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A Snapshot on the Current Status of Alzheimer's Disease, Treatment Perspectives, In-Vitro and In-Vivo Research Studies and Future Opportunities

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Abstract

Alzheimer's Disease (AD) is one of the most challenging diseases faced by humankind. AD is still not classified as curable because of the complex structure of pathologies underlying it. As the mean life expectancy of the world population constantly increases, the prevalence of AD and treatment costs for AD also grow rapidly. Current state of the art for AD treatment mainly consists of palliative therapy aimed at providing symptomatic relief and improving the standard of living in patients with AD. However, different research groups are working on more effective and safe drug delivery options aimed at both symptomatic relief and treatment of the underlying mechanisms. In this review, the current prevalence of AD, health costs, pathologies, and available treatment options including the ones in the market and/or under trial have been reviewed. Data in the existing literature have been presented, and future opportunities have been discussed. It is our belief that these nanotechnological products provide the required efficacy and safety profiles to enable these formulations go through phase studies and enter the market after regulatory authority approval, as with cancer. Last, but not the least the metabolomic studies will be providing useful informative data on the early diagnosis of AD, thus may be clinical implications might be delayed with the administration of therapeutic agents at the initial state of the disease.

Key Words: Alzheimer's disease; Amyloid-β peptide; Tau; Neurodegeneration; Metabolomics; Cell Culture

1. Snapshot on the Current Status of Alzheimer's Disease

The primary cause of Alzheimer's Disease (AD) is still not known, although there are a few hypotheses on how to treat the disease. Several research groups are working on a spectrum of new ideas and treatment opportunities. However, they still lack a complete and standardized operational procedure for AD. Most of the drugs currently in the market for treatment of AD are used only for symptomatic relief or recovery.

AD is one of the most frequently occurring diseases, especially in those over 60 years. It is a neurodegenerative disease of the central nervous system, and is mainly classified under dementia [1]. Dementia, in general terms, is a disorder that is mainly characterized by cognitive decline, including symptoms such as impairments in learning and memory, language, executive function, complex attention, perceptual-motor skills, and social cognition. These domains have been revised in The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) in 2013 to rename dementia as a *major neurocognitive disorder*. Although AD is the leading cause of dementia, accounting for 60-80% of cases, the revised definition allows the diagnosis of dementia arising from other causes including dementia with Lewy bodies (DLB), frontotemporal dementia (FTD), vascular (multi-infarct) dementia (VaD) and Parkinson's disease with dementia (PDD) [2]. As the global population is aging constantly and the mean life duration is increasing, there is a parallel increase in the prevalence of the AD. Therefore, the need to understand the background mechanisms of AD and its treatment opportunities is vital for humankind. Currently, 35.6 million patients suffer from dementia and/or AD [3]. It has been forecasted that the number of patients with dementia will double over the next 20 years [4, 5]. This will result in the number of patients with dementia increasing to 65.7 million in 2030 and 115.4 million in 2050. It is expected that the greatest increase in AD diagnoses will be in highly developed countries, due to the higher life expectancy and

income of citizens in these countries. These patients will be in need of professional health care that will likely result in elevated costs for health care authorities of the individual countries. The actual cost of the treatment/care of dementia was USD 604 billion in 2010, which significantly overshadows cancer and cardiovascular disease costs [6, 7]. The number of patients living in the United States (US) with Alzheimer's dementia has been reported to be 5.7 million, with 5.5 millions of those being over 65 years [8, 9]. Therefore, it has been forecasted that the total care costs of AD patients will increase from USD 257 billion (in 2017) to USD 1.1 trillion (in 2050), which makes studying AD essential to provide new insights into the treatment of this disorder [8].

In most AD cases, the initiation of the symptoms starts with the disruption of recent memories. During the progression of the disease, patients start to experience problems in visual and spatial domains, a lack of attention, cognitive dysfunctions such as executive dysfunctions, praxia, and other problems in daily life [10]. The major problem for AD is the unidentified etiology. During the last three decades, many drugs have been tested for the treatment of AD; however, only five showed satisfactory efficacy and have been approved by the regulatory authorities. Despite this, approved drugs only exert their effect through replacement of the cholinergic deficiency induced by the decreased level of acethylcholine in brains of AD and are generally termed as cholinesterase inhibitors. In addition to these replacement therapies, after the approval of memantine (NMDA receptor antagonist) by the US Food and Drug Administration (FDA) regulatory authority in 2003, no other novel treatment option has entered the market and many trials have been unsuccessful [11]. Since 1990, there have been many trials attempting to treat AD. There has been an especially large number of trials to remove amyloid plaques and an increased number of patent applications in this area [12, 13].

2. Basic Pathology of Alzheimer's Disease

AD is a disease of the elderly. The daily life characteristics and factors that the patients are exposed to throughout their lives may affect the history and course of the disease. This is the main reason for the uncertainty in the identification of an exact cause. Etiologic factors affecting AD can be classified as lifestyle and genetic factors [5]. However, the current basic pathophysiology is mainly focused on the accumulation of amyloid-β and tau proteins in brain parenchyma [14]. This was first illustrated by Alzheimer in 1907. Histological examination of a number of patients revealed that amyloid-β peptide and hyperphosphorylated tau were major components of the plaques and neurofibrillary tangles, respectively [15, 16]. These findings led Hardy *et al* to theorize that amyloid-β protein, which is a peptide product of amyloid precursor protein (APP), was the main component of the plaques that are responsible for the Alzheimer's pathology [17]. This hypothesis has been further supported by studies on the autosomal dominant mutations leading to AD in patients. The major components of these mutations have been extensively investigated by many research groups. One of these is the gene coding for APP. APP is the haloprotein from which amyloid-β peptide is excised via sequential scission by the β-APP, cleaving enzyme and γ-secretase [14, 18-24]. Further supporting data for these genetic mutations comes from the autosomal dominant mutations in presenilin 1 and presenilin 2 [14, 25-27]. Apolipoprotein E (APOE) genes are also a major risk factor for AD. The naturally existing alleles APOE2, APOE3, and APOE4 can protect against, have no impact on, and accelerate the onset of AD, respectively [14, 28, 29]. The mechanism of action for these effects is still not known, although it has been suggested by Kim *et al*. that these genes mediate the clearance of amyloid-β with a decreasing efficacy as follows: APOE2, APOE3, and APOE4 [30]. In summary, the studies focusing on the production of amyloid-β plaques, on decreasing the aggregation of these

plaques, or on improving their clearance can help slow down the progression of AD. The failures of clinical studies, which are focused entirely on amyloid-β peptides, provoke questioning of this perspective and result in considering other strategies besides combination opportunity [31, 32]. In studies where researchers have demonstrated the aggregation of amyloid plaques in the hippocampal region in AD patients, leakage of blood through the blood-brain barrier (BBB) was also demonstrated [33, 34]. Preventing this leakage through care and restoration of the BBB may be another optional perspective for the prevention of AD. The only drugs able to offer this form of treatment are corticosteroids; however, their efficiency is not sufficient [35].

In addition to the aforementioned perspectives, it was shown that the levels of brain derived neurotrophic factor (BDNF) and its receptor tyrosine kinase B (TrkB) are decreased in the hippocampus and cerebral cortex of patients experiencing early stages of AD [36-39]. BDNF's regulation of synaptic activity is important for neuronal plasticity, memory formation, and storage. As a result of these data, it was postulated that the BDNF-TrkB pathway is active in cases of amyloid-β stimulated neurotoxicity, synaptic dysfunction, and worsening of memory [40]. In these studies, stimulation of TrkB improved cognitive function and increased synapse intensity. Studies have also shown that the effects of TrkB activation are independent of amyloid-β and tau pathologies [41, 42].

As mentioned in a recent review by Lv *et al*., [1] amyloid-β and tau protein are still considered to be the two key peptides resulting in senile plaques and neurofibrillary tangles in AD. It was proposed that senile plaques might also be observed in the absence of neurodegeneration, however, this is thought to be related to soluble amyloid-β oligomers, which are observed in the early stages of AD [43]. The reason for the formation of senile plaques in AD patients is related to the spreading of amyloid-β across different regions of brain. The cleavage of APP

results in the formation of the hydrophobic protein Aβ42 which rapidly aggregates [44]. Disrupted mechanisms for the production and clearance of Aβ42 in AD patients result in the formation of senile plaques [1, 45].

With new generation of the state art of technologies, different omics technologies provide a comprehensive quantitative monitoring of various biological molecules including genes, RNA, proteins, metabolites and lipids etc. Comparisons of the omics profiles of treated versus untreated or heathy versus sick are powerful and useful approaches for a better understanding of molecular patho-mechanisms. Indeed, a massive amount of information produced from the omics technologies can be used for early diagnosis of diseases, identifying the mechanism behind the disease or understanding system biology. This comprehensive information may improve the clarification of a phenotype regarding the pathophysological changes. Therefore, in recent years many diseases including AD are subjected to omics analyses.

Highlighting the mechanism behind the AD pathophysiology at the early stage of the disease helps better understanding the etiopathogenesis of AD and this may lead to personalized based treatment in AD by selecting better therapeutic targets. However, the hardest challenge behind to AD is the long time period between pathophysiological changes and clinical symptoms emergence. In AD, the most of the treatment perspectives have focused on the mechanism of amyloid-beta (Aβ) conformation[1, 45].

Different omics technologies can be used for the monitoring of AD progress; but, of these, metabolomics is pioneering in many studies because of its simplicity, cost, efficiency and the most important one is reflecting a true phenotype of the living organism at the sample taken out. Since it is providing a complete picture of the organism including not only genotype but also other actors such as living style, environment and microbiota of the organism. Therefore,

in recent years many studies based on metabolomics approaches are running on different disease studies to better understand the phenotypes. Metabolomics is the analysis of small molecules in organism and they represent accurate molecular fingerprint profiles of an organism. Metabolomic analysis can be performed on different specimens such as cell, tissues, blood, urine etc. [46-53].

Metabolomics studies on AD in human mostly aimed to identify disease progression to find suitable markers for early diagnosis. In these studies, plasma and cerebrospinal fluid (CSF) were used as biospecimen in order to differentiate AD patients from cognitively normal individuals [46-53]. Up to date, in metabolomics studies of AD many different biomarkers were identified to discriminate individuals. In a study by Kaddurah-Daouk et al., postmortem ventricular cerebrospinal fluid has been analyzed for the investigation of alterations in tyrosine, tryptophan, purine, and tocopherol pathways in patients with AD [54]. It was found that norepinephrine was significantly decreased in AD patients ($p=0.0001$) with respect to control group. In addition to that, nominally significant differences between groups mostly in tyrosine, tryptophan, purine and tocopherol pathways have been shown through several metabolites. In another study by Sato et al., a new plasma biomarker of AD has been investigated by metabolomics technology [50]. In this study, it was found that plasma demesterol and demesterol/cholesterol ratio significantly decreased in AD patients with respect to the healthy subjects ($p>0.001$). Cerebrospinal fluid and plasma levels of cholesterol and its precursors lanosterol, lathosterol and desmosterol have been investigated for the evaluation of alterations in AD patients [52]. As a result of this study, cerebrospinal fluid level of cholesterol ($p = 0.011$), absolute levels of all investigated cholesterol precursors (each $p <0.001$) were found to be significantly lower in AD patients. Similarly, plasma levels of lanosterol ($p = 0.026$) and lathosterol ($p < 0.001$) and the ratio of lathosterol/cholesterol ($p = 0.002$) were attenuated in AD patients with respect to control. The levels of more than 800

molecular species of lipids have been investigated by Han et al. using shotgun lipidomics [53]. For this purpose, plasma samples of 26 AD patients were analyzed for correlation between diagnosis, apolipoprotein E4 genotype and cognitive performance. At the end of this study, 8 molecular species out of 33, which contained long aliphatic chains such as 22 and 24 carbon atoms, were significantly lower ($p<0.05$) in AD compared to control group. The plasma levels of 2 ceramide species were higher in AD patients ($p<0.05$) with a comparative having a weaker trend for 5 other species. Taking the role of brain sphingolipids in neuronal functioning into account, new perspectives on the AD sphingolipidome and possible usage of metabolomic signatures metabolomic signatures as peripheral markers have been underlined.

On the other hand, some cell culture and animal studies performed for mechanistic studies [55, 56]. One of them, performed on familial AD mice models, showed that mitochondrial function and cellular energy metabolism in human and animal models precedes amyloid deposits and memory impairments [56]. Alterations in mitochondrial activity and morphology correspond to augmented production of reactive oxygen species leading to increases in oxidative stress. This can gradually accelerate cellular damage and lead to multiple cellular dysfunctions, further accelerating cellular damage, and ultimately cell death [55, 56]. This new mechanism may also be considered for the treatment or prevention of AD and a target for new drug or drug-delivery systems [55-57].

3. Treatment Perspectives in Alzheimer's Disease

3.1. Conventional Approaches

The incurable nature of AD makes it one of the greatest challenges for health care professionals. Many research groups are working on different strategies for developing novel drugs for AD patients. A number of these fail either in Phase II or Phase III trials. Currently applied treatment choices mainly focus on the basic characteristics of AD, the degeneration

of acetylcholine-containing forebrain neurons [58]. Therefore, the first treatment option for AD patients is to increase acetylcholine levels in the brain tissue using cholinesterase inhibitors [59]. Clinical studies show that these drugs can improve cognitive function of AD patients and 5 commercial products have been introduced into the market containing active pharmaceutical ingredients such as tacrine hydrochloride, rivastigmine, donepezil, galantamine, and a combination product of memantine which is NMDA receptor antagonist and donepezil after FDA approval [60-65].The pharmacokinetic properties and approval years of the fore-mentioned drugs for AD is summarized in Table 1. Of these, tacrine, which was introduced in 1993 as the first cholinesterase inhibitor, has been discontinued due to its severe side effects in AD patients and its very low bioavailability [8, 66, 67].

Donepezil, which is the active pharmaceutical ingredient of Aricept®, was first used for the treatment of AD in 1996. It is used to treat mild to moderate and moderate to severe AD with different doses for daily use. In a meta-analysis of donepezil studies, it was observed that this drug significantly improves cognitive function of AD patients [68, 69]. The approved doses of donepezil are 5 mg, 10 mg, and 23 mg/day, according to the severity of the disease. In clinical studies with 5-10 mg/day doses, donepezil showed a statistically improved difference scores in the Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog) of patients after 24 weeks [70, 71]. In another study, a phase III trial with 1371 patients, the efficacy of high dose (23 mg/day) donepezil was compared with a moderate dose (10 mg/day) [72]. The measures of this phase III study were the Severe Impairment Battery (SIB) [73, 74] and Clinician's Interview-Based Impression of Change Plus Caregiver Input scale (CIBIC+). SIB scores ranged from 0, which is the most impaired, up to 100, which is the least impaired. CIBIC+ scores were evaluated according to a 7-point scale [75, 76]. The secondary measures for this study were the Alzheimer's Disease Cooperative Study–Activities of Daily Living scale (severe version) (ADCS-ADL) [77] and the Mini-Mental State Examination (MMSE) [78]. At the

end of the study, it was found that the cognitive and global functioning of patients receiving 23 mg/day Donepezil was associated with enhanced benefits in cognition when compared to donepezil 10 mg/day ($p < 0.001$). SIB and CIBIC+ scores of more advanced AD patients were higher than those of less impaired AD patients. Adverse effects of nausea, vomiting, and diarrhea were more prevalent in those given the 23 mg/daily dose. The study concluded that the 23 mg dose significantly improved cognition in moderate to severe AD patients, but not global functioning of the overall population [72].

Table 1. Table of pharmacokinetic parameters and approval years of conventional Alzheimer Drugs

Active Pharmaceutical Ingredient	Approval Date	Mechanism of Action	Half -life ($t_{1/2}$-h)	Protein Binding (%)	Peak Plasma Time (h)	Bioavailability (%)
Tacrine	1993	CoA inhibitor	2-4	75	1-2	17
Donepezil	1996	CoA inhibitor	70	96	3-4	100
Rivastigmine	2000	CoA and butylnylcholinesterase inhibitor	p.o.: 1.5 patch: 3	40	p.o.: 1 patch: 8	36
Galantamine	2001	Selective and competitive CoA inhibitor	7	18	1	90
Memantine	2003	NMDA receptor blockage (non-competitive)	60-80	45	3-7	100

Rivastigmine, which was approved in 2000, is the active pharmaceutical ingredient of Exelon®. It is a pseudo-irreversible inhibitor of acetylcholinesterase and butyrylcholinesterase. Although its half-life is short (1-2 hours), its activity lasts for a longer period on these enzymes, 3.5-8.5 hours [79] and in another study, up to 10-12 hours [80]. The

most significant advantage of rivastigmine is its low protein binding. This means that it is unlikely to interact with other drugs, a key consideration given that elderly patients may take several drugs for various purposes [81]. In two studies which were performed on mild to moderate AD patients, 6-12 mg/day dosing of rivastigmine for 26 weeks improved the cognitive functions in a dose-related fashion [58, 82, 83]. It is currently available in the market under different names and in the form of oral capsule, oral solution, and transdermal patch at different doses [84]. The option to use a transdermal patch makes rivastigmine superior from the other treatment options for mild to moderate AD, since this route allows for sustained blockage of enzymes and patient compliance due to ease of use [85-87]. Another study evaluated the efficacy of transdermal and oral rivastigmine in 2252 patients with mild to moderate AD. Transdermal application improved dose titration and the required plasma concentration is met in a shorter time [88]. Interestingly, a drop out study has been performed on the patients who discontinued treatment with rivastigmine. The cognitive functions were investigated after the washout period and compared against placebo controls. The results of patients who had been off rivastigmine (6-12 mg /day) for 102 days, were compared with those who had been off placebo for 68 days. At 26 weeks the ADA-Cog score of the placebo group was 4-7 points lower than that of the rivastigmine group, suggesting a significant effect of rivastigmine treatment on AD symptoms[89].

Galantamine, which was approved by FDA in 2001, is the active pharmaceutical ingredient of Razadyne®. It is a tertiary alkaloid first derived from plants and is also synthetically available for selective and competitive inhibition of acetylcholinesterase. In addition, it can enhance cholinergic nicotinic neurotransmission by modulating nicotinic cholinergic receptor sites [90, 91]. It is available in tablet, solution, and extended release capsule forms in dose of 4 to 24 mg under different commercial names. It is used in the treatment of mild-to-moderate AD patients [92]. When the clinical pharmacokinetics are considered, the bioavailability of

galantamine is 90%, with a large volume of distribution and low protein binding. The overall tolerability is good, although there are some reports of mild -to-moderate gastrointestinal events in clinical trials [93]. To determine the optimum dosing, the efficacy and safety of galantamine (16 mg/day-24 mg/day) has been investigated in patients (n = 838) experiencing mild-to-moderate AD by Aronson *et al*. [94]. For patients with mild AD, mean ADAS-Cog scores improved for both doses in comparison to placebo controls ($p < 0.001$). 24 mg/day was effective for the patients with moderate AD ($p = 0.009$). Patients treated with 16 mg/day of galantamine demonstrated a higher treatment response in comparison to both those receiving 24 mg/day and placebo controls, with patients in this group showing improved/maintained ADAS-Cog scores. 16 mg/daily dose is recommended since comparable results were observed with 24 mg/daily dosing [94]. In another study, Hager *et al*. have investigated the efficacy of galantamine after 2 years with a range of doses from 8-24 mg/day, depending on the clinical outcomes. The major measure in this study was the cognitive change (Mini-Mental State Examination score) in comparison to time point 0 and up to 24 months later. Mini-Mental State Examination scores were decreased in the placebo group in comparison to the treatment group ($p < 0.001$; -2.14-placebo versus -1.41-galantamine). Functional impairment was also significantly better in galantamine treated group ($p = 0.002$). As a result, galantamine treatment in mild to moderate AD patients was found to be effective in comparison to the placebo group [94].

In addition to the aforementioned acetylcholinesterase inhibitors, one of the recently approved drugs for neurodegenerative diseases including AD is memantine. It is the active ingredient of Namenda®, which has been in the market since 2003 following approval from the FDA. The second mechanism, aside from being an acetylcholinesterase inhibitor, is the blockage of N-methyl-d-aspartate (NMDA) receptors upon over-activation. NMDA over-activation is one of the reasons for neuronal degenerative diseases including AD [95].

Memantine is used for the non-competitive inhibition of NMDA receptors and appears to provide neuroprotection with a rapid and two-way inhibition mechanism [96-98]. In a study by Reisberg *et al.*, efficacy of memantine in patients experiencing moderate to severe AD was tested over a period of 28 weeks. Patients received 20 mg of memantine and according to the outcomes of the study, was well tolerated. Switching to memantine from placebo significantly improved functional, clinical and cognitive measures in this double-blind placebo study ($p < 0.05$) [98]. In another 24-week-long, double-blind study in Europe (470 subjects), patients with mild to moderate AD were administered 20 mg memantine per day. The outcome measures were evaluated using ADAS-Cog and CIBIC+ scores. Statistically significant improvements were observed in comparison to placebo controls at 12- and 18-week timepoints. This was followed by superior outcome measures at the 24th week [99]. Memantine is recommended at doses of 10 mg twice a day and 28 mg/day in the form of extended release capsules. A meta-analysis of six different Phase III studies in a total of patients 1826 with moderate to severe AD has been conducted by Winblad *et al.* The results suggest memantine is well tolerated and its clinically significant efficacy is well-established [100]. The second option for memantine treatment in AD is the combination with an acetylcholinesterase inhibitor. In recent meta-analysis studies, combination of memantine with acetylcholinesterase inhibitors provided a significantly improved cognitive functions and scores in a neuropsychiatric inventory ($p = 0.00001$ for both) [101]. Seven randomized controlled trials were included in another meta-analysis study that investigated the efficacy of combining memantine with acetylcholinesterase inhibitors. Considerable improvements in cognitive function were reported, in addition to improvements in behavioral disturbances and activities of daily living [102]. Despite these findings and other supportive meta-analysis studies [70, 103-105], some studies indicate that combination of memantine with donepezil has no significant effect on moderate to severe AD patients [106].

All the conventional active ingredients and products mentioned above have various limitations in the treatment of AD. Acetylcholine inhibitors have many side effects like nausea, vomiting, diarrhea and confusion that affect patient's life negatively. In addition to that, when pharmacokinetics of the conventional drugs is overviewed (Table 1), one can see that Tacrine and Rivastigmin have low bioavailability. Donepezil binds proteins very high percent, this can be cause interactions and side effects. Galantamine has very short half-life and as a result, these limitations have led to the search for new and more effective ways for treating AD.

3.2. Novel Trends and Opportunities

The treatment of AD is still a challenge and none of the treatment options currently available result in the complete removal of the disease. The major boundary for the treatment of AD is the BBB. In addition to that, Blood-Cerebrospinal Fluid Barrier keeps to be secondary challenge for neurodegenerative and neurological disorders; thus, these mechanisms are to be fully understood for creating novel therapeutics options for these disorders. In addition to the multivariate nature of AD, none of the strategies could combine the view of neuroregeneration with the prevention of neurodegeneration [107]; likewise in other studies that combines them in a single formulation [108]. Due to the natural structure of the BBB, the passage of the active molecules to the brain is limited. BBB is composed of vascular endothelial cellular structures that involve tight junctions. Therefore, these structures limit the passage of active pharmaceutical ingredients from systemic circulation to the brain. However, novel techniques in drug delivery science provide many different solutions to this problem. One possibility is nanotechnology, that brings new insights for localized drug delivery options at the desired site of action [109-112]. In this approach drug loaded nanoparticles penetrate through BBB and deliver drug to brain, which is site of action. There are a number

of new strategies to help overcome the BBB as an obstacle for drug delivery in central nervous system disorders [113], but none of have completed the phase trials for AD.

Another recently proposed approach uses long-circulating nanoparticles with high affinity for the amyloid peptide as potential amyloid scavenging or detoxifying agents. Although this approach has been now investigated with several particles, the most advanced is based on liposomes. Recent works describe the design of functionalized liposomes (ApoE-derived peptide, phosphatidic acid, or cardiolipin) that have a high affinity against the amyloid peptide and can scavenge it from the bloodstream, leading to a measurable reduction in amyloid levels and, most importantly, a recovery of the memory impairment in AD transgenic animal models. Interestingly, the authors proposed, and were able to demonstrate *in vitro*, that the liposomes do not need to reach the brain, which would be one of the main challenges in drug delivery. Indeed, by scavenging the peptide from the brain, the liposomes can promote the diffusion of peptide from the brain parenchyma to the blood [114-117]. In both approaches nanocarriers penetrate through BBB, which is the major reason for using nanoparticles; however, movement direction is different. Since nanosystems are drug delivery systems, they may both be used for delivering drug to brain for targeted therapy or carrying amyloid plaques from brain to the blood for provide clearance. Other groups used a similar approach using other classes of particles. For example, Le Droumaguet *et al*. described the design of polymeric nanoparticles functionalized with anti-Aβ antibodies, which have a very high affinity for the amyloid peptide [118]. However, there is no *in vivo* data using this particle system. Although larger studies in more relevant animal models are required, the work reported so far seem to suggest a promising approach which could translate to other diseases.

The preparation of nanoparticular systems containing conventional treatment options, as such they may provide a sustained and localized delivery at the site of action, is vital for the

treatment of AD. Using this approach, researchers have prepared nanoparticular dosage forms containing acetylcholinesterase inhibitors. Nanoparticles, which are prepared with different polymeric materials and coated for better penetration through the BBB, have been tested by many groups. Notable examples include tacrine encapsulated chitosan and poly(n-butylcyanoacrylate) nanoparticles [119, 120], rivastigmine loaded polysorbate 80 coated poly(n-butylcyanoacrylate) nanoparticles [121], albumin nanoparticles carrying cyclodextrin for nasal delivery of tacrine [122], poly(D,L-lactide-co-glycolide) microparticles containing tacrine [123], Huperzine A encapsulated in poly(D,L-lactide-co-glycolide) microparticles [124], Thioflavin-T and Thioflavin-S encapsulated in poly(butyl-2-cyanoacrylate) nanoparticles [125], quercetin loaded zein nanoparticles [126], dexibuprofen loaded nanoparticles [127], and siRNA against BACE1 (a key enzyme in the amyloid peptide production process) loaded solid lipid nanoparticles [128] . Detailed information regarding the purpose of these nano-systems are summarized in Table 2.

These studies outline either characterization of the drug delivery systems, biodistribution or brain localization of the active ingredients. However, pharmacodynamic studies demonstrating effectiveness for AD are still lacking. On the other hand, one significant example of the application of nanotechnology for the delivery of Coenzyme Q10 in AD animal models via trimethylated surface-modified poly(d,l-lactide-co-glycolide) nanoparticles were prepared by Wang *et al*. The authors characterized the *in vivo* efficacy of nanoparticles in APP/PS1 transgenic mice with behavioral testing of animals performed after nanoparticle administration. This study reported improved memory impairments, reduced concentration of Aβ, and inhibition of Aβ fibril formation [129].

Other possibility in novel trends for the AD treatment is antibody-based immunotherapy but this option is not as effective as nanotechnology. Antibody-based immunotherapy against Aβ

is using for triggering its clearance or mitigate its neurotoxicity. Unfortunately, this immunotherapy has been unsuccessful so far [130, 131]. But a recent study has used aducanumab, which is a human monoclonal antibody which selectively targets to amyloid-β aggregates and this study has good results[132]. In the Tg2576 transgenic mouse model of AD, it was shown that aducanumab could enter the brain, bind to parenchymal Amyloid-β plaques, and attenuate the soluble and insoluble form of Amyloid-β plaques in a dose-dependent fashion. In addition, in patients experiencing prodromal or mild AD, monthly i.v. aducanumab infusions for one year (3, 6 and 10 mg/kg) significantly reduced amyloid-β plaques, as confirmed by PET imaging. As noted by the authors, formation of these plaques takes approximately 20 years, yet after one year of treatment, the patients' symptoms improved [132].

Table 2. Micro and nano drug delivery systems containing various active pharmaceutical ingredients for AD

	Active Pharmaceutical Ingredient	Nanoparticle Type	Carrier	Purpose	Advantages	Reference
Tacrine encapsulated chitosan	Tacrine	Polymeric nanoparticles	chitosan & polysorbate 80	Maintenance of diffusion-controlled release of the drug.	Ability to control the release of active agents and avoidance of the use of hazardous organic solvents	[119]
Poly(n-butylcyanoacrylate) nanoparticles	Tacrine	Polymeric nanoparticles	poly(n-butylcyanoacrylate)	Targeting tacrine into the brain.	These nanoparticles reduce the total dose required for the therapy with concurrent	[120]

					reduction in dose related toxicity.	
Rivastigmine loaded polysorbate 80 coated poly(n-butylcyanoacrylate) nanoparticles	Rivastigmine	Polymeric nanoparticles	polysorbate 80 & poly(n-butylcyanoacrylate)	Maintenance of drug transport across BBB.	Significant transport of the drug rivastigmine in comparison with the free drug to the brain in this study.	[121]
Albumin nanoparticles carrying cyclodextrin for nasal delivery of tacrine	Tacrine hydrochloride	Albumin nanoparticles	β-cyclodextrin	Attenuation of the degree of problems like low bioavailability, short elimination half-life and hepatotoxicity	Albumin nanoparticles carrying native and hydrophilic derivatives β-cyclodextrin can be employed for the formulation of	[122]

					mucoadhesive nasal formulations with interesting drug permeation properties	
Poly(D,L-lactide-co-glycolide) microparticles containing tacrine	Tacrine	Microsphere	poly(D,L-lactide-co-glycolide)	Delivery of tacrine systemically for a prolonged period of time and with lower concentrations.	That would release the drug for weeks in treating Alzheimer's disease so patient compliance will be increase. Hepatic adverse reactions will reduce and drug can by-pass the gastrointestinal route.	[123]

Huperzine A encapsulated in poly(D,L-lactide-co-glycolide) microparticles	Huperzine A	Microsphere	poly(D,L-lactide-co-glycolide	Reduction of initial burst and extending the sustained release.	It indicates the potential of a 2-week sustained release system of Huperzine A.	[124]
Thioflavin-T and Thioflavin-S encapsulated in poly(butyl-2-cyanoacrylate) nanoparticles	Thioflavin-T and Thioflavin-S	Polymeric nanoparticles	poly(butyl-2-cyanoacrylate)	Aβ targeting	Increased transport of the fluorescent drugs through the BBB.	[125]
Quercetin loaded zein nanoparticles	Quercetin	Polymeric nanoparticles	2-hydroxypropyl-β-cyclodextrin (HP-β-CD)	Improvement of oral absorption and bioavailability of the quercetin.	Zein nanoparticles would offer a prolonged residence in close contact with the gut mucosa.	[126]

					Reduced level of inflammatory markers and increased bioavailability.	
Dexibuprofen loaded nanoparticles	Dexibuprofen	Polymeric nanoparticles	poly(lactic-co-glycolic) & PEG	Enhancement of dexibuprofen brain delivery and reduction of systemic side effects.	These nanoparticles transported across the BBB to treat and prevent inflammation associated with Alzheimer Disease.	[127]
siRNA against BACE1 (a key enzyme in the amyloid peptide production	Fluorescence-labeled siRNA,	Solid Lipid Nanoparticles	Chitosan	The aim of the study is to deliver therapeutically	Gene therapy, like silencing of the β-secretase	[128]

process) loaded solid lipid nanoparticles				relevant amounts of drugs directly from the nasal cavity to the central nervous system (CNS) and to provide direct access. For this purpose, BACE 1 siRNA was complexed with RVG-9R and encapsulated in solid lipid nanoparticles.	gene, BACE1, leads to an improvement in the pathogenesis of Alzheimer's disease. BACE 1 siRNA influences the β-cleavage of amyloid precursor proteins. Using the intra-nasal route in this therapy provides direct access to the CNS without the need to cross the	

					BBB.	

4. Research Studies for Alzheimer's Disease

4.1. In-Vitro Models

The recent progress in cell culture techniques has facilitated the modeling of numerous diseases in vitro, enabling the studies on mechanism and treatment options. The cell loss and neuronal disfunction mechanism of neurodegenerative diseases like AD could be enlighten using neuronal cell lines as in wild-type or transfected form to provide the microenvironment of the neurodegeneration with related protein formation, which are produced in brain during the prognosis of the disease [133].

Although there are widely used and generally accepted transgenic animal models for AD, in vitro cell culture models are also widely preferred tool for AD studies, since the time needed for the symptoms to occur in animal models are time-consuming [134]. The advantage of using cell culture models also comes from the ease of possible combinations with immunocytochemical or biochemical techniques for investigation of the underlaying mechanisms of AD [134].

The examples of most common cell lines used in AD studies are PC12, HEK293, and SH-SY5Y. In addition to these cell lines, primary cell culture models are also used to study the pathogenesis of AD. The use of primary cells from transgenic animals could also be utilized [134]. The contribution of glial cells and astrocytes to the mechanism of the disease is also an aspect to be considered because of their key role in neurodegenerative diseases [133, 135]. Primary glial cells as well as glial cell lines such as BV-2 cell line are widely used for the investigation of the features of neurodegenerative diseases [133]. The

neuronal cell cultures could be preferred to be used alone, however the co-cultures of neuron and glia cells are encouraged to investigate the interaction between these different cell groups of CNS [135].

The underlying mechanism of AD as well as the treatment options have become subject to numerous studies. The neuroprotective and neurotrophic effects of memantine, a NMDA receptor blocker, were investigated using primary midbrain neuron-glia cultures. The studies showed that memantine acts depending on the cell type as neuroprotective effects were found to be related with the inhibition of microglia metabolism, whereas neurotrophic effect occurs with the induced levels of neurotrophic factors releasing from astroglia [135].

For the evaluation of acute and chronic exposure to memantine HCl treatment, mitochondrial function was assessed using human teratocarcinoma Ntera/D1 (NT2) neuronal precursor cells. The isolated mitochondria were evaluated in terms of oxidative stress, cytochrome oxidase, citrate synthase, and complex I Vmax activities. The results showed that mitochondrial peroxide levels differ according to the duration of drug exposure [136].

Human neuroblastoma cells (SK-N-SH) and primary rat cortical neurons were used to investigate the Aβ precursor protein (APP) metabolism, which leads to amyloid-beta (Aβ) production. Following the treatment with therapeutically relevant memantine concentrations, reduced levels of APP and Aβ peptide were found in both cell groups, which was determined using denaturing polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) followed by Western blotting as well as ELISA analysis [137].

Currently, human induced pluripotent stem cells (iPSC) draw considerable interest for neurodegenerative disease investigations [138]. The stem cells from patients with either monogenic familial Alzheimer's disease (fAD) or Down's syndrome could be utilized for studying the mechanism of AD [139]. The studies employed to enlighten the genetics of the disease implied that the pathology occurs with mutations in amyloid precursor protein (APP), Presenilin 1 (PSEN1) and Presenilin 2 (PSEN2) in APP processing pathway, and mutation of these genes are widely investigated by different groups using iPSC derived neurons [139, 140]. Although there are some limitations in 2D cell culture models of these cells and the tendency of iPSC-derived models for mutations should be considered, the brain environment in AD could be generated using iPSC cell culture models as reported [141]. It must be also considered that although these models could mimic the prognosis of early stage AD with low level Aβ species accumulation and slight tauopathy, no excessive β-amyloid plaque aggregation and neurofibrillary tangles were observed recapitulating AD pathology in patients [142].

Three-dimensional (3D) cell culture models could also be utilized using human stem cells for studying the cascade of the disease formation. Recently, 3D human neuronal cell culture models are gaining attention for the research of the hallmarks of AD. The relation between Aβ accumulation and neurofibrillary tangle formation following aggregation of hyperphosphorylated tau has been demonstrated for the first time using a model of familial AD (fAD) mutation in human neural stem cell-derived three-dimensional (3D) culture system. The 3D cell culture models allow to simulate Aβ accumulation in brain, which will result in tauopathy, owing to the diffusion limiting factors in 3D cell cultures, which

mimics the brain tissue. In addition, the differentiation of neuronal and glial cells was found to be promoted when compared to 2D cell cultures, providing a useful platform for drug investigation [143]. Zhang et al. demonstrated the potential of 3D cell culture model using human iPSC derived neuroepithelial stem cells (It-NES) line AF22 to study AD [144]. p21-activated kinase mediated sensing of Aβ oligomers were investigated using 3D matrix compared to 2D culture, and it was stated that 3D neuronal cell culture could be utilized to mimic pathological changes occurred in AD. In another 3D cell culture model protocol designed for the evaluation of AD was described to be consisted of human neural progenitor cells (hNPCs) with familial AD mutations and a Matrigel used as scaffold [145]. It was reported that the formation of Aβ aggregates and phosphorylated tau protein accumulation was occurred after 6 and 10 weeks, respectively.

3D cell culture model is also applied to a microfluidic chip, which allows to mimic the brain environment using uniform 3D neurospheroid cells together with a constant flow of fluid [146]. The effect of the flow on cell culturing and Amyloid-β treatment was investigated, and it was reported that static conditions provided with slow and diffusion-based fluid flow could be utilized as an in vivo-like microenvironment to study AD.

There are still lots of ongoing studies on neurodegenerative disease models to generate physiologically relevant in vitro models for therapeutic drug screening, and together with the advances in cell culture techniques, number of strategies have been introduced recently. One should note that each model comes with its own advantage and limitations, and the most appropriate model for a particular study could be determined according to the objective of the study together with the stage of the disease to be investigated.

4.2. In-Vivo Models

Various animal models have been proposed for the full characterization of biomarkers and ultrastructural changes in AD in order to elucidate the basic mechanisms that underlie AD and bridge the gap between clinical outcomes and neuropathologic diagnosis of AD [147]. The major standing point for characterization of AD is the brain atrophy. The most commonly used animal model is the transgenic mouse lines that consist of familial mutations that result in the development of brain amyloidosis [147]. Amyloid plaques may be visualized by a various set of techniques such as Multiphoton Microscopy Imaging [148-150], Positron Emission Tomography Imaging [151-155], Magnetic Resonance Imaging [156-159] and Near Infrared Fluorescence [160, 161] methods. Although each of the fore-mentioned techniques has some drawbacks or limitations (lack of similarity with respect to clinical human outcomes), they might be used for the characterization of AD status and prognosis in the animal models. The golden standard for in-vivo evaluation of AD is still the follow up of Aβ accumulation in in-vivo experiments. In addition to that various researchers have reported some additional alterations in transgenic animal models such as abnormalities in water diffusion [162-164] and decrease of N- acetylaspartate (NAA) in the brains of APPxPS1 transgenics [165]; but it keeps to be uncertain that treatment options might not rely on such kind of biomarkers for therapeutic evaluations [147].

Keeping these facts in mind, the animal models only try to mimic the clinical outcomes of AD such as amyloid plaque formation and accumulation; but not the whole disease with neuronal loss. This point is considered to be the main reason of failure for translation of animal experiments to clinical studies [166-168]. The majority of animal research studies in AD

are conducted with transgenic mouse models that are expressing either human amyloid precursor protein and presenilin 1 with FAD mutations or tau [169-172]. There are only a few studies in which transgenic mice with expression of both plaques and tangles exist [173-175].

In models with APP expression, plaques are formed in the frontal, temporal and entorhinal cortices, hippocampus and cerebellum in addition to congophilic amyloid angiopathy, memory impairment and synaptic impairment [169]. The expression of APP is a necessity for amyloid plaque formation; thus, the promoter type and mouse strain play an important role for the exact phenotype of each strain. The most common types of transgenic mice models for AD are Tg2576 [176] and APP23 [177], J20 [178], 5xFAD [179], that have different type of mutations; thus, leading to different pathophysiological outcomes of AD.

In addition to fore-mentioned transgenic mice models, knock-in mice model has been recently introduced for the in-vivo modeling of AD for a better physiological modelling depending on the humanization of Aβ and knocking in specific APP FAD mutations [180, 181]. The major advantage of these animal models with respect to other classical transgenic ones is the avoidance of confounding effects of APP over-expression. However, it must be noted that these animals exert pathological changes after knocking-in of a combination of specific multiple FAD mutations [169].

Transgenic rat models are also available for a better understanding of AD pathology as a limited alternative although they are much more similar to human genetics, physiology and morphology [182-185]. Their larger brain tissue enables better imaging of the changes and collection of samples. Amyloid plaque expression is observed in transgenic rat models as

well as neurofibrillary tangles in TgF344-AD rats, indicating that that rat originated tau is very similar to human tau [182-185].

One of the drawbacks of transgenic mice models is the nature of AD that is formed; which is familial AD type. This is only <1% of AD patients in humans; thus, this lacking characteristic might cause mistranslation to clinical trials. Besides, formation of neurofibrillary tangles, which is considered to be an important histopathological hallmark of AD formed upon intraneuronal aggregation of hyperphosphorylated tau protein is lacking in most of the transgenic models [186]. These neurofibrillary tangles are mainly responsible for the loss of cognitive functions of the brain by impaired synaptic plasticity [187]. Last, but not the least progressive neuronal loss in human AD cannot be reflected in transgenic animal models as it is humans [188]. On the contrary, precious data on role of inflammation, mitochondrial dysfunction and oxidative stress is provided by such kind of animal models [189, 190].

In addition to the transgenic animal models, other experiments on AD has been evaluated with dogs [191], mouse lemurs [192] and rhesus monkeys [193, 194] have been done by different research groups; but in each case models were not to be classified as a complete suitable model for translation of AD.

5. Current Status of Clinical Trials

Information about the clinical trials was obtained from the web site www.clinicaltrials.gov, which is a service of the U. S. National Institute of Health. Although, there is no obligation by law for clinical studies to register into this database in the US, most of the researchers

register and share information about ongoing studies. We used this database to provide an outline of the clinical trials on AD and provide general information about the study types and the phase that the study is in. For this purpose, we searched for "Alzheimer's Disease" as the condition/disease keyword and filtered the results for the status of the clinical studies, which are "recruiting" and "enrolling by invitation". We selected all sexes and included the studies "with and without results" (Table 3).

The results clearly indicate that new molecules or the different doses of conventional molecules are currently under investigation in the treatment of AD. A total of 168 studies are in active status with tested drug molecules. Interestingly, only 6 compounds are under investigation. The devices under investigation are either for diagnosis or for the palliative treatment of AD. Some dietary supplemental studies, alongside behavioral studies, are currently being investigated for the benefit of patients experiencing AD.

Table 3. Search results of the clinical trials that are registered to www.clinicaltrials.gov [195]

Type / Phase	Early Phase I	Phase I	Phase II	Phase III	Phase IV
Drug	6	35	81	37	9
Device	1	3	3	2	1
Biological	------	7	4	1	------
Radiation	1	1	1	1	------
Imaging	1	------	1	1	------
Dietary Supplement	------	1	5	1	------
Behavioral	------	------	6	4	------
TOTAL	**9**	**47**	**101**	**47**	**10**

5. Conclusion

Despite all *precious* improvements in the diagnosis and palliative treatment opportunities, there is still no option for the complete treatment of AD. Most of the drug development research studies fail in trials due to ineffective efficacy or safety profiles [196]. However, nanotech products, which will provide effective delivery across the BBB and maintain localized and sustained delivery of various active pharmaceutical agents, seem to be promising alternatives for complete eradication of the pathological alterations underlying AD. Information on the pharmacodynamic efficacy of nanotechnology-based drugs is currently lacking; this may only be achieved with investigations using transgenic animal

models. Within the last decade the effectiveness of biotechnology products has been a particular focus for researchers investigating AD. Nanotechnology based drug delivery systems enable the transport through the BBB, but it is clear that the high cost of these drugs is a challenge when the large number of patients is taken into account. Despite this, biologically derived drugs may deliver more specific therapy options and provide rapid improvements in the progression of AD. It is our belief that these nanotechnological products provide the required efficacy and safety profiles to enable these formulations go through phase studies and enter the market after regulatory authority approval, as with cancer. Last, but not the least the metabolomic studies will be providing useful informative data on the early diagnosis of AD, thus may be clinical implications might be delayed with the administration of therapeutic agents at the initial state of the disease.

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7. Conflict of Interest

The authors declare no conflict of interest.

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