

Review

Translation of Pre-Clinical Studies into Successful Clinical Trials for Alzheimer's Disease: What are the Roadblocks and How Can They Be Overcome?¹

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Abstract. Preclinical studies are essential for translation to disease treatments and effective use in clinical practice. An undue emphasis on single approaches to Alzheimer's disease (AD) appears to have retarded the pace of translation in the field, and there is much frustration in the public about the lack of an effective treatment. We critically reviewed past literature (1990–2014), analyzed numerous data, and discussed key issues at a consensus conference on Brain Ageing and Dementia to identify and overcome roadblocks in studies intended for translation. We highlight various factors that influence the translation of preclinical research and highlight specific preclinical strategies that have failed to demonstrate efficacy in clinical trials. The field has been hindered by the domination of the amyloid hypothesis in AD pathogenesis while the causative pathways in disease pathology are widely considered to be multifactorial. Understanding the causative events and mechanisms in the pathogenesis are equally important for translation. Greater efforts are necessary to fill in the gaps and overcome a variety of confounds in the generation, study design, testing, and evaluation of animal models and the application to future novel anti-dementia drug trials. A greater variety of potential disease mechanisms must be entertained to enhance progress.

Keywords: Alzheimer's disease, animal model, dementia, memory disorder, pre-clinical, treatment

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia. Over 7% of the world's population above 65 years of age (over 24 million people) suffer from dementia, with the number of cases estimated to double every twenty years [1–3]. In the most populous countries of the world, India and China, these numbers are estimated to be 3–8 million and are expected to double by the year 2030 [4, 5].

Translational medicine has emerged as a reaction to the slow speed by which new medical research findings are transformed to improved therapies of human disorders. The intent of this movement is to help bench and clinical researchers learn from each other and thus benefit patients [6] forming a “translational cycle” [7]. The most common use of the term translational research describes a “bench-to-bedside” flow, occasionally distinguishing two translational phases. T1 referring to “transfer of new understanding of disease mechanisms gained in the laboratory into development of new methods for diagnosis, therapy, and prevention and their first testing in humans”; T2, involves two phases, T2a, which describes the translation to patients or clinical care and T2b as translation to practice and health decision making [8, 9] (Fig. 1). The focus of this review is on this arm of the translational research cycle; the contribution of recent studies on animals and more direct systems in the development of therapeutic targets and drugs for treatment of AD.

AD is a complex disease and more of a syndrome. The global threat of this syndrome and its associated social and economic burden has tremendously increased over last two decades [4, 5]. A variety of etiological factors has been proposed to contribute the pathogenesis of the disease. However, there is almost universal acceptance that proliferation of amyloid- β (A β) deposition is the root of AD. The central prob-

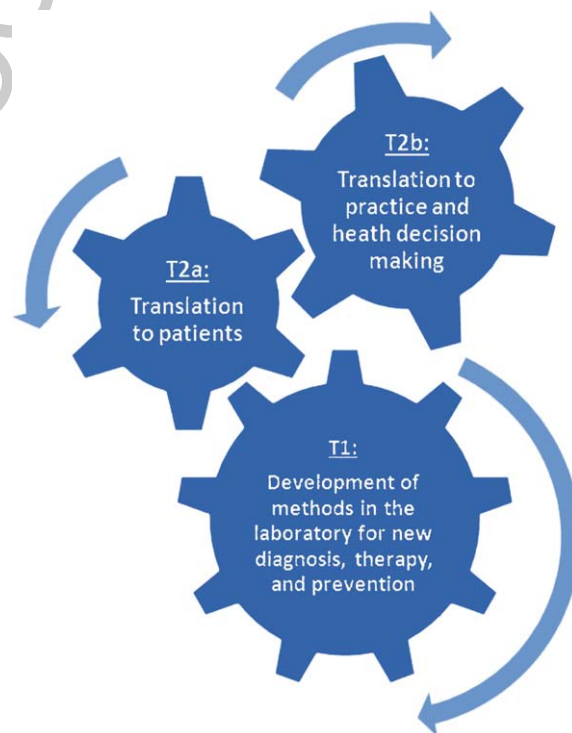


Fig. 1. Pathways from basic discoveries at the bench to clinical practice via essentially three stages (T1 to T2b).

lem confronting AD research today is the failure to recognize other important causative pathological factors in parallel with A β pathology. Perhaps this alone has contributed to the failure of multiple drug trials. A new consensus hypothesis to replace one based on A β may not necessarily solve the problem, rather replicate the problem all over again. It is certain that A β plays a role in AD pathology but other factors ought to be equally investigated simultaneously for better pre-clinical outcomes. Such an approach may also provide better biomarkers for trials and result in patient cohorts that may be less variable in their response to treatments.

New treatment strategies such as cell transplantation and immune-modulation have shown promising outcomes in various disease models. Their translation, however, into effective clinical treatments has generally not met expectations [10]. For example, the application of traditional herbal extracts such as *Bacopa monniera* [11–13] and traditional Chinese medicines [14] in animal models appear promising but these findings require further refining for translation to successful human trials [15–17]. Similarly, other studies based on phytochemicals, such as Nanocurcumin and S-Allyl-Cysteine (SAC), have also shown promising results in animal models [18, 19]. However, there is a general reluctance to launch new clinical trials based on herbal extracts as there are concerns about their safety, efficacy, and mode of action [20, 21].

The absolute reliance on animal models has been cited as a major factor in the impedance of drug discovery [22]. There are no animal models of any human

disease, in particular of those that affect specifically human behavior, cognition, changes in mood and similar, and chronic in nature [23, 24]. Animal models at best mimic certain elements of human pathology. While it is imperative that animal models have added valuable information to our current understanding, the limitations in humanized disease models argue that the critical step in translation is the understanding of human disease pathology as a prerequisite for designing informative animal experiments.

Discovery of successful new drugs for clinical benefit of AD requires stringent analysis for safety and efficacy in suitable animal and cell-culture models. Over the last three decades, several animal models of AD have been developed to understand disease mechanisms and identify therapeutic targets, as well as screen novel drugs derived from synthetic chemistry and/or traditional herbal formulations [25]. Based upon different theories, multiple approaches have been used



Fig. 2. Road blocks in the translation of preclinical outcomes in Alzheimer's disease (AD) from animal to human.

for developing animal models ranging from traumatic brain injury [26] and neuronal cell death induced by intracranial delivery of neurotoxins to specific brain areas [27] and the generation of transgenic mouse models by genetic manipulation which impact upon molecular pathways involved in the pathogenesis of the disease [28, 29]. Naturally, these models need to be widely reproducible and validated, and have the characteristic neuropathological and behavioral/cognitive features of AD. However, it seems a daunting task to decipher which model(s) among the numerous produced may best simulate preclinical disease.

Given these impediments in translating basic research from animal models to treatments, a critical re-evaluation of current animal models as well as clinical trials methodology is imperative to understand the road blocks in the translation of preclinical knowledge for human benefit [30–32]. This review identifies key obstacles (Fig. 2) by use of examples where either central dogmas have been challenged or previous inexact research has established flaws in current understanding. We briefly review the impending limitations in diagnostic as well as treatment strategies, the weaknesses of the amyloid hypothesis of AD, and discuss why some other AD-related pathology should be considered. We then discuss the animal models and their inherent problems in recapitulating human disease, and the need for better integration of basic and clinical studies and for controlled and validated methods for design and analysis of clinical trials.

SLOW PROGRESS IN DIAGNOSIS OF AD

There have been increased efforts to discover unique biomarkers for early diagnosis and identify populations at high risk [33]. An optimal biomarker not only enhances the chance of early detection of the disease but also strengthens attempts to determine the prognosis, enabling disease monitoring. The current strategies do not focus on different biomarkers for different purposes but lump them together. Instead, it should depend upon the use at different stages of the disease management such as, detection of pathology, prediction of progression, surrogate marker of efficacy, or disease modification. Furthermore, if multiple biomarkers are found to reflect different cohorts of AD patients, they can be used to establish the optimal population for use in clinical trials of targeted therapeutics. There are several studies directed at identifying and validating biomarkers of AD in the blood or cerebrospinal fluid (CSF) [34–36] and at seeking neuroimaging, genomic,

and epigenetic markers [37, 38], as would be expected of a multifactorial disease, but no single biomarker is reliable and valid [39]. The existing clinical accuracy, including sensitivity and specificity of the markers, remains relatively low [40].

CSF levels of the A β , total Tau, and phosphorylated Tau (pTau) in AD subjects are measured as potential diagnostic biomarkers [40–43]. Although these CSF markers enable categorization of the patients as mild cognitive impairment (MCI) or AD [43], they could only be established naturally by utilizing neuropsychological testing followed by biomarker evaluation and not *vice versa*. A β profiles may overlap considerably between non-cognitively impaired and AD subjects, even in subjects with the A673T mutation in A β PP gene. This suggests that it is not the amount of A β generated but post-cleavage processing that might be contributing to the differences between plaque formation (perhaps a beneficial factor to rid the brain of the soluble forms of A β) and processed soluble species (perhaps oxidized forms) in causing dementia [44, 45]. Hence, there is confusion whether the blood and CSF based biomarkers should be used for diagnosis or prognosis of the disease. Nevertheless, reliable biomarkers, which can differentiate AD from MCI, are warranted for appropriate treatment administered early in disease progression [46–48]. Standardization and validation of biomarkers thus play a critical role in the drug discovery process.

It is acknowledged that there is a high variability in the accuracy of CSF biomarkers which have been tested in various centers worldwide [39, 49]. While an association between disease progression and increased level of total Tau and pTau with concomitant decrease in A β _{1–42} concentrations in CSF has been documented, the optimal reference range has not been defined due to variability in their levels. This variation could be due to the multifactorial nature of the disease in different populations or simply the use of various antibodies and ELISA sources in different laboratories. However, concerted efforts are needed to standardize procedures for biomarker assays and improve reproducibility between laboratories [39, 50].

There is also need for reliable clinically acceptable neuroimaging markers. Position emission tomography (PET) imaging biomarkers such as ¹¹C-labelled PiB (Pittsburgh Compound B) have been useful for *in vivo* imaging of A β distribution as a research tool [51]. The wide use and applicability of this compound in PET imaging is, however, limited due to its cost and short half-life (20 minutes), which mandates the availability of a cyclotron on-site for production of the isotope.

On the other hand, ^{18}F ligands (florbetapir, florbetaben, and flutemetamol) with a half-life of 110 minutes make $\text{A}\beta$ PET imaging more attractive. A multicenter study has shown that florbetapir PET can identify individuals at increased risk of progressive cognitive decline [52]. All the three ligands are now approved by the US Food and Drug Administration (FDA) [53]. Subsequently, two more ^{18}F ligands, florbetaben and flutemetamol, were approved. Although these agents do not establish a positive diagnosis of AD due to their ability to identify individuals at high risk, they have potential for use in new drug development (Chase A, 2014). The use of these ligands along with other biomarkers including as magnetic resonance imaging (MRI), fluorodeoxyglucose (FDG) PET, CSF protein and clinical score of Alzheimer's Disease Assessment Scale (ADAS-Cog) will improve accuracy of diagnosis and predicting conversion from MCI to AD [54]. Combining the neuroimaging traits with epigenetic biomarkers is also under serious consideration to diagnose the disease even at the early stage, but these efforts are still in their infancy [55, 56].

Amyloid imaging and CSF screens for $\text{A}\beta_{1-42}$ and Tau protein levels could be of value in defining cognitively normal subjects who do not have preclinical AD pathology. For the past 50 years, studies have used controls that include subjects who had these early stages of the disease [57]. The use of "super-controls" could be of value in assessing premorbid anatomical and functional changes.

One problem in diagnosing AD is that the pathology changes over time, resulting in different stages of AD pathology [58], and the biomarkers used to detect AD may need to be matched to the pathological stages of AD [59, 60]. Measuring longitudinal patterns of changes in a set of different biomarkers may be the most reliable way to diagnose AD and measure its progression [60]. Relevant mouse models could provide vital information for translation to humans [61]. The use of both fluid (CSF and blood) biomarkers in combination with brain imaging and correlating changes with cognitive deficits would provide a means for the early detection of AD and for predicting which patients with MCI develop AD.

TREATMENT FOR AD: FOCUS BEYOND NEUROTRANSMITTERS

After several decades of research in the field of AD, there are, only two classes of FDA approved drugs for AD, namely acetylcholinesterase (AChE)

inhibitors (donepezil, rivastigmine, and galantamine) and N-methyl-D-aspartate (NMDA) receptor antagonists (memantine). These are safe and efficacious but only offer short symptomatic relief without altering underlying disease pathology [62, 63].

Recent developments in treatment strategies include immune therapy consist of three approaches: active immunization, passive immunization, and immunomodulation. These approaches are mostly targeted against the insoluble oligomeric or fibrillar forms of the $\text{A}\beta$ peptide. In addition, there are developments to counteract Tau pathology [64–66]. The humanized antibodies raised against these aggregated proteins or peptides are administered in passive immunization, whereas in active immunization the vaccine contains antigens, which generate antibodies in the recipient. The immunomodulation therapy consists of cytokine administration, which is able to alter the immune response in host against $\text{A}\beta$ processing. Several animal studies have shown promising results after introduction of the immune therapies [66–68]. The positive outcome from preclinical immunomodulation studies has led to investigation of $\text{A}\beta$ targeting molecules such as tarenfluril (Myriad Genetics, USA), semagacestat (Eli Lilly and Company, USA), tramiprosate (Neurochem Inc., Canada), ELND006 and AN1792 (Elan Corporation, Ireland), and ponezumab (Pfizer, USA) in randomized, controlled trials but most of them could not successfully satisfy the safety and efficacy issues in human. None of these trials has proceeded beyond phase III due to negative primary outcomes [69]. Immune therapies showed worsening of cognitive performance and poor amyloid clearance [66]. These were accompanied by adverse events of microhemorrhages and increased deposition of $\text{A}\beta$ in the vasculature causing harmful effects within the parenchyma [70]. The challenge of immunotherapy therefore, lies in the identification of the relevant antibody variants that can successfully clear the forms of $\text{A}\beta$ responsible for the synaptic dysfunction with minimal adversity [70]. This approach requires the identification of the $\text{A}\beta$ species, for which there is no current consensus.

There are also other strategies such as kinase inhibition [71], microtubule stabilization [72], vitamin supplementation [73], aggregate disintegration [74], among others, which have been tested in preclinical settings. Application of metal chelators such as clioquinol and PBT2 in arresting $\text{A}\beta$ pathology also showed promising results in animal models [75]. Phase II clinical trials with PBT2 also improved cognitive functions in human subjects [76, 77]. It was further demonstrated that these metal ionophores have a strong

Table 1
Current list of clinical trials in Alzheimer's disease on compounds or strategies other than amyloid immunotherapy, acetylcholinesterase inhibition or glutamate antagonism (memantine). Data obtained from the site maintained by National Institute of Health; Web: www.clinicaltrials.gov

Molecule	Purpose	Sponsor/ Collaborator Countries	Study Design	Subject	Phase	Duration	Current Status	Outcome
TRx0237 [Trial ID: NCT01689233]	To assess the safety and efficacy of this Tau based new molecule	TauRx Therapeutics Ltd., USA	Double-blind, placebo-controlled, randomized	Mild to Moderate AD	Phase III	2012-	Ongoing but not recruiting participants	Study result not available
CERE-110 [Trial ID: NCT00876863]	An Adeno-associated virus to transfer the gene to make NGF in brain to protect nerve cells	Ceregene, USA	Double-blind, placebo-controlled (sham surgery)	Mild to Moderate AD	Phase II	2009-	Unknown	Study result not available
Semagacestat (LY450139) [Trial ID: NCT00762411]	A gamma-secretase inhibitor to lower beta amyloid in blood, spinal fluid and brain	Eli Lilly and Company, Asia, Europe	Double-blind, placebo-controlled, randomized	AD with MMSE score of 16	Phase III	2008-2011	Completed	Could not slow down the disease progression and worsened symptomatic outcomes compared to placebo Study result not available
Exendin-4 [Trial ID: NCT01255163]	To assess safety and efficacy of this syntheticglucagon-like peptide-1 agonist to protect nerve cells	National Institute on Aging (NIA), USA	Double blind, randomized, placebo-controlled	Early-stage AD	Phase II	2010-	Recruiting participants	Study result not available
Bryostatin 1 [Trial No: NCT00606164]	To evaluate the safety, efficacy and pharmacokinetics of this PKC modulator	Blanchette Rockefeller Neurosciences Institute, USA	Randomized, double-blind, placebo-controlled	Mild to Moderate AD	Phase II	2008-	Unknown	Study result not available
EVP-6124 [Trial ID: NCT01073228]	To evaluate the safety and efficacy of this selective alpha7 nicotinic receptor agonist through cholinergic neurotransmission	EnVivo Pharmaceuticals, USA, Russia	Randomized, double-blind, placebo-controlled	Mild to Moderate probable AD	Phase II	2010-2012	Completed	The dose was safe and well tolerated and did not show any gender, age or food effects
ST101 (ZSET1446) [Trial ID: NCT00842673]	To evaluate the safety and efficacy of this azaindolinone derivative compound	Sonexa Therapeutics, Inc. USA, Canada	Randomized, double-blind, placebo-controlled	AD	Phase II	2009-2011	Completed	Study result not available
Resveratrol (trans-3,4',5-trihydroxy stilbene) [Trial ID: NCT01504854]	To evaluate the impact of this natural phenol extracted from plants in delaying or altering the mechanism of aging and dementia	Alzheimer's Disease Cooperative Study, National Institute on Aging, USA	Randomized, double-blind, placebo-controlled	Mild to Moderate AD	Phase II	2012-2014	Completed	Study result not available
Octagam [Trial ID: NCT00812565]	To evaluate the effect of intravenous immunoglobulin infusion on Aβ peptide levels in CSF and blood plasma	Octapharma, USA, Germany	Double-blind, randomized, placebo-controlled	Mild to Moderate AD	Phase II	2008-2011	Completed	Plasma Aβ level was found to be insignificant between placebo and treated among 5 out of 6 intervention groups

LY450139 Dihydrate (gamma-secretase inhibitor) [Trial ID: NCT00244322]	To evaluate the safety, tolerability and level of Aβ in plasma and CSF	Eli Lilly and Company USA	Randomized, double-blind, dose-escalation, placebo-controlled	Mild to moderate AD	Phase II	2005-2007	Completed	Doses well tolerated with reported adverse events. Decreased plasma Aβ concentration but no cognitive difference
Semagacestat (LY450139) (gamma-secretase inhibitor) [Trial ID: NCT00594568]	To evaluate the effect of γ-Secretase Inhibition on the progression of AD	Eli Lilly and Company USA	Randomized, double-blind, placebo-controlled safety/efficacy study	AD	Phase III	2008-2011	Completed	Did not slow disease progression and cognitive worsening with severe adverse events reported
NIC5-15 (Pintol) [Trial ID: NCT01928420]	To evaluate the safety and efficacy of this plant product with mild insulin sensitizing effects	James J. Peters Veterans Affairs Medical Center, USA; National Center for Complementary and Integrative Health (NCCIH), USA; Humanetics Corporation, USA	Randomized, double-blind, placebo controlled	AD	Phase II	2010-	Recruiting	Study result not available
ELND005 (scyllo-inositol) [Trial ID: NCT00568776]	To evaluate the dose-related safety and efficacy of this naturally occurring plant sugar alcohol	Transition Therapeutics Ireland Limited; Multi-center	Randomized, double-blind, placebo-controlled, dose-ranging	Mild to Moderate AD	Phase II	2007-2010	Completed	There was no significant improvement in cognitive measures compared to placebo
AAVvac1 [Trial ID: NCT01850238]	To assess the safety and tolerability of this Tau peptide-KLH-conjugate active vaccine	Axon Neuroscience SE, Multi-center	Randomized, double-blind, placebo-controlled	Mild to Moderate AD	Phase I	2013-	Ongoing but not recruiting participants	Study result not available
Leukine/Sagramostim [Trial ID: NCT01409915]	To assess the safety, tolerability and efficacy on cognitive function of this granulocyte-macrophage colony-stimulating factor	University of Colorado, Denver; The Dana Foundation, USA	Randomized, double-blind, placebo-controlled	Mild to Moderate AD	Phase II	2011-	Recruiting	Study result not available
Benfotiamine [Trial ID: NCT02292238]	To assess the efficacy of this synthetic S-acyl derivative of thiamine on cognitive function by minimizing the decrease in glucose utilization in brain	Burke Medical Research Institute, Columbia University, National Institute on Aging (NIA), Alzheimer's Drug Discovery Foundation, USA	Randomized, double-blind, placebo-controlled	Amnesic mild cognitive impairment and mild AD	Phase II	2014-	Recruiting	Study result not available
Azeltragon (TTP488) [Trial ID: NCT02080364]	To evaluate the safety and efficacy of this RAGE (receptor for advanced glycation endproducts) inhibitor	TransTech Pharma, LLC, USA, Canada	Randomized, double-blind, placebo-controlled	Mild AD	Phase III	2015-	Recruiting	Study result not available
Rebif (Interferon Beta-1a) [Trial ID: NCT01075763]	To evaluate safety, tolerability and clinical efficacy of this cytokine	Merck KGaA, Merck Serono S.P.A., Italy	Randomized, double-blind, placebo-controlled	Early-onset AD	Phase II	2004-2008	Completed	Dose well tolerated. No major side effects. No significant improvement reported in neuropsychological conditions

Table 1
(Continued)

Molecule	Purpose	Sponsor/ Collaborator Countries	Study Design	Subject	Phase	Duration	Current Status	Outcome
Dimebon [Trial ID: NCT00829374]	To evaluate safety, tolerability and clinical efficacy of this antihistamine drug	Medivation, Inc. Pfizer, Multi-center	Randomized, double-blind, placebo-controlled	Mild to Moderate AD	Phase III	2009-2011	Completed	Study result not available
Vitamin E and Selenium [Trial ID: NCT00040378]	To evaluate clinical efficacy of these anti-oxidants	University of Kentucky, National Institute on Aging (NIA), National Cancer Institute (NCI), USA, Canada, Puerto Rico	Randomized, double-blind, placebo-controlled	AD	Phase III	2002-	Current status unknown (Active but not recruiting in 2013)	Study result not available
E2609 [Trial ID: NCT02322021]	To evaluate safety, tolerability, and efficacy of this BACE1 inhibitor	Eisai Inc. Biogen Idec, USA	Randomized, double-blind, placebo-controlled	Prodromal AD and Mild AD	Phase II	2014-	Recruiting	Study result not available
AZD3293 [Trial ID: NCT02245731]	To evaluate safety and efficacy of this BACE1 inhibitor	AstraZeneca, Eli Lilly and Company, Multi-center	Randomized, double-blind, placebo-controlled	Early AD	Phase II Phase III	2014-	Recruiting	Study result not available
Resveratrol With Glucose and Malate (RCM) [Trial ID: NCT00678431]	To evaluate the efficacy of dietary supplement in slowing progression of AD	Department of Veterans Affairs, Alzheimer's Association, Mount Sinai School of Medicine, USA	Randomized, double-blind, placebo-controlled	AD	Phase III	2008-2011	Completed	Study result not available

affinity for synaptic metal ions and may restore the ion imbalance in the extracellular spaces of A β deposits and in turn restore the cognitive impairment [78]. However, the outcomes from animal studies remain mostly unreplicated in human trials [79].

Given the multifactorial nature of AD, it is likely that multiple approaches or a polypill is warranted for patient treatment until such a time as biomarkers allow for the selection of the appropriate cohort for new therapies. Additionally, in the absence of a reference anti-AD drug, even one that works on a limited cohort of patients, the comparative analysis of the efficacy of new therapeutic strategies remains difficult [80, 81]. There are several new molecules and compounds being tested on animal AD models and subsequently tested in human for their safety and efficacy (Table 1). It is pertinent that most of the clinical trials for AD are registered in the official site of the U.S. National Institute of Health, which has a tab for “results posted” but they are seldom reported (<http://www.clinicaltrials.gov>). Ironically, only a few of these new agents have reached the stage of successful clinical translation. With the existing translational gap, a key question which remains unanswered is why many drugs which work in animals do not work in humans [32].

THE BLOOD-BRAIN BARRIER: OBSTACLE IN DRUG DISCOVERY

Delivery of therapeutic agents for most brain disorders remains a major cause of concern due to central nervous system (CNS) penetration across the blood-brain barrier (BBB) [82, 83]. The individual variability in absorption and dysfunction of the BBB [84, 85] could prove to be another significant barrier in the efficacy and brain bioavailability of novel CNS drugs. The CNS penetrability of drugs, their routes, and doses must be considered when formulating new treatment strategies. The same molecule may also not be equally effective in different pharmaceutical preparations. The current treatment strategy for AD overstates the systemic approach to the CNS rather than other approaches such as transdermal patches [86]. Other approaches including intranasal and intracerebroventricular delivery of therapeutic molecules, which can bypass the BBB, have been tested utilizing experimental animals to evaluate their safety and efficacy when compared to conventional approaches [87, 88]. The intranasal route was recently tested for its safety and efficacy in AD subjects [89]. It was also shown that intranasal delivery of insulin in AD and MCI

patients improved memory performance and levels of CSF biomarkers [90]. Adeno-associated virus- and nanoparticle-based drug delivery systems also have been tested as alternative approaches for drug delivery to the brain with limited adverse effects [91]. Other animal studies have shown that drugs can be delivered orally in a hydration gel diet [92, 93]. These reports highlight the beneficial effects of novel drug delivery systems, albeit in animal models, but they need rigorous evaluation in humans. The outcome also looks promising but there are only a few studies in man. Further efforts are needed to validate other approaches in drug delivery.

TIME FOR REASSESSMENT OF THE ROLE OF A β

A β peptide has been implicated as a central feature in AD for more than two decades. The prevailing view is that A β peptide accumulation as etiological and implies that abnormal accumulation of A β_{42} is an early event in the pathophysiologic cascade leading to the disease. Detergent extractable A β peptide and its fragments may also accumulate in different age-related dementias, particularly the oldest old, irrespective of the primary diagnosis [93]. There is also an overlap in the profiles of A β peptides pertaining to peptide solubility and oligomeric assemblies in brains from AD and normal aging subjects without any history of dementia [45]. A β subunits were shown to play a role in nucleation of A β aggregates, which has led to the proposal of AD being a prion disorder [94]. However, this is still controversial [95]. Recent studies on at least two mutations in human amyloid- β protein precursor (A β PP) have modified our understanding of the role of A β in brain aging and cognitive decline. The A β PP A673T mutation (an A2T change in A β) decreases A β production and confers resistance to AD and possibly to age-related cognitive decline, suggesting that such decline may also be A β -related [44]. The A β PP E693 Δ mutation (E21 Δ in A β) was discovered in subjects exhibiting AD-like dementia, but who were not diagnosed as AD because they lacked amyloid plaques [96]. Although total A β in E693 Δ brain extracts was lower than from cognitively normal subjects, the majority of A β that was present was in the form of an SDS-stable dimer [96]. SDS-stable dimers are a major synaptotoxic form found in AD brain extracts [97] and, along with increased soluble A β , these correlate with the severity of dementia [98].

Rat and mouse A β differs from human A β by three residues, gly for arg at residue 5, phe for tyr at residue 10, and arg for his at residue 13. The three histidine residues at positions 6, 13, and 14 of A β provide the primary metal binding site of A β [99]. Binding of Cu¹⁺ to this site is of very high affinity and its redox cycling between Cu¹⁺ and Cu²⁺ can provide a source of reactive oxygen species (ROS) [100]. Furthermore, Cu depletion has been shown to down-regulate expression of the A β PP gene, which has an obvious consequence on amyloid deposition [101]. Aging related early changes in human brain associated with MCI include the accumulation of markers for oxidative stress [102]. Thus, it is plausible that increased ROS production contribute to synapse loss that accompanies early changes in cognitive ability. These early biochemical changes are predominant in pathological aging and validated by reduced expression of synaptic proteins such as synaptobrevin and synaptotagmin leading to cognitive decline [103].

Exposure of human but not rodent A β to low levels of Cu²⁺ (levels similar to those found associated with human A β plaques) in the presence of ROS, results in the formation of dityrosine linked A β covalent dimers. The dityrosine cross-linking may play significant role in Cu-mediated toxicity of A β [104]. The lack of tyrosine in rat and mouse A β explains its inability to form this product and its lack of neurotoxicity. The A β sequence from the naked mole rat differs from human A β in only a single residue, arg for his at position 13 [105]. This rodent species is very long lived (>30 years), has levels of A β in its brain that are equal or greater to those in the transgenic mouse AD models expressing mutant human A β PP, but the naked mole rats develop no cognitive behavioral deficits in assays for which the humanized transgenic mouse models show defects. Even though the naked mole rat A β has tyr10, an arg in place of the Cu²⁺-chelating his13 would likely reduce its ability to form the A β dityrosine dimer. Thus, it is likely that specific modified forms of A β , and not simple A β oligomers or fibrils, are responsible for the early synaptic deficits that can be tested electrophysiologically in hippocampal slices for a loss of long-term potentiation [106]. Moreover, the Cu binding ability to A β differs from rat to human due to their differences in peptide sequences leading to a varied range of Cu²⁺-induced A β aggregation and neurotoxicity in these species. The three amino acid residual changes at 5, 10, and 13 positions from human to rat A β peptides are also the Cu²⁺ binding domains influencing the aggregation process [107]. These examples show that we may need to redress

the forms of A β that may be mediators of variable synaptotoxicity in AD in human and rodents.

In addition to better understanding of the forms of A β that mediate synapse loss, the targets of A β interaction leading to synapse dysfunction need to be fully evaluated. A β is a promiscuous protein with many different membrane proteins identified as binding partners [108]. Surprisingly, transgenic mice overexpressing human A β without one of the highly divergent A β receptors (e.g., cellular prion protein (PrP^C) [109]; PirB [110]; metabotropic glutamate receptor mGluR5 [111]) become resistant to memory and learning deficits. It is likely that the different receptors for A β are working through a common signaling pathway or a neuronal population involved in different aspects of memory and learning. The involvement of PrP^C, which is linked to the outer membrane leaflet via glycosylphosphatidylinositol, suggests that the different A β -binding transmembrane receptors participate in a membrane complex to generate a final common response. Indeed, a complex containing PrP^C and mGluR5 has been identified, and activation of the fyn non-receptor tyrosine kinase is one downstream target of the signaling pathway [111]. Although our current understanding on A β toxicity has contributed significantly toward corroborating the disease pathology, there is still much to investigate about the functional and structural differences in the peptide and their level of neurotoxicity in different species.

BEYOND A β HYPOTHESIS: OTHER CAUSATIVE AD PATHOLOGIES

Recent studies have found that amyloid deposits fail to induce AD-like symptoms in some mouse models [112, 113], leading to the hypothesis that non-amyloid targets may also underlie AD pathogenesis [114]. Over the past three decades, disease mechanisms other than those involving the amyloid hypothesis have been implicated in the pathogenesis of AD. The putative role of inflammatory-immune mechanisms in AD brain pathology has long been debated [115]. An increase in neuroinflammatory cytokines has been observed in AD subjects and considered to be another hallmark of AD pathogenesis, based on a careful analysis of the neuroinflammatory protein markers' phenotypes in sera of AD patients [116]. In keeping with the nomenclature used in phenotyping macrophages in which the M1 phenotype produces proinflammatory cytokines (e.g., IL-1 β , IL-6, TNF α) and the M2 phenotype produces wound repair mediators (e.g., arginase-1) and

anti-inflammatory cytokines (e.g., IL-4 and IL-10), early AD subjects could be classified quite distinctly into each of these cohorts with 11 of 23 subjects assigned to the M1 cohort and remaining 12 to the M2 group, based upon 5 distinct markers for each group including interleukins-1 β , -6, and -12, interferon- γ , and tumor necrosis factor- α [117]. The frontal cortex was used for these analyses, and the cerebellum, which showed no significant changes in more than a single marker, was used as control tissue. For late-stage AD subjects, both sets of markers were greatly increased and no distinction could be made. These results again point to the multifactorial nature of AD and the need to be able to categorize patients using biomarkers to test different therapeutic approaches.

Another example of change found in AD brain and explored in rodent neurons and organotypic slice cultures is that of cofilin-actin rods. These structures, detected by immunostaining for cofilin [118], contain linear arrays of cofilin-saturated actin filaments [119]. They form in both dendrites and axons and require oxidative stress to accompany the hyperactivation (dephosphorylation) of cofilin to form cofilin disulfide linked dimers [120]. Many changes that occur in AD brain, such as the increased production of A β [121], the decline in the p21 activated protein kinase (Pak1), which works upstream of cofilin and helps regulate its activity [122], the loss of two microRNAs (miR103 and 107) that control the expression of cofilin [123], the decrease in glutamate transporters leading to increased extracellular glutamate [124], and increased oxidative stress [102], all lead to cofilin-actin rod formation when studied in neuronal culture. Because rods sequester virtually all of the cofilin within the local region of the neurite [118], they inhibit synaptic function [125] because cofilin plays an important role in both AMPA channel insertion and dendritic spine enlargement associated with long-term potentiation [126]. Although rods are induced by oligomers of synthetic human A β ₁₋₄₂ [121], Cu²⁺-peroxide oxidation of synthetic human A β increases its rod inducing efficacy by about 600 fold [127]. The cofilin-actin rods contribute to synaptic loss as evident from cultured neurons subjected to excitotoxic stress and A β exposure. Exposure to proinflammatory cytokines also induced rod formation in these neurons. They have experimentally shown that both A β and proinflammatory cytokines induced rod formation in neurons are mediated through activation of prion protein-dependent NADPH oxidase pathway. This study links the A β and cytokine hypothesis in AD pathology and explains how complex and diverse

mechanisms mediate synapse loss in AD [128]. Since rod pathology may be a common downstream effector of synapse loss for both familial and sporadic forms of AD, it is surprising that more studies have not examined the therapeutic potential for the elimination of cofilin-actin rods [129].

ANIMAL MODELS FOR AD: IN NEED OF BETTER CHARACTERIZATION

A range of animal species are used as experimental models for AD pathologies [129]. These models vary from invertebrate animals such as *Drosophila* [130], *C. elegans* [131], and zebrafish [132] to rodents [133, 134] and non-human primates [135, 136], although mice are most often used to investigate AD pathogenesis. Even the most resilient transgenic mouse models for AD exhibit the rarer familial form of the disease, whereas sporadic AD is the most common [137]. Overall, no animal model is able to reproduce disease onset, progression, and relapse, reminiscent of AD patients [138]. The use of transgenic tau mice, either on their own or in association with A β PP mutations has not wholly enabled their purpose; characteristics of the phenotype depend on the transgenome and fall short of fully reflecting features of AD pathology. Thus, some of the available transgenic tau mice models, e.g., Tau4R-P301L and P301S, have shorter lifespan of 9 months, with most not surviving beyond 12 months. Others have age-associated impairment in retention of spatial memory, associative memory and cognition, or no abnormal behavior. Furthermore, they may also differ in respect to their motor phenotypes, which include severe and early onset of motor, gait, balance and behavioral disturbances, and the neuropathological features [139]. The highlighted cognitive behavioral, motor, and neuropathological heterogeneity of the transgenic tau animal models make them difficult to use in AD translational research, and much attention also needs to be paid in selecting an all-encompassing tau model to investigate the properties of novel drugs for treatment or neuroradiological tracers to diagnose AD [2, 140, 141].

Variables such as the strain, age, and expression level of the transgene and gender of animal models influence the disease progression and its pathophysiology, thereby confounding the results of studies seeking to discover novel biomarkers and therapies for AD. There are neural and behavioral differences between background strains used to produce transgenic mice [142, 143]. Strain differences in anxiety-like behavior

have been documented in mice tested in the open field and elevated plus maze [144]. For example, female C57BL/6J mice exhibit less anxiety-like behavior and more exploratory activity, compared to BALB/cJ mice [145]. Therefore, background strain effects should be considered when using transgenic mice to model neurodegenerative diseases (Table 2).

Since neurodegenerative disorders are age-dependent and physiological deficits may be age-specific [146], the age of the mice is another variable that must be considered. When age differences are taken into considerations in a transgenic (Tg2576) mice, FDG-PET reveals greater glucose metabolism in 7-month-old than in 19-month-old mice. However these differences were not supported by hemodynamic parameters when cerebral blood volume was measured by functional MRI [147]. One solution to this problem is to use senescence accelerated mice [148].

Some may argue that gender of animals does not pose differences in their brain architecture and related behavioral performances, but the current literature is contrary to this belief. There are gender differences in the brain and behavior of rodents [149] including transgenic mouse models of AD [150]. For example, mood disorders are reported to be more striking in female rodents [146, 151] and therefore the majority of behavioral experiments are performed using males. Some studies have reported differences in the biochemical parameters in the blood of Wistar rats as a function of gender and age [152]. The resulting gender bias in animal research is a serious concern. Researchers are encouraged to study both males and females of each animal model [153]. In many studies significant gender differences are reported [154], but these are not taken into consideration in the design of subsequent clinical trials [155]. Therefore, when developing an animal model of AD, either by inducing a specific genetic mutation or by electrical, mechanical, or pharmacological interventions, the validation of the model must consider the genetics of the strain used, expression level of the transgene, age, and gender differences. Such models should also address pathologies that are independent of or supplemental to the centrality of A β hypothesis. Given numerous existing models with none of them representing the true molecular, pathological, and behavioral traits of the human disease, it is unclear which one would best fit for evaluation. Considering the multifactorial nature of the disease, it is challenging to develop an AD model without any limitations. Therefore, it is imperative to test the safety and efficacy of any candidate molecule in multiple AD models before undertaking human studies.

PHYLOGENETIC DIVERSITY BETWEEN RODENTS AND HUMANS

As rodent models are extensively used in understanding the pathophysiology of AD, it is important to corroborate the basic differences between mouse and human nervous systems. The inter-species phylogenetic comparisons may reveal deeper insights into the cellular and genetic differences specific to neurodegenerative disorders [156]. For example, drug doses tested and validated in experimental animal models are not easy to extrapolate to humans [157]. In addition, the network of connections in the brain transcriptome differs among species and this is little considered. Although targeted gene mutations in animal models may mimic certain comparable phenotypes in humans, a single gene mutation in mouse models is often considered incapable of translating the same behavioral and pathological parameters observed in the human phenotype. While there is genetic similarity between mouse and human species, only a 10% homology for co-expressed genes has been reported [158]. A transcriptome analysis across human and mouse brains was developed by analyzing more than 1000 gene microarrays in both species. Among them, the species-specific gene modules described some highly conserved transcriptomes with an overlap of genetic networks between the two species [159]. When AD and other neurodegenerative disorders were specifically examined, the transcriptome revealed that the recruitment of microglial cells appeared to play a significant role in the development of AD. For instance, the human presenilin 1 mutation (PS1), which increases A β production, can increase the severity of the disease in man but was found to have a limited effect in mouse mutants. There is also a correlation between presenilin 1 and oligodendrocytes, which is selective in humans. It is evident that the number of oligodendrocyte progenitors increase significantly in brains of A β PP/PS1 transgenic mice while these cells are found to be limited in postmortem AD brains. Thus in similar pathological conditions, these cells have different repair mechanisms in humans and mice [160].

As microglia are an important sources of proinflammatory cytokines and major players in AD pathogenesis [116] that can trigger stress enhancing oxidation of the amyloid peptide, the differences between mice and humans could be dramatic for disease progression. Regardless of the dissimilarities, it is still possible to improve such animal models to incorporate the elements of AD pathogenesis. For example, oxidative stress can be co-induced in an AD transgenic model. Therefore, changes in expression patterns

Table 2
 Impending limitations and plausible solutions in translation of pre-clinical studies in AD

Several animal models for AD but unable to completely reproduce disease onset, progression, relapse and reminiscent of human AD patients	Variables such as the strain, age, gender, genetics and expression level of the transgene should be taken into consideration
AD Models such as lesion induced by targeted brain injury or A β injection may only mimic the cognitive impairment and may not resemble the molecular mechanism behind disease pathogenesis	Such models must be critically examined for their pathological and molecular roles behind associated cognitive impairment
Transgenic mouse models mimic the familiar form of the disease, even though AD is mostly of sporadic origin	Expression patterns of such transgenes and their interrelated activities at cellular levels in comparison to man must be considered in animal testing
Most transgenic models focus on either the amyloid pathology or the neurofibrillary tangle (NFT) pathology alone and seldom both together	It must consider both A β and NFT at transgenic level and their synergistic effects in the progression of neural and behavioral pathologies
Rodent A β differs from human A β in structure as well as toxicity	Need to readdress the precise form and role of A β in AD pathology
Ignorance of associated risk factors on the occurrence of AD and its progression	Factors including increasing age, diet, cardiovascular disorders, diabetes, obesity, hypertension and other environmental factors must be evaluated in parallel to AD pathology
Ignorance of confounding effects in neurobehavioral studies	Variables such as genetic manipulation, stress, predatory effect, housing and test room environment, experimental design, test apparatus and experimenter effects should be taken into account
Compromised quality and reliability of the data obtained from animal studies	OECD Principles of Good Laboratory Practices must be applied to authenticate the data generated by establishment of Quality Assurance procedures in the test facilities
Due to lack of randomization and blinding in animal study design negative results are rarely published in journals, resulting in a bias in pre-clinical data	Standardized reporting through validated guidelines for animal experiments can improve the quality of the research and also contribute effectively to future research
Lack of consultation with statisticians with regards to the study design, conduct and analysis of animal trials	The statistical analysis plan (SAP) should be carefully developed before the initiation of the study. The SAP should determine various study protocol such as power analysis, sample size, randomization method, duration of trial and dropout animals information, etc.
Majority of investigations in animal models are cross-sectional in on the nature leading to lack of evidence for epigenetic influence pathogenesis of AD	Longitudinal animal studies, characterized by long-term follow up of exposure to drugs, nutrition, biotherapeutics or other non-pharmacological interventions can provide crucial evidence to delineate the causal factors in disease pathogenesis

of several genes related to AD and their interrelated activities at cellular levels play a unique and significant role in pathogenesis of disease in mouse models and patients, and these pose a serious challenge for experimental neurologists [159, 161–163].

PATHOPHYSIOLOGICAL DISSIMILARITIES

The pathological signature for AD is widely acknowledged to be the intracellular accumulation of hyperphosphorylated tau proteins into neurofibrillary tangles and neuropil threads and the extracellular insoluble deposits of A β peptide into neuritic plaques [164]. However, there is lack of a robust relationship between burden of senile plaques and cognitive impairment, but McDonald et al. [98, 165] have shown that the soluble forms of A β , particularly the SDS-stable covalent A β dimers, have a strong correlation with dementia progression. The Osaka mutation in A β PP (E693 Δ) that gives rise to dementia in the absence of plaques

[95], suggests that plaque formation, which is used to define AD, should not be highlighted as a critical pathological parameter. Mouse models expressing E693 Δ mutant A β PP develop impaired hippocampal synaptic plasticity and memory impairment with no extracellular plaque formation [166]. Reduced total amount of A β but greater amounts of A β dimers are found in subjects with the Osaka mutation, again suggesting that it is the form of the A β present and not the total amount, which drive the synaptic dysfunction. Some animals develop plaques and tangles spontaneously, but for the majority of animal models, AD-like symptoms are induced by either pharmacological, neurochemical, electrolytic lesions, A β infusion, or genetic manipulations [127]. These manipulations are an attempt to induce the behavioral and pathological symptoms of AD in animal models without validating the critical pathophysiological details in the disease mechanism. For example, the lesion induced by a targeted brain injury or by A β injection may only mimic the behavior or cognitive impairment and may not resemble the molec-

ular mechanism responsible for disease pathogenesis. Similarly, injection of neurotoxins like amyloid peptide or ibotenic acid, which induce neuronal loss in defined regions of the brain, might mimic neuronal loss without providing reliable information about the pathophysiological mechanisms such as apoptosis and deposition of amyloid peptides [6]. Even the most promising transgenic models of AD have limitations [137, 167]. Although these models attempt to reproduce all the three aspects of the biological mechanisms for the pathogenesis of the disease viz. cause, symptoms, and pathology, the complexity of disease is difficult to capture through molecular pathways in a single model. For instance, the vast majority of the transgenic mouse models focus on either the amyloid pathology or the neurofibrillary tangle pathology alone and not both together which is more representative of the disease state [168]. Therefore, it is clear that such animal models cannot address all the aspects of the disease [169].

It is vital to understand the interaction between tau protein and amyloid peptide and their synergistic effect in the progression of neural and behavioral pathologies in transgenic animal models [170]. Nevertheless, newer transgenic rodent models that capture more of the pathology of human AD have been developed from which we can hope to learn more about the interactions between tau protein and amyloid peptide and their synergistic effects on the progression of neural and behavioral pathologies. However, even these models fail to include additional epigenetic influences that will help us better understand sporadic AD, the most common form. It is also pertinent to mention that these models have contributed enormously to the current progress of AD research and therefore the focus could be more on representing the human AD pathology and expressing newer human mutant proteins in mouse brain.

ANIMAL STUDIES AND ASSOCIATED RISK FACTORS OF AD

Proper assessment of risk factors has been instrumental in the discovery of disease mechanisms and drug discovery. By using case-control and disease cohort studies, multiple risk factors have been identified and assessed for their effects on the occurrence of the disease and its progression [171]. Increasing age, dietary factors, apolipoprotein E gene isoform 4 (*APOE ϵ 4*), cardiovascular disorders, diabetes, Down syndrome, MCI, traumatic brain injury, and multiple environmental factors are reported to be associated

with pathogenesis of AD [172–176]. Unfortunately, the majority of these studies are retrospective in nature and none of these potential risk factors is routinely considered in experimental designs of animal studies as well as human trials.

Increasing age is considered the most important risk factor for sporadic AD [177]. However, old age is not the sole factor for development of disease. The vitality of all organs in the body during normal aging to ward off cardiovascular disease, hypertension, diabetes, and obesity plays an important role [178]. There appear to be seven key risk factors associated with AD. It is projected that an estimated 10–25% reduction in diabetes, obesity, hypertension, depression, smoking, and cognitive and physical inactivity could prevent around 3 million cases of AD in the global population [179].

Non-neuronal cells such as lymphocytes also show a high degree of susceptibility toward cellular apoptosis with increasing age [180]. Dietary deficiencies in folate, vitamin B6, and B12, which result in an increase in the level of circulating homocysteine, may also be a risk factor for developing AD. Transgenic A β PP mice fed on diets deficient in folate and vitamins B6 and B12 have an increase in the AD progression and pathological levels of amyloid in their brains [181]. Even wild-type mice develop atrophy and reduced metabolism in the hippocampus after long-term cerebral hypoperfusion [182].

The most prevalent genetic risk factor associated with sporadic AD is the inheritance of the ϵ 4 allele of *APOE*. It is reported that the population carrying *APOE ϵ 4* alleles, increases the likelihood that they will eventually develop AD [183]. There are studies estimating *APOE* allele frequency to be about 12–14% for Caucasians and varies by ethnicity [184]. Type 2 diabetic patients are also at high risk of developing AD. Double transgenic mice for AD and diabetes (A β PP⁺-ob/ob mice) exhibit significant cerebrovascular inflammation and extreme amyloid angiopathy compared to single A β PP⁺ transgenic mice [185], and insulin deficiency may alter A β PP processing to result in an increase in A β plaques [186]. Among other factors, MCI is a significant risk factor for AD. In a multicenter study, Mattsson et al. [187] have shown that around 36% of the MCI population recruited in the study was eventually diagnosed with AD within 2 years follow up.

Individuals with risk factors identified for AD can modify their lifestyles to reduce the chances of developing AD. Some of the lifestyle factors include diet and burden of hyperglycemia as these may enhance the susceptibility for AD. The Mediterranean

diet has a significant role in lowering the risks of neurodegenerative disorders. This balanced diet, consisting of high amount of plant foods and olive oil and low consumption of dairy products and meat, when adhered to for a long time reduces the chance of developing AD [188]. Exercise and environmental enrichment may reduce the cognitive impairments caused by high fat diets in A β PP transgenic mice [189]. Future studies should, therefore, focus on rigorously identifying the lifestyle changes that reduce the risk factors for developing AD [178, 190–194]. In order to address such issues, many investigators have suggested the introduction of stress in transgenic animals across their lifespan.

LIMITED LONG TERM PROSPECTIVE STUDIES IN ANIMALS

The majority of investigations in both animal models and AD research are cross-sectional in nature. Longitudinal animal studies, characterized by long-term follow up of exposure to drugs, nutrition, biotherapeutics, or other non-pharmacological interventions, can provide crucial evidence to delineate the causal factors in disease pathogenesis. There is growing evidence that early exposure to environmental stimuli such as toxins, metals, and nutritional or educational exposure can exert epigenetic influence on the pathogenesis of AD [195–198]. Lahiri and colleagues [199, 200] have proposed the LEARN (Latent Early-life Associated Regulation) model highlighting the significance of long term follow up of animals exposed to subtoxic levels of lead in early life which resulted in the development of AD as they aged. A better understanding of epigenetic influences on neural disorders has come from studies in monkeys exposed to lead for 400 days in their infancy and evaluated 23 years later [201]. The expression of neuropathological genes related to AD, such as, A β PP, BACE1, and their transcriptional regulator (Sp1) were upregulated in the brains of the exposed monkeys compared to their control counterparts. Furthermore, the elevated levels of intracellular amyloid plaques and their increased distribution in cortical regions were observed in these monkeys. They concluded that alteration in DNA methylation levels through oxidative damage in early life resulted in the greater expression of AD related genes and disease pathogenesis at a later stage of life.

These findings signify the importance of early life regulation in AD. The FDA now prioritizes discovery of new drugs and institution of clinical trials in the

early stages of AD. The Center for Drug Evaluation and Research, a US-FDA body, has recently released a draft guideline to assist the pharmaceutical industry in selecting early AD patients or patients with determined risk of developing AD for enrolment into the trials thus emphasizing the significance of early detection of the disease for effective intervention. A key reason for the failure in clinical trial of drugs effective in animal models is the recruitment of patients at an advanced disease state, further highlighting the need for early diagnosis and intervention. Therefore, the desirable characteristics of an animal model of late onset (sporadic) AD should incorporate not only the risk alleles but also the “age-related and environmentally induced epigenetic dysregulation” of AD [189] for effective translation of preclinical studies into tangible therapies.

CONFOUNDS IN THE NEUROBEHAVIORAL STUDY OF ANIMAL MODELS

AD is diagnosed on the basis of age-related cognitive and behavioral deficits of the patients as described in the DSM-IVR [202] and in more recent diagnostic manuals [203, 204], which can be correlated with biomarkers [60] as well as postmortem neuropathological abnormalities including A β plaques and neurofibrillary tangles [205]. Thus, mouse models of AD must demonstrate age-related cognitive and behavioral deficits of the type found in human AD, and these deficits must be shown to arise from neural degeneration of the AD type and no other confounding factors such as sensory-motor deficits or laboratory artifacts [206, 207]. However, many argue that animal models may not reproduce cognitive features of human disorders but only the pathological changes similar to human AD. The aim of using transgenic mouse models of AD is to demonstrate that AD transgenes and not some other confounding factors cause AD-like deterioration of behavior and brain. These confounding variables include the genetic manipulation and the housing environment of the mice, stress and the design of the experiments and tests conducted, the test room environment, test apparatus, and experimenter effects [206, 207].

When mice are genetically engineered as transgenic models of AD, genes are transfected into the mice and the behavioral changes of the transgenic mice are assumed to be due to these genes, but they can also be due to the background strain of mice used, to flanking genes or genes disrupted by transgene insertion

or to errors in genetic manipulation. Thus, one must know how the genome of the background strains of mice affects their behavior and be able to detect the effects of unwanted genes transfected along with the genes of interest. Since some strains of transgenic AD mice undergo retinal degeneration, using these as background strains means that the transgenic mice will be blind [208] or have age-related blindness which is not detected until mice are over 9 months of age [209]. Obviously, such age-related changes in vision will have profound effects on cognitive behavior measured using visual cues. Since water maze is often used to test memory in transgenic AD mice and to predict treatment response, it is important to analyze the effect of vision loss as a confounding effect on their cognitive performance. There can be profound effects of blindness on spatial memory deficits as demonstrated in a B6/SJL genetic background of widely used transgenic mice of AD [210]. The Tg2576 mice with visual impairment were unable to perform during acquisition trials in water maze when compared to transgenic alone. This confound was of no consequence when analyzed in memory retention trials [210]. One must also ensure that the mice being tested actually bear the genetic manipulation that they are supposed to have [211]. The source of the mice may also be a factor as mice from different breeders are not always the same [212]. Health status is also an important consideration as mice which have a peripheral infection of some sort may show abnormal behavior which is independent of the transgenes they express, or which exacerbate the effects of the transgene they express. The housing environment of mice is often ignored, but differences in the vivarium environment, home cage features, diet, social versus individual housing, and cage enrichment can significantly affect the behavior of mice. In addition, predatory stress of rat to mice (due to long-term housing of rats and mice in the same secondary enclosure) and some housing conditions may induce selective stress among the animals, altering their behavioral performance [212–214]. Even changing the mice from social housing to individual housing may be particularly stressful [215].

When testing mice behavior, the design of the experimental test is crucial. Which tests should be given, and in which order? Which strain of mice should be tested and what is the appropriate control strain? What sex and age of mice should be tested? Should tests at different ages be done longitudinally, with the same mice tested at each age, or in a cross-sectional study? How many subjects should be used in each group and what statistical analyses should be done? Behavioral testing

is done on an apparatus in a test room. How should the test room be designed? How should animals be transported from their housing room to the test room, and when during the light-dark cycle, should mice be tested? Are all tests performed at the same time of the day? The type of apparatus used must be carefully considered as mice behave differently in different designs of the same apparatus. For example, different designs of the Barnes maze result in different types of learning and memory [216, 217]. Finally, one must ensure that the behavioral measures represent the psychological construct that they are meant to measure. For example, there are many tests of anxiety in mice but they may not be measuring the same psychological state or trait [145].

Many types of errors can occur. Thus test results must be examined for observer effects, observer error, and observer bias. Testing should be performed such that the observer is blind to the genotype of the mice being tested. Videotaping of each test is advised to check for procedural errors, equipment setup errors, and animal handling errors. Data recording errors must also be checked. To reduce experimenter error, test procedures may be automated, but automation may also introduce unexpected errors into the behavioral testing. Thus, uncontrolled and undetected confounds in the neurobehavioral study of mouse models may be a significant reason for the problems in the translation of pre-clinical studies into clinical trials [206].

NEED FOR RANDOMIZATION AND BLINDING IN STUDY DESIGN

Randomization of subjects to groups and blinding of experimenters as to which group each subject is allocated are essential elements of clinical trials, assuring quality trial performance and unbiased reporting of clinical outcomes. Even though the fundamental rationale for the animal experiments is similar to that of human randomized controlled trials, often the reporting of the methodology of animal studies is not sufficiently detailed to determine if randomization and blinding were appropriately done. Thus, the validity of the outcomes may be questionable, widening the chasm between preclinical and clinical investigations [218]. Rarely are negative results published in journals, resulting in a bias in reporting positive pre-clinical data [219, 220]. This is often argued as the primary reason why despite more than 1000 preclinical animal studies with positive neuroprotective interventions in stroke, none of the results could successfully achieve

clinical translation [221]. The situation is similar for preclinical studies in AD. A retrospective survey of 290 abstracts presented at a meeting reported that animal experiments without randomization and blinding are 5.3 times more likely to report positive outcomes [222]. Many surveys mention that most studies reveal inadequate reporting of the study design, sample size, randomization of the sample, blinding of the experimenter and statistical methods [218, 223, 224]. The sample size of experimental groups in a preclinical study rarely exceeds 10. It is difficult to imagine publishing a study on human AD with 6–10 patients.

Incomplete or inappropriate reporting may cause serious scientific, ethical, and economical implications. The situation is compounded by lack of guidelines for proper reporting of animal experiments unlike the CONSORT (Consolidated Standard of reporting trials) statement which is followed in most of the randomized controlled trials [225]. Standardized reporting through validated guidelines for animal experiments can improve the quality of the research and contribute effectively to future research. Kilkenny et al. [226] have taken the initiative to propose a draft guideline called ARRIVE (Animal Research: Reporting *In Vivo* Experiments) in order to strengthen preclinical reporting of data. Taking their cue from the CONSORT statement and after consultation with several eminent scientists, funding agencies and high impact factor journal editors have included essential requirements in study design and reporting such as randomization, blinding, sample size, inclusion and exclusion criteria, co-morbidities, and missing data. Compliance to new methods of reporting might increase the quality of investigations and hence the pace of clinical translation. Journals such as *Genes*, *Brain and Behavior* and *Nature Neuroscience* [227] have instituted new standards for reporting animal studies.

UNIVERSALIZATION OF GOOD LABORATORY AND CLINICAL PRACTICES

Although we have progressed reasonably in AD research, there is not enough information regarding key events in its causation so that they can be exploited for identifying suitable biomarkers for diagnosis or targets for its treatment. It is also obvious that animal studies have their own limitations in extrapolation of results to human. However, there is a third issue of quality and reliability of the animal and clinical studies which

is essential for making these results meaningful for clinical translation. In a complex situation like the one being posed by AD, animal studies ought to be made more acceptable by applying the OECD Principles of Good Laboratory Practices (GLPs) [228, 229]. Briefly, these quality principles require use of “demonstrably” appropriate resources (manpower, infrastructure, equipment, laboratory facility, clinical facility, animal facility, and dose formulation facility) along with the use of “demonstrably” well characterized animals and dosing materials. Further, there should be well-written study plans, followed accurately with the help of documented standard operation procedures supported by detailed documentation of findings of the study [230] suitably archived in organized formats. GLPs also enjoin carefully secured and retrievable archiving of all study-related and facility-related data and materials so that any reasons for discrepancy in the findings of further studies can be traced back to their causation. Finally, the GLPs recommend establishment of Quality Assurance procedures in the test facilities with the help of staff not directly involved in the conduct of studies to ensure that all the work is done as per prior commitment, with true representation of all the study data in the final reports and acknowledgement of deviations from the planned activities and procedures (if any) [231]. It is hoped that if researchers and clinical scientists utilize GLP principles in planning, performing, recording, analyzing, and archiving of the animal and clinical studies with constant vigil of internal quality assurance, and make an averment to this effect while publishing their results, their data will be more reliable and acceptable for successful clinical translation.

CHALLENGES AND OPPORTUNITIES OF INTEGRATING GENETIC AND GENOMIC INFORMATION INTO CLINICAL PRACTICE

AD comprises familial and sporadic forms. The early-onset familial AD is an inherited disorder, caused by mutations in three major genes (*A β PP*, *PSEN1*, and *PSEN2*). The late-onset sporadic AD is a complex disease caused by multiple genetic and environmental factors. Great efforts have been made to understand the genetic causes of sporadic AD in the past decade [232, 233]. Recent candidate gene and genome-wide association studies (GWAS) have identified multiple genes associated with AD, such as *APOE*, bridging integrator 1 (*BINI*), clusterin (*CLU*), complement receptor (*CR1*), and phosphatidylinositol clathrin assembly

lymphoid-Myeloid Leukemia (*PICALM*). To increase study sample size, consortium meta-analysis combined multiple studies to further expand the gene list [234].

Despite the great progress on identifying disease-associated variants, it is still not clear how these identified genes affect the initiation and progression of AD pathology in the brain. The identified single nucleotide polymorphisms (SNPs) often fall into non-coding regions and have no obvious functional implication. With improving genotyping tools and technologies, there have been a series of SNP studies in AD conducted but the risk effects of these SNPs are small and have little contribution to disease prediction or diagnosis. Instead, these findings have led to a pre-debated possibility of highlighting false positive signals among the true disease polymorphisms [235].

At this stage, genetic information has not been widely integrated in clinical practice. Next-generation sequencing will be the next step to identify additional variants with less frequency (rare variants) but large effects [236]. Besides DNA variation, there is accumulating evidence of epigenetic effect contributing to AD, which implies the complex interplay between genetic and environmental factors [237–239]. However, most epigenetic studies so far in AD are limited by the sample size and the genome coverage. New technologies (e.g., whole-genome methylation chip) will help researchers assess the epigenetic mechanism systematically and bring new important insights to enhance our understanding of the pathogenesis of AD.

A potential benefit of emerging findings from genetic studies is to directly facilitate the design of clinical trials. Hu et al. [239] proposed a framework to integrate genetic risk scores that are based on the findings from GWAS, in clinical trials to reduce trial cost. The rationale is that using genetic information to enroll a subgroup of individuals can increase the disease rate, and thus reduce study duration and trial cost [240]. A few common complex diseases, such as type 1 and 2 diabetes, myocardial infarction, and macular degeneration have been examined. This approach can be applied analogously to AD. Furthermore, individual genetic profiles provide potentials for researchers to identify a subgroup of patients that have better drug response than general populations. Given the lack of new drug development, many researchers are examining how effective new pharmacological treatments are discovered [241] and returning to phenotypic screening in addition to target based drug discovery [242–244]. Studies of pharmacogenetics and pharmacogenomics on AD patients will help optimize drug use and improve drug efficacy [245]. We anticipate that the

combined information of genetics, genomics, environmental factors, and drug response will yield a major change in clinical practice and facilitate the success of personalized medicine in near future.

AD DRUG TRIALS: GAPS IN DESIGN AND METHODS

Preclinical studies are generally not as highly regulated as clinical studies. For many preclinical studies, there is limited consultation with statisticians with regard to study design (such as sample size determination, randomization method, and duration of trial) and statistical analysis and interpretation of the data. Statistical support is believed to be less important and therefore less appreciated in the preclinical research phase. In the recommendations on best practice for animal studies of AD, jointly published by Alzheimer's Drug Discovery Foundation (ADDF) and Charles River Discovery Research Services [31], lack of standards in design, conduct, and analysis of animal trials is considered as one of the key challenges in translating preclinical studies to clinical trials for AD. Establishing rigorous preclinical standards cannot be accomplished without significant statistical contributions, especially for research on such a complex disease. Similar to the clinical studies, the hypotheses and objectives of the preclinical study must be pre-specified using preferable statistical language so that they can be formally tested and evaluated. The statistical analysis plan (SAP) should be carefully developed before the initiation of the study. Power analysis and sample size estimation are recommended prior to the study even for the exploratory experiment. Randomization methods and blinding procedures should be carefully considered and stated in SAP. Appropriate procedures of handling the dropout animals (e.g., due to death or other adverse events) need to be specified in the SAP. By implementing these enhancements in the preclinical studies, the quality of the findings and the predictive value of the preclinical research can be improved and the translation to the clinical trials can be more reliable and efficient.

The design and implementation of clinical trials have also generated numerous obstacles (Table 3). According to ClinicalTrials.gov, there have been over 330 trials to understand and treat AD and about 30 among these are in Phase 3. To improve the probability of success in large late phase trials, AD clinical think-tank leaders have faced challenges on how best to design and evaluate early phase trials [246]. Although

some efforts have been already made to improve the quality of early clinical studies on AD, there is still considerable scope to introduce emerging statistical methods and advanced trial designs. Most of the AD clinical trials have used cognition as the primary outcome measure. However, it has been suggested that ADAS-Cog (Alzheimer's Disease Assessment Scale – Cognitive) performs less than adequately in detecting patients at the mild stage of AD [247, 248]. The FDA Guidance Document on Alzheimer's Disease reiterates that most clinical evaluation criteria can only detect the disease in the presence of cognitive impairment, when it is generally late to prevent disease onset [249]. In addition, the primary outcomes in some trials are analyzed by univariate statistical methods to compare differences between treated and control groups that greatly limits the scope to interpret the complex multivariate data [247]. The outcomes of phase III bapineuzumab and solanezumab trials were critically reviewed by the EU/US/CTAD Task Force Members to evaluate the design methods and outcomes particularly for insights on future clinical trials. Remarkably, other factors contributing to the lack of efficacy include significant differences in actual binding and the cross-reactivity of anti-A β antibodies to amyloid and other proteins in humans. The lack of target engagement raises questions as to whether some of the monoclonal antibodies are suitable drug candidates for the preventative clinical trials for AD [250].

The task force came to a broad consensus that AD should be treated at early stages and a line of secondary prevention should be incorporated in the disease management. Based on the trial result, it is also realized that the trial outcomes should be measured primarily based on the cognitive outcomes irrespective of the changes in disease pathology. Other interesting recommendations made in this meeting were consideration of combining phase II and phase III trials, targeted and adaptive mode of trials, and measures of biomarkers at downstream levels for enhanced chance of success in trials [251].

AD is a complicated disease and there is considerable variability in disease symptoms, progression profiles, and responses to interventions among different populations. It is unlikely that a treatment can be effective in all populations. Dubois and colleagues [251] have suggested a revised definition of AD. One major impact from this new definition applies to the clinical trial design, indicating more targeted subpopulations of AD should be considered. This also implies that, as a result of targeted clinical trials, more advanced statistical methods for subgroup identifica-

tion and evaluations have to be implemented in the analysis of such targeted trials. All of these could yield substantial improvements for assessing the efficacy of AD interventions.

COLLABORATIVE EFFORTS BETWEEN RESEARCH SCIENTISTS AND CLINICIANS

The crosstalk between basic scientists and clinicians is prerequisite for successful translation of preclinical findings into clinical prospects [252]. Unfortunately, this has not been the practice in most of the clinical or preclinical settings, creating a knowledge gap among the scientists and clinicians, and dampening the hope of promising clinical translation. Involvement of both can increase the transparency of the study design in animal models as well as clinical trials. Clinicians can have the opportunity to inform the animal modelers what kind and in which form they need the information from animal studies to benefit human trials. As an exemplary case, a group of basic scientists and clinical oncologists recently met at the Wistar Institute, Philadelphia, PA to discuss the outcome of preclinical mouse models of human melanoma for facilitating improved clinical trials [253]. The outcome of this exchange indicated that no human trial in melanoma should be planned without strong evidence of beneficial effects from progressively designed animal studies. Such meetings are important for ensuring optimal patient selection—many drugs have failed in clinical trials because the patients selected for trial were far too advanced for any disease-modifying therapies to be effective. This has clearly been demonstrated in numerous animal models but is not taken on board by investigators of clinical trials [254].

To tackle the problems of translation from animal studies of neuropsychiatric and neurodegenerative diseases, a number of new programs have been initiated. These include programs such as MITACS [255], CNTRICS [256], and Pivital [257]. Others [258] have been developed to facilitate the development of new treatments for AD. The National Alzheimer's Coordinating Center (NACC) [259] has developed a "uniform data set" to submit the information related to neuropathological and epidemiological details of AD and propagate them among the basic researchers for a better development of preclinical studies. The Mary S. Easton Center for Alzheimer's Disease Research at UCLA has also been developed to co-ordinate research among the AD researchers and clinicians [260]. New programs such as these will train researchers who can integrate

Table 3
Limitations and plausible solutions in designing clinical studies

AD research has predominantly focused on A β pathology neglecting several other mechanisms	Consideration of other AD pathologies such as neuroinflammatory cytokines, cofilin-actin rods pathology
Pathological variations among different cohorts of AD patients are not considered in human trials	Variations such as A β PP mutation and plaque formation, which alter from cohort to cohort, should not be highlighted alone as a major pathological parameter
There are no standard biomarkers identified for early diagnosis of the disease and for the identification of populations with high risk or differentiate MCI from AD	Because of the multifactorial nature of AD, efforts should be made to establish a standardized diagnostic protocol by combining genetic analysis with neuroimaging traits and epigenetic biomarkers
AD pathology changes over time. The different stages of the disease are not considered in diagnostic criteria	Measuring longitudinal patterns of changes in a set of different biomarkers may be the most reliable way to diagnose AD and measure its progression
Majority of investigations in human trials are not longitudinal and patients recruited at an advanced disease state leading to lack of evidence for epigenetic influence on the pathogenesis of AD	Now FDA prioritizes the institution of clinical trials in the early stages of AD emphasizing the significance of early diagnosis for effective intervention
Conventional drug delivery system in AD remains a major cause of concern due to poor CNS penetration across the blood-brain barrier	Efficacious brain bioavailability by other approaches should be tested such as transdermal patches, intranasal, intracerebroventricular, adeno-associated virus- and nanoparticle-based drug delivery
Lack of crosstalk between basic researchers and clinicians creating a knowledge gap among them, dampening the hope of clinical translation	Involvement of both can increase the transparency and rationality of the study design in animal models as well as clinical trials
Recent genome-wide association studies (GWAS) have identified multiple genes associated with AD but these are not widely integrated in clinical practice	Emerging findings from GWAS studies should be integrated into the design of clinical trials, which may substantially reduce study duration and trial cost

basic research and mechanistic studies with clinical problems posed by patients as well as public-private partnerships [261].

CONCLUSIONS

Several factors have impeded the translation of basic bench research to effective treatment for AD (Tables 2, 3). It is indisputable that the development of animal models has paved the way to understanding the neurobehavioral outcomes, pathophysiology, and molecular events involved in the disease. Still it is apparent that human disease pathology cannot be replicated in animal models. The pathophysiological and phylogenetic differences between rodents and humans have made translation difficult. Preclinical studies involving animals seldom consider confounds, randomization, and blinding in their study designs. A variety of confounds in the generation, study design, and testing and evaluation of the models have also contributed to the limited success in the clinical translation of these findings (Fig. 2). In addition, the overemphasis on centrality of amyloid hypothesis to the exclusion of the non-amyloid mechanisms including early transgene changes, synapse loss, neuroinflammation, microvascular abnormalities that may trigger the cascade of cognitive decline, has hampered progress. The outcomes of the anti-A β drug trials in AD resonate with the outcome of Parkinson's disease trials, five

decades ago, when the central hypothesis underlying Parkinson's disease research was tested: drugs that reversed the characteristic dopamine depletion in nigrostriatal neurons effectively ameliorated Parkinsonian signs and symptoms in most patients, even though the drugs had no discernible effect on the underlying disease process. Some hypotheses turn out to be correct; others do not. Hence, a broad range of putative underlying pathological mechanisms could be targeted for AD. A more balanced approach to disease treatment and prevention that includes the impact of nutritional and lifestyle changes should be considered in future direction for AD research.

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