Advanced Drug Discovery for Alzheimer's Disease: Challenges and Strategies

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2.1 An Introduction to Alzheimer's Disease

The progressive loss of organization and function of neurons leading to the death of neurons collectively constitutes the Neurodegenerative Diseases (NDs). Age-associated NDs have been a cause of significant health burden because of lack of treatment. NDs pose a great challenge for the elderly population, healthcare providers and caregivers. These diseases result from progressive loss of structure and/or function of neurons. Neuronal death within specific areas of brain predominantly cerebral cortex, hippocampus, and spinal cord results in deficiency of key neurotransmitters further affecting motor functions/movement (known as ataxia), and non-motor functions/mental functioning (known as dementias). Neurons in general don't reproduce or substitute themselves, when they are damaged they cannot be replaced in abundance under normal circumstances though recent studies on neurogenesis provide some hope on neuronal recovery too. NDs are incurable and debilitating conditions that result in progressive degeneration and/or death of nerve cells. A striking number of more than 600 disorders have been reported that affect the nervous system. The most common disorders include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, Spinocerebellar ataxia, Prion disease, and Amyotrophic Lateral Sclerosis (ALS). The cause of each one being believed to be dependent on a number of factors, some most important wherein causes range from particularly genetic or environmental factors [1]. The most common among all NDs is AD with an annual death toll of more than 500,000 people [2]. According to the World Health Organization (WHO) Global Burden of Disease Study in 2012, AD and other dementias are the top fourth cause of death in high income countries after heart disease, stroke, and lung cancer [3]. A 2014 report

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submitted by the US organization reported that more than 5.2 million Americans are currently living with the disease, which includes five million people above the age of 65 years (late-onset AD) and roughly 2 lakh individuals below 65 years of age (early-onset AD) [2], thus making AD the most expensive disease condition in the United States with an estimated $214 billion cost to the American Society. Worldwide, currently more than 25 million people are affected by dementia, most suffering from AD with five million new cases accruing up each year [4]. In Europe, the age-standardized prevalence in people more than 65 years of age is 6.4% for dementia and 4.4% for AD [5]. A new study predicts that the AD in the United States will get doubled by 2050 and the cost of caring will rise to $1.5 trillion per year.

AD has been named after a German physician Alois Alzheimer. On 3rd November 1906, while presenting his findings at the "37th meeting of the Society of Southwest German Psychiatrists" in Tübingen Germany, Alois Alzheimer for the first time described the symptoms of progressive cognitive impairment, focal symptoms, hallucinations, delusions, and psychosocial incompetence changes in a patient called Auguste D, a 51-year-old woman from Frankfurt hospital [6]. The disease was later named by a German psychiatrist Emil Kraepelin as "Alzheimer's Disease." AD is an age-related disorder which affects the population over 65 years of age (elderly population) and is not to be confused with the "normal ageing" phenomenon. Clinically, AD is characterized by progressive and irreversible decline in memory and cognitive functions. In later stages, motor and sensory functions are compromised which leads to drastic personality changes like aggression, apathy, agitation, paranoia, insensitivity to others, lack of initiative, delusional thinking, loss of interest in activities they previously enjoyed, inability to make decisions, and finally the person is socially withdrawn. The cognitive defects are reflected neuropathologically by demise of specific neuronal populations, synaptic loss, and brain atrophy in specific brain areas [7–9] and most importantly by the presence of senile plaques (amyloid plaques) and neurofibrillary tangles (Tau protein) which are formed by improperly processed proteins. These improperly processed proteins tend to form aggregates which are toxic to the neurons and ultimately result in their degeneration [10]. The diagnosis of AD can only be confirmed by autopsy after the death and in living patients it can be done on the basis of some cognitive tests [11]. Patients affected with AD tend to show cognitive decline which includes gradual memory loss, difficulty in performing daily tasks, declining physical coordination, lack of judgment making, personality changes, difficulty in learning, and loss of communication skills [12]. The disease eventually leaves its victims unable to care for themselves and in the final stages; victims are bedridden and normally die due to secondary infections like urinary tract infection, pneumonia, and/or bedsores. The molecular mechanism of the disease progression of AD has been a topic of debate for last several years, and there are two cardinal theories prevailing in the scientific community regarding mechanism of AD. Factors governing neuronal loss can be grouped into genetic, environmental, and endogenous ones. The main culprit is known to be the accumulation of abnormal extracellular protein plaques and neurofibrillary tangles of the microtubules formed by amyloid beta (Aβ) and tau protein, respectively. Aβ is a 40 or 42 amino acid peptide with approximate size of 4 kDa, derived from the precursor protein, namely amyloid

precursor protein (APP). In mid-1980s, APP was first reported to be the core component of amyloid deposits in AD and Down's syndrome patients [13–15]. APP is considered to play a protective role in glutamate excitotoxicity induced by neuronal stress or injury [16]. In diseased individuals APP undergoes abnormal cleavage by enzymes beta-secretase and gamma-secretase sequentially resulting in either a soluble 40 amino acid residue (Aβ40) or an insoluble 42 amino acid peptide (Aβ42) [17]. The resulting hydrophobic 42 amino acid fragments stick together to form clumps of senile plaque outside the neuronal surface causing the death of neurons. The other theory revolves around the tau protein, which plays a role in providing structural integrity to microtubules within neurons [18]. The hyperphosphorylated forms of tau have a tendency to bind among themselves rather than with the microtubules as a result hyperphosphorylated tau leads to formation of flame-shaped neurofibrillary tangles (a paired helical filament) thereby killing the neurons. Both the theories are equally recognized in the scientific world, as each of them is backed up by very important findings. It is possible that both theories are interconnected to each other via some junction points which are still to be uncovered. AD is subdivided into two forms: the rare early-onset AD, which affects individuals before the age of 65 usually in their 30s or 40s; and the second more common late-onset AD, where symptoms develop after 65 years of age. Early-onset AD tends to cluster within families affecting several generations and hence gets its name as "familial AD." These subforms of the disease show different patterns of genetic inheritance and genes associated with them. The familial forms of AD are reported to be linked with many genetic factors which are extremely rare mutations with the occurrence of 1 in 1000 cases. The genes implicated in causing early-onset AD include mutation in *PSEN1* (Presenilin 1) on chromosome 14, *PSEN2* (Presenilin 2) on chromosome 1, and APP on chromosome 21. Research findings suggest that *PSEN1* encodes for one of the four proteins in presenilin complex, *PSEN1/PSEN2* both function to regulate proteolytic cleavage of gamma-secretase [19], a predominant enzyme that cleaves the APP. Mutations in either *PSEN1/PSEN2* influence proteolytic property of gamma-secretase enzyme causing more Aβ42 peptide formation. APP is highly conserved protein whose function is yet to be discovered although it has been suspected to regulate synapse formation [20] and neural plasticity [21]. The gene for APP is located on chromosome 21 (triplicate in Down's syndrome) and mutation in APP gene results in abnormal APP protein that is preferentially cleaved by proteases to form more Aß42 [22]. It is likely that any person having mutant versions of *PSEN1, PSEN2*, or APP will develop AD at comparatively early age of 30s or 40s and will pass on these genes to their offsprings. The inheritance of late-onset forms of AD follows a more complex pattern because of its unpredictable behavior. At that place are some modest but rising gene variations that have been identified which affect—to different degrees—the prospects of developing late-onset Alzheimer's. The effects of these genes are subtle, with variations altering the likeliness of getting AD. The most important gene that is associated with and greatly influences the risk of developing late-onset AD is apolipoprotein E (APOE). APOE, primarily produced by the liver and macrophages, is a major cholesterol carrier that corroborates lipid transport through the bloodstream. Any deviation from the normal levels of cholesterol may cause disease like heart attack and stroke. The human APOE gene is found on chromosome 19 and occurs in

three common forms (polymorphic alleles): APOE ε2, APOE ε3, and APOE ε4. People carrying the ε4 allele are at expanding danger of getting AD, as compared with those carrying the more commonly found ε3 allele, conversely the ε2 allele is reported to diminish the hazard. Presence of APOE ε4 influences deposition of Aβ to form senile plaques and play as a risk factor for developing cerebral amyloid angiopathy. Aβ deposits as decrepit plaques are more plenteous in APOE ε4 transporters than in noncarriers [23]. Nowadays, scientists are targeting APOE ε4 which might offer an attractive alternative target for AD therapy in the near future [24].

2.2 Diagnostics and Treatments Available

AD follows a complex etiology, additionally the cause of the disease is governed by multiple factors. It is believed that the clinical symptoms of AD are manifested many years after the initiation of pathological hallmark governing the disease. Researchers believe that since it is a complex ailment, it is impossible that a single treatment will avert or cure it. Broad exploration is creating and testing an assortment of conceivable medications for AD. While doctors can quite often figure out whether a person has dementia, it might be hard to decide the definite cause. There is no single test that can indicate or rule out, if a person has Alzheimer's. AD is analyzed through a complete medicinal appraisal; it is typically diagnosed with the help of healthcare providers that include neurologists, psychiatrists, and psychologists. They look at the patient's health history to figure out if a person's memory issue or other mental abilities have been declining over time. The first diagnostic test may include mental status testing via physical examination of memory, verbal skills, problem solving, thinking skills, and mood stability. The cognitive tests can be carried out by two commonly used tests; the mini-mental state exam (MMSE) [25] and the mini-cog test [26]. These tests depend upon a series of questions and simple remembering exercises intended to test the scope of ordinary mental abilities of the patient. Secondly, a physical and neurological exam of patient's urine, blood, and spinal fluid is carried out. Lastly, brain imaging techniques like computed tomography (CT) scan or magnetic resonance imaging (MRI) tests are carried out to rule out other causes of dementia-like symptoms that include strokes, tumors, or blood clots that might be the cause of dementia. Apart from this, there are some genetic tests available that check for some causative genes like Apo-E4, but this routine genetic testing is still not recommended for AD. This is because these genetic testings don't show whether a person will develop Alzheimer's or whether a person already has Alzheimer's. Furthermore, genetic tests may give us an idea of familial AD, which only accounts for 5% of all cases. The 100% confirmation of AD can only be done by performing autopsy that displays the amyloid plaques after the death of the patient [36]. In year 2012, the National Institute on Aging and the Alzheimer's Association published an article on the diagnostic guidelines for AD. In this article, they have defined the factors (molecular biomarker, epidemiological and neuropsychological evidence) which best predict the risk of progression from "normal" cognition to mild cognitive impairment and AD dementia, but this is only for research purposes and they do not have any clinical implications so far [27].

Table 2.1 List of drugs approved for Alzheimer's disease

Drug name	Brand name	Approved for	Function	Year of FDA approval
1. Donepezil	Aricept	All stages	Cholinesterase inhibitor	1996
2. Galantamine	Razadyne	Mild to moderate	Cholinesterase inhibitor	2001
3. Memantine	Namenda	Moderate to severe	NMDA (*N*-methyl-D-aspartate) receptor antagonist	2003
4. Rivastigmine	Exelon	All stages	Cholinesterase inhibitor	2000
5. Donepezil and memantine	Namzaric	Moderate to severe	Cholinesterase inhibitor + NMDA (*N*-methyl-D-aspartate) receptor antagonist	2014

2.3 Current Treatments

There is currently no absolute cure for AD, multiple FDA-approved drugs currently being prescribed (listed in Table 2.1) merely slow down the disease progression or bring down the symptoms. Pharmacological agents available in the market to treat AD provide only short-term symptomatic relief to the patients towards helping them in taking care of daily problems like Amnesia (memory loss), thinking, and judgment making. They either improve the cholinergic transmission within CNS or prevent the excitotoxic action that results from overstimulation of NMDA-glutamate receptors in selected areas of the brain. These drugs are characterized under acetylcholinesterase inhibitors and NMDA receptor antagonists.

2.4 Cholinesterase Inhibitors

Acetylcholine is the major neurotransmitter, a chemical messenger of the parasympathetic system that transmits signals across a synapse (the junction) from one neuron to another neuron, muscle cell, or gland cell. A defect in this cholinergic neurotransmission leads to the destruction of synapses and killing of neurons which is the characteristic feature of AD. Acetylcholinesterase enzymes are a class of serine hydrolase enzymes found mainly in neuromuscular junction and within cholinergic synapse that are involved in rapid hydrolysis of neurotransmitter Acetylcholine into choline and acetic acid, thus leading to its termination within the central and peripheral nervous system. Its active site contains two subunits—the anionic site that corresponds to catalytic machinery and its esteric site that holds choline binding pocket, on which many drugs target. Along these lines, inhibition of this protein Acetylcholinesterase is utilized as a key target towards managing the diminished acetylcholine in AD patients. Inhibition of Acetylcholinesterase leads to accumulation of the neurotransmitter acetylcholine and enhanced stimulation of postsynaptic cholinergic receptor. Currently, three drugs available in the market that include Donepezil, Rivastigmine, and Galantamine, work on the principle of inhibition of acetylcholinesterase. Another

acetylcholinesterase inhibitor, Tacrine, was the first drug that was used in the treatment of AD. It was potent in improving the memory and cognition but also resulted in cholinergic associated side effects like nausea, abdominal cramps, and hepatotoxicity because of which its utilization was suspended in the United States in 2013, due to concerns over safety. Donepezil acts as a reversible acetylcholinesterase inhibitor, provides symptomatic relief, and delays deposition of amyloid plaques within the brain [28]. It also imparts some cholinergic associated side effects like malaise, appetite loss, weight loss, sleep problems (insomnia), muscle cramps, weakness, nausea, vomiting, or diarrhea. Donepezil is prescribed for severe Alzheimer's dementia cases. Recently, it has also been used to improve speech in children with autism. Rivastigmine is a slow reversible carbamate inhibitor that blocks cholinesterase activity by binding to esteric part of the acetylcholinesterase. A transdermal rivastigmine patch with lesser side effects is available in the market that can be applied to the skin. Transdermal rivastigmine is also used to treat lewy bodies and dementia associated with PD. Galantamine is an alkaloid isolated from the plant *Galanthus woronowii.* Galantamine is a selective, competitive, rapidly reversible AChE inhibitor that acts at the anionic subunit of acetylcholinesterase. Due to the allosteric potentiating effect of nicotinic receptors, it has perturbing role on cholinergic, glutamate, GABA as well as monoamine neurotransmitter systems. Galantamine improves cognitive dysfunction and psychiatric disorder in patients of schizophrenia, depression, bipolar disorder, and alcohol abuse. Despite providing symptomatic relief none of the above medication imparts long-term benefits with superior efficacy to patients. AchE inhibitors have marked pharmacological application in various other neurodegenerative disorders but cholinergic side effects associated with them don't strongly support their prominent use. Thus, effective pharmacotherapy is still needed to combat AD that could target both cholinergic transmission hindrance and the protein aggregation associated with it.

2.5 NMDA (*N*-Methyl-D-Aspartate) Receptor Antagonists

NMDA (*N*-methyl-D-aspartate) receptors are glutamate receptors and ion channel proteins found inside the nerve cells. NMDA receptor is very important for regulating synaptic plasticity and memory function. It gets activated by docking of the glutamate present at cell surface which further allows influx of Calcium (Ca^{2+}) ion through the cell membrane. Over-activation of NMDA-type glutamate receptors results in excessive Ca^{2+} influx that leads to excitotoxicity leading to neuronal injury and death. Agents that block the NMDA receptor activity like partial antagonist Memantine are used for treatment of moderate-to-severe AD. Memantine, discovered as an antiviral drug, serves as an uncompetitive, open-channel blocker of excessive NMDA receptors without disturbing normal activity. The way to Memantine's restorative activity lies in its uncompetitive binding to the NMDA receptor. Its low affinity and rapid off-rate kinetics retain the normal physiological function of the receptor. Memantine is being

prescribed to patients of moderate and severe Alzheimer's dementia. Memantine is also in clinical trials, for additional neurological disorders that include dementia, depression, and severe neuropathic pain. In addition to this, researchers are currently working on a series of second-generation Memantine derivatives that may have better and safe therapeutic intervention properties as compared to Memantine.

2.6 Drugs in Clinical Trials

There are many new drugs that are under clinical trials for the treatment, prevention, immunization, or towards slowing the progress of AD. Past trials have tested an assortment of medications/drugs in individuals but no significant improvements have yet been demonstrated. The paradigm of drug discovery for AD has shifted from providing mere symptomatic relief towards targeting other parameters like inhibiting Aβ/tau aggregation, combinatorial drug therapy, inhibition of other enzymes and receptors involved in AD manifestation, modulation of pathways and vaccination/immunization therapy, and so on. Many drugs, as listed in Table 2.2, are in pipeline of phase III trials; these drugs focus on ways to treat the disease. These drugs may provide a critical opportunity for therapeutic intervention of AD in the near future.

Table 2.2 Drugs in phase III clinical trials

S.No.	Agents	Mechanism of action	Manufacturer
1.	Aducanumab	Antibody to protofibrillar Aβ	Biogen
2.	ALZT-OP1	Drug combination	AZTherapeutics
3.	AZD3293	BACE inhibitor	Astrazeneca
4.	Azeliragon	Inhibits receptor for advanced glycation end products	TransTech Pharma
5.	CAD-106	Vaccine against Aβ	Novartis
6.	CNP520	BACE inhibitor	Novartis
7.	Gantenerumab	Monoclonal antibody against Aβ	Hoffman-La Roche
8.	Insulin	Intravenous immunoglobulin	Grifols
9.	JNJ-54861911	BACE inhibitor	Janssen
10.	LU AE58054	5HT6 receptor antagonist	H. Lundbeck
11.	Masitinib	Inhibitor of c-KIT cell signaling	AB Science
12.	Nilvadipine	Calcium Channel Blocker	St. James Hospital
13.	Pioglitazone	PPAR-gamma activator	Takeda
14.	Sodium Oligo-mannurate	Inhibits Aβ aggregation	Shanghai Greenvalley Pharmaceuticals
15.	Solanezumab	Humanized antibody against Aβ	Eli Lilly
16.	TRx0237	Tau aggregation inhibitor	TauRX
17.	Verubecestat	BACE inhibitor	Merck

2.7 Challenges in Treating the Disease

With an astonishing improvement in science and medication, human life span has increased significantly; yet this has led to an increase in the age-related incessant ailment and AD is one of them. AD is a standout among the various forms of dementia in the elderly and seeking of an effective therapy against the disease is a serious concern. It is not a simple undertaking; however, a number of factors, as detailed below, make the goal of complete treatment hard to achieve:

1. *Complex etiology of the disease*: Scientists are trying to comprehend the actual cause for AD. There are several combinations of causes that contribute in the development of the disease but none of them are responsible for the disease progression as a sole factor.
   (a) *Genetic causes*: Pharmacogenetic profiling of AD patients has portrayed the inherited genetic differences in response to therapeutic potential of drugs. Variation in APOE gene greatly influences the development of late-onset AD. Presence or absence or a particular polymorphic form of APOE alleles greatly affects the therapeutic potential of the drugs. Similar drugs respond differently in APOE ε4 carrier and noncarrier. For example, bapineuzumab, a new immunotherapy against AD prevents Aβ deposition in the brains of APOE ε4 carriers with mild or moderate AD, but not noncarriers [29]. APOE ε3 noncarriers responded better to donepezil treatment than E3 carriers in Han Chinese patients with AD [30]. Thus, genetic make-up can increase the complication of therapeutic targets. People with different alleles respond to the same treatment differently. There are some specific genes that decide how effective a therapy would be, especially a gene which is involved in the drug metabolism and transportation, but none can be associated with the disease with certainty.
   (b) *Age*: Age is the biggest risk factor for AD. Age-related changes in the brain like atrophy, inflammation, mitochondrial dysfunctioning, and formation of free radicals may contribute to AD development. In AD, old brain is the easiest victim. Scientists are still trying to find answers of what factors and their amount is to be considered for the therapeutic development.
   (c) *Factors other than age*: Factors other than age may also play a role in the development of the disease, for example, gender, education, obesity, and other diseased conditions. These factors also decide the therapy that should be applied to a person, male or female, lean or fat, immunocompromised or hypersensitive. For example, if a person is immunocompromised, anti-inflammatory drugs are not prescribed.
2. *Multifactorial nature of the disease*: The conventional approach of the drug development has been "one molecule one target" but AD is a syndrome and has many contributing factors. There is no unitary theory to explain the molecular basis of the disease. There are varied opinions regarding different processes being the primary reason, some researchers believe in cholinergic hypothesis, some in tau hypothesis and others in amyloid hypothesis. Any of these or all of

them may contribute to the development of the disease, so we require a drug that could interact with several molecular targets of the cascade. All the presently marketed drugs although improve cognitive, functional, and behavioral impairments yet none of them inhibits disease progression, hence remain ineffective.

3. *Sensitivity of nervous system*: Our nervous system is highly sensitive. Researcher's trying to develop any CNS therapy have to be extra cautious. Neurons control almost every primal activity of our body be it movement, be it cognition, all senses and their related aspects are managed by neurons. Any interference in any of its function may result into serious damage; therefore, CNS drugs are more likely to fail than other therapeutic drugs. Most CNS drugs have side effects, 75% of all CNS drugs available to date have side effects. CNS drug takes longer to get into the market than other drugs. All these factors make it difficult to develop a therapy of Alzheimer's disease.
4. *Blood–brain barrier*: A huge number of 100 billion nerve cells communicate with each other in a very efficient way. In order to do so, it is very important to maintain its own microenvironment and integrity. Our body goes through chemical fluctuations all the time, for example, hormonal fluctuations, fluctuations in ions and others components, but to keep our brain unaffected from all these fluctuations our brain is provided with an additional protection that is blood–brain barrier. A network of blood vessels with tight junctions that is produced by several transmembrane proteins inhibit the entry of almost every molecule except the entry of essential nutrients like glucose, some amino acid, insulin, and other precursor molecules. Even if any molecule crosses the blood–brain barrier, it cannot stay inside for long, because of transporter proteins that extrude compounds from the brain, for example, P-glycoprotein which is a member of ABC transporter protein, it most effectively extrudes molecules from the brain. Even though we somehow develop any drug to cure AD, the problem is to deliver the drug into the brain.
5. *Cross talk with other diseases*: AD and its cross talk with other diseases make AD treatment more challenging. Some disease conditions increase the risk factors of AD, for example, diabetes significantly increases the risk of developing AD [31]. Insulin is the common factor that plays an important role in both of these conditions. Insulin regulates energy metabolism and homeostasis in various cells. It reaches brain by cerebral spinal fluid and transporters present at blood–brain barrier. It is thought to increase cognitive ability by activating insulin receptor in hippocampus region of the brain. The binding of insulin to its receptor activates extracellular signal-related kinase, mitogen-activated protein kinase, PI3 kinase/AKT pathway and inhibits GSK-3. Transforming growth factor-β signaling cascade also modulates Aβ aggregation and associated outcome as demonstrated in studies employing varius animal models [32, 33]. Dysregulation of these pathways may also lead to cardiovascular abnormalities, inflammation, and neuropathy. All of these have been associated with AD and further complicate the development of therapy against AD.

2.8 Progress Being Made

Transgenic mouse models for AD have been developed based on amyloid hypothesis and further improved with the understanding of specific genetic mutations. Like other disease models, AD mouse model also helps in better understanding of pathophysiology of the disease and has emerged as an invaluable tool in preclinical testing of potential therapeutics as well. Mouse serves as an efficient, evolutionarily close, and robust model for several diseases including AD. It has short life span as compared to other vertebrates. Invertebrate models of AD such as *Drosophila melanogaster* and *Caenorhabditis elegans* offer certain advantages over mouse model and have aiding in obtaining significant understanding of the disease; however, the downside of these models is that they are evolutionarily distant from the humans as compared to mice.

Triple transgenic mouse model of AD: The neuropathology of AD mainly revolves around the correlation of Aβ plaques and tangles. Therefore, in order to develop a better understanding of this correlation and its effect in synaptic function, triple transgenic mouse model (3×Tg-AD) has been developed having three transgenes $PS1_{MI46V}$, APP_{Swe}, and tau_{P301} [34]. Instead of crossing three independent lines, 3×Tg-AD is created by introducing two transgenes human APP_{Swe} and tau_{P301} (under the control of mouse Thy1.2 regulatory element) inside the single cell embryo isolated from $PS1_{MI46V}$ knock-in mice. The 3×Tg-AD mice deposit Aβ extracellularly prior to tangle formation mimicking amyloid cascade hypothesis [34]. These mice exhibit synaptic disjunction and deficit in long-term potentiation (LTR) in an age-related manner before plaque and tangle deposition which is found associated with intracellular Aβ immunoreactivity. Comparatively, double transgenic mice (2×Tg-AD) lack human APP as a result they neither deposit extracellular Aβ plaques nor exhibit intracellular Aβ immunoreactivity. Therefore, these 3×Tg-AD mice are very useful in studying the effect of Aβ and tau deposition in synaptic philology and to access anti-AD therapies in a more reliable and mechanistic way mediated by both hallmark lesions.

5XFAD mouse model: The 5XFAD model is a double transgenic mouse model of APP and PS1, which co-expresses five mutations (three mutations in APP and two mutations in PS1) which elicit overall Aβ production with $Aβ_{X-42}$ toxicity [35]. The model was developed by introducing APP Swedish (mutation at β-secretase cleavage site), London, and Florida mutations (mutation at γ-secretase cleavage site, APP 717 and APP 716, respectively) with human PS1 having M146V and L286V mutation under the control of mouse Thy-1 promoter. The Swedish mutation results into the higher level of total Aβ, whereas Florida, London, M146V, and L286V mutation enhances the production of $Aβ_{X-42.}$ This process is observed as an early onset of plaque deposition with astrocytosis and microgliosis parallely in 5XFAD mice. Interestingly, a gender-specific response is observed in this model system in response to stress, where female mice show significant deposition of plaques in the hippocampal area of brain as compared to male [36]. Apart from plaque pathology age-dependent synaptic degradation is observed in them. The 5XFAD model is also among the few models that show neuronal loss at the cortex and subiculum (by 9 months of age) mimicking AD pathophysiology relatively better.

A worm as AD model system: *Caenorhabditis elegans* (*C. elegans*), a nematode (round worm), is a very useful transgenic model to study neurodegenerative disease related to basic toxic mechanism [37]. The transparent nature of this worm facilitates to study cellular differentiation and other developmental processes as well. In the context of neurodegenerative diseases, using *C. elegans* confers many advantages like short life span; as a result, construction of transgenic model and assessment of any experiment can be done relatively faster. Researchers have developed transgenic AD model of *C. elegans* which expresses human Aβ fragment in the muscle cells [38]. A construct called PCL12 having DNA fragment which encodes for human $A\beta_{1\text{-}4}$ under the control of UNC-54 promoter was prepared . This construct was introduced inside the gonads of nematodes via microinjection to produce Aβ constitutively making animals undergoing paralysis. The assessment of transgenic strain was done by co-injecting pRF4 plasmid which encodes for a mutant collagen gene whose expression leads to the onset of roller mutation. The strain is temperature sensitive and maintained at 15 °C. Onset of paralysis and egg laying deficiency is introduced by maintaining the strain at 20 °C.

Drosophila as a model organism for AD: Comparative genomics has revealed that around 70% of disease-causing genes in humans have orthologs in the fly including the orthologs associated with AD genes with functional conservation. Drosophila has APP orthologs dAPPl, γ-secretase complex, and β-secretase-like enzyme which shows very low β-secretase activity; as a result, there is no endogenous production of Aβ in the fly. Overproduction of β-secretase-like enzyme has shown the cleavage of dAPPl which corresponds to the human Aβ which is able to aggregate and can induce behavioral and neurological impairments in an age-dependent manner.

Apart from endogenous Aβ production transgenic flies have also been generated to understand the AD in a better way. Greeve and coworkers have developed a transgenic fly which expresses human APP, human β-secretase (hBACE), and Drosophila presenilin (dpsn) with point mutations N14I, L235P, and E280A in order to mimic familial AD mutations [39]. These flies exhibit a correlatable association between neurodegeneration and age progression. Using Gal4/UAS inducible system another transgenic fly has been developed carrying Gal4-driven construct which encodes human APP and human BACE which is able to generate Aβ peptide in a tissue-specific manner. In this system, by using specific promoter yeast Gal4 protein is expressed in particular cells. As Gal4 is a transcription factor; it binds to the UAS and induces the expression of the gene of interest which is upstream to UAS in the construct. Such complex Aβ is ideal for the better understanding of APP processing. Such models are easy to handle as compared to models, where Aβ is fused with downstream of secretory peptide. Fly models overexpressing tau have also been developed, where human tau is overexpressed with increased activity of glycogen synthase kinase 3β (GSK3β) activity to form intracellular inclusions which resemble neurofibrillary tangles; this finding also confirms the previous notion that tau toxicity requires hyperphosphorylation and aggregation. Thus, Drosophila AD models provide a better insight of mechanism with two important AD hallmarks.

Newer imaging techniques for diagnosis: Molecular imaging techniques have grown rapidly in the past few years. The advancements allow us to measure brain structural changes (like atrophy, volume, cortical thickness), pathology of the brain (fibrillary Aβ and tau), and functional changes (glucose metabolism and neurotransmitter activity). These new imaging techniques help us in diagnosing AD early and in evaluating the medical therapy in a better way [40]. Nowadays, the most commonly used molecular technique for diagnosis and treatment follow-up in AD is positron emission tomography (PET). This technique helps physicians in assessing abnormalities of the brain via a painless and safe method. PET scanning is a technique where three-dimensional images at both cellular and molecular level can be obtained. In a PET scan, very small amount of radioactive tracer is injected into the patient's body which has either affinity to bind to the desired target, usually a particular protein, to show the presence and extent of its deposition in the brain or the tracer can be metabolized to study the functional changes inside the brain.

1. *Aβ imaging in AD patients*: Pittsburgh Compound B (^{11}C-PIB) was among the first Aβ PET tracers. It has been found that as compared to the healthy individual in AD patients high ^{11}C-PIB is observed in cortical and subcortical areas of the brain [41]. Nowadays, ^{18}F-labeled tracers are thought to be more suitable because of their long half-life. The first ^{18}F-PET tracer that is used for visualizing Aβ plaque was ^{18}F-FDDNP. Though it was showing low affinity to Aβ as compared to ^{11}C-PIB but observations suggested that it also binds to neurofibrillary tangles. ^{18}F-flutemetamol, ^{18}F-florbetapir, and ^{18}F-florbetaben have been found as promising ^{18}F-PET tracer in AD patients [42].
2. *Imaging functional changes in AD brain*: In order to measure regional cerebral glucose metabolism rCMRglc, 2-[^{18}F]-fluoro-2-deoxy-d-glucose (^{18}F -FDG) is widely used [43]. Reduction in rCMRglc is observed in very specific areas of the brain (as the brain with specific dementia will consume less energy at specific area and therefore less sugar) which is more prevalent in early onset as compared to late-onset AD; whereas retention of ^{11}C-PIB is observed in larger areas of the brain with no variation in the regions specific in its retention in both early and late-onset AD. With the progression of the disease, a decline in the rCMRglc is observed.
3. *Imaging neuroreceptor and neurotransmitter*: Wreckage in cholinergic, dopaminergic, and serotonergic neurotransmitter system is usually observed in AD brains. Many PET tracers have been developed to study the different neurotransmitter levels and the receptors in the AD patient. Decline in cholinergic neurotransmission and nicotinic receptor has always been correlated to the reduction in attention and cognitive function.
4. *Tau imaging*: After successful implications of PET-Aβ imaging, substantial research is going on towards developing PET tracer that can detect tau inclusion bodies in order to understand the tau pathophysiology better. There are lots of hurdles in the development of such PET tracers highly specific for tau because of its structural variation and severe posttranslational modification. Despite these hindrances, several compounds have been developed which show tau binding ability [44]. Such compounds are under thorough investigation before they can enter into the clinical practice.

2.9 Strategies Ahead

Despite the fact that the pathophysiology of AD is not fully understood, it is majorly exhibited by the neurotoxic effect of amyloid plaques (Aβ) and deposition of neurofibrillary tangles (tau protein). Clinical advantages of the drugs available for the treatment of AD (to be specific cholinesterase inhibitors and NMDA receptor antagonist) are obvious, albeit they are merely restricted towards providing symptomatic and psychological treatment only. Over the past few decades, enormous amount of research worldwide has been directed towards the development of newer strategies in order to tackle or even prevent the pivotal pathological processes in AD. More than 200 pharmaceutical compounds are currently being tested in phase II and III clinical trials. The following are the cases of promising focuses for cutting-edge drug treatments under scrutiny in current research studies:

Anti-amyloid strategies: Misfolding of the Aβ protein leading to its aggregation is the characteristic hallmark of AD. During the past years, a great understanding has been developed about the molecular mechanisms through which they are formed. Drugs are being designed that are aimed at inhibition, prevention from overproduction or aggregation of Aβ or facilitating the clearance of Aβ from the brain. As shown in Table II numerous drugs that are in phase III clinical trials for their mechanistic ability to handle Aβ with the possibility of diminishing its load in the brain. The reported limitation is that these anti-amyloid agents should be administered in the early events of AD progression. Anti-Aβ vaccine, AN1792 has been proved effective for removal of brain Aβ via the use of anti-Aβ antibodies but its use has been shut down due to its side effects of causing acute meningoencephalitis. These vaccines work by eliciting immunological response against Aβ. The newer active and passive Aβ immunotherapies like bapineuzumab, humanized anti-Aβ monoclonal antibodies, aducanumab, CAD-106, and gantenerumab have been developed which are under clinical investigation with the aim of accelerating Aβ clearance from the brain of the AD patients [45]. For further reading, anti-amyloid agent has been reviewed in the work of Aprahamian et al. [46].

Beta-secretase (BACE) inhibitors: Apart from vaccines, therapeutic agents are being vigorously pursued that are aimed to block the action of β-site amyloid precursor protein cleaving enzyme (BACE) which is responsible for the production of the neurotoxic Aβ (reviewed in [47]). These therapeutic agents inhibit the ability of the BACE to make Aβ thus preventing Aβ clustering into plaques. The only difficult part for the researchers is to tackle the blood–brain barrier as BACE inhibitor drugs cannot pass through it and there also is toxicity associated with the molecules. Much of the BACE inhibitor drugs have been dropped out because of their toxicity. BACE inhibitor drugs, namely AZD3293 [48], CNP520 [49], JNJ-54861911 [50], and Verubecestat [51] are currently in phase III clinical trials.

Anti-tau strategies: Tau protein is the main component of neurofibrillary tangles, another hallmark associated with Alzheimer pathology. Tau proteins stabilize the microtubule and help in maintaining the structural integrity of the neurons. Analysts are developing drugs that can prevent formation, phosphorylation, and aggregation of tau protein to form neurofibrillary tangles. Strategies involved are the use of active and passive immunotherapy, tau protein kinase inhibitors,

microtubule-stabilizing agents, and tau aggregation inhibitors. Drug TRx0237, currently in phase III clinical trials, is a second-generation tau protein aggregation inhibitor [52]. TRx0237 is a more advanced formulation for Rember®, a formulation of methylthioninium chloride which is the purified form of methylene blue. TRx0237 is much more improved formulation than Rember® because it renders greater absorption, bioavailability, and tolerability. Another class of compounds is lithium salts that have been reported to prevent tau hyperphosphorylation and reduce its concentration; mode of their action is via inhibition of glycogen synthase kinase (GSK-3β), axonal degeneration, and release of transforming growth factor beta 1 (TGF-β1) [53–55]. Prolonged chronic lithium treatment may result in renal impairment and hypothyroidism. Safe treatment limits of lithium salts are still to be determined. Additionally, AADvac1, a vaccine against an abnormal form of tau protein is under research and may prove beneficial for the treatment of AD in the near future.

Anti-oxidative agents: In recent years, considerable amount of research data has provided evidence towards increased oxidative stress in the brains of AD patients. The theory of increased oxidative stress is supported by the fact that there is increased free radical generation, lipid peroxidation, protein and DNA oxidation, advanced glycation end products (AGE), and SOD-1 in neurofibrillary tangles present in AD patient brain. The increased ROS levels may be due to mitochondrial dysfunction, accumulation of transition metals, and accumulation of Aβ and tau proteins. These increased free radicals further lead to neuronal degeneration and death. Theoretically, therapeutic drugs aimed at getting rid of ROS are being designed to prevent propagation of tissue damage by ROS and improve neuronal survivals. Administration of very few antioxidants in the diet has shown some efficacy but its protective role against AD is still in question. The ROS formation may be a secondary event but its contribution to the initiation and progression of AD is not denied; hence, targeting it may be fruitful.

Anti-inflammatory agents: In the AD patient brain, Aβ and tau deposits are reported to elicit stimuli for inflammation, which result in degeneration and death of neurons. Inflammation results in activation of immune response in the brain primarily recruiting microglial cells. The presence of Aβ and tau microglial cells may become overactive and secrete toxic compounds thus damaging nearby neuronal cells. Recent clinical trials of readily available anti-inflammatory drugs like celecoxib and naproxen which were expected to improve cognitive function in elderly individuals were rendered insignificant along with slight adverse effects. In a new theory, inflammation may play a beneficial role towards the clearance of Aβ. CSP-1103 is another inflammatory drug in research which has recently undergone phase II clinical testing. CSP-1103 also acts via modulation of microglial cells in order to reduce inflammation in the brain [56]. Preliminary studies have been proven effective towards lowering Aβ levels and increased memory in phase II clinical trials. CSP-1103 is still in exploration and not accessible to people in general.

Targeting brain insulin signaling: There is an impairment of insulin signaling in AD patient brain. Decline in insulin level has been correlated with cognitive decline and the development of AD. A great interaction is reported to be present between

diabetes and Alzheimer's to an extent that some researchers are calling AD as type 3 diabetes [31]. An important role of insulin and insulin signaling in the treatment of AD has been proven. AD brain tends to show resistance to the normal effects of insulin known as insulin resistance, which may occur due to decreased expression of insulin receptors, or decreased levels of insulin or insulin-like growth factor in the brain. The insensitivity of insulin may also be due to impairment in the transport of insulin across the blood–brain barrier. Thus, targeting "brain" insulin signaling for the treatment of AD has now pulled in much consideration in the field of AD research and therapy [57]. Current drug in research that targets insulin resistance is the insulin molecule itself and drugs that improve insulin sensitivity, which include drugs like incretins, dipeptidyl peptidase IV inhibitors, thiazolidinediones, and metformin [58–61]. Intranasal insulin is given to counter the problem related to blood–brain barrier. Intranasal insulin therapy is currently under phase III clinical trials. Use of intranasal insulin is supporting therapeutic potential for patients with amnestic mild cognitive impairment and patients with AD [62].

Exploring of novel delivery routes: AD pathology is very specifically limited to the person's brain as the neurons degenerate in that area. Numerous drugs that have shown good efficacy in rodent models in the treatment of AD have failed in clinical trials. This may be attributed to the process of passing therapeutically active molecules across the blood–brain barrier. Passing of the drugs across the blood–brain barrier is a complex process that is mediated by special tight junctions present at blood–brain barrier. Several strategies have been employed to enhance the efficacy of the drugs being administered to AD patients. Some strategies include structural damage to the blood–brain barrier, nanobiotechnology transport/carriers-based delivery methods, and alternative route of delivery such as intranasal administration. Structural damage to the blood–brain barrier is done by forcibly opening it to allow diffusion of drugs or by direct introduction of drugs in the brain by surgical procedures. The drawbacks of structural damage to blood–brain barrier are that it results in damage to the barrier permanently. Another delivery method is the use of nanoscale particles transport/carriers [63]. The blood–brain barrier allows transport of molecules that are less than 1 nm. The advancement of nanobiotechnology has enabled us to synthesize very small nanoparticles/receptor-tagged nanoparticles that may just pass through the blood–brain barrier. In this approach, drugs are bound to a nanoparticle making the drug capable of passing through blood–brain barrier. The only limitation is the uncontrolled passage into the brain which may not be desirable. Nanobiotechnology researchers are exploring controlled ways of delivering nanoparticle-based drugs. The last approach is practical, simple, and noninvasive to tackle the blood–brain barrier by delivering drugs through nasal route [64]. The nasal cavity is rich in olfactory and trigeminal nerves that are involved in sensing smell. This olfactory epithelium in between the nasal mucosa and the brain serves as a link between brain and external environment that provides portal of entry of molecules directly to the brain. This route also allows drugs which do not cross the blood–brain barrier to enter the brain. A wide variety of therapeutic agents like insulin molecule are being rapidly delivered to the CNS using this intranasal approach.

Combinatorial therapies: AD is a multifactorial disease and is linked to the formation of aggregates of misfolded proteins (Aβ, tau) in neurons, degradation of neurotransmitters, increase in oxidative stress, mitochondrial dysfunction, excitotoxicity of neurons, genetic factors, insulin resistance, etc. As AD is governed by various factors, no single therapy has so far proven to be satisfactory for the effective treatment. One of the hypotheses for combating AD is the use of multiple agents/novel agents working through different mechanisms for targeting multiple factors associated with the disease [65]. The proposed hypothesis may offer the best hope for a future neuroprotective therapy. A combination of bioactive components, used against different pathomechanisms in neurodegenerative diseases may be fruitful. In addition to combinatorial therapy, researchers are also developing multitarget ligands, for example, benzofuran and chalcone hybrids bearing hybrid structures with a capacity to simultaneously interact with multiple targets against AD [66]. Phytomedicines are widely used across the globe as economical, effective, and safer alternatives to synthetic drugs. Phytomedicines exhibit the property of neuroprotection via free radical scavenging, abating misfolded protein aggregation, and enhancing dopamine (a neurotransmitter) level. Use of phytomedicines, multiple molecules or hybrid molecules may help in countering AD. It is still too early to comment whether combinatorial therapies will be effective for the prevention or treatment of AD.

Advent of early diagnostic tools via *employing newer technologies, looking for noninvasive/sensitive diagnostic tools*: The early diagnostic tools include high resolution neuro-imaging via MRI which is used for the quantification of the loss of brain volume. Analysis of Aβ and tau levels in cerebrospinal fluid by high resolution imaging, computed tomography (CT) may be used as an alternative for MRI when it is contraindicated [67]. Better tracer molecules like Amyvid (florbetapir F-18), Vizamyl (flutametamol F18), and Neuraceq (florbetaben F18) that bind to Aβ/plaques in the brain are being developed for use in living patients. Newer computerized cognitive testing is also being used for early detection of mild cognitive impairment via detection of changes in walking speed that reflect defects in motor neurons [68]. Current research is going on towards delivering an early diagnostic tool for AD via identification of several potential biomarkers that may appear before clinical symptoms [69]. Biomarkers are used to identify the state of disease, their progression, and predict possible treatment. BACE1 enzyme, still in its infancy, is considered as a biomarker in blood as one report suggests that it is enhanced in AD patients [70]. The plasma lipid, a lipid pathway in the brain particularly in cholesterol biosynthesis, is used as a noninvasive AD biomarker [71]. Small RNAs mainly miRNAs may serve as an upcoming class of biomarkers for AD as the regulation of amyloid production has been linked with some specific miRNAs [72, 73]. In spite of intense research efforts, currently there are no validated biomarkers for AD; however, significant progress has been made in the field. A very preliminary study known as saliva test has been presented at the 2015 Alzheimer's Association International Conference in Washington for the early detection of AD. In addition to this, identification of new markers, newer amyloid imaging, technology like smaller sensors, wireless technology, combining home computer, and use of proteomics assessment of protein signatures in the brain are underway for the early detection of AD.

Popularizing ways of increasing brain plasticity, encouraging healthy lifestyle: A standout among the most energizing science improvements of late years is the generation of knowledge regarding the plasticity of brain. The ability of the brain to form new neural connections is called neuroplasticity and the process to make new connections between neurons continues throughout the duration of our lives. A new study suggests that utilizing memory techniques can help the brain develop new neural pathways for learning and enhance memory, notwithstanding for individuals with early signs of AD. Nowadays, more and more neuroplasticity exercises like brain program, exercise, or game are becoming available on the Internet to enhance brain working. Brain's neural connections can be strengthened and regenerated by performing mental exercises on daily basis. Ongoing study shows that brains of people with MCI/Alzheimer's have plasticity and routine brain exercise increased their memory score by 33% [74]. Popularizing ways of increasing brain plasticity is a promising approach for delaying the effects of AD. Encouraging healthy lifestyle is another way of influencing AD improvement. Adherence to a healthy lifestyle throughout life may directly or indirectly help in preventing AD by reducing modifiable risk factors [75]. Promoting this knowledge can also help people and their families from the perils of AD.

Using information technology-based approaches towards recruiting larger number of patients for carrying out clinical studies: For carrying out clinical trials, recruitment of participants (human subjects) plays a vital role in the success of the research. Most of the studies fail due to less participation of human subjects. The failure of research frequently takes longer than foreseen that may be attributed to longer recruitment time which further increases the cost of projects than expected. The process usually involves identification of eligible candidate, obtaining informed consent, holding members until study fulfillment and following ethical norms. In one of the statistical surveys, it is reported that only 3–20% of the pool human subjects participate in the clinical trials [76]. The reduction in sample size diminishes the statistical analysis of the study and may give us a wrong impression of the drug under research. Among several ways, one way to tackle this situation is the use of information technology-based approaches towards recruiting larger number of patients for carrying out clinical studies. The approaches may include the use of automated clinical trial recruitment [77] that include digital platforms, social media, and mobile technologies. Internet is used to gather/share information about medical condition, experiences, and illness between caregivers and patients directly which minimizes the time and is less expensive. There are many online patient communities such as PatientsLikeMe®, TrialMatch®, and Ben's Friends which work on these principles to significantly boost success rates [78, 79]. These portals are used for phone calls, emails, or sending messages directly which minimize the burden on participants and make their participation more efficient and convenient for them. In fact, dynamic organizations have started to utilize e-enrollment systems effectively.

Improving outreach in hard-to-reach areas: One of the strategies to support people with AD and their families is by providing greater understanding of the disease and its symptoms so that it can be diagnosed and treated early.

Early detection may help the AD patients to slow down the disease by coordination of care and treatment. Recently, huge budget government projects have been sanctioned aimed at improving outreach in hard-to-reach areas. Outreach also helps the healthcare providers in recruiting human subjects for the clinical trials. It likewise increases support for individuals with AD and caregivers in the community with improved data collection and analysis to better comprehend the effect of AD on individuals with the disease, families and the well-being, and long-term care systems.

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