YEAR
2023

UNIVERSITY OF MEDICINE AND PHARMACY
„CAROL DAVILA" BUCHAREST
DOCTORAL SCHOOL
MEDICINE

Theoretical and experimental contributions to amyloid beta dynamics in neurodegeneration models

PhD THESIS SUMMARY

PhD Coordinator:
PROF. UNIV. DR. POPESCU
BOGDAN OVIDIU

PhD Student:
SCHREINER
THOMAS GABRIEL

YEAR
2023

Content

List of abbreviations and symbols

AD – Alzheimer’s disease

APP - amyloid precursor protein

Aβ – amyloid beta

BBB – blood-brain barrier

CNS – central nervous system

CSF – cerebrospinal fluid

HHV-6 - Human herpesvirus 6

HSV - Herpes simplex virus

Introduction

Neurodegenerative diseases represent an important field of 21st century neurology, being characterized by a continuously increasing incidence and an increased interest from researchers, as evidenced by the large number of scientific studies in recent years. Alzheimer's disease (AD), the most common neurodegenerative disorder worldwide, is a relevant example of the challenges that neurodegenerative diseases bring to the neurologist. Thus, despite the many researches carried out to date, there are still many unknown elements regarding the etiology and pathophysiology of the disease, as well as the difficulty of early detection and curative treatment of the disease.

The lack of an effective treatment to stop (or even reverse) the neurodegeneration process is mainly due to insufficient knowledge of the cellular and molecular mechanisms involved in the onset and evolution of neurodegenerative pathologies. Starting with the example of Alzheimer's disease, which was originally described in 1906, even after 100 years of exhaustive research, the hypotheses circulated remain incomplete. Currently, research focuses on several directions, the most important being represented by the pathological accumulation of beta amyloid, the impact of oxidative stress on neurons and chronic neuroinflammation. However, the development of therapies to counteract these pathological mechanisms did not have the expected effect, i.e. the significant improvement of symptoms, the reduction of brain injury or the increase of the patient's life expectancy. In

this sense, for a better understanding of AD, another approach is needed in fundamental research, which is initially based on the reinterpretation of some intensely debated theories (such as the amyloid hypothesis), and later, on the assembly of heterogeneous theoretical information into an unitary pathophysiological picture.

Amyloid beta (Aβ), a peptide resulting from the pathological cleavage of the amyloid precursor protein (APP), although initially described several decades ago in the brains of AD patients, currently remains one of the essential biomarkers in the diagnosis of this pathology. Moreover, in recent years, Aβ has also become a genuine therapeutic target for AD, but also for other neurodegenerative diseases, with the very recent appearance of monoclonal antibodies directed against this molecule, with potential beneficial effect for patients. Aβ plays a primary role in the pathophysiology of AD, the toxicity of different forms of Aβ found in the brain being linked to the rest of the destructive processes (chronic inflammation, oxidative stress, accumulation of other proteins with a pathological role, neuronal apoptosis) encountered in the central nervous system (CNS) that is affected by neurodegeneration. According to the latest research, Aβ seems to have multiple roles also in physiological conditions, targeting this molecule from the preclinical stages of AD becoming a possible therapeutic and preventive option.

I. General part

Chapter 1: Preliminary considerations on neurodegeneration and neurodegenerative diseases. Alzheimer's disease

1.1. Neurodegeneration in the context of neurodegenerative diseases

Neurodegenerative diseases represent a heterogeneous group of neurological disorders that usually affect the elderly population. The most common neurodegenerative pathology is Alzheimer's disease (AD) which, according to the most recent epidemiological data, currently affects more than 6 million Americans over the age of 65 (Alzheimer's Association, 2021). Currently, the exact etiology of AD is not known, with only a few risk factors associated with neurodegeneration being observed (Hu & Octave, 2019). In addition, the treatments available at present are mostly symptomatic therapies, which have limited effects on improving the clinical picture, but fail to stop the progression of the disease (Wareham et al., 2022).

The central process that causes AD, as well as other neurodegenerative pathologies, is neurodegeneration. This process affecting the central nervous system (CNS) is characterized by the disruption of connectivity between neurons located in different areas of the brain, by the progressive degradation of interneuronal synapses and axons, culminating in extensive neuronal loss (Jellinger, 2010). The clinical consequence of this phenomenon is represented by the impairment of numerous cognitive, sensory and motor processes, including mainly language, memory, motility, vision and hearing (Taipa et al., 2022). This explains the clinical picture found in patients with AD, where memory, language and attention are predominantly affected. However, other cognitive dysfunctions such as agnosia or apraxia can be encountered.

Although neurodegeneration is centered on the death of neurons, this phenomenon would not be possible without the presence of triggering factors, supporting factors and, last but not least, the simultaneous damage to other cells in the CNS (Valori et al., 2021). In addition, according to recent research, CNS neurodegeneration would also be the result of imbalances in the peripheral nervous system (Ma et al., 2021) and the immune system acting in the periphery (Zang et al., 2022).

1.2.Risk factors in neurodegenerative diseases

Multiple studies conducted in the last decades have highlighted genetic factors and environmental factors that increase the predisposition to the onset of neurodegenerative diseases (Wang et al., 2021). Human genome sequencing studies were needed to identify genes that confer an increased risk of AD. This research revealed common molecular pathways associated with genes such as *APOE4* or *CLU*, which encode apolipoprotein E and apolipoprotein J (also called clusterin) (An et al., 2021). APOE4 is an intensively studied risk factor for AD, modulating diverse mechanisms involving pathological protein aggregation, supporting inflammation and neurodegeneration (Husain et al., 2021). Other genes that undergo autosomal dominant mutations in AD patients, especially in early onset Alzheimer's disease, are the *APP*, *PSEN1* and *PSEN2* genes (Xiao et al., 2021). These genes encode key elements of the amyloid beta cascade, such as amyloid precursor protein (APP), presenilin-1 and presenilin-2, components of the gamma secretase complex.

It is clear that the mere presence of genetic factors is not a certainty for the appearance of AD, there is a process on several stages, some still unknown, through which a person possessing a certain allele develops clinical manifestations compatible with CNS degeneration (Avila & Perry, 2021). Thus, perhaps of greater importance are the

environmental factors, many of them modifiable, that have been associated with an increased risk for the onset of AD.

Over time, numerous environmental factors have been studied in association with AD, including age, diet, exercise, and exposure to neurotoxic substances, as triggers or exacerbating factors of the neurodegeneration cascade. Advanced age is an essential risk factor in numerous pathologies, aging having an even more significant influence on CNS neurons (Sikora et al., 2021). Among demographic factors, it has been observed that a patient has an increased risk of developing AD in case of a lower educational and social level (Bodryzlova et al., 2022), while the impact of gender or race still remains incompletely elucidated (Mielke et al. al., 2022). As environmental neurotoxic factors, the debate remains open regarding the true impact of metal particles in the pathogenesis of AD, through various mechanisms such as interaction with APP metabolism or APOE modulation. Most research has focused on studying the effects of aluminum, zinc, mercury, copper or magnesium in neurodegeneration (Bakulski et al., 2020). In addition, studies have also shown the cumulative effect in the case of the association of several metals, the exact mechanisms remaining incompletely elucidated (Islam et al., 2022).

An interesting association is noted between craniocerebral trauma and AD, especially in patients with a history of multiple traumatic injuries to the cephalic extremity (Ramos-Cejudo et al., 2018). The most recent data point towards the fact that the etiopathogenesis of AD is influenced by the diet, people with an excessive intake of saturated fats or those with a deficiency of vitamin E having an increased risk of developing this disease (Bello-Corral et al., 2021). Malnutrition, obesity and diabetes have been associated with neurodegeneration, particularly AD. Also, as an exogenous factor with potential implications in the appearance and/or evolution could be infections with different pathogens, most of them viral. Among the relevant studies on this topic, worth mentioning are the results regarding herpes simplex virus (HSV), Epstein-Barr virus or human herpes virus type 6 (HHV-6) and the increased risk of developing AD (Itzhaki et al., 2021).

As observed, there is a mosaic of endogenous and exogenous elements that, through their cumulative effect and most likely based on an additional mechanism not elucidated to date, induce and maintain the neurodegeneration characteristic of AD.

1.3.Theories of neurodegeneration

Currently, there are several accepted theories regarding the occurrence and evolution of neurodegeneration in AD. The temporal criterion is an essential one in the evolution of the

degenerative process, diseases characterized by neurodegeneration being the result of the accumulation of a toxic substance (endo- or exogenous) or the time-dependent effect of a toxic substance previously present in a certain concentration (Modgil et al., 2014). The mechanism of action of the risk factors is not a direct one, but a cumulative one, dependent on age. One of the common ways of these promoters of neurodegeneration is the increased release of oxygen free radicals that, in the case of aging, can no longer be optimally countered by physiological neutralization mechanisms, causing disease (Ionescu-Tucker & Cotman, 2021).

Another theoretical approach is based on the “double hit” hypothesis, in which there are two distinct key moments that lead to the emergence of the pathological condition (Zhu et al., 2007). Similarly, the "multiple hits" hypothesis was generated, in which its authors tried to explain the emergence of neurodegenerative pathologies based on multiple and complex interactions between bacterial infections, immune dysfunctions and CNS pathology (Patrick et al., 2019) . Finally, another interesting, more recent theory is based on the concept of the body's "allostatic load" (Guidi et al., 2021). According to this concept, the main risk factor is the rate of aging, the age-dependent dysfunction of anatomical systems causing a "wear and tear of the body" that includes the loss of synapses and neurons.

Chapter 2: Amyloid beta – from normal to pathological

2.1. The formation and elimination of amyloid beta

Amyloid beta (Aβ) is a peptide resulting from the pathological cleavage of the amyloid precursor protein (APP) in the amyloidogenic pathway, under the action of β-secretase and the gamma-secretase complex. The impact (toxicity) of Aβ in the pathophysiology of AD is mainly due to changes in the quaternary structure of the molecule. Along with the neurotoxicity of amyloid plaques, monomers and oligomers also play increasingly important roles in neurotoxicity leading to neurodegeneration.

The accumulation of Aβ at the brain level remains the central element in the pathophysiology of AD, but not only its excess production, the more the elimination deficit has been associated with the late-onset form of AD ("late-onset Alzheimer's disease") (Rosas-Hernandez et al., 2020). There are several ways in which Aβ is cleared from the brain parenchyma, by intra- and extracellular degradation or by transport to the systemic and lymphatic circulation (Tarasoff-Conway et al., 2015). The elimination of cerebral Aβ is done in a significant proportion at the BBB level directly in the blood stream, under the action of

some specialized transport systems. Mainly responsible for soluble Aβ efflux from the brain parenchyma are members of the LDL receptor (LDLR) family, such as LRP1, and ATP-binding cassette transporters (ABC transporters). There are other pathways for Aβ elimination from the CNS, such as intracellular and extracellular degradation, perivascular and glymphatic drainage, or absorption from the cerebrospinal fluid (CSF).

2.2. The roles of amyloid beta in physiological conditions

In recent years, increasing evidence has emerged suggesting that Aβ monomers and oligomers have beneficial roles under physiological conditions. In this regard, five main directions have been proposed to explain the "protective" role of Aβ under physiological conditions: antimicrobial effect, tumor suppressive activity, blood-brain barrier sealing ability, promoter of brain damage repair, and regulator of synaptic functioning.

In vitro cell culture studies have shown that Aβ can reduce the proliferation of several bacterial species (Soscia et al., 2010). Aβ reduced the viability of fungal cultures in vitro, such as Candida albicans cultures that were inhibited by brain homogenates of AD patients (Vojtechova et al., 2022). The antitumor role of Aβ peptide was initially suggested based on the inverse correlation between AD and tumor risk. Numerous epidemiological studies have shown that patients with AD have a much lower risk of developing cancer compared to healthy subjects (Lanni et al., 2021). Regarding the connection between Aβ and the blood-brain barrier, it is already well known the role that the structural alteration of BBB has in supporting the neurodegenerative cascade, leading to the pathological accumulation of Aβ in the brain. There are some studies that have shown an increased accumulation of Aβ several hours after craniocerebral trauma, with higher accumulations being correlated with better recovery (Plummer et al., 2016). Finally, recent research has demonstrated the role of the APP molecule in the axonal transport and release of Aβ in the synaptic cleft during neuronal activity (Fagiani et al., 2021). Aβ acts on the presynaptic neuron, stimulating the release of neurotransmitters, thus supporting the physiological processes that ensure the correct functioning of neural memory circuits (Fanutza et al., 2015).

2.3. The roles of amyloid beta in pathological conditions of neurodegeneration

In the case of AD, Aβ has a negative impact on numerous processes in the CNS, generating and sustaining neurodegeneration. Synapse dysfunction is one of the main consequences of toxic Aβ plaques and is the result of increased synaptic loss without

adequate compensatory synaptogenesis (Sciaccaluga et al., 2021). Aβ also negatively influences other brain structures, such as Tau protein or α-synuclein. Being closely related to Aβ, these proteins express abnormal behavior in neurodegenerative diseases, undergoing hyperphosphorylation or other structural changes on the basis of which pathological aggregation is sustained. Aβ also has a negative impact at the intracellular level, numerous signaling processes being affected, resulting in ionic imbalances, especially the one related to calcium (Ca2+). Chronic cellular stress causes reduced metabolic activity, and with regard to mitochondria, there is a deficient energy balance, with reduced production of adenosine triphosphate (ATP) and a decrease in mitochondrial enzyme activity (Ryu et al., 2021). Mitochondrial damage, along with the alteration of cellular homeostasis, also means damage to cellular respiration, as the role of mitochondria in glycolysis and maintaining the balance of reactive oxygen species is well known (Bell et al., 2021).

Multiple studies have shown that Aβ activates microglia and astrocytes, cells that subsequently produce pro-inflammatory cytokines and chemokines that support a chronic inflammatory state (Jung et al., 2022). It also remains important to mention the bidirectional link between Aβ and the blood-brain barrier (BBB), the BBB being strongly altered in AD. The increased permeability of the capillary endothelium, the degradation of tight junctions and the increase in the rate of transcytosis, the detachment of the pericyte from the capillary endothelium and the transformation into a phagocytic cell or fibroblast, the activation of astrocytes and microglia, these being the most relevant changes observable at the BBB level.

2.4. Amyloid beta as a therapeutic target in Alzheimer's disease

Considering the importance of Aβ in the complex process of neurodegeneration, with Aβ being one of the key molecules that generate and sustain this phenomenon, it is easy to understand why researchers have considered Aβ a suitable therapeutic target in anti-dementia treatment. From a summarized perspective, the control of Aβ balance at the brain level can be achieved by modulating two distinct processes: Aβ production (which is increased especially in early-onset forms of AD, and in this case, it is necessary to decrease it), and clearance of Aβ from the CNS (which is particularly defective in late-onset forms of AD, and in this case its increase is required).

Given that therapeutic attempts based on reducing Aβ production have not led to the expected results, recent approaches focus on improving Aβ drainage, both at the cerebral and systemic level. Two distinct approaches based on immune reactivity have been used, active and passive immunotherapy, which neutralize monomeric and/or oligomeric Aβ

molecules, favoring the elimination of the compound. Finally, improving the elimination of Aβ from the brain (but also systemic) can also be done through minimally invasive methods, such as plasmapheresis, dialysis, or filtering Aβ from different biological compartments (including the CSF compartment) and neutralizing Aβ by using antibodies specific.

II. Personal contributions

Chapter 3: Working hypotheses and general objectives

The personal contribution section can be fragmented within three distinct studies, the first theoretical and the other two practical. The general objectives of the thesis include two objectives for each of the three aforementioned studies:

I. Generation of a new hypothesis regarding the role of Aβ dynamics in the onset and evolution of Alzheimer's disease, starting from the classic amyloid hypothesis

II. Generating a hypothesis called the "therapeutic strategy of the cerebrospinal fluid compartment" that opens new therapeutic perspectives in Alzheimer's disease and other neurodegenerative diseases

III. Development of a nanoporous membrane capable of filtering soluble Aβ molecules (such as monomers and oligomers)

IV. Development of a biocompatible filtration device capable of filtrating and eliminating Aβ from the cerebrospinal fluid compartment

V. Practical validation of the theoretical hypotheses of study 1 by implantation of the Aβ filtration device in a murine model of Alzheimer's disease, with good survival of the animal for 4 weeks

VI. Quantifying the therapeutic effects of the Aβ filtration device in a murine model of Alzheimer's disease based on improvement in clinical symptomatology and paraclinical parameters

Chapter 4: General research methodology

To achieve the objectives of the thesis, various laboratory and bioengineering methods were applied. Thus, several steps were required for the development of biocompatible nanoporous filtration membranes:

- membrane synthesis through a two-stage anodization process in oxalic acid-based electrolytes starting from aluminum discs,

- the functionalization of the alumina membrane through the ALD (atomic layer deposition) technique, through which several atomic layers were deposited,

- quality control of the functionalization which was carried out by different measurement methods: X-ray diffraction, Raman spectroscopy, infrared spectroscopy, scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS) and a simultaneous thermal analysis consisting of thermogravimetry and differential scanning calorimetry,

- membrane selectivity measurements to confirm the selectivity of the nanoporous membrane for Aβ,

- evaluation of membrane biocompatibility based on MTT tests.

Later, after obtaining the biocompatible nanoporous membrane, we imagined and assembled an experimental CSF filtration device, consisting of three main components: a catheter with the role of connecting the lateral ventricle to the filtration module, the filtration module containing the nanoporous membrane with the role of selective filtration of Aβ from CSF, and a reservoir where Aβ-rich CSF is sequestered based on an antigen-antibody reaction. All the components that make up the device have been individually tested for biocompatibility through MTT tests.

The device thus assembled was implanted on a total of 20 animals, representing 4 batches of 5 animals each. Specifically, to perform the in vivo studies, 10 C57BL/6 mice and 10 APP/PS1 mice were used and administered at well-established time intervals (1 week before implantation and 4 weeks after treatment) behavioral tests of the elevated plus maze and the Y maze test, and at the end of the treatment period, they were sacrificed, and biological samples (blood, CSF, brain and liver tissue) were collected , which were analyzed by immunohistochemical methods. Additionally, 10 more animals, 5 C57BL/6 mice and 5 APP/PS1 mice, were used as negative controls that did not receive treatment.

Chapter 5: Study I: Theory of amyloid beta dynamics and derived therapeutic strategies

5.3. Results

The first study within the thesis resulted in the production of a review-type article (Schreiner et al., 2022a), in which the authors realize two important aspects:

- first of all, I review the specialized literature existing up to that moment and explain,

based on the information already known and demonstrated, the importance of knowing the dynamics of Aβ between the compartments of the human body for the appearance and evolution of AD;

- generate a new hypothesis called the "therapeutic strategy of the cerebrospinal fluid compartment" with significant therapeutic impact, which they will use as the theoretical basis and fundamental principle for the experimental studies in study 3.

Starting from the hypothesis of Aβ dynamics, it is observed that the common point of cerebral drainage of Aβ, along with other residual compounds, is the cerebrospinal fluid compartment, from where subsequently a significant part of protein residues is directed to the peripheral circulation. The cerebrospinal fluid thus becomes an essential therapeutic target, this compartment communicating mainly with the cerebral interstitial fluid, but also with the internal environment. Through various interventions designed to modify the biochemical characteristics of the cerebrospinal fluid (reduction of certain solutes), reducing Aβ concentration from this level could also lead to significant changes in brain Aβ peptide concentration. Thus, the foundations of the "therapeutic strategy of the cerebrospinal fluid compartment" are laid, starting from the dynamic balance of soluble substances between the three distinct biological compartments, the cerebral interstitial fluid, the cerebrospinal fluid and the peripheral blood. This hypothesis is valid and useful from a practical point of view also due to the fact that Aβ (predominantly in the form of monomer and small oligomer) in various solutions (CSF or blood under in vivo conditions) is filterable through filtration membranes with specific parameters, this being and the main reason for making a filtration device capable of separating Aβ from a colloidal solution (see study 2 for details).

Chapter 6: Study II: Development of a biocompatible filtration device capable of purifying Aβ from a solution

6.3. Results

The first stage of study II consisted in the development of a nanoporous filtration membrane capable of filtering soluble Aβ molecules (such as monomers and oligomers). Obtaining this membrane was possible following the synthesis process of the alumina membrane and the subsequent functionalization of the surface of this structure. All stages are presented in detail in the "Material and method" section. Subsequently, an essential step is the validation of the membrane functionalization which was performed by spectroscopic

analysis based on X-ray diffraction, Raman spectroscopy, infrared spectroscopy and SEM-EDS analysis. The results proved that the functionalized membrane falls within the desired parameters (see graphs below).

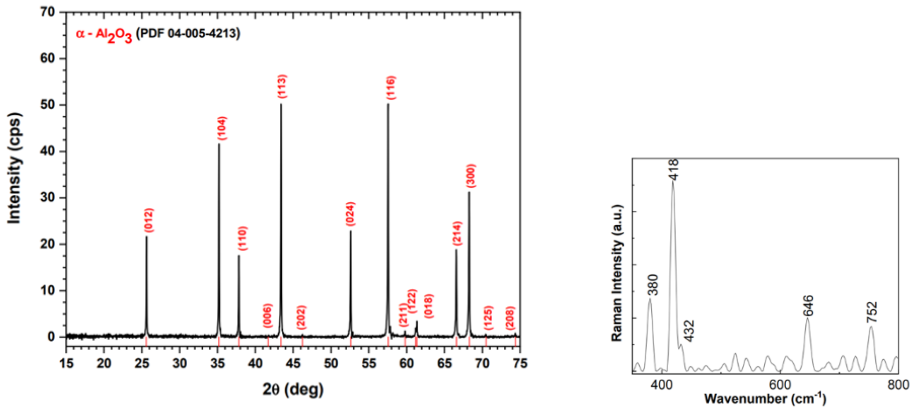

Figure 1: Nanoporous membrane analysis by X-ray spectroscopy and Raman spectroscopy

The SEM-EDS analysis provides a much more detailed picture regarding the structure of the nanoporous membrane. This analysis showed a uniform pore distribution and quasi-equal sizes of pore diameters. The technological process (mainly the functionalization process) to obtain the nanoporous membrane was well done, as the pores of the final product had an average size of approx. 17 nm, ideal for Aβ filtration. Theoretically, a pore size below 25 nm (ideally below 20 nm) is sufficient for selective filtration of Aβ. In addition, by strictly controlling the technological process of membrane formation, the obtained pores were much more uniform and spatially closer than what was originally considered in the theoretical design, that is, an interpore distance of less than 50 nm. The direct impact is the increase in filtration efficiency, and given that the required filtration surface can be smaller, further miniaturization of the device can be considered.

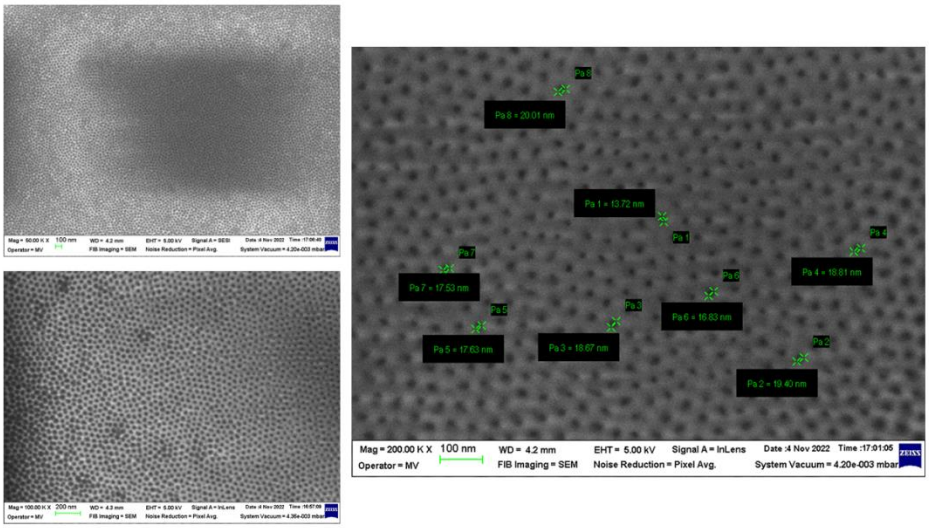

Figure 2: SEM-EDS analysis of the nanoporous membrane at different magnifications (top left 50,000X, bottom left 100,000X and right 200,000X)

Finally, a simultaneous thermal analysis consisting of thermogravimetry (TG) and differential scanning calorimetry (DSC) was also performed (Figure 3). The sample was initially analyzed by DSC in nitrogen up to a temperature of 300°C to observe for organic/polymeric contamination, an essential aspect of filters designed to separate organic molecules. A weak endothermic process was observed at about 82.5°C, probably due to removal of residual moisture. Otherwise, the graph obtained is characteristic for uncontaminated inorganic materials. In DSC analysis performed in air up to 500°C, there are some weak exothermic processes from 290°C to 375°C, explained by the oxidation of traces of carbon and/or phosphorus. No change is observed in TG analysis in nitrogen up to 700°C, thus reconfirming that the inorganic nanoporous membrane is uncontaminated with organic substances.

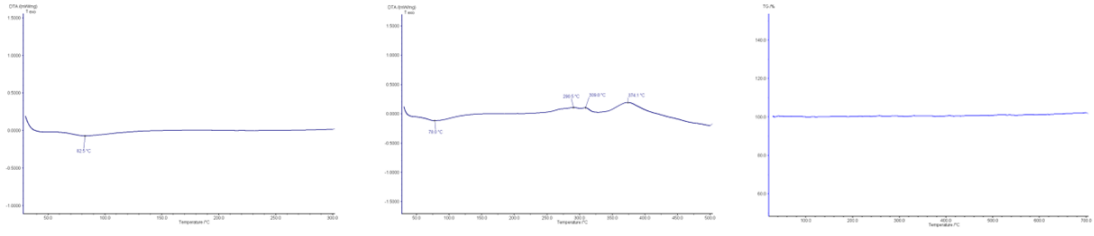

Figure 3: Simultaneous thermal analysis of the nanoporous membrane (left – DSC analysis in nitrogen up to 300°C, middle – DSC analysis in air up to 500°C, right – TG analysis in nitrogen up to 700°C)

The use of the nanoporous membrane under in vivo conditions requires mandatory the demonstration of its biocompatibility.

The alumina membrane did not influence the viability of MCF-7 cells, the only visible effect being the increase in cell number (viable cells). Crude extract increased cell count/viability by 13.58%, 1:2 dilution by 17.74% and 1:4 dilution by 19.03%. Furthermore, in the case of MDA-MB-231 cells, the alumina membrane extract did not influence cell viability; however, there was an increase in the number of live cells. The amplitude was lower than in MDA-MB-231 cells, but exceeded the 100% viability of the control group by 9.45% (crude extract), 10.71% (1:2 dilution) and 13.52 % (1:4 dilution). Statistical significance calculated by Student's t-test showed that all differences in cell viability were statistically relevant (* $\leq$0.05, ** $\leq$0.01). Thus, in this preliminary biocompatibility evaluation test, the tested membrane proved to be fully compatible with the two cell cultures tested. The results in terms of biocompatibility/toxicity were also promising for the other components that, along with the nanoporous membrane, make up the filtration module of the prototype.

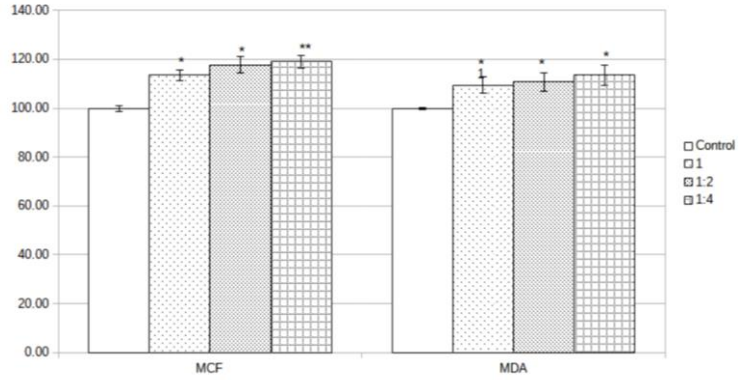

Figure 4: Viability of MCF-7 and MDA-MB-231 cell lines determined by MTT assay after 48 hours of incubation with the membrane extract tested in different dilutions: 1 concentrated extract, 1:2—1 part extract and 1 part DMEM, 1:4 —1 part extract and 3 parts DMEM.

This study provides a detailed description of the mandatory steps to achieve an efficient and biocompatible device capable of filtering Aβ from the cerebrospinal fluid compartment. The nanopore filtration membrane remains the central component of the device, and obtaining a functionalized alumina nanostructure by the ALD technique is a viable and preferable option, especially considering the technological advances in the field

of bioengineering. Finally, this study opens new perspectives for improving the design of experimental cerebrospinal fluid filtration devices and for the development of new biocompatible, miniaturized prototypes that can be easily and cost-effectively produced on an industrial scale.

Chapter 7: Study III: Clinical and paraclinical effects of Aβ filtration from the cerebrospinal fluid in a murine model of Alzheimer's disease

7.3. Results

Starting from the hypotheses detailed in study I, this third study had three main objectives. First of all, we wanted to validate the theoretical notions in practice by implanting the experimental Aβ filtration device in a murine model for Alzheimer's disease (APP/PS1 mouse). Along with the successful implantation of the Aβ cerebrospinal fluid filtration prototype, the aim of the study is to maintain long-term viability (minimum 4 weeks) of the mouse with the mounted device. Secondly, at the end of the trial period, based on clinical (behavioral studies) and paraclinical (study of biomarkers in various biological samples) analyzes, the therapeutic effect of this method was quantified, conclusions being drawn regarding the possibility of using this method in daily neurological practice in the future. Finally, after sacrificing the mice, a number of five filtration devices were recovered, and the effect of 4 weeks of treatment on the nanoporous membrane was studied. Tracking post-treatment membrane structural changes such as protein residue accumulation and pore clogging can be considered an indirect method of tracking the efficiency of this device.

Except for the two L0 groups that were used as control groups, the remaining 20 mice benefited from implantation of the Aβ filtration device. The surgical procedure (detailed in the "Material and method" chapter) involved several steps, each with its own peri- and post-procedural risks. However, for the groups in the present study, surgery had a 100% success rate (no intraoperative deaths). Moreover, during the 4 weeks after the intervention, the animals were followed daily in order to detect as early as possible any alteration of the general condition or the appearance of complications. With the exception of some skin dehiscences in the areas adjacent to the cannula implantation, which occurred in 5 of the 20 animals during the first week after the procedure, we did not report any other adverse effects such as infections, changes in the general status or death. The main causes for changes in the

integrity of the skin are diverse, from reasons related to the reduced thickness of the skin in the area of the cephalic extremity, the individualized rate of postoperative recovery, elements related to the device such as the increased dimensions of the cannula compared to the cephalic extremity of the mouse, the high tension of the catheter located immediately subcutaneous, to problems related to the quality of the materials and the technique used in performing the final suture.

In order to demonstrate the efficiency of the method of removing Aβ from the cerebrospinal fluid with the help of the filtration device developed previously, we quantified both from a clinical and a paraclinical point of view the results obtained within the groups of mice that benefited from this treatment.

On the clinical side, we looked at two distinct but correlated parameters, namely the degree of anxiety and short-term memory. Both parameters are closely related to the cognitive deficit found in Alzheimer's disease. The results obtained in the case of groups that benefited from treatment with antibody solution are encouraging, observing a decrease in the general degree of anxiety in these animals. The same results cannot be found in the case of the control groups, further evidence for the therapeutic effect of anti-Aβ antibodies in the context of CSF filtration. Also, from the behavioral point of view, a slight improvement of the memory deficit is observed only in the group of APP/PS1 mice that benefited from treatment with antibody solution. The cognitive decline recorded in the other groups further suggests a negative effect of this treatment in WT mice, with excess clearance of Aβ from the CSF leading to cognitive impairment. It should be noted that the results were obtained from small study groups, and studies with more subjects are needed to confirm these trends.

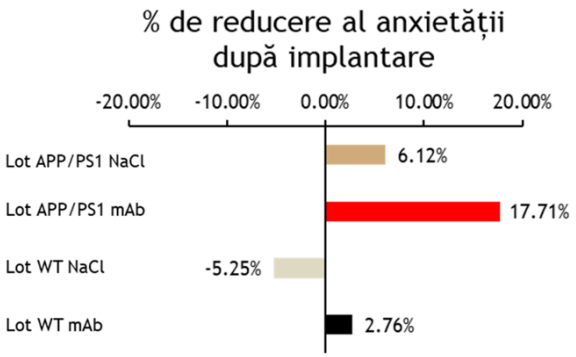

Figure 5: The final data on the reduction of the degree of anxiety in the tested mice after the treatment period

Regarding the paraclinical data, relevant results could only be obtained in the case of brain tissue samples. Limitations due to the small amount of CSF and blood in the mice used, as well as contamination during sample collection, resulted in a very small number of adequate liquid samples for subsequent analysis. Although not statistically significant, the partial results support the efficacy of the experimental treatment in decreasing CSF Aβ levels in APP/PS1 mice. Similarly, a (statistically insignificant) decrease was also observed in the brain Aβ concentration, demonstrating the dynamic balance of Aβ between the central compartment and the peripheral compartment. It should be noted that this experimental treatment has negative effects in healthy control subjects, where cognitive decline and excess accumulation of Aβ in the brain were recorded.

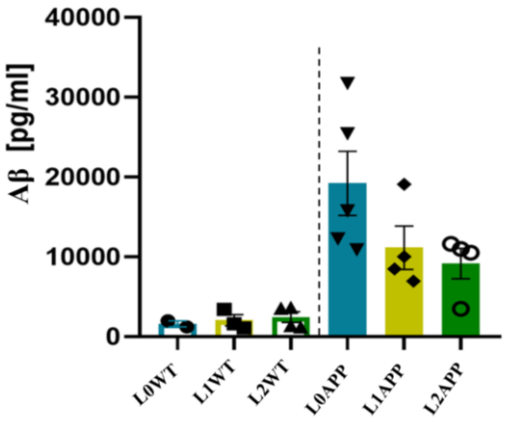

Figure 6: Brain Aβ concentration before and after treatment

Finally, the five filtration devices used in batch L2APP were recovered, from which the nanoporous membranes were taken for further tests. Based on the results of the combined SEM and EDS analysis, significant accumulations of proteins were visualized leading to the reduction of the pore size, their incomplete clogging, ultimately diminishing the filtration efficiency of the nanomembrane. The reduction in pore diameter, from 17 nm to an average of 11 nm, was inhomogeneous, affecting the nanomembranes differently and unsystematically. Despite the protein residues, the membrane fouling was not total, it retained an efficiency of about 60-75% even after continuous filtration for one month.

Limiting the clogging of the filtration membrane is another important objective in the process of obtaining an ideal membrane, with bio-medical applications. Moreover, avoiding the accumulation of residues maintains its efficiency and ensures a longer life of the membrane. Membrane polarization is a common and unavoidable phenomenon that supports

the unwanted accumulation of organic residue within osmotic membrane processes, leading to the unwanted reduction of transmembrane flux. Current strategies to limit concentration bias include adjusting hydrodynamics, flux management, and membrane design optimization. The optimization of the filtration membrane can be done based on the precise adjustment of both the chemical nature and the physical nature of the membrane pore surface. In this sense, the ALD technology, also used in the case of the membranes in the present study, is one of the solutions for improving the membrane surface and reducing the polarization phenomenon.

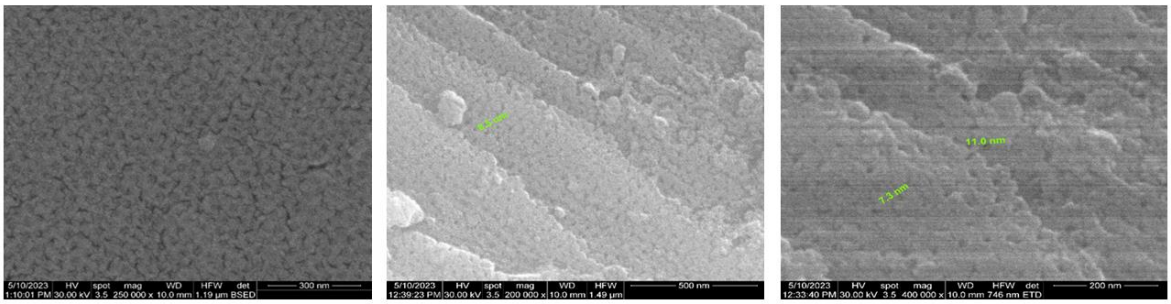

Figure 7: SEM analysis of the nanoporous filtration membrane after the 4-week treatment period: a) basal surface, 250,000X magnification; b) apical surface, 200,000X magnification; c) apical surface, 400,000X magnification.

The high degree of organic matter (protein) accumulation at the level of the nanoporous membrane was additionally found in the simultaneous thermal analysis (TG and DSC). Two thermal phenomena were detected at 221°C and 300°C, characteristic of the decomposition of protein substances, specifically the Aβ residues clogged at the level of the nanopores. These results lead to two conclusions: on the one hand, they are an indirect way that attests, through the accumulation within the pores of the nanomembrane, the good functioning and effectiveness of the filtration device, and on the other hand, they show the fact that a period of approximately 4 weeks is optimal lifetime of the nanoporous membrane used in this study. It is thus required to change the nanoporous membrane after one month of use if the device is to be used longer term in future studies.

8. Conclusion and personal contribution

Alzheimer's disease remains the most common neurodegenerative pathology in

Romania and worldwide, and its prevalence is expected to increase in the coming decades. Even though a lot of research is being done on the etiology, pathophysiology and treatment of this disease, the results are still modest.

Among the currently accepted theories regarding the onset and progression of Alzheimer's disease, the amyloid hypothesis is the most consistent. Based on the anatomopathological results, the main marker is considered to be the pathological accumulation of Aβ in the brain, in the form of senile plaques. Aβ thus becomes a key element for both experimental research and a genuine therapeutic target.

Starting from the classical version of the amyloid hypothesis, we reinterpreted the classical view based on the most recent findings. Thus, we generated a new hypothesis, based on the role of Aβ dynamics between the different compartments of the human body (CNS, CSF and peripheral circulation). Alteration of Aβ dynamics, mainly a defective clearance, lead to the onset and progression of Alzheimer's disease, especially the late-onset form.

Based on the impairment of Aβ dynamics in neurodegeneration, studies have demonstrated the superiority of therapies based on improving Aβ clearance from the brain compared to treatment based on slowing/stopping production. In this context, we generated a new hypothesis that may have therapeutic importance, which we termed the "therapeutic strategy of the cerebrospinal fluid compartment". According to this new approach, starting from the hydrodynamic equilibrium of soluble Aβ between the brain interstitial compartment and the CSF compartment, the removal of Aβ from the CSF would cause a decrease in the level of Aβ from the brain. Furthermore, clearance of Aβ from the CSF has a superior impact on brain Aβ levels compared to clearance from the systemic circulation.

To put into practice the previously mentioned theoretical concepts, the central part of the thesis was the development of a biocompatible device capable of filtering the cerebrospinal fluid and purifying Aβ from this level. The main component of this device is the nanopore filtration membrane, capable of selectively filtering soluble Aβ molecules (such as monomers and oligomers).

Obtaining the nanoporous membrane capable of filtering Aβ is the result of two distinct processes: initially, the synthesis of the alumina membrane and subsequently the surface functionalization of this structure through the ALD technique. Once obtained, the membrane must undergo a careful check to confirm the parameters, quality and biocompatibility of the nanopore structure.

The first aspect consists in confirming that the membrane functionalization process has proceeded according to specifications, in this sense the following techniques are used:

X-ray diffraction spectroscopy, Raman spectroscopy, infrared spectroscopy, scanning electron microscopy (SEM) with spectroscopy energy dispersive X-ray (EDS) and a simultaneous thermal analysis consisting of thermogravimetry and differential scanning calorimetry. Then, the selectivity of the final product for Aβ molecules and finally the biocompatibility based on the MTT assay were checked. Finally, after validation, the nanoporous membrane was incorporated into the experimental CSF filtration device.

Demonstration of the theoretical concepts was possible by implanting the previously developed device in a murine model simulating Alzheimer's disease. In the study, we presented in detail the surgical technique of implanting the device, as well as the methods of collecting biological samples after the treatment period.

The surgical technique had a 100% success rate (no peri- or post-surgical deaths). Afterwards, throughout the treatment period totaling 4 weeks, the animals were carefully monitored, with no major local or general complications.

Demonstration of the efficiency of the CSF filtration method using the experimental device was done by quantifying the clinical and paraclinical effects. Regarding the clinical side, we followed two parameters, the degree of anxiety and short-term memory, both parameters being closely related to the cognitive deficit found in Alzheimer's disease. In the groups of APP/PS1 mice that received treatment with the antibody solution, a decrease in overall anxiety and a slight improvement in short-term memory deficits were observed.

Regarding the paraclinical data, relevant results could only be obtained in the case of brain tissue samples. After treatment, a (statistically insignificant) decrease in brain Aβ concentration in APP/PS1 mice was observed, demonstrating the dynamic balance of Aβ between the central and the peripheral compartment, thus the validity of the therapeutic procedure.

In addition, the five filtration devices used in mice from the L2APP group were recovered, from which the nanoporous membranes were retrieved for retesting. Based on the results of the combined SEM and EDS analysis, significant accumulations of proteins were visualized leading to the reduction of the pore size, their incomplete clogging, ultimately diminishing the filtration efficiency of the nanomembrane. This high degree of accumulation of organic matter (protein) was additionally found in the simultaneous thermal analysis (TG and DSC). These results indirectly show the correct and efficient operation of the filtration device.

In conclusion, the treatment based on the forced removal of Aβ from the CSF compartment is a viable therapeutic modality with promising perspectives in the treatment

of Alzheimer's disease. Based on the results of the present study, the optimal lifetime of the prototype used in this research is 4 weeks. It is thus required to change the nanomembrane (or the whole assembly if possible) after one month of use, if it is desired in future studies to use the device for a longer period.

Selective bibliography

Alzheimer’s Association. 2021 Alzheimer's disease facts and figures. *Alzheimers Dement.* 2021;17(3):327-406. doi:10.1002/alz.12328

An N, Fu Y, Shi J, et al. Synergistic Effects of APOE and CLU May Increase the Risk of Alzheimer's Disease: Acceleration of Atrophy in the Volumes and Shapes of the Hippocampus and Amygdala. *J Alzheimers Dis*. 2021;80(3):1311-1327. doi:10.3233/JAD-201162

Avila J, Perry G. A Multilevel View of the Development of Alzheimer's Disease. *Neuroscience*. 2021;457:283-293. doi:10.1016/j.neuroscience.2020.11.015

Bakulski KM, Seo YA, Hickman RC, et al. Heavy Metals Exposure and Alzheimer's Disease and Related Dementias. *J Alzheimers Dis*. 2020;76(4):1215-1242. doi:10.3233/JAD-200282

Bell SM, Barnes K, De Marco M, Shaw PJ, Ferraiuolo L, Blackburn DJ, Venneri A, Mortiboys H. Mitochondrial Dysfunction in Alzheimer’s Disease: A Biomarker of the Future? *Biomedicines* 2021, 9, 63

Bello-Corral L, Sánchez-Valdeón L, Casado-Verdejo I, Seco-Calvo JÁ, Antonio Fernández-Fernández J, Nélida Fernández-Martínez M. The Influence of Nutrition in Alzheimer's Disease: Neuroinflammation and the Microbiome vs. Transmissible Prion. *Front Neurosci*. 2021;15:677777. doi:10.3389/fnins.2021.677777

Bodryzlova Y, Kim A, Michaud X, André C, Bélanger E, Moullec G. Social class and the risk of dementia: A systematic review and meta-analysis of the prospective longitudinal studies. *Scand J Public Health*. 2022;14034948221110019.

Fagiani F, Lanni C, Racchi M, Govoni S. (Dys)regulation of Synaptic Activity and Neurotransmitter Release by β-Amyloid: A Look Beyond Alzheimer's Disease Pathogenesis. *Front Mol Neurosci*. 2021;14:635880. doi:10.3389/fnmol.2021.635880

Fanutza T, Del Prete D, Ford MJ, Castillo PE, D'Adamio L. APP and APLP2 interact with the synaptic release machinery and facilitate transmitter release at hippocampal synapses. *Elife*. 2015;4:e09743. doi:10.7554/eLife.09743

Guidi J, Lucente M, Sonino N, Fava GA. Allostatic Load and Its Impact on Health: A Systematic Review. *Psychother Psychosom*. 2021;90(1):11-27. doi:10.1159/000510696

Hu CJ, Octave JN. Editorial: Risk Factors and Outcome Predicating Biomarker of Neurodegenerative Diseases. *Front Neurol*. 2019;10:45. doi:10.3389/fneur.2019.00045

Hussain B, Fang C, Chang J. Blood-Brain Barrier Breakdown: An Emerging Biomarker of Cognitive Impairment in Normal Aging and Dementia. *Front Neurosci*. 2021;15:688090. doi:10.3389/fnins.2021.688090

Ionescu-Tucker A, Cotman CW. Emerging roles of oxidative stress in brain aging and Alzheimer's disease. *Neurobiol Aging*. 2021;107:86-95. doi:10.1016/j.neurobiolaging.2021.07.014

Islam F, Shohag S, Akhter S, et al. Exposure of metal toxicity in Alzheimer's disease: An extensive review. *Front Pharmacol*. 2022;13:903099. doi:10.3389/fphar.2022.903099

Itzhaki RF. Overwhelming Evidence for a Major Role for Herpes Simplex Virus Type 1 (HSV1) in Alzheimer's Disease (AD); Underwhelming Evidence against. *Vaccines (Basel)*. 2021;9(6):679. doi:10.3390/vaccines9060679

Jellinger KA. Basic mechanisms of neurodegeneration: a critical update. *J Cell Mol Med*. 2010;14(3):457-487. doi:10.1111/j.1582-4934.2010.01010.x

Jung ES, Suh K, Han J, Kim H, Kang HS, Choi WS, Mook-Jung I. Amyloid-β activates NLRP3 inflammasomes by affecting microglial immunometabolism through the Syk-AMPK pathway. *Aging Cell*. 2022, 21, e13623

Lanni C, Masi M, Racchi M, Govoni S. Cancer and Alzheimer's disease inverse relationship: an age-associated diverging derailment of shared pathways. *Mol Psychiatry*. 2021;26(1):280-295. doi:10.1038/s41380-020-0760-2

Ma C, Zhang W, Cao M. Role of the Peripheral Nervous System in PD Pathology, Diagnosis, and Treatment. *Front Neurosci*. 2021;15:598457. doi:10.3389/fnins.2021.598457

Mielke MM, Aggarwal NT, Vila-Castelar C, et al. Consideration of sex and gender in Alzheimer's disease and related disorders from a global perspective. *Alzheimers Dement*. 2022;10.1002/alz.12662. doi:10.1002/alz.12662

Modgil S, Lahiri DK, Sharma VL, Anand A. Role of early life exposure and environment on neurodegeneration: implications on brain disorders. *Transl Neurodegener*. 2014;3:9. doi:10.1186/2047-9158-3-9

Patrick KL, Bell SL, Weindel CG, Watson RO. Exploring the "Multiple-Hit Hypothesis" of Neurodegenerative Disease: Bacterial Infection Comes Up to Bat. *Front Cell*

Infect Microbiol. 2019;9:138. doi:10.3389/fcimb.2019.00138

Plummer S, Van den Heuvel C, Thornton E, Corrigan F, Cappai R. The Neuroprotective Properties of the Amyloid Pre-cursor Protein Following Traumatic Brain Injury. *Aging Dis*. 2016, 7, 163–179

Ramos-Cejudo J, Wisniewski T, Marmar C, et al. Traumatic Brain Injury and Alzheimer's Disease: The Cerebrovascular Link. *EBioMedicine*. 2018;28:21-30. doi:10.1016/j.ebiom.2018.01.021

Rosas-Hernandez H, Cuevas E, Raymick JB, Robinson BL, Sarkar S. Impaired Amyloid Beta Clearance and Brain Microvascular Dysfunction are Present in the Tg-SwDI Mouse Model of Alzheimer's Disease. *Neuroscience*. 2020;440:48-55. doi:10.1016/j.neuroscience.2020.05.024

Ryu WI, Bormann MK, Shen M, Kim D, Forester B, Park Y, So J, Seo H, Sonntag KC, Cohen BM. Brain cells derived from Alzheimer's disease patients have multiple specific innate abnormalities in energy metabolism. *Mol. Psychiatry* 2021, 26, 5702–5714

Sciaccaluga M, Megaro A, Bellomo G, et al. An Unbalanced Synaptic Transmission: Cause or Consequence of the Amyloid Oligomers Neurotoxicity?. *Int J Mol Sci*. 2021;22(11):5991. doi:10.3390/ijms22115991

Sikora E, Bielak-Zmijewska A, Dudkowska M, et al. Cellular Senescence in Brain Aging. *Front Aging Neurosci*. 2021;13:646924. doi:10.3389/fnagi.2021.646924

Soscia SJ, Kirby JE, Washicosky KJ, et al. The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide. *PLoS One*. 2010;5(3):e9505. doi:10.1371/journal.pone.0009505

Taipa R, Pinho J, Melo-Pires M. Clinico-pathological correlations of the most common neurodegenerative dementias. *Front Neurol*. 2012;3:68. doi:10.3389/fneur.2012.00068

Tarasoff-Conway JM, Carare RO, Osorio RS, et al. Clearance systems in the brain-implications for Alzheimer disease [published correction appears in Nat Rev Neurol. 2016 Apr;12(4):248]. *Nat Rev Neurol*. 2015;11(8):457-470. doi:10.1038/nrneurol.2015.119

Valori CF, Possenti A, Brambilla L, Rossi D. Challenges and Opportunities of Targeting Astrocytes to Halt Neurodegenerative Disorders. *Cells*. 2021; 10(8):2019. https://doi.org/10.3390/cells10082019

Vojtechova I, Machacek T, Kristofikova Z, Stuchlik A, Petrasek T. Infectious origin of Alzheimer's disease: Amyloid beta as a component of brain antimicrobial immunity. *PLoS Pathog*. 2022;18(11):e1010929. doi:10.1371/journal.ppat.1010929

Wang H, Yang F, Zhang S, Xin R, Sun Y. Genetic and environmental factors in Alzheimer's and Parkinson's diseases and promising therapeutic intervention via fecal microbiota transplantation. *NPJ Parkinsons Dis*. 2021;7(1):70. doi:10.1038/s41531-021-00213-7

Wareham LK, Liddelow SA, Temple S, et al. Solving neurodegeneration: common mechanisms and strategies for new treatments. *Mol Neurodegener*. 2022;17(1):23. doi:10.1186/s13024-022-00524-0

Xiao X, Liu H, Liu X, Zhang W, Zhang S, Jiao B. *APP*, *PSEN1*, and *PSEN2* Variants in Alzheimer's Disease: Systematic Re-evaluation According to ACMG Guidelines. *Front Aging Neurosci*. 2021;13:695808. doi:10.3389/fnagi.2021.695808

Zang X, Chen S, Zhu J, Ma J, Zhai Y. The Emerging Role of Central and Peripheral Immune Systems in Neurodegenerative Diseases. *Front Aging Neurosci*. 2022;14:872134. doi:10.3389/fnagi.2022.872134

Zhu X, Lee HG, Perry G, Smith MA. Alzheimer disease, the two-hit hypothesis: an update. *Biochim Biophys Acta*. 2007;1772(4):494-502. doi:10.1016/j.bbadis.2006.10.014

List of published articles

Schreiner TG, Menéndez-González M, Popescu BO. The "Cerebrospinal Fluid Sink Therapeutic Strategy" in Alzheimer's Disease—From Theory to Design of Applied Systems. *Biomedicines* 2022, 10, 1509. https://doi.org/10.3390/biomedicines10071509 (Chapter 5, pages 38-43)

Schreiner TG, Popescu BO. Amyloid Beta Dynamics in Biological Fluids—Therapeutic Impact. *J. Clin. Med*. 2021, 10, 5986. https://doi.org/10.3390/jcm10245986 (Chapter 2, Subchapter 2.1. and Subchapter 2.4. pages 18-22 și 31-34)

Schreiner TG, Schreiner OD, Adam M, Popescu BO. The Roles of the Amyloid Beta Monomers in Physiological and Pathological Conditions. *Biomedicines* 2023, 11, 1411. https://doi.org/10.3390/biomedicines11051411 (Chapter 2, Subchapter 2.2 and Subchapter 2.3, pages 23-30)

Schreiner TG, Tamba BI, Mihai CT, Lorinczi A, Baibarac M, Ciobanu RC, Popescu BO. Nanoporous Membranes for the Filtration of Proteins from Biological Fluids: Biocompatibility Tests on Cell Cultures and Suggested Applications for the Treatment of Alzheimer's Disease. *J. Clin. Med*. 2022, 11, 5846. https://doi.org/10.3390/jcm11195846 (Chapter 6, pages 43-66)