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Alzheimer's disease: etiological and risk factors, pathophysiological mechanisms and therapeutic approaches

Alzheimerjeva bolezen: etiološki in rizični dejavniki, patofiziološki mehanizmi in terapevtski pristopi

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Abstract

Alzheimer's disease is a chronic progressive neurodegenerative disease that affects an increasing number of people worldwide. It is the most common and severe form of dementia (50–80% of all patients), characterized by cognitive decline and behavioural disorders, mainly in people over 65 years of age. Nowadays, the disease's pathogenesis remains obscure. However, several hypotheses involve a combination of genetic and environmental factors and people's lifestyle. Depending on the onset, the disease is divided into an early-onset form and a late-onset form. A rare early-onset form is associated with mutations in the gene for amyloid precursor protein and genes for the presenilin 1 and 2. The genetic risk factor for a late-onset form is the ε4 allele of the apolipoprotein E gene. Besides, there are several causal hypotheses for the onset of the late-onset form: i) cholinergic hypothesis, ii) amyloid hypothesis, iii) tau hyperphosphorylation and propagation hypothesis, iv) mitochondrial cascade hypothesis, v) inflammatory hypothesis, vi) neurovascular hypothesis, vii) metal ion hypothesis and viii) lymphatic system hypothesis. Also, there are metabolic and other risk factors for Alzheimer's disease, such as hypertension, hypercholesterolemia, obesity, type 2 diabetes mellitus, sleep disorders, and many others. Despite many studies, the cause and mechanism of the disease have not been fully explained. Furthermore, we do not know the medicine to prevent the onset or stop the disease's progression. Alzheimer's disease treatment is primarily pharmacological and is based on the known disease's aetiology. Recently, much attention has been directed to immunotherapy with anti-amyloid antibodies.

Izvleček

Alzheimerjeva bolezen je kronična progresivna nevrodegenerativna bolezen, za katero oboleva vse večje število ljudi po vsem svetu. Je najpogostejši vzrok za demenco (pri 50–80 % vseh primerov) in obenem tudi najhujša oblika demence, za

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katero so značilne kognitivne in vedenjske motnje pri ljudeh, večinoma starejših od 65 let. Patogeneza bolezni še ni popolnoma pojasnjena. Obstaja več hipotez, ki vključujejo kombinacijo genetskih in okoljskih dejavnikov ter načina življenja. Glede na začetek bolezni lahko bolezen opredelimo kot obliko z zgodnjim začetkom, običajno pred 65. letom starosti, in obliko s poznim začetkom, običajno po 65. letu starosti. Redka oblika bolezni z zgodnjim začetkom je povezana z mutacijami v genu za amiloidni prekurzorski protein ter v genih za presenilin 1 in 2. Genetski dejavnik tveganja za nastanek bolezni s poznim začetkom je prisotnost alela ε4 za apolipoprotein E. Za nastanek oblike s poznim začetkom obstaja več vzročnih hipotez: a) holinergična hipoteza, b) amiloidna hipoteza, c) hipoteza o hiperfosforilaciji proteina tau, č) hipoteza o mitohondrijski kaskadi, d) vnetna hipoteza, e) nevro-vaskularna hipoteza, f) hipoteza o kovinskih ionih ter g) hipoteza limfnega sistema. Poleg tega obstajajo tudi presnovni in drugi dejavniki tveganja za nastanek Alzheimerjeve bolezni, med katere sodijo: zvišana raven holesterola v krvi, debelost, zvišan krvni tlak, sladkorna bolezen tipa 2, motnje spanja idr. Kljub velikemu številu raziskav etiopatogeneza do danes še ni dokončno pojasnjena, prav tako ne poznamo zdravila, ki bi preprečilo nastanek bolezni ali ustavilo njeno napredovanje. Zdravljenje Alzheimerjeve bolezni trenutno temelji na farmakološkem zdravljenju, ki poteka na osnovi doslej znane etiologije bolezni. Veliko obeta tudi imunoterapija z antiamiloidnimi protitelesi.

1 Introduction

More than a decade ago, demographic studies predicted an increase in life expectancy and the proportion of the elderly (over 65) in the population. In Slovenia, the proportion of people over the age of 65 in 2012 was 16.8%; by 2020, this had increased to 20.5%. By 2057, this age group is projected to represent as much as 31% of Slovenia's population. Various geriatric diseases, including Alzheimer's disease (AD), are also directly linked to population aging (1-4). AD is a chronic neurodegenerative disease that currently affects 50 million people worldwide (5). According to projections, the number of patients will increase to 74.7 million by 2030 and even to 131.5 million by 2050 (5,6). Asia has the highest AD incidence in the world (49%), followed by Europe (25%) and the United States of America (USA) (18%). Data from the World Alzheimer Report show that the highest incidence in Europe and the USA is between the ages of 80 and 89, in Asia between the ages of 75 and 84 and in Africa between the ages of 65 and 74. AD is the most common cause of dementia (50–80% of all cases), manifesting itself in the form of cognitive and behavioural disorders, most commonly in people over 65 years of age (6); it is also the most severe type of dementia (7). In addition to AD, other, less common forms of dementia are known: vascular dementia, Lewy body dementia, frontotemporal dementia (FTD), dementia in Parkinson's disease and mixed dementia (8,9). In 2018, the estimated total costs, which include healthcare, social and informal care for patients with AD, was about one trillion US dollars, expected to rise to about two trillion US dollars by 2030 (6). Based on the patient's age, disease onset and genetic component, AD is divided into two forms: a) early-onset or familial AD (early-onset Alzheimer disease, EOAD), in which clinical signs appear before the age of 65 and b) late-onset or sporadic AD (late-onset Alzheimer disease, LOAD) with the appearance of clinical signs after the age of 65 (10-13). Approximately 95% of patients are diagnosed with LOAD and only 5% have EOAD (10). Both environmental factors and genetic predisposition contribute to the development of LOAD, making it a multifactorial disease. EOAD is caused by mutations in one of the following three genes: a) the amyloid precursor protein (APP) gene on chromosome 21, b) the presenilin 1 (*PSEN1*) gene on chromosome 14, and c) the presenilin 2 (*PSEN2*) gene on chromosome 1 (12). AD causes gradual loss of memory and cognitive abilities in patients, until their daily lives are severely affected (14). The most characteristic neuropathological signs of AD are the deposition of extracellular senile plaques and formation of intracellular neurofibrillary tangles (NFTs) (8). Extracellular amyloid-β (Aβ) plaques result from increased formation and deposition of Aβ peptides, which are formed after cleavage of transmembrane APP by sequential action of β and γ secretase enzymes. NFTs are formed by hyperphosphorylation of the microtubule-associated tau protein. Hyperphosphorylation of the tau protein serine and threonine amino acid residues causes the tau protein to accumulate within cells and form NFTs (15). Additionally, the disease is characterized by: a) decreased levels of acetylcholine (ACh), b) initially increased acetylcholinesterase (AChE) activity, which begins to decline with AD progression (decrease by 55–67% from normal); at this time, butyrylcholinesterase (BChE) activity increases (by 40–90% above normal), c) oxidative stress, c) disturbance of metal ion homeostasis, d) impairment of cholinergic neuron function or their degeneration and loss of cholinergic synapses, and e) reactive gliosis (6,14-16). The neurotransmitter ACh

is crucial, playing an important role in memory, thinking and learning processes. Patients with AD have been found to have severely reduced cholinergic innervation of the cerebral cortex and hippocampus (14). It follows that AD is a very complex multifactorial disease, for which several causal hypotheses have been described. Recently, in addition to the possible causes, risks and mechanisms, much of the research has focused on the possible link between LOAD and type 2 diabetes mellitus. Due to the not yet fully explained mechanism of AD development and progression, several different, demanding and, above all, very complex pharmacological approaches to treatment have been developed. In the following sections, we will focus on the main hypotheses, risk factors, causes, mechanisms, and associations that may lead to AD development, and on the most important pharmacological approaches to symptomatic treatment.

2 Aetiological factors, risk factors and pathophysiological mechanisms of Alzheimer's disease

Currently, the disease pathophysiology is not yet fully understood. Several hypotheses, risk factors, causes and mechanisms that may lead to its development have been described. Macroscopic and microscopic markers and other tissue markers help us to characterize the disease and understand its pathophysiology. At the macroscopic level, atrophy of the cerebral cortex and hippocampus is observed, and among microscopic changes, the most visible are the formation of extracellular senile plaques, intracellular NFTs, extensive selective loss of cholinergic neurons and reactive gliosis (6). As has been mentioned, AD is clinically divided into two types: EOAD or familial and LOAD or sporadic. EOAD is associated with mutations in the APP, *PSEN1* and *PSEN2* genes, with a low overall incidence (5%). In addition to these mutations, ε4 allele of the apolipoprotein E (ApoE) gene should also be mentioned as an important genetic risk factor for AD development. In the case of sporadic or LOAD, several hypotheses have been described based on the presumed disease causes: cholinergic hypothesis, amyloid hypothesis, tau hypothesis, mitochondrial cascade hypothesis, inflammatory hypothesis, neurovascular hypothesis, metals hypothesis and lymphatic system hypothesis (17). However, there are also metabolic and non-genetic AD risk factors, including elevated blood cholesterol levels, obesity, high blood pressure, type 2 diabetes mellitus, sleep disorders, oxidative stress, and many others. In the following subsections, we will discuss the main hypotheses, risk factors, causes, mechanisms and associations that can lead to AD development.

2.1 Genetic causes and risk factors

2.1.1 Mutations in presenilins 1 and 2 and the APP gene

Presenilin (PSEN) regulates the proteolytic activity of γ-secretase, as it is a catalytic subunit of the enzyme with two aspartate residues responsible for substrate cleavage (18). Presenilin 1 (PSEN1, *PSEN1* gene - chromosome 14) and presenilin 2 (PSEN2, *PSEN2* gene - chromosome 1) are homologous proteins with 66% sequence similarity (5). Mutations in the PSEN genes are associated with EOADor familial AD and account for as much as 87% (81% have the *PSEN1* gene mutation and 6% have the *PSEN2* gene mutation) of this AD type. In the remaining 13% of cases, the mutations are in the gene for APP (17,18). The mutation in these genes results in the malfunction of the γ-secretase enzyme in the APP proteolysis process, which is reflected in the partial degradation of Aβ peptides and the formation of large amounts of $Aβ_{42}$ peptides in EOAD. To date, 121 *PSEN1* mutations have been detected and only 13 *PSEN2* mutations. Studies have shown that deletions of the *PSEN1* and *PSEN2* genes cause memory and learning disorders and neuronal death due to the lack of interaction between PSEN1 and BCl-2 antiapoptotic proteins (5).

2.1.2 Genetic risk factor allele ε4 for apolipoprotein E

ApoE, along with ApoJ and ApoA1, is the primary cholesterol carrier in the brain. It is formed in astrocytes and acts as a lipid transporter. Additionally, it is involved in the process of repairing brain damage and carrier-mediated lipoproteins transport, such as low density lipoprotein receptor-related protein 1 (LRP1). LRP1 also plays a role in removing Aβ peptides. ApoE encodes the *ApoE* gene, in which three alleles were identified: *ε2*, *ε3* and *ε4*. Carriers of the *ε4* allele of *ApoE* were found to have an increased risk of developing AD due to increased $Aβ_{42}$ peptide production, Aβ aggregation or decreased Aβ clearance. In addition, carriers of the *ε4* allele of *ApoE* encoding ApoE4 have been shown to have decreased expression of insulin degrading enzyme (IDE) in the brain, which affects the breakdown of extracellular Aβ (5). People who are homozygous for the *ε4* allele (*ε4*/*ε4*) have up to 15 times the risk of AD compared to people with only one ε4 allele, in whom the risk of developing the disease is only three times higher. On the

other hand, the *ε2* allele has been found to be protective and positively associated with cognition in aging, as it has been linked to improved glucose uptake and glycolysis in the brain (7). The *ε4* allele of *ApoE* has also been studied as a risk factor for LOAD associated with type 2 diabetes mellitus. The ApoE genotype is known to affect glucose and insulin metabolism, as ApoE4 has been found to affect insulin receptors (IR). ApoE4 interferes with the binding of IR-containing endosomes to the plasma membrane, resulting in the capture of IR in the endosome. Ultimately, this leads to impaired insulin signalling and insulin resistance. The association between type 2 diabetes mellitus and LOAD is specific for people with the *ε4* allele of *ApoE* gene compared to those with the *ε2* and *ε3* alleles of *ApoE*. These data support the metabolic hypothesis that includes type 2 diabetes mellitus as a key factor in the development of LOAD (10,11).

2.2 Alzheimer disease pathogenesis hypotheses

2.2.1 Cholinergic hypothesis

The cholinergic hypothesis is the first theory related to AD pathogenesis. Among the mechanisms of AD cause and development, it is also one of the most proven (6). Findings of reduced choline uptake, decreased ACh secretion and loss of cholinergic neurons in the nucleus basalis of Meynert confirmed the hypothesis of presynaptic ACh deficiency. The described studies, together with the discovery of the ACh role in the processes of learning and memorization, led to the cholinergic hypothesis of AD. This suggests that the degeneration of cholinergic neurons in the basal forebrain structures and the associated loss of cholinergic connections in the cerebral cortex and other areas significantly contributes to the deterioration of cognitive functions in patients with AD (19). Degeneration or loss of cholinergic neurons, loss of synapses and disturbances in the transmission of nerve signals are observed in patients with AD. In the initial stage of the disease, the loss of neurons is noticeable mainly in the nucleus basalis of Meynert and entorhinal cortex. In advanced stages, however, the loss of cholinergic neurons in the nucleus basalis of Meynert can exceed 90%. Decreased expression of choline acetyltransferase in neurons of cortex, amygdala, hippocampus, and nucleus basalis of Meynert has also been reported. Decreased ACh levels in these areas can be detected, because choline acetyltransferase is crucial in ACh synthesis. The expression and activity of other enzymes that are crucial for the synthesis of other neurotransmitters (gamma-aminobutyric acid, dopamine, adrenaline, noradrenaline and 5-hydroxytryptamine) are within physiological limits in patients with AD (17). The cholinergic hypothesis confirms that the loss of cholinergic neurons leads to ACh deficiency (6). Decreased levels of choline acetyltransferase and ACh have been observed not only in patients with AD but also in patients with Parkinson's disease and depression (17).

2.2.2 Amyloid hypothesis

The amyloid hypothesis was first proposed by Hardy and Allsop in 1991 (20). They found a mutation in the gene encoding APP, present on chromosome 21. They hypothesized that impairment of Aβ metabolism and deposition is the primary factor in AD. This is followed by hyperphosphorylation of the tau protein, NFTs formation and neuronal death (17). A characteristic pathological sign of AD is the presence of extracellular senile plaques formed by Aβ peptides (21). Aβ peptides are formed by cleavage of transmembrane APP. The latter has an extensive glycosylated extracellular N-terminal and a short intracellular C-terminal domain (5). There are two pathways of proteolytic APP degradation, the non-amyloidogenic (the most common) and amyloidogenic. In the non-amyloidogenic pathway, α-secretase first cleaves APP, releasing sAPPα. The γ-secretase complex then proteolytically cleaves the part of the protein on the inside of the neuron membrane. This degradation does not result in the formation of Aβ but creates a soluble p3 fragment (22). In amyloidogenic degradation, the Aβ peptide is formed after the cleavage of APP by the membrane-bound β- and γ-secretases (5). β-secretase is found in two forms as BACE1 and 2, between which there is a 65% similarity. BACE1 is important in Aβ formation, which is strongly expressed in the brain. Its expression in other organs is low. BACE1 first cleaves sAPPβ from APP. The C-terminal fragment β (CTFβ), which remains bound to the membrane after cleavage with BACE1, is then cleaved by γ-secretase to form the Aβ peptide. In cleavage, γ-secretase is inaccurate, resulting in C-terminal heterogeneity of Aβ peptides. Therefore, there are different types of Aβ peptides, the most common being $Aβ_{40}$ (approximately 80-90%) and $Aβ_{42}$ (approximately 5-10%) (5,18,21). Longer peptides are more hydrophobic and fibrillogenic, so they are also the most common type deposited in the brain (21). Under physiological conditions, a very small portion of APP cleavage takes place via the amyloidogenic pathway. The small amounts of Aβ that are formed are removed by the immune system cells (17). Under pathological conditions, the relationship between the formation and removal of

Aβ peptide is disrupted, which is the reason for its deposition in the form of oligomers, protofibrils, fibrils and extracellular senile Aβ plaques (5). Certain mutations in APP (Lys670Asn/Met671Leu and Ala673Val) occurring near the BACE1 enzyme cleavage site are characterized by a tendency of APP to cleave via the amyloidogenic pathway, which is reflected in increased Aβ formation and deposition (17). According to the amyloid hypothesis, Aβ plaques and/or their precursors trigger a cascade of events leading to synaptic dysfunction, microgliosis, and loss of cholinergic neurons. Senile plaques are not consistently associated with cognitive impairment. Therefore, some believe that soluble Aβ oligomers are responsible for neuronal death (23), while others believe that senile plaques are responsible (24). The degree of cognitive impairment correlates with the amount of Aβ oligomers in the brain and not with the total amount of Aβ. The latter confirms that soluble oligomers are the most toxic to neurons (25). $A\beta_{42}$ oligomers disrupt neuronal calcium homeostasis and trigger oxidative stress. Increased activity of intracellular Ca^{2+} activates endogenous phospholipases, leading to lysophospholipid formation and plasmalemmal damage. The passage of Ca^{2+} ions into the mitochondria inhibits the respiratory chain and reduces ATP production. Many processes, including the preservation of antioxidant potential, are dependent on ATP. Additionally, $A\beta_{42}$ oligomers indirectly increase tau protein hyperphosphorylation, leading to the expansion or opening of transitional pores in the inner mitochondrial membrane, the release of cytochrome C, increased levels of reactive oxygen species, and consequent apoptosis (5).

2.2.3 Tau protein hyperphosphorilation

In addition to senile plaques, NFTs formation is also a characteristic pathological sign of AD. These are filamentous intracellular neuronal inclusions of hyperphosphorylated tau protein in patients with AD (26). Clinical syndromes caused by tau protein aggregation over a long period of time are called taupathies (8). These include various neurodegenerative diseases: FTD, Pick's disease and corticobasal degeneration (8,26). The tau protein is a microtubule-associated protein, playing a key role in the polymerization, stabilization and function of microtubules (27). In addition to phosphorylation, it undergoes various posttranslational changes such as acetylation, nitration, glycation, O-glycosylation, transglutaminase cross-linking, isomerization, conformational change and proteolytic cleavage. All these changes can affect tau protein function and position, although their importance in the process of neurodegeneration is not yet fully known (8,15). According to studies to date, some posttranslational tau protein changes may reduce its phosphorylation or increase its detrimental effects. Serine and threonine O-glycosylation, which may reduce the extent of phosphorylation, is thought to be useful (17). In the central nervous system, the neuronal tau protein content is high. It is also found in smaller amounts in astrocytes and oligodendrocytes. Due to its involvement in the process of regulating the dynamics of microtubules, it also participates in the functions of cell signalling, synaptic plasticity and regulation of genomic stability. The tau protein mRNA is encoded by the *MAPT* gene. Due to the alternative fusion of exons 2, 3 and 10 in humans, six major isoforms are known, creating tau proteins with three or four binding domains for microtubules (3R or 4R). In healthy people, the same amounts of tau protein with 3R and 4R are present in the brain, although with regional differences. Importantly, an increase in 4R expression involving exon 10 may lead to increased tau protein aggregation and NFTs formation. One of the most likely causes of tau protein hyperphosphorylation is a disrupted ratio between kinase and phosphatase activity. Activation of glycogen synthase kinase 3 (GSK-3) has been shown to lead to tau protein hyperphosphorylation (5). Hyperphosphorylated tau protein has reduced affinity for binding to microtubules, leading to destabilization and disruption of microtubule structure (8). Insulin has the opposite effect by preventing tau protein hyperphosphorylation and thus neurotoxicity by regulating various cellular metabolic processes. Insulin and insulin-like growth factor 1 (IGF-1) are known to inhibit excessive tau protein hyperphosphorylation through GSK-3 inactivation caused by protein kinase B (PKB), thereby stabilizing the microtubule structure (5). Additionally, posttranslational modifications, in particular tau protein hyperphosphorylation, are a key factor in the dimerization of tau monomers and the formation of irregularly folded tau oligomers, leading to their deposition and aggregation into paired helical filaments. NFTs are formed by the formation of bundles of paired helical filaments that accumulate in the neuronal cytoplasm (28). NFTs alone are unlikely to cause neurotoxicity, as some studies suggest that tau oligomers are the most toxic form of tau protein, triggering interneuronal signalling disturbances in AD (29). Additionally, *in vivo* and *in vitro* studies have shown the presence of previously described changes in different areas of the brain (30). It has also been suggested that fibrillar aggregates and improperly folded tau protein, like prions, may eventually spread between cells and therefore to

other areas of the brain in patients with AD (17). Tau protein hyperphosphorylation is ultimately reflected in loss of microtubule structural integrity, impaired axonal transport, mitochondrial dysfunction and altered synaptic structure and function (23). It has been found that a mutation in the tau protein gene can lead to dementia, in contrast to a mutation in the *APP* gene, which more often leads to AD. Two models are used to interpret the latter. The series model states that increased levels of Aβ peptides lead to tau hyperphosphorylation. The dual model, however, states that both Aβ peptides and tau protein hyperphosphorylation lead to neuronal loss in AD (5). Additionally, a study conducted a few years ago found a link between disorders of insulin signal transmission and pathological tau protein changes. This association is described in more detail in the chapter "The effect of IR inactivation on tau protein hyperphosphorylation".

2.2.4 The neuroinflammatory process and oxidative stress

2.2.4.1 Neuroinflammatory process

Aβ deposition in the brain results in an inflammatory response that leads to the activation of the complement cascade and microglia cells and astrocytes recruitment. (31). In the areas surrounding Aβ deposits, astrocytes undergo activation and release a number of signalling molecules. In addition to the activation of astrocytes, microglia activation has also been found near Aβ aggregates (32). Under physiological conditions, microglia play a key role in maintaining homeostasis and plasticity of the central nervous system. Microglia are also the first line of defence in the brain (33). Activated microglia are involved in many cellular processes. Key pathways include cell proliferation, migration in response to signalling molecules and release of cytotoxic and inflammatory mediators. By releasing substances such as proteases, oxygen reactive species, nitric oxide and inflammatory cytokines, microglia can function in the same way as cytotoxic T cells. In addition, activated microglia can phagocytose Aβ and smaller Aβ aggregates, but it is not yet fully understood whether it can successfully remove larger aggregates and fibrils (32). This response likely helps to remove Aβ aggregates but may also stimulate the secretion of damage-causing inflammatory mediators (22). Studies show that fibrillar forms of Aβ are of great importance in activating microglia. In patients with AD, Aβ binding to microglia cells occurs via the CD36-TLR4-TLR6 receptor complex or via the inflammatory NLRP3 complex, leading to the secretion of inflammatory mediators (e.g. TNFα) and ultimately leading to an inflammatory response, during which chemokines, oxygen reactive species, and inflammatory cytokines are formed, which deregulate the initial immune response and ultimately lead to neurodegeneration. In patients with AD, increased levels of TNFα, IL1-β, TNFβ, IL12 and IL18 correlate with disease progression and increased rates of brain injury or involvement (17). Additionally, activated microglia can interfere with synapse formation and neuronal plasticity in an inflammatory environment (22). Many studies have shown that tau pathologies worsen significantly during acute and chronic inflammatory processes (6). Activations of the inflammatory cascade may involve changes in tau phosphorylation simultaneously with oxidative damage to neurons (22). Given all these influences, Aβ deposition in the brain plays an important role in AD pathogenesis. The result is a chronic inflammatory response that contributes to neurodegeneration.

2.2.4.2 Oxidative stress

Oxidative stress represents an imbalance in pro-oxidants and antioxidants with associated disruption of redox circuitry and macromolecular damage. Oxidative stress is caused by increased production of reactive oxygen and/or nitrogen species and/or reduced production of antioxidants (17). AD development is thought to be a direct result of increased oxidative stress in the brain (22). Oxidative stress is involved in a variety of pathophysiological conditions, including neurodegenerative disorders. Oxidative stress-induced inflammation has been shown to actively coincide with the aetiopathology of AD. The inflammatory process is associated with the formation of oxygen reactive species that act as signals to activate inflammatory genes. Minimum physiological levels of oxygen reactive species are present in the cell as by-products of aerobic metabolism, as well as secondary message molecules in many signalling pathways. Under normal conditions, there is a dynamic balance between prooxidants and antioxidants (31). Oxidative stress can be caused by: a) increased deposition of mercury, iron and aluminium in the brain, thereby stimulating the formation of free radicals through Fenton and Haber-Weiss reactions, b) increased lipid peroxidation, c) increased protein and DNA oxidation, d) decreased energy metabolism and d) reduced cytochrome c oxidase activity. Senile plaques and NFTs contribute to oxidative stress through the formation of glycation end products, malondialdehydes, carbonyls, peroxynitrites, hemoxygenase 1 and superoxide dismutase 1 and, last but not least, the ability to generate free radicals via Aβ (22,34). The main source of oxygen reactive species is

mitochondrial electronic transmission chain dysfunction, inflammatory response and activated microglia (31). Aβ causes oxidative stress by triggering the formation of superoxide anions, hydrogen peroxide and hydroxyl anions. Through the Haber-Weiss and Fenton reactions, highly reactive hydroxyl anions are formed, which are key oxygen reactive species that damage membrane lipids, proteins, and other biological molecules. Oxidative stress caused by Aβ is thus caused by increased synthesis of free radicals in the presence of iron, copper and zinc, which are concentrated inside the nucleus and on the periphery of Aβ aggregates (22). Aβ is also excitotoxic. Increased excitation leads to increased influx of Ca^{2+} and intracellular activity. This can result in the passage of Ca^{2+} into mitochondria and respiratory chain impairment, activation of phospholipase A_2 and plasma membrane damage, increased $3Na^{+}/Ca2^{+}$ exchanger activity and activation of intracellular proteases. In addition, Aβ also activates oxidative stress-related signalling pathways through oxidative stress or energy metabolism impairment (17). The reactive oxygen species themselves increase Aβ formation and aggregation and tau protein hyperphosphorylation, starting the destructive cycle (5,22). Antioxidant systems in the brain play an important role in AD prevention. The cellular system that protects against oxidative stress is the thioredoxin system. Its proteins, with their redox properties, modulate the function and expression of other proteins, including various transcription factors that are crucial for the development and control of cell survival or death (22). Additionally, patients with AD showed a marked increase in mitochondrial DNA and cytochrome c oxidase levels and a decrease in intact mitochondria and some key oxidative metabolic enzymes (17).

2.2.5 The association between type 2 diabetes mellitus and Alzheimer's disease

In the previous century, Hoyer presented the non-functioning insulin signalling theory in LOAD and the development of central insulin resistance (35). Initially, a possible association between type 2 diabetes mellitus and LOAD was only theoretical, but was later confirmed with epidemiological and clinical studies. These showed that hyperglycaemia and hyperinsulinemia correlated with LOAD (11). In humans, the importance of insulin signalling in AD was confirmed by intracerebroventricular administration of streptozotocin (a diabetogenic agent), which resulted in the development of various pathological features resembling those seen in patients with LOAD. Additionally, insulin and glucose have been shown to enhance learning and memory in patients with AD (17). The metabolic hypothesis associated with LOAD is based on the fact that IRs are widespread throughout the brain, with high levels of expression mainly in the olfactory bulb, hypothalamus, hippocampus, cerebral cortex, striatum, and cerebellum (11,36). Studies to date have shown: a) a significant reduction in brain IR in patients with late-onset AD, b) a significant reduction in IR and IGF1R in the hippocampus, hypothalamus and frontal cortex in patients with LOAD, c) that insulin resistance in the hippocampus impairs memory formation (causes cognitive impairment) and d) that the binding of Aβ oligomers to IR leads to internalization of IR and insulin resistance in LOAD (11).

2.2.5.1 Influence of Aβ oligomers on central insulin resistance

As type 2 diabetes mellitus progresses, both Aβ formation and tau protein hyperphosphorylation increase. Type 2 diabetes mellitus and Aβ are involved in the development of neurodegenerative disorders in LOAD. Soluble Aβ oligomers are thought to act as synaptotoxins. Additionally, both Aβ and insulin are amyloidogenic peptides that share a common sequence recognition motif. Therefore, it is likely that Aβ, like insulin, may bind to IR. Given this assumption, Aβ may also potentiate insulin resistance through antagonistic effects, blocking the downstream pathway and facilitating the phosphorylation of GSK-3β. Both processes, Aβ formation and tau protein hyperphosphorylation, have a synergistic effect leading to neuronal dysfunction. Studies have shown that the formation of $Aβ_{42}$ induces insulin resistance in the liver through the activation of Janus kinase 2 (JAK2). Additionally, Aβ peptides produced in the brain reach the hypothalamus, which acts as a control centre for metabolic processes. There, through neuro-inflammatory mechanisms, they disrupt metabolic processes and alter the body's energy balance, which further promotes the progression of type 2 diabetes mellitus. It is also known that Aβ oligomers in the hippocampus lead to astrocyte activation, which is reflected in the increased secretion of cytokines, particularly TNFα, which activates c-Jun N-terminal kinase 1 (JNK1). This leads to insulin resistance and tau protein hyperphosphorylation (11).

2.2.5.2 Influence of IR inactivation on tau protein hyperphosphorylation

Recent studies have found an association between insulin signalling disorders and pathological tau protein changes. Insulin binding activates IR, leading to the activation of insulin receptor substrate -1 and -2 (IRS-1 and

-2)), which establish several signalling pathways. Phosphatidylinositol 3-kinase (PI3K) converts phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-phosphate (PIP_3), which is involved in PKB activation. This leads to the translocation of the type 4 glucose transporter (GLUT4) to the plasma membrane (11). At the same time, PKB activation, which causes phosphorylation of many intracellular proteins (mTOR, FOXO, GSK3, CREB, filamin A, NO synthase), affects various cellular responses, including tau protein phosphorylation (11,36). Under physiological conditions, many kinases and phosphatases regulate the balance between phosphorylation and dephosphorylation of substrates to maintain neuronal homeostasis. Numerous kinases (GSK3-β, CDK5, MARK, PKA, PKB, ERK1/2, JNK) are involved in tau protein phosphorylation. Glycogen synthase kinase 3-β (GSK3-β) is particularly important (10). Its activation is inhibited by PKB-stimulated phosphorylation. With the onset of insulin resistance, its activation increases tau protein phosphorylation and the intracellular NFTs production, as evidenced by genetically modified NIRKO mice with reduced PKB activity. In them, increased GSK3-β activity and tau protein hyperphosphorylation were found in regions associated with LO-AD. Additionally, phosphorylation of IRS-1 serine residues has been shown to inactivate IRS-1 and induce insulin resistance in the hippocampus. Therefore, the development of insulin resistance leads to changes in tau protein (hyperphosphorylation) and LOAD (11). In addition to insulin resistance stimulating hyperphosphorylation and tau protein aggregation, reflected in the formation of NFTs, recent studies also show that hyperphosphorylated tau protein and its aggregates accumulate insulin (10).

3 Pharmacological approaches to Alzheimer's disease treatment

Despite efforts, the mechanism of AD development and progression has not yet been explained. An active ingredient that would prevent the development or completely stop the disease progression has also not yet been found. The currently known treatments for AD offer only symptomatic relief.

3.1 Cholinesterase inhibitors

According to the cholinergic hypothesis, decreased activity of the enzyme choline acetyltransferase and therefore lower ACh levels were found in certain brain regions in patients with AD. To increase the availability of ACh in the synaptic cleft, and thus to improve synaptic cholinergic function, cholinesterase inhibitors are clinically very commonly used for symptomatic AD treatment, reducing the symptoms of cognitive impairment for a short period. These inhibit the action of AChE and/or BChE, which are responsible for ACh breakdown (6). Compounds that inhibit the action of these enzymes may be of natural or synthetic origin. Inthe Laboratory of Physiology of the Veterinary Faculty, University of Ljubljana, we studied the effects of various, both natural and synthetic, cholinesterase inhibitors on the functioning of the peripheral neuromuscular system as part of preclinical studies (37-42). Despite numerous studies around the world aimed at discovering new drugs with this mode of action, the European Medicines Agency (EMA) has so far approved only four cholinesterase inhibitors : tacrine (1995), donepezil (1998), rivastigmine (1998) in galantamine (2000) (43). Tacrine is a non-competitive, non-selective, reversible AChE inhibitor with a short half-life. Due to poor bioavailability and side effects (especially hepatotoxicity), it was withdrawn from the market in 2012 (44). Donepezil, a piperidine derivative, is a non-competitive, selective, reversible AChE inhibitor (with poor BChE inhibitory activity). It easily crosses the blood-brain barrier, has an inhibitory effect even at nanomolar concentrations and significantly improves cognitive functions. As it is metabolised via the cytochrome P450 system, it may interact with other drugs, so caution should be exercised (6,45-47). Rivastigmine inhibits both AChE and BChE (48). It is a potent and slow reversible inhibitor (pseudo-reversible inhibitor) of AChE (49). Among the approved active ingredients is galantamine, a natural alkaloid isolated from the green snowdrop (*Galanthus woronowii*). It is a competitive, selective, rapidly reversible AChE inhibitor (46,50,51). It also binds to nicotinic acetylcholine receptors (nAChR - α4β2, α3, α6β4 and α7) and acts as a sensitizer or positive allosteric modulator of nAChR (binding to sites other than ACh and other nicotinic agonists and antagonists) (45,52,53). By allosteric binding to nAChR, galantamine may potentiate the action of agonists (e.g. ACh) of these receptors (45). It should be noted that donepezil, rivastigmine and galantamine improve cognitive function in patients with mild to moderate AD, but do not prevent disease development or progression (17).

3.2 N-methyl-D-aspartate receptor antagonists

N-methyl-D-aspartate (NMDA) receptor antagonists are also used for symptomatic AD treatment. Memantine (1-Amino-3,5-dimethyladamantane) is a

Table 1: Classes of β-secretase inhibitors - BACE1 and their compounds.

of BACE1 inhibitors	Compounds
Naturally occurring BACE1 inhibitors	• 2,2',4'-Trihydroxychalcone; liquorice (Glycyrrhiza glabra) (59), • Ginsenoside Rg1; Panax notoginseng (59), • Polymethoxyflavones (5,7-dimethoxyflavone (DMF), 5,7,4'-trimethoxyflavone (TMF), and 3,5,7,3',4'-pentamethoxyflavone (PMF)); Kaempferia parviflora (58), • Triflavonoids derivatives; Selaginella doederleinii (58), • Ursolic acid and lupeol; Leea indica (58).
Peptidomimetic BACE1 inhibitors	Compounds based on: • Isophthalamide, • Hydroxyethylamine (HEA), • Hydroxyethylamine isostere (58).
Non-peptidomimetic BACE1 inhibitors	• Compounds based on: • Acyl guanidine, • 2-aminopyridine, • Aminoimidazole, • Amino/iminohydantoin, • Aminothiazoline, • Aminoxazoline, • Dihydroquinazoline, • Aminoquinoline, • Aminopyrrolidine (58).
BACE1 inhibitors, included in clinical trials	• MK-8931 (Verubecestat), • E2609 (Elenbecestat), • AZD3293 (Lanabecestat), • JNJ-54861911 (Atabecestat), • Umibecestat (17).
BACE1 inhibitors, included in phase III clinical trials	• MK-8931 (Verubecestat), • E2609 (Elenbecestat) (17,58).

non-competitive NMDA receptor antagonist approved by the EMA in 2002 (54). It is used to treat both mild and severe AD. It reduces excitotoxicity and glutamate-induced neurodegeneration. It can reduce tau protein hyperphosphorylation and protects against the toxic effects of Aβ peptides by preventing the binding of glutamate to NMDA receptors (55,56). Studies have shown that it reduces neuropsychiatric AD symptoms compared to placebo. It does not significantly alleviate anxiety symptoms in people with moderate to severe AD (57).

3.3 Pharmacological agents that prevent the formation and aggregation of insoluble Aβ peptides or promote their elimination

The current main treatment strategies based on the amyloid hypothesis are: a) β-secretase (BACE1) and γ-secretase inhibitors, b) substances that prevent Aβ aggregation, c) substances that regulate protease activities (increase Aβ removal), and d) immunotherapy. In this chapter, we will focus mainly on BACE1 inhibitors and Aβ-targeting monoclonal antibodies (AD immunotherapy) (17). The main enzymes involved in the amyloidogenic pathway of APP cleavage are BACE1 and γ-secretase. Secretase inhibitors inhibit the action of these enzymes in the amyloidogenic pathway of APP cleavage, thereby preventing the formation of insoluble Aβ peptides and the formation of senile plaques (6). Due to the serious side effects of γ-secretase inhibitors, more attention is paid to the development of BACE1 inhibitors. These are divided into three groups, namely peptidomimetic, non-peptidomimetic and natural (Table 1) (58).

Among BACE1 inhibitors, special mention should be made of compounds included in clinical trials: MK-8931 (verubecestat), E2609 (elenbecestat), AZD3293 (lanabecestat), JNJ-54861911 (atabecestat) and umibecestat (17). None of them were included in phase IV clinical trials. However, two compounds have entered phase III clinical trials, i.e. MK-8931 and E2609. None of these compounds, however, have so far successfully

completed phase III clinical trials (58). In addition to BACE1 inhibitors, which prevent the formation of insoluble Aβ peptides, monoclonal antibodies to already formed Aβ peptides have been developed. Solanezumab, gentenerumab, crenezumab and aducanumab are Aβ-targeting monoclonal antibodies which have been included in clinical trials. Solanezumab may bind to Aβ monomers and soluble peptides. Gentenerumab binds to Aβ oligomers and fibrils and can also trigger plaque phagocytosis via microglia. There are also crenezumab and aducanumab; the target of the first are Aβ monomers, oligomers and fibrils, and the target of the second are Aβ aggregates. Aducanumab has also been shown to reduce Aβ deposition. Clinical trials of the first three preparations finished in phase III at the latest (17), while in the case of aducanumab, in March 2019, an independent monitoring committee found that there was insufficient evidence to support its effectiveness in treating AD. The biotechnology company Biogen therefore stopped all clinical trials. Subsequent additional date analyses showed that aducanumab at the highest doses reduced the progression of cognitive and functional impairment in patients with early-stage AD. Based on this finding, in August 2020 the USA Food and Drug Administration (FDA) accepted the application for marketing authorization for aducanumab; it was granted Fast Track designation. Aducanumab is a high-affinity preparation of human IgG1 monoclonal antibodies that target the conformational epitope present on Aβ oligomers. They are derived from autoimmune B lymphocytes from elderly individuals with normal cognitive function. Treatment of early-stage patients with aducanumab has been shown to reduce Aβ deposition in the brain and slow the decline in cognitive function in a dose-dependent fashion. Studies suggest that aducanumab is very likely to be equally effective in removing Aβ, regardless of ApoE genotype. Other monoclonal antibodies were largely directed against the N-terminal region of Aβ and recognized only monomers. Monoclonal antibody preparations directed against the N-terminal region of Aβ were very effective in the treatment of transgenic mouse models with AD, but, unlike aducanumab, did not show a therapeutic effect in humans (60,61). If aducanumab is approved, it will be the first drug to show that Aβ removal is associated with better AD clinical outcomes.

3.4 Pharmacological agents targeting tau protein

In AD development, the tau protein hyperphosphorylation and proliferation hypothesis is one of the most important. Pharmacological agents targeting tau protein can be divided into several categories: a) tau aggregation inhibitors, b) tau kinase inhibitors, c) O-GlcNAc inhibitors, d) microtubule stabilizers and d) immunotherapy. At this point, it should be emphasized that GSK-3β inhibitors are very important therapeutic agents (17). GSK-3 is a serine/threonine protein kinase. There are two isoforms: GSK-3α and GSK-3β. The latter is divided into two subtypes, namely GSK-3β1 and GSK-3β2. GSK-3β1 is present in numerous organs, while GSK-3β2 is found only in the central nervous system (6). GSK-3 is responsible for phosphorylation and therefore inactivation of glycogen synthase, control of glycogen metabolism and regulation of cell proliferation and the cell cycle. In AD, activation of GSK-3β triggers tau protein hyperphosphorylation, resulting in its deposition, aggregation into paired helical filaments, NFTs formation and disruption of physiological processes within neurons (23,28). This process can be slowed down by using inhibitors. These may act indirectly by regulating phosphatases or primary kinases that affect GSK-3 substrates. GSK-3 activity is thus regulated by kinases (PKB, PKC, PKA, p38) or phosphatases (PP2A, PP1). However, the reduction of GSK-3 activity is directly influenced using small molecules as highly specific inhibitors (62). Depending on their origin, we divide inhibitors from this group into those isolated from natural sources, cations and small synthetic molecules. Depending on the inhibition mechanism, we distinguish between ATP-competitive and non-ATP-competitive inhibitors and substrate-competitive inhibitors (Table 2).

The results of a 24-week trial of NP031112 (tideglusib) showed an improvement in cognitive performance in patients with mild or moderate AD (63). In addition to GSK-3β inhibitors, tau protein aggregation inhibitors, heat-shock protein 90 (hsp 90) inhibitorsand vaccines targeting hyperphosphorylated tau protein should also be mentioned. Some of the tau protein aggregation inhibitors, such as the active substance TRx0237, have reached the clinical stage of testing. Phase III clinical trial results have shown that this drug reduces brain atrophy in patients with AD. Two vaccines, ACI-35 and AADvac1, targeting hyperphosphorylated tau protein are also included in clinical trials (17).

3.5 Anti-inflammatory drugs

The neuroinflammatory process that can be detected in patients with AD has, in addition to many negative

Table 2: Classes of glycogen synthase kinase-3 (GSK-3) inhibitors and their compounds.

Classes of GSK-3 inhibitors	Compounds
Metal cations as GSK-3 inhibitors	• Lithium (6,63), • Beryllium (63), • Zinc (63), • Mercury (63), • Copper (63).
ATP-competitive GSK-3 inhibitors from natural resources	• Bis-indole indirubin, • Indorubin analogues, • Debromohymenialdisine (DBH), • Hymenialdisine (HD), • Meridianins (6,63).
Synthetic, ATP-competitive GSK-3 inhibitors	• Aminopyrimidines developed by Chiron – CHIR98014, CHIR98023, CHIR99021, • Arylindolemaleimides – SB-216763 and SB-415286, • Amino thiazole – AR-A014418, • Paullone compounds – kenpaullone and alsterpaullone (63).
Non-ATP-competitive GSK-3 inhibitors from natural resources	• Manzamine A, • Extracts and compounds obtained from the marine organism *Ircinia sp* (63).
Synthetic, non-ATP-competitive GSK-3 inhibitors	• Thiadiazolidinones (TDZD) - NP00111, NP031112, NP03115, • Halomethylketone (HMK) derivatives (6,63).
GSK-3 inhibitor included in a phase III clinical trial	• NP031112 (Tideglusib) (63).

Legend: GSK3 – glycogen synthase kinase-3; ATP – adenosine triphosphate.

effects, also some positive effects, such as removal of Aβ aggregates. The negative effects of inflammation are associated with the formation of substances during the inflammatory process: chemokines, oxygen reactive species and inflammatory cytokines, which deregulate the innate initial immune response and ultimately lead to neuronal degeneration (22). The results of several studies indicate the potential importance of anti-inflammatory drugsin patients with AD; celecoxib, naproxen and lornoxicam were included in clinical trials. Therapeutic efficacy analysis showed that naproxen and celecoxib failed to provide benefit compared to placebo while also causing side effects, leading to discontinuation of the clinical trial in phase III (17). Clinical trials of lornoxicam have also been discontinued due to ineffectiveness in patients with AD. All these facts indicate the need for further research on the clinical use of anti-inflammatory drugs in AD.

Prevention, detection and treatment of metabolic and non-genetic AD risk factors, including type 2 diabetes mellitus, elevated cholesterol and high blood pressure, are also important. Associations which can affect AD development or progression exist between these diseases and AD.

4 Conclusion

More than 100 years ago, Alois Alzheimer was the first to publish a report on the disease now named after him. Despite the huge amount of AD research in recent decades and the large financial investment in it, many questions regarding the aetiology, pathogenesis and treatment of this disease remain unresolved. Recently, the use of advanced technology in various genetic, molecular and cellular studies provides us with new insights into the disease pathogenesis, potentially contributing to the discovery of new therapeutic targets, drugs or therapeutic approaches that would not only slow down but also successfully prevent disease development or progression.

Conflict of interest

None declared.

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