microtubule-associated proteins. A single gene, named *MAPT* located at position 17q21 encoded for several isoforms resulting from alternative splicing of exons 2, 3 and 10 in the human adult brain. Tau proteins constitute a family of six isoforms (six mRNAs) that range from 352 to 441 amino acids. The Tau isoforms differ from each other by the presence of either three (3R) or four repeat-regions (4R) in the carboxy-terminal (C-terminal) part of the molecule and the absence or presence of one or two inserts (29 or 58 amino acids) in the amino-terminal (N-terminal) part[271-274]. Tau isoforms are likely to have specific functions related to absence or presence of regions encoded by cassette exons 2, 3 and 10. Tau proteins bind microtubules through repetitive regions in their C-terminal part. These repetitive regions are the repeat domains (R1-R4) encoded by exons 9 to 12[275]. Besides its major microtubule-binding, -stabilizing, paralleled-ordering functions, Tau also has other functions. Tau proteins bind to cytoskeleton protein components such as actin, neurofilament, spectrin[276-280]. There is also evidence that Tau proteins interact with cytoplasmic organelles. Such interactions may allow for binding between microtubules and mitochondria[281]. The Tau N-terminal projection domain also permits interactions with neural plasma membrane[282].

Tau proteins are subjected to multiple control mechanisms, particularly phosphorylation. There are 85 potential phosphorylation sites on the longest Tau isoform (See Figure 3). 71 among the 85 putative phosphorylation sites can be phosphorylated in physiological or pathological conditions (For review see[274]. Most of the phosphorylation sites are surrounding the microtubule-binding domains, in the proline-rich region and carboxy-terminal region of Tau. A total of more than 20 protein kinases can phosphorylate Tau proteins. Recent evidence supports a role for the microtubule-binding domain in the modulation of the phosphorylation state of Tau proteins. A direct and competitive binding has been demonstrated between residues 224-236 (according to the numbering of the longest isoform) and microtubules on one hand, and residues 224-236 and protein phosphatase 2A (PP2A) on the other hand[283]. As a consequence, microtubules could inhibit PP2A activity by competing for binding to Tau at the microtubule-binding domains. The lysine residue 280 is crucial for microtubule-binding of 4R Tau. This lysine is mutated in heritable form of FTDP-17 and promotes Tau aggregation *in vitro* and in vivo[284, 285]. Much more recently, acetylation of Tau has been shown also to regulate the binding of Tau to microtubules[286, 287]. Histone acetyl transferase P300 or CREB-binding protein (CBP) and deacetylase SIRT1 or HDAC6 likely regulate the acetylation of Tau[286, 287]. Moreover, Tau-acetylation is suggested to promote Tau aggregation and is observed in animal models, Alzheimer's disease and brain tissues of patients with Tauopathies, at the exception of Pick's disease where Pick bodies are negative for acetylated lys-280 as well as phosphorylation at ser-262[286, 288, 289]; . However, due to selective aggregation of 3R Tau in Pick's disease[289, 290], acetylation of 3R Tau on another lysine residue cannot be precluded.

• Peripheral amyloidosis. What we can learn from sporadic inclusion body myositis? (O Benveniste)

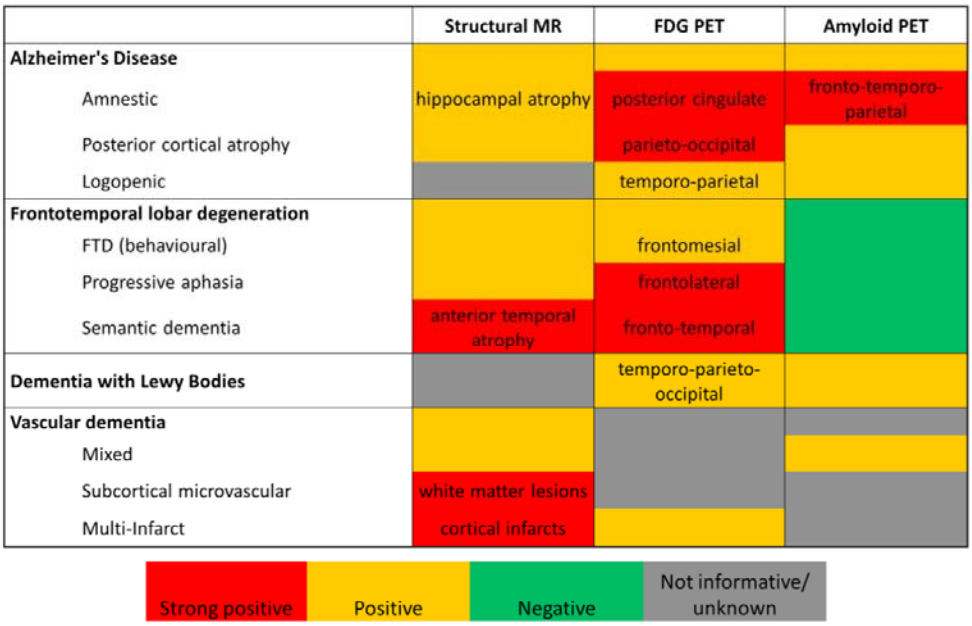
Sporadic inclusion-body myositis (s-IBM) is a disabling (but not lethal) muscle disease in persons over 50 years (median age of first symptoms: 61 years), which features deposition of Aβ and hyperphosphorylated tau[291]. Can we learn from s-IBM? No specific treatments are currently recommended and immunosuppressant drug therapies could even exacerbate progression of disability[291]. The s-IBM muscle biopsy exhibits an unusual and specific pathologic phenotype, which combines multifaceted muscle fiber degeneration with an extracellular T-cell inflammation. s-IBM muscle-fiber degeneration is characterized by vacuolization and intra-muscle-fiber accumulation of ubiquitinated, congophilic multiple-protein aggregates such as Aβ and phosphorylated tau deposits[292].

By immunohistochemical study with antibodies directed against ubiquitin, amyloid-β precursor protein (AβPP), Aβ ,SMI-31, SMI-310, Tar-DNA binding protein-43 (TDP-43) and p62, the two latter were shown to be the most sensitive markers[293]. These deposits are accompanied with protein degradation dysfunctions (at both, proteasome and autophagy levels)[292]. From different experiments in mouse models, it appears that the forced over-expression of different kind of amyloid proteins (βAPP42, gelsolin) leads to muscle weakness, appearance of vacuoles with, in parallel, increase of proteasome and autophagy markers[294, 295]. It seems that, when the protein degradation systems are overloaded, amyloids appear within muscle fibres, *e.g.* as misfolded and/or ubiquinated protein accumulations. This situation may also exist in patients with a hereditary inclusion body myopathy due to a p97/VCP mutation, i.e. in a complex responsible of the regulation of protein degradation through the proteasome and autophagy.

The second hallmark of sIBM is the presence of inflammatory infiltrates. Effector cells can be clonally expanded and mostly consist of CD8+, CD28- T cells which can exert cytotoxic activity and are found surrounding or invading muscle fibres[296]. The latter may present so far unknown auto-antigens to these effector cells in a MHC class I restricted manner. MHC class I overexpression at the surface of muscle fibres has become a surrogate marker of inflammation. Most of these immune abnormalities are not only observable in muscle but also in the peripheral blood[297]. Apart from the cellular immune response, auto-antibodies start to be also described in sIBM[298]. In this context, the cytokines IFN-γ and IL-1β have been demonstrated to be important inducers of accumulation of Aβ in muscle cells[299]. The lessons of inflammation in s-IBM may be useful for AD.

The key question still remaining is the following: Are the amyloid deposits a cause or a consequence of inflammation? Indeed, if amyloids are a consequence of a primary immune reaction including secretion of cytokines that increase MHC class I expression to such an extent that protein degradation capabilities are overloaded, then a targeted immune intervention (such as by biotherapies) may be useful. On the contrary, if sIBM is a degenerative disease, where the accumulation of unfolded proteins causes a secondary immune reaction, an immunointervention may be of limited effect, if at all. However the possibility of biopsies and the peripheral localisation of the disease encourage much further research.

Figure S1. Diagnostic features provided by imaging biomarker techniques.



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