UNIVERSITY OF LATVIA

FACULTY OF MEDICINE AND LIFE SCIENCES

INVESTIGATION OF A NOVEL ACTIVE IMMUNIZATION APPROACH IN A TRANSGENIC MOUSE MODEL OF ALZHEIMER´S DISEASE

DIPLOMA THESIS

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RĪGA 2025

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ABSTRACT

Title: Investigation of a novel active immunization approach in a transgenic mouse model of Alzheimer´s disease

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Background: Alzheimer's disease (AD) is the predominant type of age-related dementia, affecting million individuals globally. Around 5-10% of AD cases exhibit autosomal-dominant inheritance, leading to familial Alzheimer's disease (FAD). Conventional therapies slow down disease progression without reversing pre-existing neurodegeneration. Novel therapeutic strategies are being developed to target Alzheimer's disease-specific pathological formations via active immunization to amyloid β plaques (Aβ) in early disease stages.

Objective: In this study, a virus-like particle (VLP) bacteriophage platform was used to obtain a vaccine candidate and test it as an active immunization approach against AD-related pathological alterations in a female 5xFAD mouse model.

Material and Methods: Behavioral tests were conducted in six- and eight-month-old female 5xFAD mice to evaluate motor and cognitive impairments. Using immunofluorescence, the effects of vaccination on Aβ numbers, the density of activated astroglial cells, and the number of ionized calcium binding adaptor molecule 1 (Iba-1)-positive cells were determined in the neocortex (NCTX) and dentate gyrus (DG) of the mice.

Results: VLP-based immunization significantly reduced the Aβ plaque count and the astroglial density in the NCTX of female 5xFAD mice but did not alter Iba-1-positive cell number in either the NCTX or the DG. No cognitive and motor improvements were observed in the treatment group.

Conclusion: VLP-based vaccine candidate only provided modest preclinical efficacy. Further research of VLP-based vaccine displaying Aβ or tau epitopes in both sexes of 5xFAD mice is required to investigate the full treatment potential of this approach.

Keywords: Alzheimer´s disease, Virus-like particle vaccine, Active immunization, Amyloid β, 5xFAD mouse model, Behavioral tests, Immunofluorescence

ANOTĀCIJA

Nosaukums: Jaunās aktīvās imunizācijas pieejas pārbaude transgēnajā Alcheimera slimības peļu modelī

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Ievads: Alcheimera slimība (AD) ir visizplatītākais ar vecumu saistītās demences veids, kas skar miljoniem cilvēku visā pasaulē. Apmēram 5–10% AS gadījumu ir autosomāli dominanti pārmantoti, izraisot ģimenes Alcheimera slimību (FAD). Tradicionālās terapijas spēj palēnināt slimības progresēšanu, bet nespēj atjaunot jau esošo neirodeģenerāciju. Jaunas terapeitiskās imunizācijas stratēģijas mērķis ir vērsties pret specifiskām AS intracerebrālām patoloģiskām formācijām, lai potenciāli uzlabotu kognitīvās funkcijas agrīnās slimības stadijās.

Mērķis: Šajā pētījumā vīrusam līdzīga daļiņa (VLP) – bakteriofāga platforma tika izmantota, lai iegūtu vakcīnas kandidātu un pārbaudītu to kā aktīvas imunizācijas pieeju pret ar Alcheimera slimību (AD) saistītām patoloģiskām izmaiņām sieviešu dzimuma 5xFAD peļu modelī.

Materiāli un metodes: Uzvedības testi tika veikti sešus un astoņus mēnešus vecām 5xFAD mātītēm, lai novērtētu motoros un kognitīvos traucējumus. Izmantojot imūnfluorescenci, tika noteikta vakcinācijas ietekme uz Aβ daudzumu, aktivēto astrogliju šūnu blīvumu un ar jonu saistītā kalcija adaptera molekulas 1 (Iba-1) pozitīvo šūnu skaitu peles neokorteksā (NCTX) un dantatā girā (DG).

Rezultāti: VLP balstītā imunizācija būtiski samazināja Aβ plāksnīšu skaitu un astrogliālo blīvumu sieviešu dzimuma 5xFAD pelēm NCTX, taču neizraisīja izmaiņas Iba-1 pozitīvo šūnu skaitā ne NCTX, ne DG. Ārstēšanas grupā netika novēroti uzlabojumi ne kognitīvajās, ne motoriskajās funkcijās.

Secinjumi: Uz VLP balstītais vakcīnas kandidāts sniedza tikai mērenu preklīnisko efektivitāti. Lai pilnībā izpētītu šīs pieejas ārstniecisko potenciālu, nepieciešami turpmāki pētījumi ar uz Aβ vai tau epitopiem balstītu VLP vakcīnu abiem dzimumiem 5xFAD peļu modelī.

Atslēgvārdi: Alcheimera slimība, Vīrusam līdzīga daļiņu vakcīna, Aktīvā imunizācija, Amiloīds-β, 5xFAD peļu modelis, Uzvedības testi, Imūnfluorescence

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholine esterase
AChEi	Acetylcholine esterase inhibitors
AD	Alzheimer´s disease
ADRD	Alzheimer´s disease related dementia
ANOVA	Analysis of variance
ARD	Age-related dementia
ATP	Adenosine triphosphate
APO	Apolipoprotein
APP	Amyloid precursor protein
APP	Amyloid precursor protein gene
Aβ	Amyloid beta
BACE	Beta-amyloid converting enzyme
CA	Cornu ammonis
ChAT	Choline-acetyltransferase
CMR	Cerebral metabolic rate
CNS	Central nervous system
CSF	Cerebrospinal fluid
DG	Dentate gyrus
DM	Diabetes mellitus
DMT	Disease-modifying therapy
EMA	European Medicines Agency
EOAD	Early-onset Alzheimer´s disease
EZM	Elevated Zero Maze test
FAD	Familial Alzheimer´s disease
FDA	U.S. Food and Drug Administration
GDS	Global Deterioration Scale
GFAP	Glial fibrillary acidic protein
GLUT	Glucose transporter
Iba-1	Ionized calcium binding adaptor molecule 1
ICD-10	10th revision of the International Statistical Classification of Diseases and Related Health Problems
IGF	Insulin-like growth factor
IgG	Immunoglobulin G

LOAD	Late-onset Alzheimer´s disease
MAB	Monoclonal antibody
MCI	Mild cognitive impairment
MRI	Magnetic resonance tomography
Ms	Mouse
NCTX	Neocortex
NFT	Neurofibrillary tangle
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
OD	Optical density
OF	Open Field test
PBS	Phosphate-buffered saline
PBST	1X PBS containing 0.3% Tween® 20
PET	Positron emission tomography
PFA	Paraformaldehyde
PS	Presenilin
PSEN	Presenilin gene
Rb	Rabbit
ROS	Reactive oxygen species
SAD	Sporadic Alzheimer´s disease
TNF	Tumor necrosis factor
VLP	Virus-like particle
WHO	World Health Organization
WMS	Wechsler Memory Scale
YM	Y-Maze test
^{18}F-FDG	^{18}F-Fluorodeoxyglucose

INTRODUCTION

In 1906, the German physician Dr. Alois Alzheimer was the first one to describe the typical clinical picture of what was then an unknown disease, now known as Alzheimer´s disease (AD), in the case of his patient Auguste Deter. She experienced progressive memory loss and confusion and later passed away at the relatively early age of 56. *Postmortem*, Alzheimer examined her brain tissues and observed severe thinning of the cerebral cortical mass and accumulations of plaque formations and neurofibrillary tangles (NFTs) compared to healthy brain tissues**.** Four years later, the renowned psychiatrist and Alzheimer's superior, Dr. Emil Kraepelin, included his findings in the academically important textbook for students and physicians, referring to the disease as Alzheimer´s disease (Schuchart, 2017).

Over a century ago, AD was considered a rarely occurring pathology. However, due to gradually ongoing demographic changes and increased life expectancy, it became clear that AD is a widespread, epidemic-like disease affecting older individuals worldwide (Polis and Samson, 2019). With 60–70% of dementia cases, AD is the most common form of age-related dementia (ARD) (Carrillo, Thies and Bain, 2012). It is a chronically progressing, irreversible neurodegenerative disorder causing gradual cognitive decline and memory loss (Polis and Samson, 2019). Dementia ranks as the seventh leading cause of mortality among the elderly worldwide (Greenblat, 2025).

Currently, over 55 million individuals are diagnosed with dementia, with around 10 million new cases occurring each year (Greenblat, 2025). It causes a considerable strain on patients, their families, and healthcare systems, necessitating extensive medical and financial resources. In 2019, the worldwide economic load of Alzheimer's disease and related dementias (ADRDs) was estimated at around 2.8 trillion USD, with projections indicating an increase due to population growth and enhanced life expectancy (Nandi *et al.*, 2022). The increasing incidence of AD cases and their related expenses underscore the pressing necessity for additional research into treatment and prevention methodologies.

To date, the primary treatment strategy for AD involves the administration of antidementia medications, such as acetylcholinesterase (AChE) inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists, which target specific metabolic pathways associated with the disease's principal pathologies. These medications are used to slow down the disease progression; however, they cannot reverse pre-existing structural alterations in the cerebral tissues of individuals.

A novel strategy for AD treatment involves the utilization of active immunization through Virus-like particle (VLP) vaccines as targeted therapy against pre-existing pathological

formations in cerebral tissues, such as amyloid β (Aβ) plaques. Until now, only a limited number of studies have been conducted on this topic, yet all have demonstrated efficacy and promising results.

This work focused on active VLP vaccination targeting brain Aβ plaques in a mouse model of familial AD (FAD).

FAD represents a rare, early-onset form of AD, providing a well-predictable genetic background, making it particularly suitable for preclinical research on this topic. Autosomal dominant gene mutations, leading to an increased production and accumulation of Aβ are causing FAD formation (Karve *et al.*, 2012; Kumar, Kim and Bishayee, 2022).

Hypothesis of this study: H_1: Active VLP-immunization can reduce the amount of cerebral Aβ plaques and prevent gliosis in the brains of female 5xFAD mice. **H_2:** Active immunization with a VLP based vaccine can reduce AD-associated behavioral impairments.

Aim of this study: To determine the effects of VLP-based immunization on behavior and disease-characteristic pathological cerebral changes in a 5xFAD mouse model.

Objectives of this study:

- Performing behavioral tests in 5xFAD mice to determine changes in working memory (Y-Maze test), general locomotor activity (Open Field test), anxiety-related behaviors (Open Field and Elevated Zero Maze test)
- Assessing the Aβ-plaque burden and the amount of astro- and microgliosis in the neocortex and hippocampus of 5xFAD mice with immunofluorescence
- Obtain behavioral and biochemical data, perform statistical analysis and interpretation of obtained results

I. LITERATURE REVIEW

I.1. Alzheimer´s disease

AD is the most common type of ARD worldwide. The term dementia does not describe a certain disease but rather characterizes a syndrome that can be caused by several progressive or chronic cerebral diseases (Greenblat, 2025). The syndrome results in deficits of several higher brain functions as disturbances in memory, orientation, language capabilities, learning, general thinking processes, decision-making, comprehension, calculations, and orientation in time, space, and person (WHO, 2016). Dementia syndromes are often seen in individuals over the age of 65, which are not considered to be part of the normal aging process. Major risk factors for developing any kind of dementia include aging, which resembles the greatest known risk factor, family history, race and ethnicity, poor general health condition, as well as traumatic head and brain injuries (Omura *et al.*, 2022). Starting from age 60, an individual's risk of developing dementia approximately doubles every five years (Klimaschewski, 2021).

Predominantly marked by the shrinkage of hippocampal tissues and cerebral cortex, along with a simultaneous buildup of Aβ plaques and NFTs, AD is a chronic, progressive, and irreversible neurodegenerative disease (Sheppard and Coleman, 2020). In 2010, Desikan discovered significant correlations between neocortical area thickness and hippocampal volume in the early stages of AD (Desikan *et al.*, 2010).

An examination of the 2019 edition of the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) by the World Health Organization (WHO) indicates that AD can be categorized into different types based on disease onset and progression (WHO, 2016). The ICD-10 code includes early-onset (EOAD): G30.0 and late-onset AD (LOAD): G30.1. EAOD occurs when the disease affects individuals below the age of 65 years, comprising approximately 5% of all AD cases (Sheppard and Coleman, 2020). These patients often have a significant genetic predisposition as well as a higher polygenic risk but typically present fewer numbers of comorbidities compared to LOAD patients (Mendez, 2019). LOAD refers to patients who develop AD symptoms after the age of 65, accounting for around 95% of all AD cases (Rabinovici, 2019; Sheppard and Coleman, 2020).The majority of EOAD cases are linked to genetic mutations known as FAD (Sheppard and Coleman, 2020). In contrast, LOAD typically lacks direct hereditary risk factors and is also referred to as sporadic AD (SAD) (Peters, Connor and Meadowcroft, 2015; Sheppard and Coleman, 2020).

I.1.1. Etiology

The etiology of AD is multifactorial, including several variables such as the genetic background, environmental, and lifestyle factors. Genetically, AD can be divided into two major forms: FAD and SAD. This thesis exclusively focuses on FAD, hence, additional elaboration on SAD will not occur. While SAD is more prevalent, FAD constitutes around 5% of all AD cases and is attributed to autosomal dominant Mendelian inheritance resulting from mutations in genes such as presenilin (*PSEN*)-1 and -2, or amyloid precursor protein (*APP*) (Karve et al., 2012; Sheppard and Coleman, 2020; Kumar, Kim and Bishayee, 2022). Mutations within these mentioned genes are causing alterations in the proteolytic processing of APP (Kumar, Kim and Bishayee, 2022). In patients with FAD, around 10-15% can be attributed to variations in *PSEN1*, *PSEN2,* and *APP (National Institute on Aging, 2023)*

Numerous additional risk factors promote AD development. The most important ones to mention are increasing age, together with other demographic factors like gender, ethnicity, and social status (Armstrong, 2019). Acquired risk factors include cerebrovascular diseases, arterial hypertension, Diabetes mellitus (DM) type 2, obesity, dyslipidemia, tobacco smoking, as well as marital status, level of stress, depression, and inadequate sleep (Silva *et al.*, 2019). According to Barnes and Yaffe, potentially modifiable risk factors include low educational level, tobacco smoking, physical inactivity, depression, mid-life arterial hypertension, Diabetes mellitus, and mid-life obesity (Barnes and Yaffe, 2011).

I.1.2. Epidemiology

An estimated 416 million individuals globally are impacted by the Alzheimer's disease continuum, constituting 22% of those aged 50 and above (Gustavsson *et al.*, 2023). From 57.4 million in 2019, the incidence of dementia is estimated to increase up to 152.8 million by 2050 (Nichols *et al.*, 2022).

In 2021, ADRDs were ranked the seventh leading cause of death globally. Of the deaths caused by ADRDs, 68% affected female individuals. ADRDs were found to be the second-leading cause of death in high-income countries and the eighth leading cause of death worldwide in upper-middle-income countries. In the year 2021, these diseases were responsible for the deaths of 1.8 million people (WHO, 2024).

1.2.1. Age-related prevalence

The number of AD dementia patients is increasing exponentially, starting from the age of 60 years. As seen in *Fig. 1.2.1.1*, in Europe, around 0.6% of all individuals are suffering from AD dementia in the age group between 60-64 years. Within the age group of 80-84 years, already

12.1% of the European population are affected. Among the population aged 90 years and older, 40.8% of people are affected in total (Alzheimer Europe, 2019).

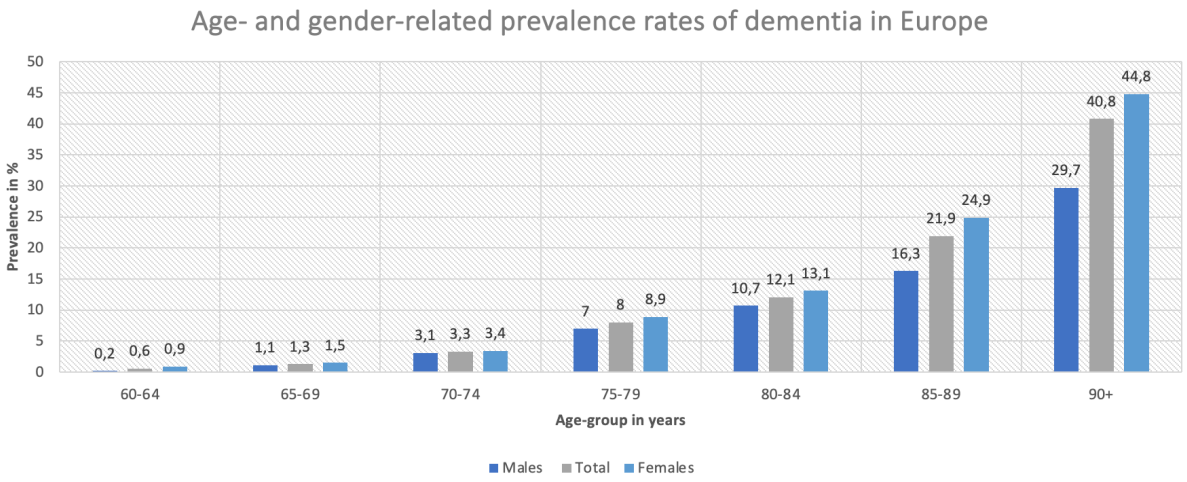

Figure 1.2.1.1: **Age- and gender-related prevalence rates of dementia in Alzheimer´s disease in Europe.** Adapted and modified form (Alzheimer Europe, 2019).

1.2.2. Gender-related prevalence

AD prevalence generally increases with age and is higher in females, especially in Europe and North America (Gustavsson *et al.*, 2023). In 2019, the female-to-male ratio was expected to be 1.69 and will likely maintain a similar trend up to the year 2050. This trend is expected to persist until 2025. This may be attributed to the generally higher life expectancy of females (Nichols *et al.*, 2022). As displayed in *Fig. 1.2.1.1*, in the age group of 60-64 years, males have a prevalence of 0.2% compared to 0.9% in females within the same age group. The prevalence increases to 44.8% in females and only 29.7% in males among the age group of 90 years and older (Alzheimer Europe, 2019).

I.1.3. Pathogenesis

AD is characterized by cerebral atrophy, synaptic loss, cortical shrinkage, as well as the accumulation of senile Aβ plaques and NFTs (Sheppard and Coleman, 2020).

AD is a complex condition arising from various interrelated pathogenic mechanisms. These processes include oxidative damage, endothelial dysfunction, traumatic brain injuries, inflammatory processes, insulin resistance, or other events causing neuroinflammation (Polis and Samson, 2019).

1.3.1. Neuroanatomy

Alzheimer's disease is generally responsible for degeneration in cortical brain areas. The limbic system and the multimodal association cortices are the most impacted structures. Typically, a macroscopic reduction in cerebral mass and weight can be observed. It manifests as expanded sulcal gaps, diminished size of the precuneus and posterior cingulate gyrus, and enlargement of the frontal and temporal horns of the lateral ventricles. However, these changes are fairly nonspecific for Alzheimer's disease. They may also be observed in healthy persons experiencing modest cortical atrophy. Atrophy in the medial temporal lobe regions, impacting the amygdala and hippocampus, along with growth of the temporal horns, are distinctive indicators of this neurodegenerative disorder (DeTure and Dickson, 2019). The limbic system is made up of cortical and subcortical elements. It consists of the limbic cortex, which includes the cingulate gyrus and the parahippocampal gyrus, the hippocampal formations comprising the dentate gyrus (DG), the hippocampus proper, and the subiculum, as well as the amygdala, the septal area, and the hypothalamus. These particular alterations in the cerebral tissues result in significantly enlarged ventricular formations within the brain of an affected individual. *Fig. 1.3.1.1* illustrates selected parts of the previously mentioned neuroanatomical structures.

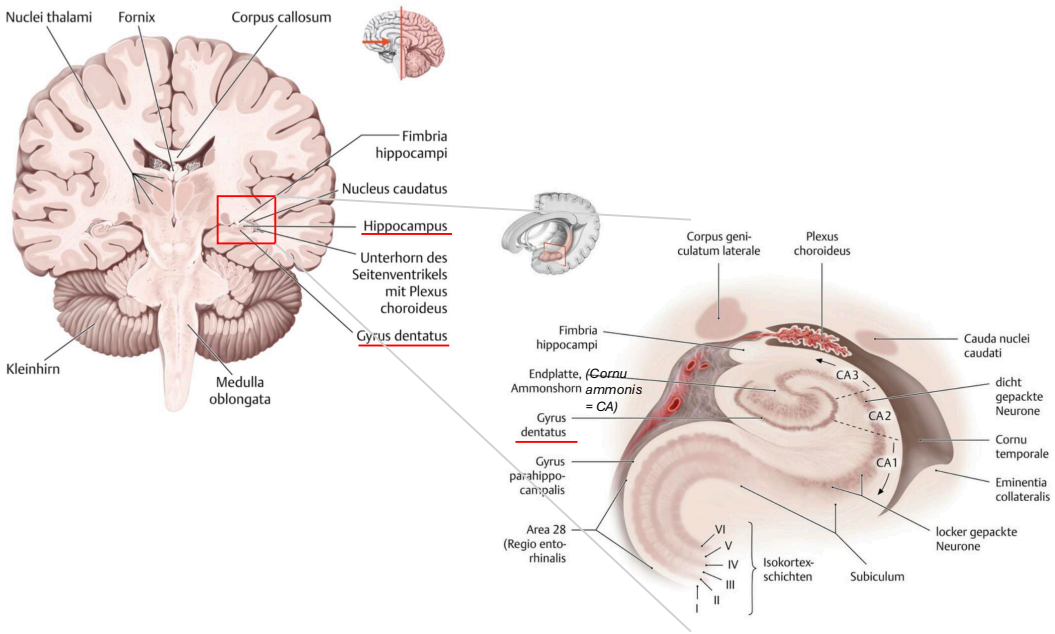

Figure 1.3.1.1: **Frontal section of the human brain (left) and cytoarchitecture of the hippocampal formation (right).** Adapted and modified from (Schünke, Schulte and Schumacher, 2018a, 2018b).

The structures are interconnected by the Papez circuit. The limbic system is accountable for numerous functions. It enhances the capacity to recognize, assess, process, and retain various emotions. It also contributes to the regulation of motor and autonomic activities. It largely

engages in memory formation, learning processes, and the regulation of primitive urges such as hunger and sexual behavior. The hippocampal structures control autonomic functioning, cognition, attention, emotional processing, spatial memory creation, and information transmission from short-term to long-term memory. It mostly involves the retention of information and occurrences, particularly orientation. This information will be retained in declarative memory. The amygdala regulates emotional reactions such as anxiety, fear conditioning, emotional memory, and social cognition. Finally, the hypothalamic hormones regulate the autonomic nerve system, influencing many vegetative functions related to eating, sleeping, and sexual behaviors. AD significantly affects the hippocampus, DG, and basal ganglia within the limbic system, leading to the characteristic symptoms of dementia (Rajmohan and Mohandas, 2007).

1.3.2. Amyloid pathology

The presence of Aβ aggregations within cerebral tissues is one of the key pathological hallmarks in AD patients. Already in 1906, Dr. Alois Alzheimer first described the typical findings of Aβ plaque deposition in the brain tissues of Auguste Deter. These senile plaques are spherical microscopical lesions consisting of an extracellular Aβ peptide core structure surrounded by enlarged degenerated axonal endings (Kumar, Kim and Bishayee, 2022). Those peptides, comprising chains of 39-43 amino acids in length, develop from flawed proteolysis of APP (Klimaschewski, 2021). These amino acid chains may form several new formations, so-called protein fibrils, reaching lengths from up to 10nm. Together with apolipoprotein (APO) ε4, proteoglycans, and α1-antichymotripsin, senile plaques can form, ranging from ten to several hundred micrometers in diameter. Those plaques exhibit distorted dendrites and neuronal axons containing abnormal intracellular neurofibrils (Silbernagl and Lang, 2020).

APP is as type 1 transmembrane glycoprotein encoded by a single gene located on the 21st chromosome (Pfundstein, Nikonenko and Sytnyk, 2022). APP is usually cleaved by the proteolytic enzymes (proteases) named α-, β-, and γ-secretase. The proteolytic cleavage of APP can follow either an amyloidogenic or non-amyloidogenic pathway, as demonstrated in *Fig. 1.3.2.1* (Schu *et al.*, 2012). Since β-secretase was identified as being responsible for Aβ peptide formation, it is also known as beta-amyloid converting enzyme (BACE) 1 (Decourt *et al.*, 2022) γ-secretase contains presenilin (PS) 1 or PS-2 as its catalytic subunit (Klimaschewski, 2021).

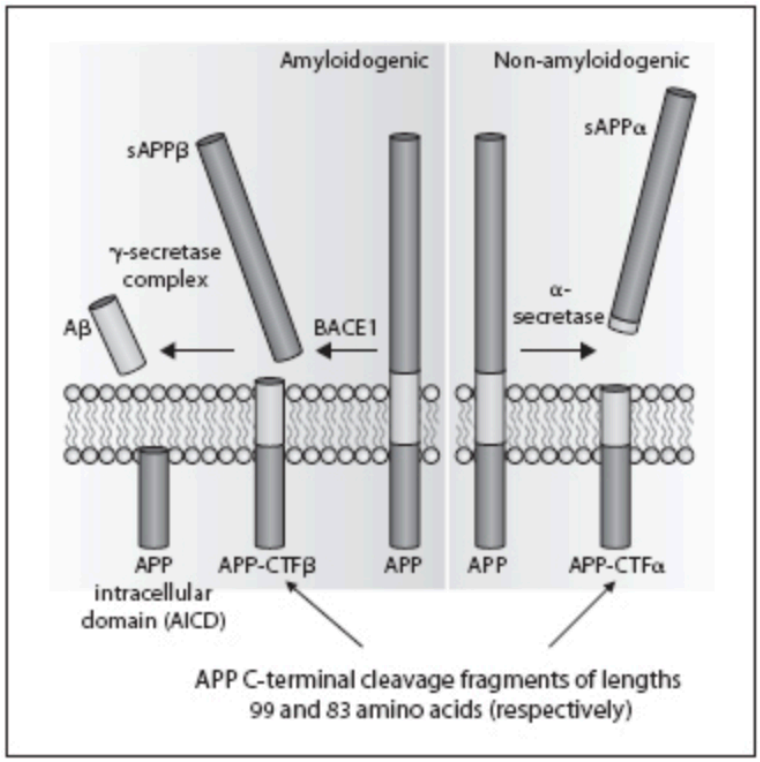

Figure 1.3.2.1: **Amyloid precursor protein degradation**. Aβ = amyloid β, APP = amyloid precursor protein, APP-CTF= C-terminal fragments of the amyloid precursor protein, BACE1 = beta-amyloid converting enzyme 1, sAPPβ = soluble amyloid precursor protein β. Adapted form (Schu *et al.*, 2012).

In most cases, APP is cleaved by either α- or β-secretase, creating non-toxic protein fragments. In the amyloidogenic pathway, sequential cleavage of APP by β- and later γ-secretase causes neurotoxic Aβ particles to develop and to accumulate (Kumar, Kim and Bishayee, 2022). Due to cleavage of APP by BACE (Alzheimer Europe, 2019) soluble APPβ peptide forms, which are later released by γ-secretase from APP transmembranous fragments. As a result of FAD missense mutations in *PSEN1* and *PSEN2* genes, the APP cleavage products are changed in their composition, leading to the accumulation of longer, non-soluble Aβ dimers or oligomers, creating primary neurotoxic formations within the extracellular spaces. The Aβ peptides, containing 40 and 42 amino acids each, play an important role in AD pathophysiology. Both are generally soluble substances but possess the tendency to aggregate and form insoluble complexes, which can interact with other peptides like amyloid aeta. The Aβ42 isoform is most commonly found in senile plaques, while Aβ40, constituting 60% of soluble peptides within the cerebrospinal fluid (CSF), is the most common isoform in the fluid (Klimaschewski, 2021). Some amyloid isoforms possess a greater potential for conformational changes than others. Generally, longer and more insoluble molecules are more likely to undergo these changes (Haass and Selkoe, 2022). The term “amyloidogenic propensity” describes the tendency of

amyloid to form potentially neurotoxic aggregations. Aβ42 was found to have a higher amyloidogenic propensity than Aβ40 (Hampel *et al.*, 2021; Alraawi *et al.*, 2022). These abnormally shaped Aβ oligomers are likely to induce structural changes in tau proteins, causing cellular stress responses by triggering downstream cascades, including phosphorylation of protein tau, aggregation of caspases, cofilin, and other substances. This results in general disturbances of mitochondrial function, synaptic structure, and plasticity, ultimately leading to progressive cognitive decline (Tu *et al.*, 2014).

1.3.3. Tau pathology

The chronically occurring pathological intraneuronal buildup of hyperphosphorylated tau protein additionally characterizes AD as a progressive neurodegenerative disease. Due to this characteristic, AD can also be classified as tauopathy (Zhang *et al.*, 2022).

Located inside the neuronal cytoplasms and axons, tau is a microtubule-associated protein. The microtubule-associated protein tau gene, which is located on the 17th chromosome, encodes it. The physiological role of tau involves binding tubulin, leading to polymerization and stabilization of microtubules (Zhang *et al.*, 2022).

Generally, six isoforms of the protein exist, being generated by alternative splicing processes of the number of exons two, three, and ten. Each isoform has different properties regarding how tightly the protein binds to microtubules, and the tendency is to form aggregations. In AD, tau protein becomes hyperphosphorylated before NFT formation occurs (Sheppard and Coleman, 2020). According to Klimaschewski, this hyperphosphorylation can result from the activity of enzymes such as glycogen-synthase-kinase, protein kinase (PK)-C, and PK-N1, from the reduced activity of protein-phosphatase 2A, which normally removes phosphate groups. Those events negatively affect neuronal metabolism (Klimaschewski, 2021). The accumulation of hyperphosphorylated tau protein is more strongly correlated with cognitive decline in AD patients than the amount of Aβ plaques within cerebral tissues (Walker, 2020; Boccalini *et al.*, 2024; Jarek *et al.*, 2024).

1.3.4. Mitochondrial dysfunction and oxidative stress hypothesis

1.3.4.1. Mitochondrial physiology and cerebral O_2 concentration in humans

The physiological purpose of mitochondria is to generate the high-energy molecule adenosine triphosphate (ATP) during cellular respiration. This metabolic process consists of glycolysis, oxidative decarboxylation, the Krebs cycle, and the respiratory chain, resulting in ATP production. In brief, ATP gets generated through oxidative phosphorylation of glucose, which

produces ATP, water, and carbon dioxide (Sadavaet al., 2011). The high-energy source ATP is essential for various metabolic processes throughout mammalian bodies, such as maintaining homeostasis, tissue growth, and movement facilitation. Impairments or dysfunction in any of these specific pathways can lead to the development of pathological processes causing cellular aging and stress.

The brain makes up around 20% of the total oxygen consumption in the human body, and with that making it the heaviest consumer. Despite this, the normal O_2 concentration in the adult brain is comparatively low, with a suggested cortical partial pressure of 20-25 mmHg, in contrast to other tissues and the atmospheric partial pressure of oxygen within the inhaled air ($_{Atm}PO_2$=159 mmHg) (Ortiz-Prado *et al.*, 2019). Under normal conditions, the oxygen saturation within the arterial blood is around SaO_2= 95-100% (Castro *et al.*, 2024). This level of oxygen saturation is balanced throughout different tissues of the body. The oxygen uptake differs between tissues based on their individual demands. The brain requires the largest amount of oxygen within the body. The uptake is comparatively higher, causing a comparatively low oxygen concentration in the outflowing venous blood. Generally, the oxygen saturation of the blood in the *V. jugularis* is between 55-75% (Lewis *et al.*, 1996; Chieregato *et al.*, 2003). The oxygen partial pressure within the brain depends on the individual cerebral metabolic rate (CMR), the local blood perfusion, and systemic exposure to hypoxia (Ortiz-Prado *et al.*, 2019).

As highly active aerobic cells, neurons are largely dependent on the ATP supply provided by mitochondria. Adequate oxygen supply is crucial to ensure sufficient mitochondrial energy generation. Due to the critical role of oxygen, the brain is quite sensitive to changes in oxygen concentration (Zhang, Zhu and Fan, 2011). In AD, it remains to be clarified whether neuronal atrophy is caused by increased oxidative stress or if it is mainly a result of the increased death of neuronal cells.

1.3.4.2. Mitochondrial dysfunction and reactive oxygen species

During the process of ATP generation by insufficiently working mitochondria, reactive oxygen species (ROS) are formed. According to the National Cancer Institute, ROS are defined as “a type of unstable molecule that contains oxygen and […] easily reacts with other molecules in a cell. A buildup of reactive oxygen species in cells may cause damage to DNA, RNA, and proteins and may cause cell death. Reactive oxygen species are free radicals.” (National Cancer Institute, 2024). The mitochondrial free radical theory of aging includes that ROS are the main causative agents, damaging nucleic acids, proteins, and lipids. As a result of ROS-induced damage in neuronal structures, signal transmission impairments might occur, leading to cognitive dysfunctions. Scientists agree that mitochondria-generated radicals are significantly

promoting aging processes and the formation of age-related diseases, including AD (Brand *et al.*, 2013). Healthy individuals are possessing several protective mechanisms to remove dysfunctional mitochondria, protect tissues from oxidative stress, and maintain a healthy cellular environment for normal cognitive functions and neurogenesis (Song *et al.*, 2024).

Mitochondrial health and neurogenesis are closely interconnected (Ozgen *et al.*, 2022; Bonzano *et al.*, 2024). During neurogenesis, new neurons form from neural stem cells, which become neural progenitor cells and develop further into fully mature nerve cells. Neurogenesis requires a substantial amount of energy. In individuals suffering from AD, both neurogenesis and protective mechanisms are impaired, leading to the gradual progression of the disease and decline of cognitive functions.

Mary and colleagues described the process of mitophagy and disease-specific alterations in AD due to the presence of APO-E, Aβ, and tau protein. The AD-specific substances influence mitochondrial processes and result in alterations in certain pathways compared to physiological processes.

1.3.5. Neuroinflammation hypothesis

Neuroinflammation is defined by the presence of gliosis, including the activation of astroglial and microglial cells, resulting in the synthesis and liberation of inflammatory mediators such as cytokines, chemokines, tumor necrosis factors (TNFs), small molecule messengers, nitric oxide, and ROS (Wang et al., 2023). Astrocytes, which represent a subtype of glial cells, are the predominant cell type in the human central nervous system (CNS), contributing to the maintenance of CNS health by performing various functions (Wei and Morrison, 2023).Generally, disrupted cross-communication between astrocytes and microglial cells may be critical mechanisms in the pathological development of various neurodegenerative diseases (Garland, Hartnell and Boche, 2022). In AD patients, these chronic inflammatory processes are resulting in an imbalance of physiological neuronal cell homeostasis, which exacerbates brain damage progression. The activation of glial cells results in the synthesis and secretion of inflammatory agents, which accelerate neuronal degeneration and decline of cognitive functions (Al-Ghraiybah *et al.*, 2022). Microglial cells are generally considered the main source of these inflammatory processes (Wang *et al.*, 2023). Physiologically, microglial cells are resident immune cells in the CNS and are part of the innate immunity, maintaining CNS homeostasis and neuroprotection by removing pathogens, defective neurons, and plaque formations. They regulate cerebral development via two different mechanisms: phagocytosis and the secretion of diffusible factors. Phagocytosis is regulated by substances like phosphatidylserine, the complement system, calreticulin, ATP, and sialic acid, which bind to

microglial cell receptors (Lenz and Nelson, 2018). Additionally, the immunological complement system, particularly factors C1q and C3b, are instrumental in these processes. Chronic activation of microglial cells results in higher amounts of C1-complexes being secreted, which can bind to synaptic membranes and induce complement cascades, leading to synaptic phagocytosis and neuronal cell death (Klimaschewski, 2021).

Microglial activation, proliferation, and resulting inflammatory reactions often occur in close proximity to senile plaques with high concentrations of tau protein (Lee *et al.*, 2021; Sobue, Komine and Yamanaka, 2023). As previously mentioned, increased concentration of ROS not only causes oxidative stress but also negatively impacts normal astrocytic function and promotes further Aβ generation. It is believed that cellular interactions between microglia and astrocytes are mediated by inflammatory cytokines like TNFs, interleukins, and parts of the complement system induced by Aβ exposure. Lee and colleagues state that astrocyte-derived Aβ induces neuroinflammatory processes, and Aβ-induced nuclear factor kappa-light-chain-enhancer of activated B cells could be quite significant in these inflammatory processes (Lee *et al.*, 2021).

1.3.6. Glucose hypometabolism hypothesis

1.3.6.1. Glucose uptake and metabolism within the human body

It is known that the uptake and consumption of glucose in healthy humans are highest in the brain, accounting for up to 25% of the total glucose utilization within the body (Kumar, Kim and Bishayee, 2022). Glucose is crucial for sustaining neuronal activity and growth, serving as the main source of energy for mammalian brains. The majority of glucose is metabolized for neuronal computation and information processing, including the generation of electric potentials and the synthesis of neurotransmitter precursors (Mergenthaler *et al.*, 2013).

After the ingestion and digestion of carbohydrates in food, absorbable monosaccharide molecules like glucose are taken up by the brushed-border enterocytes within the small intestine via secondary-active sodium-dependent glucose transporter 1. The glucose molecules then passively move into the blood of *V. portae* through facilitated diffusion via glucose transporter (GLUT) 2. Post-absorption, water-soluble glucose molecules are distributed throughout the body via the cardiovascular circulation (Silbernagl, Despopoulos and Draguhn, 2018). In the brain and erythrocytes, insulin-independent GLUT1 and GLUT3 transporters, which have an extraordinarily high affinity for glucose, facilitate glucose uptake since this substrate is extremely essential for these types of tissues (Horn, 2020). GLUT1 further facilitates glucose uptake into astrocytes, oligodendrocytes, and microglia, whereas GLUT3 mediates uptake into

neuronal cells. The brain´s glucose dependence is mainly due to the blood-brain barrier and its selective permeability.

1.3.6.2. Glucose uptake and consumption in AD brains

After conducting a study in 2008 on advanced AD and its association with the gene expression encoding insulin, insulin-like growth factor (IGF)-1 and -2, as well as other related components, de la Monte and Wands found severely reduced levels of insulin, IGF-1, and corresponding receptors within cerebral tissues of AD patients in the absence of type 1 or type 2 DM. Since these AD-associated abnormalities in the brain mimic the effects of known forms of DM, they suggested the term "type 3 diabetes" (De La Monte and Wands, 2008). In 2016, Grieb proposed the theory of glucose hypometabolism in AD patients. It is not fully understood whether the disturbance of glucose metabolism in the cerebral tissues of AD patients is the primary cause of disease formation or a secondary side effect caused by various other pathophysiological mechanisms related to AD disease development and progression. In his "cerebral glucodepriviation hypothesis", Grieb suggests another perspective on AD pathogenesis: an unknown primary cause initiates decreased CMR and glucose consumption, which induces neurodegenerative processes and eventually results in amyloido- and tauopathies (Grieb, 2016).

1.3.7. Cholinergic hypothesis

Not only do cognitive functions progressively decrease in age-related neurodegenerative diseases like AD, but cholinergic atrophy is also a typical feature (Chen *et al.*, 2022). Within the CNS and the peripheral nervous system, all pre- and post-ganglionic parasympathetic neurons and some post-ganglionic neurons of the sympathetic nervous system use acetylcholine (ACh) as a neurotransmitter. Physiologically, ACh is generally an excitatory neurotransmitter. However, depending on the type of cholinergic receptor (nicotinic or muscarinic) and neuronal localization in which ACh is binding, it can either promote neuronal stimulation or inhibition. Since cholinergic neurotransmission is widespread across several cerebral tissues, it is crucial for the regulation and modulation of important neuronal functions like learning and memory, attention, stress response, sleep-wake cycle, and transmission and processing of sensory information (Ferreira-Vieira *et al.*, 2016).

In AD, the underproduction of ACh is caused by disease-characteristic atrophy of its production site: the *nucleus basalis of Meynert*, which is located within the hippocampus. Particularly affected are the cholinergic neurons within the hippocampal region *cornu ammonis* (CA) *1*, cells of the *subiculum*, as well as within the entorhinal cortex. ACh production is massively impaired. Severely lowered concentrations (up to 90% lower than normal) of the biosynthetic enzyme choline-acetyltransferase (ChAT) were found within the cerebral cortex and

hippocampus (Silbernagl and Lang, 2020). ChAT is essential for ACh production; therefore, reduced levels of the enzyme are simultaneously correlated with reduced ACh concentrations. ChAT synthesizes ACh by combining choline and acetyl-coenzyme A. This process takes place within the cell cytoplasm of cholinergic nerve cells. After ACh synthesis, the neurotransmitter is transported to storage vesicles within the presynaptic terminals of neurons by a vesicular ACh transporter until its liberation is induced (Breijyeh and Karaman, 2020). Physiologically, the hippocampus is a crucial structure for information registration, processing, memory development, and retrieval of information. Impairments located within this structure cause disturbed short-term memory and disturbed function in the formation of new long-term memories (Pape, Kurtz and Silbernagl, 2019; Fogwe, Reddy and Mesfin, 2023).

1.3.8. Glutamatergic hypothesis

The most prevalent excitatory neurotransmitter in the mammalian CNS is glutamate. Similar to ACh, this neurotransmitter is crucial for important cognitive activities, including learning and memory, since it significantly influences synaptic plasticity (Wang and Reddy, 2017). Increased concentrations of Aβ peptides influence neurons by inducing hyperexcitability. Aβ particles alter signal transduction cascades, leading to disturbed synaptic signal transmission, impairing memory formation, and long-term potentiation at synapses. These changes result in the excessive synaptic liberation of activated glutamate. At higher concentrations, this neurotransmitter exhibits neurotoxic properties, causing damage to surrounding tissues, which is linked to the N-methyl-D-aspartate (NMDA) receptor-associated cation channel TRPM4 (Klimaschewski, 2021). Overstimulation of NMDA receptors leads to a pathological increase in free intracellular calcium, causing calcium-driven excitotoxicity and neuronal death (Liu *et al.*, 2019). In AD, the uptake and recycling of glutamate are also disturbed, negatively impacting cellular signaling processes. This recycling system is severely weakened in AD patients, with a general decrease in the capacity of glutamate transporters and selective loss of vesicular glutamate transporters (Wang and Reddy, 2017). The characteristic Aβ pathology in AD affects various systems, including the glutamatergic one, which then results in increased liberation and decreased reuptake of the glutamatergic system, leading to increased glutamate release and decreased reuptake. This contributes to heightened neurotoxic processes and neuronal cell death, which adversely affects the individual's cognitive functions and overall abilities. Additionally, also in AD patients, the number of glutamatergic neurons is reduced, especially in the hippocampal region CA1 and within the neocortex (Gasiorowska *et al.*, 2021).

I.1.4. Clinical manifestations

AD symptoms can be broadly classified into mild, moderate, and severe phases. These phases can be further subdivided according to the Global Deterioration Scale (GDS).

1.4.1. Early and mild disease

1st stage: According to the Alzheimer Society of Canada, the early stage of AD includes GDS stages two and three. During this phase, individuals often experience normal aged forgetfulness and MCI (Reisberg *et al.*, 1982).

2nd stage: In stage two, which usually lasts between five to ten years, normal aged forgetfulness is usually noticeable only to the individual him- or herself and not to close family members or external observers.

3rd stage: As individuals progress to stage three, MCI becomes noticeable to close acquaintances. At this stage, individuals often exhibit reduced capacities to perform executive functions, difficulties with concentration, and challenges in learning or planning complex tasks. The average duration of stage three of AD typically ranges from two to seven years (Reisberg *et al.*, 1982).

1.4.2. Moderate disease

Clinical stages four to six of AD fall within the moderate phase of the disease. During these stages, memory loss and decline of cognitive abilities become more pronounced, leading to a reduction in independence due to diminished mental and physical abilities. Individuals often require supervision and assistance with daily tasks (Reisberg *et al.*, 1982). Neuropsychiatric symptoms such as false perceptions (hallucinations), content thought disturbances (delusions), affect change including depressive symptoms, euphoria, dysphoria, flattened affect and affect liability, anxiety, agitation and aggressivness, irritability, sleep distrubances, changes in eating and motor behaviors are also common (Cummings, 1997) as cited in (Schroeter *et al.*, 2012).

4th stage: Diagnosis can be made with considerable accuracy in this stage, which typically lasts around two years. Individuals begin to experience difficulties with overcoming complex tasks in everyday activities. They may have difficulties with meal preparation, recalling recent events, and knowing the correct date or day of the week. Despite these challenges, they can still remember important information such as their name, address, and recognition of close relatives and friends. Early changes in mood and behavior might include flattened emotional affect and social withdrawal. Patients are often aware of their declining cognitive abilities and may use psychological defense mechanisms such as denial and withdrawal (Reisberg *et al.*, 1982).

5th stage: This stage is marked by major difficulties with basic day-to-day activities, such as eating and taking care of personal hygiene, as well as loss of temporal and spatial orientation. Individuals may struggle to recall important personal information like their own name and residency. The average duration of this stage is approximately one and a half years (Reisberg *et al.*, 1982).

6th stage When progressing into the sixth stage of the disease, AD patients are generally not able to perform basic activities of daily life, such as dressing themselves, due to severe cognitive deficiencies. Problems with recalling basic life information and managing toileting often later proceed to urinary and fecal incontinence. Speech becomes impaired, and behavioral changes, including aggression and violent outbursts, may arise from the individual's inability to comprehend and process certain situations correctly, leading to anxiety, fear, frustration, and anger. This stage generally lasts around two and a half years (Reisberg *et al.*, 1982).

1.4.3. Severe disease

7th stage: In the final stages of AD, patients are often near the end of their lives and require constant supervision and assistance with basic activities of daily life to ensure survival. With the evolution of the seventh stage of the disease, individuals lose the ability to verbally communicate, walk, sit unsupported, smile, and hold up their heads. Due to the lack of movement, bedridden patients are prone to developing irreversible contractures and decubital ulcerations on the common pressure points of the body. Pathological "infantile" reflexes are manifesting. AD patients are usually at high risk of death at the end of the 7th stage. The most common cause of death is aspiration pneumonia. The duration of the final stage can vary widely depending on the quality of palliative care provided. With appropriate care, individuals may remain in this stage for several years (Reisberg *et al.*, 1982).

I.1.5. Diagnosis

General signs of dementia often involve impairments in higher cortical functions. Commonly occurring neurological symptoms include difficulties in comprehension or formation of language (*aphasia*), writing (*agraphia*), understanding written information (*alexia*), speech coordination (*apraxia*), and recognition and identification of persons, objects, or sounds (*agnosia*) (Payk and Brüne, 2021). According to ICD-10, ADRDs are clinically classified into early-onset (F00.0), late-onset (F00.1), atypical or mixed forms (F00.2), and unspecified types (F00.9), with typical criteria including age of onset, rate of progression, and family history (WHO, 2016).

1.5.1. Neuropsychological testing

When suspecting AD in a non-specialized setting, for example, such as a general practitioner's office or a hospital, it is recommended to perform short cognitive tests. In German outpatient memory clinics most commonly used assessment methods are the Mini-Mental State Examination (100%), the Clock Drawing Test (75%) or the Montreal Cognitive Assessment (53,57%). Also frequently applied neuropsychological tests include the Wechsler Adult Intelligence Scale/Wechsler Memory Scale (WMS) (71,43%), the California Verbal Learning test/Auditory Verbal Learning Test (67,87%) and subtests of the fourth edition of WMS (42,86%). If the results of these tests are positive or if the patient presents with dementia of unknown etiology, it is recommended to proceed further with laboratory and visual diagnostics (DGN e. V. & DGPPN e. V., 2025).

1.5.2. Laboratory tests

The S3-guideline “Demenzen” includes the recommendation for diagnostic testing of the CSF biomarkers Aβ42, total tau, and phosphorylated tau in dementia cases or patients with MCI with unknown etiology (DGN e. V. & DGPPN e. V., 2025).

According to Jack Jr. and his workgroup, the presence of the so-called early-changing core CSF biomarkers is sufficient to establish a diagnosis of AD (Jack *et al.*, 2024). These include Aβ42, p-tau181 and 217, which can be measured in CSF or as plasma analytes (Jack *et al.*, 2024).

The ratios of CSF biomarkers Aβ42 to 40, phosphorylated tau protein 181 to Aβ42, and total tau to Aβ42, correlate with the amounts of senile plaques and NFTs (Dubois *et al.*, 2023).

1.5.3. Visual diagnostics

1.5.3.1. Magnet resonance imaging

Certain cerebral atrophy patterns can be seen in magnetic resonance imaging (MRI) images of AD patients. Typically, AD is characterized by mediotemporal and parietally emphasized atrophy, with severe atrophy of hippocampal tissues being common. Atypical AD types exhibit variations of brain atrophy patterns (DGN e. V. & DGPPN e. V., 2025).

For visual evaluation and grading of the severity of atrophy patterns, specific diagnostic evaluation scores and schemes are used by diagnostic (neuro-)radiologists. Generalized visual rating scales used to evaluate cerebral MRI images of AD patients include the medial temporal lobe atrophy/Scheltens score, the entorhinal cortex atrophy score, the Koedam score, the Pons-Midbrain ratio, as well as volumetric analysis (Urbach and Egger, 2020).

1.5.3.2. ^{18}F-FDG PET

Since patterns of regional hypometabolism can be visualized with the aid of ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography (PET), a differentiation between typical and atypical AD forms is now possible (Dubois *et al.*, 2023). Similar to glucose, ^{18}F-FDG gets taken up by metabolically active cells through GLUTs.

^{18}F-FDG PET is regularly used to distinguish AD from other types of dementias or non-neurodegenerative conditions. It also plays a crucial role in the early detection of AD, as early metabolic changes of glucose consumption in brain tissues can be identified and allow an early adaptation of treatment measures (Ashraf and Goyal, 2023).

1.5.3.3. Beta-Amyloid-PET

The ability for non-invasive *in vivo* visualization of Aβ plaques and NFTs via the aid of PET radiopharmaceuticals has many advantages for correct clinical diagnosis of AD. Aβ-PET examination can help diagnose AD in the early stages of the disease, particularly in combination with the presence of MCI. It can also be used to exclude AD as the cause of dementia in case of the absence of senile plaques in cerebral tissues (Barthel *et al.*, 2015).

According to the S1-guideline of 2015 of the German Society of Nuclear Medicine ("Deutsche Gesellschaft für Nuklearmedizin e.V."), it is advised to perform Aβ-PET scans in patients with persistent or progressive cognitive impairments according to results in tests of cognitive functions, in case of non-classical clinical presentation of dementia, or in case of onset of dementia before the age of 65 (Barthel *et al.*, 2015).

1.5.3.4. Tau-PET

During Tau-PET imaging, accumulations of pathological tau protein are visualized within cerebral tissues. Since the Tau-PET examination has high sensitivity and specificity, it is a suitable imaging tool to differentiate AD from other neurodegenerative diseases. Tau-PET also showed higher specificity when used for diagnosing AD-associated dementia compared to Aβ biomarkers (Ossenkoppele *et al.*, 2018). Because the cerebral accumulation of NFTs is more strongly correlated with cognitive impairment in AD patients, Tau-PET enables individual evaluation of disease severity (Jarek *et al.*, 2024). According to Braak *et al.*, NFT distribution within cerebral tissues occurs in characteristic patterns correlating to the stage of disease severity. In the first two stages of the disease, NFTs are mainly located within the entorhinal and hippocampal cortices. Within the third and fourth stages of severity, further NFT accumulations are found in the limbic system. In the two most severe stages of AD, additional NFT bundles are also found in both mesial and neocortical tissues (Braak *et al.*, 2006).

I.1.6. Treatment and prevention

To date, AD is not curable. Symptomatic treatment is the main option available to slow disease progression, which only provides temporary relief of symptoms in AD patients. This situation, combined with the lack of a broad spectrum of effective therapies to slow down or reverse AD in Europe, makes it more important and urgent to continue researching new treatment options. This is why this thesis is dedicated to exploring a novel treatment approach for AD with a new type of active immunization.

1.6.1. General Treatment of risk factors and prevention of disease formation

Early diagnosis and appropriate treatment of vascular risk factors and diseases, as mentioned in the section "*Etiology of AD*", are considered valuable primary disease prevention measures. The S3 guideline "Demenzen" recommends following a balanced diet (e.g. Mediterranean diet), engaging in regular physical activity, and maintaining an active mental and social lifestyle (DGN e. V. & DGPPN e. V., 2025).

According to Trichopoulou and her group, a Mediterranean diet includes a high intake of fresh fruits and vegetables, nuts, legumes, and unprocessed whole grains, along with low consumption of animal products such as meat, processed meat products, and dairy (except for long-preservable cheeses) (Trichopoulou *et al.*, 2014).

To potentially reduce the risk of dementia development and slow down disease progression, Ahlskog *et al.* suggest regular physical exercise (Ahlskog *et al.*, 2011). Regular physical exercise in adults is defined by the Physical Activity Guidelines for Americans as "150-300 minutes of moderate-intensity exercise, 75-150 minutes of vigorous-intensity aerobic physical activity per week, or a combination of both" (Piercy and Troiano, 2018). Long-term physical exercise has shown positive effects on brain health, improved cognition, and reduced risk of dementia (Piercy and Troiano, 2018).

1.6.2. Pharmacological anti-dementia treatment strategies

Currently, pharmacological therapy strategies for AD are based on treating the main symptoms of dementia, such as the impairment of cognitive function and activities of daily life, as well as separately addressing psychiatric disturbances and behavioral changes, including depression, apathy, and hallucination.

In Europe, approved medications for treating the main symptoms of AD-induced dementia include antidementive drugs like acetylcholinesterase (AChE) inhibitors and the non-competitive NMDA receptor antagonist Memantine, depending on disease activity and progression. AChE inhibitors (AChEi) including donepezil, galantamine, and rivastigmine, are

recommended for managing mild to moderate forms of AD. In cases of moderate to severe AD, usage of the NMDA receptor antagonist memantine is advised (DGN e. V. & DGPPN e. V., 2025).

1.6.2.1. AChE inhibitors

By binding to the active site and inhibiting the enzymatic function of AChE, AChEi prevent the hydrolysis of ACh into acetate and choline. This increases synaptic availability and prolongs the duration of action. The prolonged presence of ACh in the synapses allows more molecules of the neurotransmitter to accumulate and activate the ACh receptors (Singh and Sadiq, 2023). The currently available AChEis donepezil, galantamine, and rivastigmine reversibly bind and block AChE activity. Since the effect of AChEi depends on dosage, it is recommended to gradually increase to the maximum dosage of the corresponding medication if tolerated by the patient (Gerwig, 2011).

1.6.2.2. N-methyl-D-aspartate receptor antagonist

Memantine non-competitively binds to NMDA receptors. By binding to specific receptor sites, it prevents the oversecretion of glutamate, thereby reducing the pathologically increased concentration of this excitatory neurotransmitter glutamate within the synaptic cleft. Memantine only attaches to activated NMDA receptors and quickly diffuses from its binding sites. It reversibly blocks the NMDA receptors, reducing increased calcium accumulation and calcium-associated neurotoxicity (Seiffert, 2021).

1.6.3. Biological disease-modifying treatments: Monoclonal antibodies

Generally, anti-amyloid monoclonal antibodies (MABs) are disease-modifying therapies (DMTs) that can positively influence the cognitive functions of AD patients by reducing the size and quantity of senile plaques within cerebral tissues. It is believed that all treatments with MABs are based on the certain mechanism of plaque reduction and removal by activated microglial cells, which phagocytize fibrillary forms of Aβ. This process leads to endosomal and lysosomal digestion and the removal of unwanted substances (Cummings *et al.*, 2023).

In the United States of America, in June 2021, the U.S. Food and Drug Administration (FDA) approved the fully human (100% human) (Wojtunik-Kulesza, Rudkowska and Orzeł-Sajdłowska, 2023). Aβ-directed immunoglobulin gamma 1 (IgG) MAB Aducanumab (Aduhelm®; Biogen) for treating AD via the Accelerated Approval Pathway (FDA, 2021). This MAB targets a broad range of Aβ types but generally has a greater affinity for high molecular weight molecules. It was approved to treat MCI or mild dementia in AD. However, in January 2024, the production of Aducanumab was discontinued due to safety concerns (Padda and Parmar, 2024).

Shortly after, the Aβ-directed humanized immunoglobulin (90% human and 10% mouse protein) (Wojtunik-Kulesza, Rudkowska and Orzeł-Sajdłowska, 2023) IgG1 MAB Lecanemab (Leqembi®; Eisai Inc. and Biogen) was FDA-approved under the Accelerated Approval Pathway in January 2023, which was converted to traditional approval in July 2023. Lecanemab reduces the disease-characteristic underlying process of Aβ plaque accumulation, leading to fewer amyloid deposits and improved cognition in AD patients. Specifically, this MAB has a 10:1 targeting preference for soluble protofibrils over insoluble forms of Aβ (Cummings *et al.*, 2023). Like Aducanumab, it is recommended to initiate treatment with Lecanemab in patients with MCI or mild dementia with confirmed Aβ pathology (FDA, 2023).

The Committee for Medicinal Products for Human Use of the European Medicines Agency (EMA) recently re-examined Lecanemab and recommended its use for patients with MCI or mild dementia in early AD stages. It concluded that the benefits of the monoclonal antibody outweigh the risks associated with treatment. As a result, Leqembi® received marketing authorization for treating patients with confirmed amyloid pathology with one or no copies of the APO-E ε4 allele, offering a new treatment option to slow disease progression (EMA, 2024).

Wojtunik-Kulesza *et al.* reviewed several recent studies in 2023 on testing anti-neurodegenerative MABs for treating AD. These studies include humanized IgG1 MABs like Donanemab, humanized IgG4 MABs like Gosuranemab, Semorinemab, or Tilavonemab, as well as the fully human IgG1 MAB Ganterumab. The results of these studies are promising (Wojtunik-Kulesza, Rudkowska and Orzeł-Sajdłowska, 2023). In a placebo-controlled trial, these DMTs significantly reduced the rate of cognitive decline. For instance, the FDA-approved agent Lecanemab showed a 27% reduction in clinical decline. Donanemab showed a 32% reduction (Cummings *et al.*, 2023). In summary, treatment with MAB technology holds promise for the future. However, further diverse studies on effectiveness and drug safety are necessary.

I.1.7. Experimental DMT: active immunization with VLP vaccines

VLP vaccines are categorized as subunit vaccines, which can be further separated into enclosed and non-enveloped variants. Generally, VLPs are nanoscale biological entities that replicate the architecture of viruses but lack infectious genetic material and the capacity to infect host cells for replication. They comprise one or many molecular components obtained from viruses. Because these particles just replicate the viral envelope, such vaccinations are regarded as also comparatively safe for immunocompromised patients. VLPs can self-assemble or reconstitute based on specific environmental circumstances. These innovative vaccinations have garnered

heightened favor in preventive medicine, particularly following the COVID-19 pandemic in 2019. They have also demonstrated effective applications in targeted medication administration and gene therapy owing to their distinctive cavity-containing shape. VLP vaccinations have superior immunogenicity relative to traditional inactivated viral vaccines. These vaccines can stimulate both humoral and cellular components of the adaptive immune response (Nooraei *et al.*, 2021).

The combination of specific components, such as antigens and adjuvants, significantly amplifies the efficacy of this vaccination type. This enhancement is connected to their improved capacity to concurrently stimulate antigen-presenting cells while also initiating humoral and cellular immunity pathways (Gogesch *et al.*, 2018). The humoral response is facilitated by B-lymphocytes that produce antigen-specific antibodies, whereas cell-mediated immunity is governed by T-lymphocytes (Roers, 2022).

Currently, certain VLP vaccines have received approval for human application. The commercially effective and widely utilized vaccines are those for the hepatitis B and E and human papillomaviruses (Chackerian, 2010; Donaldson *et al.*, 2018; Dhawan, Saied and Sharma, 2023; Kheirvari, Liu and Tumban, 2023).

To date, only a small number of studies have been done on VLP-based vaccinations in AD models. Those studies showed promising results in reducing either the Aβ plaque burden (Zamora et al., 2006; Bach et al., 2009; Chackerian, 2010) or the amount of hyperphosphorylated protein tau accumulations (Jiet al., 2018; Maphis et al., 2019) in cerebral tissues in AD mice models. These findings indicate possible disease-modifying properties (EMA, 2016; Cummings and Fox, 2017).

II. MATERIALS AND METHODS

II.1. Animals

In this study, adult female transgenic 5xFAD mice (five weeks old upon arrival, JAX Stock #006554, The Jackson Laboratory, Bar Harbour, USA) were used.

All animals were housed in a temperature- and humidity-controlled laboratory environment (25 ± 1°C; 55 ± 5%) with *ad libitum* access to food (standard pelleted chow) and filtered tap water except during the time of procedures. All subjects were housed under a regular 12h/12h light-dark cycle (7:30-19:30). Four mice each were group-housed in standard 39.5cm wide x 34.5cm long x 21.3cm high same-litter cages located in individually ventilated stainless-steel racks. To increase the animals' well-being, the cages were provided with wooden chip bedding, wooden wool, as well as a polycarbonate tunnel or a crawling ball for environmental enrichment.

At all times, we made every effort to reduce animal stress and improve their overall well-being. To reduce animals' stress levels on experimental days, subjects were left undisturbed for 30-45 minutes in their home cages prior to testing procedures to allow acclimation to the testing environment. The behavioral experiments were performed during the light phase of the light-dark cycle.

All experimental procedures of the current study were approved by the Animal Ethics Committee of the Food and Veterinary Service of the Republic of Latvia (permit no. 147) and performed in accordance with the ARRIVE guidelines 2.0.

II.2. Chemicals and antibodies

The chemicals utilized in this study included Fluoromount™ aqueous mounting medium (cat. no. F4680), Hoechst solution (cat. no. 94403), normal goat serum (NGS, cat. no. S26-M), phosphate-buffered saline (PBS, cat. no. P3813), paraformaldehyde (PFA, cat. no. P6148), and Tween® 20 (cat. no. P2287), all purchased from Sigma-Aldrich (USA).

The squalene-oil-in-water vaccine adjuvant Addavax™ (cat. no. vac-axd-10) was acquired from InvivoGen (France).

The utilized antibodies include mouse (Ms) anti-Aβ (cat. no. 803001, Biolegend, Netherlands), rabbit (Rb) anti-glial fibrillary acidic protein (GFAP) (cat. no. ab68428), goat anti-Ms AlexaFluor®488 (cat. no. ab150113), and goat anti-Rb AlexaFluor®488 (cat. no. ab150077), acquired from Abcam (UK). All antibodies were utilized at a concentration of 1:500.

The vaccine candidate was created, and along with Addavax™ provided by Prof. Kaspars Tārs and Ilva Liekniņa from the Latvian Biomedical Research Center.

II.3. Treatment schedule

The treatment schedule is summarized in *Fig. 2.3.1*. The animal groups receiving either VLP vaccination or AddaVax™ vaccine adjuvant were vaccinated on experimental day 1 and received booster administrations on days 15, 29, and 103. Enzyme-linked immunosorbent assay blood tests were routinely conducted on vaccine-receiving subjects by other researchers to quantify antibody titers and assess the immunogenicity of the VLP vaccine candidate. Booster vaccinations were administered when titer concentrations were insufficient. One week after the last vaccination was administered, the behavioral experiments were started. These included the open field test (OF) and y-maze test (YM) and both were conducted on subjects aged six and eight months (on experimental days 110 and 111, respectively). Additionally, the elevated zero maze test (EZM) was only conducted once at the animal age of eight months (experimental day 168).

On day 175, the subjects were euthanized. This was performed by inducing deep anesthesia using a ketamine/xylazine mixture (100mg/10mg)/kg body weight, followed by a transcardial approach of perfusions with ice-cold PBS followed by 4% PFA.

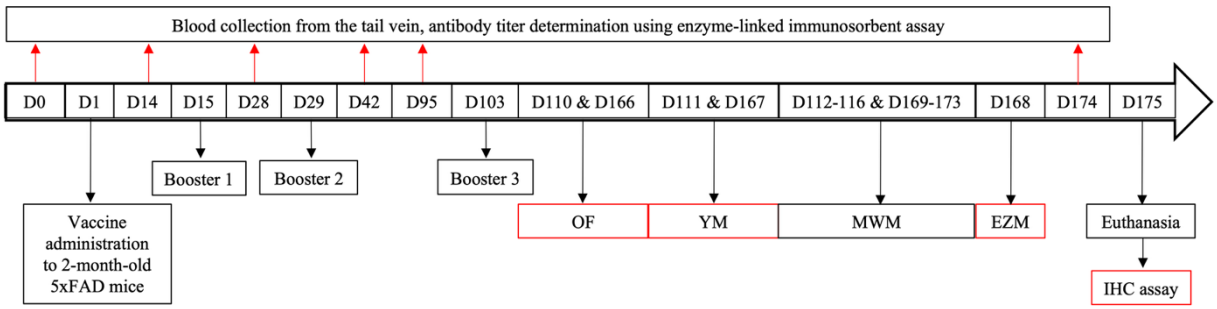

Figure 2.3.1: **Experimental design of the study.** *D: day, EZM: elevated zero maze test; IHC: immunohistochemistry; MWM: Morris-Water maze test; OF: open field test; YM: y-maze test. Red brackets indicate procedures that I helped performing.*

II.4. Behavioral tests

2.4.1. Open Field test

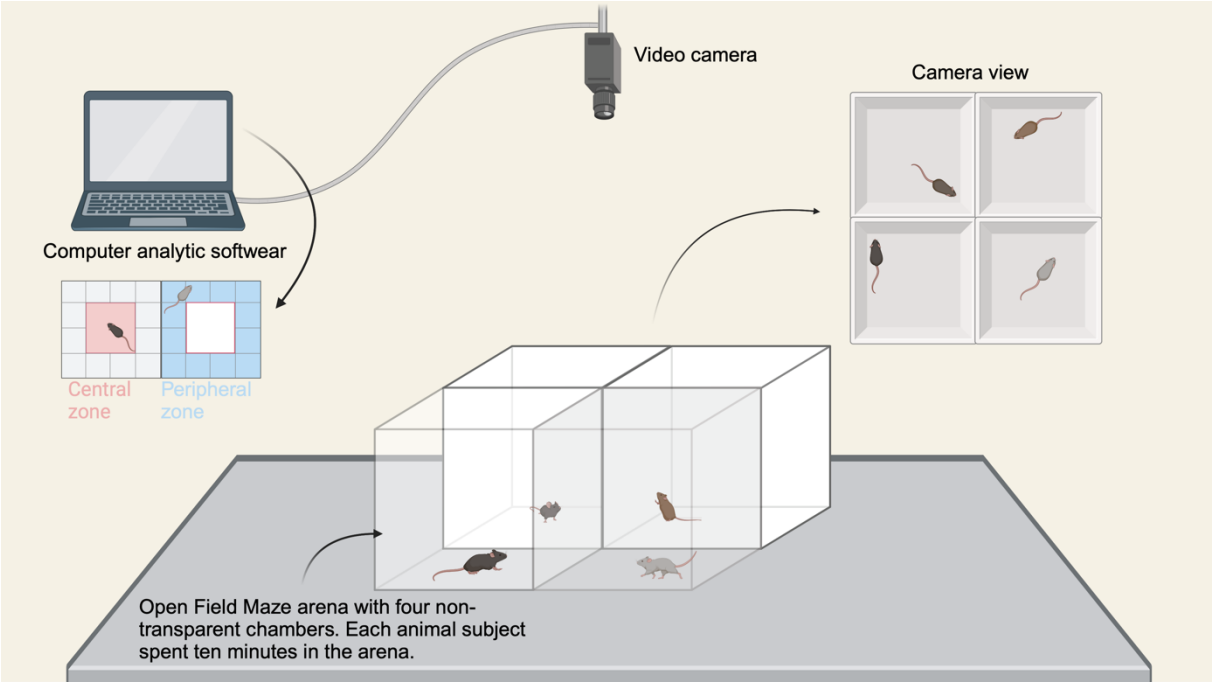

Figure 2.4.1.1: **Simplified model of the OF experimental setup.** Illustration generated with BioRender.com (accessed on 25th of August 2024).

Using the OF, we investigated the effects of VLP vaccine candidate on general locomotor activity, anxiety-related, and exploratory behavior in the 5xFAD mice. The test was conducted on the experimental days 110 and 166 (*Fig. 2.3.1*).

As seen in the simplified model in *Fig 2.4.1.1*, the OF arena consisted of four neighboring 50cm wide x 50cm long and 20cm high square boxes. For tracking and analyzing the subject's behavioral patterns, a Basler video camera, located directly above the experimental arena, was coupled to EthoVision XT 11.5 (Noldus, Wageningen, Netherlands) tracking program, recording the animal's behavior for 10 minutes each. The time started after each of the four mice was placed into their corresponding box facing the wall. During that time, they were allowed to move freely and explore their OF arena. The arena was thoroughly cleaned and disinfected with 70% ethanol and paper towels before and between each trial.

Data of interest in the OF were the total distance traveled (in cm), time spent in the central zone (in s), mean speed, and maximal velocity (in cm/s) of the animal subjects.

2.4.2. Y-Maze test

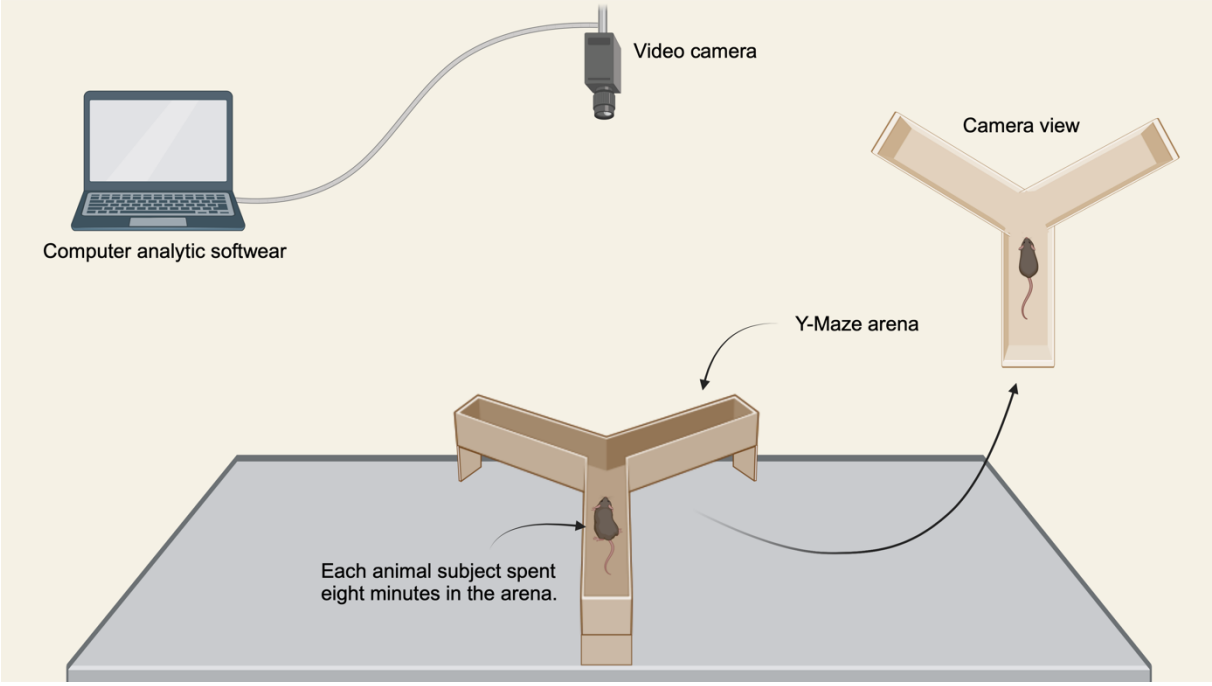

Figure 2.4.2.1: **Simplified model of the YM experimental setup.** Illustration generated with BioRender.com (accessed on 25th of August 2024).

The YM was performed to investigate the possible effects of VLPs on explorative behavior and spatial learning in animal subjects. This test was conducted on the experimental days 111 and 167 (*Fig. 2.3.1*). The same video equipment and software program were used for tracking and analyzing purposes in this behavioral experiment.

The YM arena consisted of three single arms of the same length positioned at 120° toward each other, as seen in the simplified model in *Fig. 2.4.2.1*.

Each mouse was allowed to freely explore the arena for eight minutes. During each trial, all movements were recorded and later tracked using EthoVision XT 11.5. Before and in between each trial, the arena was cleaned and disinfected with 70% ethanol and paper towels.

Here, we were interested in the number of successful arm entries, the index of spontaneous alternations (n), and the number of maximal alternations possible (n).

2.4.3. Elevated Zero Maze test

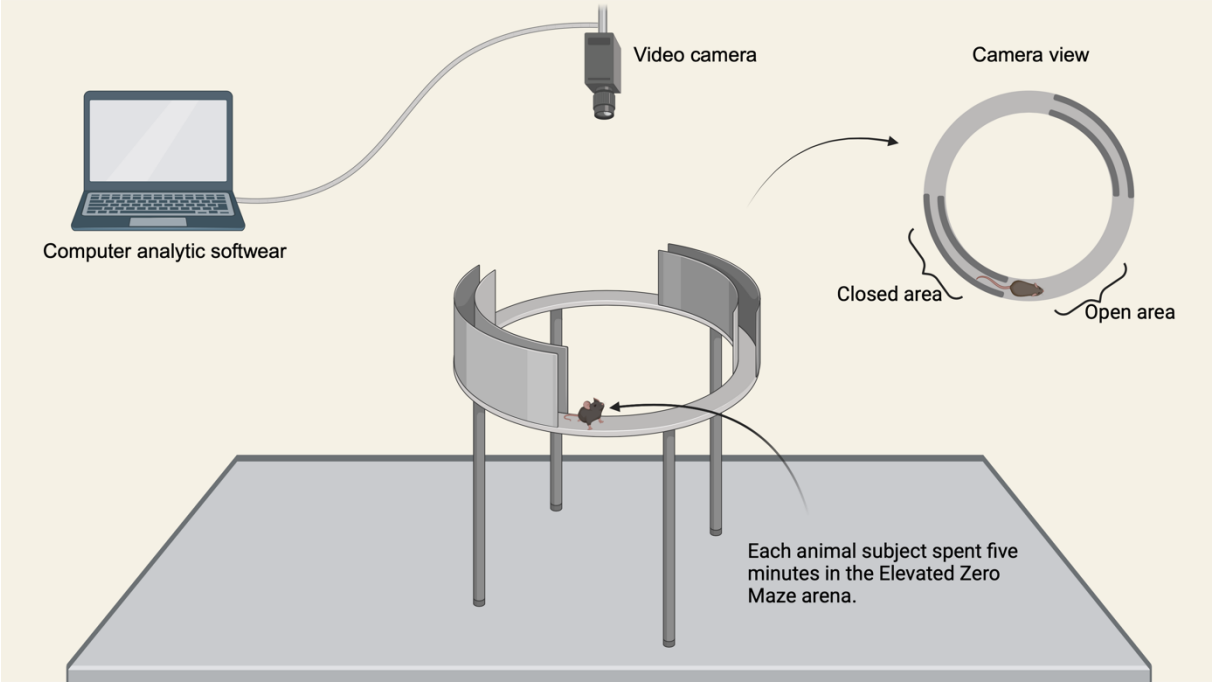

Figure 2.4.3.1: **Simplified model of the EZM experimental setup.** Illustration generated with BioRender.com (accessed on 25th of August 2024).

For investigative purposes of anxiety-like behavior following administration of multiple vaccinations with VLPs in eight-month-old 5xFAD, the EZM was performed on the 168th experimental day of this study (see *Fig. 2.3.1*).

As seen in the simplified image in *Fig. 2.4.3.1*, the EZM arena consists of a circular plexiglas ring, 70cm in diameter and 5cm in width, with a 4mm high lip attached to the open quadrants to prevent the animals from falling. The arena was 50cm elevated from the ground. The circular arena was divided into four equal parts: two open areas and two closed ones with walls 15cm in height. Two equal parts are opposite to each other. The arena was thoroughly cleaned and disinfected with 70% ethanol and paper towels before and between each trial.

The mice were placed in one of the open areas and were allowed to freely explore the arena undisturbed for five minutes each. All movements were recorded, tracked, and analyzed using EthoVision XT 11.5. We were interested in the number of entries into both the open and the closed area (n), as well as the time (in s) the subject spent in open or closed quadrants of the arena.

II.5. Immunofluorescence

Immediately after animal euthanization, whole brain excision has been performed. The brains were then fixed in 4% PFA overnight. Afterwards, they were transferred to a 30% sucrose solution in PBS for cryopreservation.

Each mouse brain was cut into 30 µm thin coronal sections at -25°C using a cryostat (cat. no. CM1850, Leica Biosystems, USA). The sections were cut according to specific coordinates ranging from +1.18 to –2.3 mm anterior-posterior to the bregma. Within these 200 µm, three sections per subject were randomly selected for further immunofluorescent assays.

Rinsing of the selected cerebral sections was performed three times for five minutes each using 1X PBS containing 0.3% Tween® 20 (PBST). Then, incubation of the previously rinsed section was done for 10 minutes in 0.01M sodium citrate buffer with a pH of 6.0 at 95°C to achieve antigen retrieval. Next, the sections were incubated for one hour at room temperature in a blocking solution consisting of 10% NGS in PBST to prevent unwanted non-specific binding of antibodies. After blocking, the brain sections were incubated overnight in a 5% NGS-PBST solution, additionally containing the primary antibody (Ms anti-Aβ (cat. no. 803001) or Rb anti-GFAP (cat. no. ab68428)). The day after, sections were rinsed again three times à five minutes with PBST, followed by incubation in PBST containing the corresponding secondary immunoglobulins (goat anti-Ms AlexaFluor®488 (cat. no. ab150113) or goat anti-Rb AlexaFluor®488 (cat. no. ab150077)). For visualization of cell nuclei, staining with Hoechst solution (concentration 1:1000) was done for five minutes. Afterwards, sections were rinsed with dH_2O. After performing all the mentioned steps, sections were transferred to gelatin-coated histological slides, where they were allowed to air-dry overnight before coverslip adhesion using Fluoromount™ aqueous mounting medium was done.

II.6. Digitalization and quantification

After all histological slides were coverslipped and completed the drying process, they were digitized by a person blinded to the experimental groups. Cerebral sections were digitally documented using the Nikon Eclipse Ti microscopy system (Nikon Europe, The Netherlands).

For data acquisition of immunofluorescence assays, we used the open-source biological image analysis software Fiji (ImageJ2, Version 2.14.0/1.54f, USA) (Schindelin *et al.*, 2012). Equal regions of interest were selected for all analyzed samples. For optical density (OD) measurements, the mean fluorescence intensity of tissue staining was calculated after software calibration using the Rodbard method. The obtained measurements were tabulated in a

Microsoft ® Office Excel 2024 (Version 16.88, Microsoft Corporation, USA) spreadsheet, and the tabulated data was further used for statistical analyses.

II.7. Statistical analysis

All data was analyzed using *GraphPad Prism* software (version *8.3.0*; GraphPad Software, San Diego, USA).

To detect differences between the groups in the performance of behavioral tests and immunofluorescence, one-way analysis of variance (ANOVA) followed by Holm-Sidak post-hoc correction for multiple comparisons was conducted, preceded by testing for Gaussian distribution to ensure that the assumption of normal distribution was met.

All data are expressed as mean ± standard deviation (SD). The level of significance for analysis was set at $p < 0.05$.

III. RESULTS

III.1. Evaluation of behavioral test performance of female 5xFAD mice

3.1.1. Performance of female 5xFAD mice in the Open Field test

No significant differences were observed across the groups, neither for mean speed nor maximum velocity, when comparing group results at the age of six and eight months (see *Fig. 3.1.1.1a, b, c, d*, and *Tab. 3.1*).

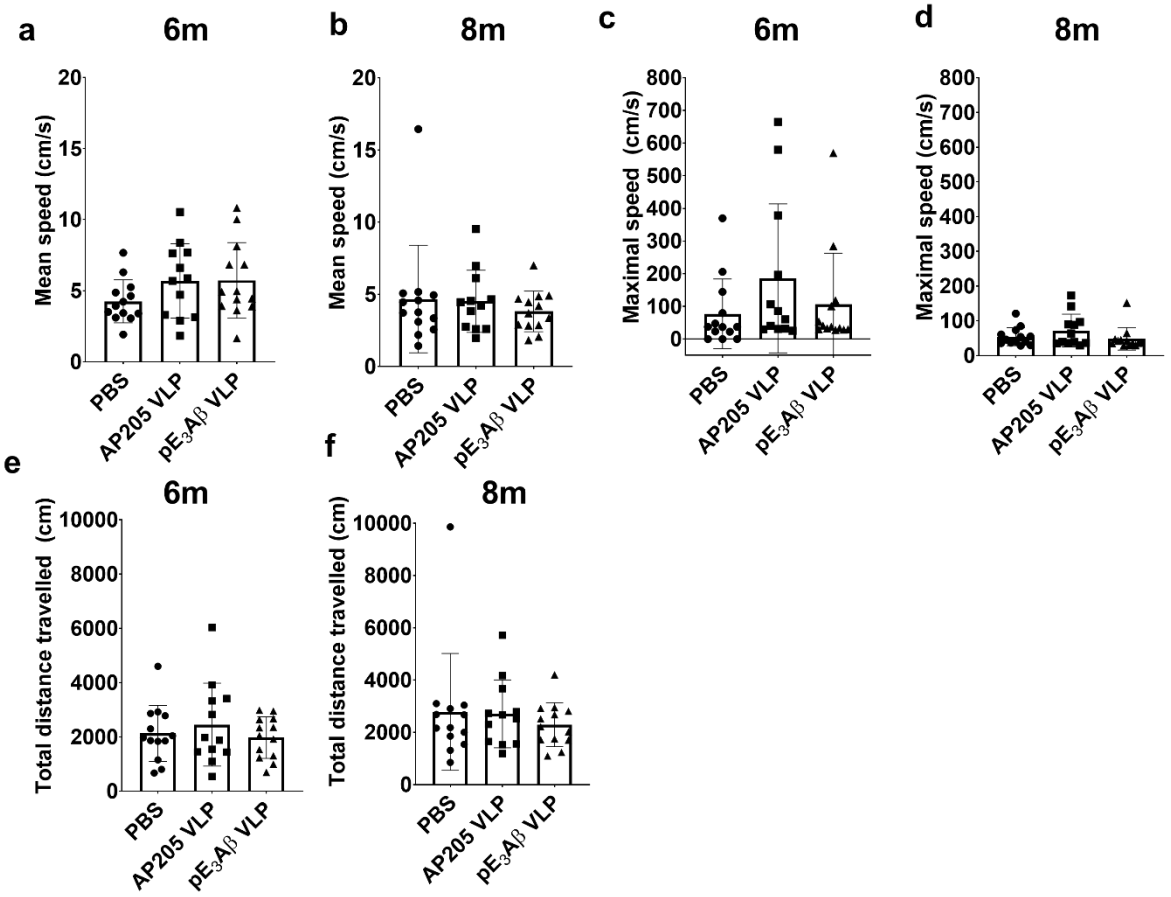

Figure 3.1.1.1: **Locomotor performance in the OF arena (mean speed: a, b; maximal speed: c, d; and total distance travelled: e, f) in six- and eight-month-old 5xFAD mice.**

Regarding total distance traveled and time spent in the central zone of the arena, no significant differences were found between the individual groups, neither at six nor at eight months.

Similar non-significant results were observed when comparing performances within the groups at different ages for the total distance traveled and the time spent in the inner zone of the OF arena. Results are visualized in *Fig. 3.1.1.1e* and *f*, *Fig. 3.1.1.2a* and *b* and *Tab. 3.1*.

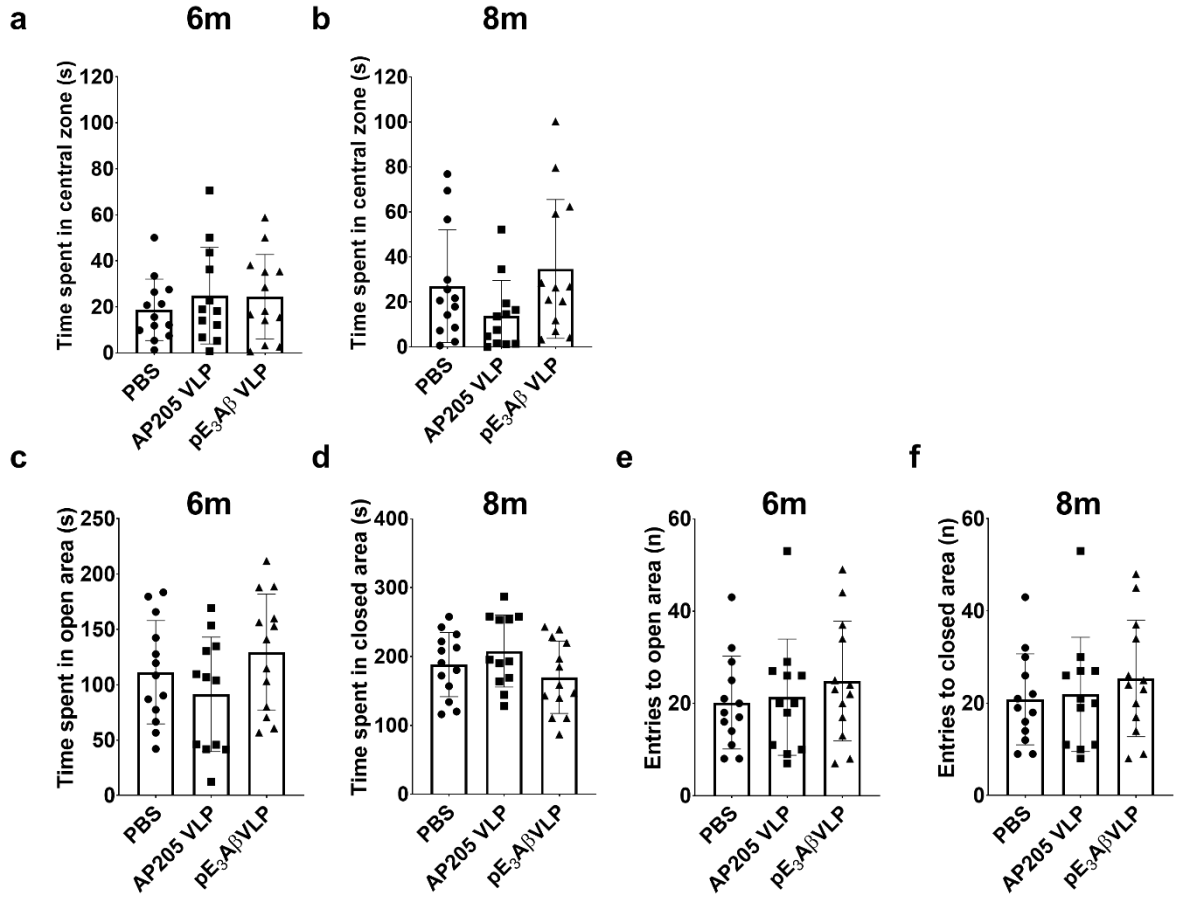

Figure 3.1.1.2: **Anxiety-related behaviors in six- and eight-month-old 5xFAD mice.** *Included are the time spent and localization within the OF arena (a, b) and the EZM arena (c, d, e, f).*

Table 3.1: **Treatment effects on anxiety-related behaviors in six- and eight-month-old female 5xFAD mice in the OF.**

Treatment group	Mean speed (cm/s)	Maximal speed (cm/s)	Total distance travelled (cm)	Time spent in inner zone (s)
PBS 6m	4.35 ± 1.52	77.28 ± 106.52	2127.50 ± 1033,98	18.69 ± 13.34
PBS 8m	4.67 ± 3.72	54.29 ± 26.00	2780.63 ± 2235.46	27.06 ± 25.05
AP205 VLP 6m	5.69 ± 2.62	185.82 ± 228.17	2453.79 ± 1528.03	24.94 ± 21.04
AP205 VLP 8m	4.51 ± 2.16	72.26 ± 49.97	2705.97 ± 1296.21	13.97 ± 15.70
pE_3Aβ VLP 6m	6.54 ± 3.96	106.76 ± 156.18	2063.69 ± 778.79	24.42 ± 17.67
pE_3Aβ VLP 8m	3.82 ± 1.41	48.11 ± 32.54	2290.18 ± 843.30	34.69 ± 30.89

Mean ± SD of OF locomotor performance parameters (mean speed, maximal speed, total distance travelled and time spent in inner zone) in six- and eight-month-old female 5xFAD mice.

3.1.2 Elevated Zero Maze performance of 5xFAD mice

The key data points in this experiment were the number of entries to and times spent in the open and closed areas of the EZM. No statistically significant differences were observed between the treatment groups, as displayed in *Fig. 3.1.1.2 c, d, e, f* and *Tab. 3.2.*

Table 3.2: **Treatment effects on anxiety-related behaviors in six- and eight-month-old female 5xFAD mice in the EZM.**

Treatment group	Entries to closed area (n)	Time spent in closed area (s)	Entries to open area (n)	Time spent in closed area (s)
PBS 8m	20.85 ± 9.86	188.17 ± 46.49	20.15 ± 10.06	111.32 ± 46.66
AP205 VLP 8m	21.92 ± 12.38	207.82 ± 52.18	21.33 ± 12.59	91.39 ± 51.71
pE$_3$Aβ VLP 8m	25.38 ± 12.63	169.89 ± 52.62	24.85 ± 12.98	129.59 ± 52.37

Mean ± SD of EZM locomotor performance parameters (entries to and time spent in open and closed area) in six- and eight-month-old female 5xFAD mice.

3.1.3. Assessment of spontaneous alternations of 5xFAD mice in the Y-Maze test

When observing the spatial learning, short-term memory, and explorative behavior of the 5xFAD mice in the YM, no significant differences were detected in the number of spontaneous alternations between different arms among the three groups at different ages, as displayed in *Fig. 3.1.3.1a, b, c, d* and *Tab. 3.3.*

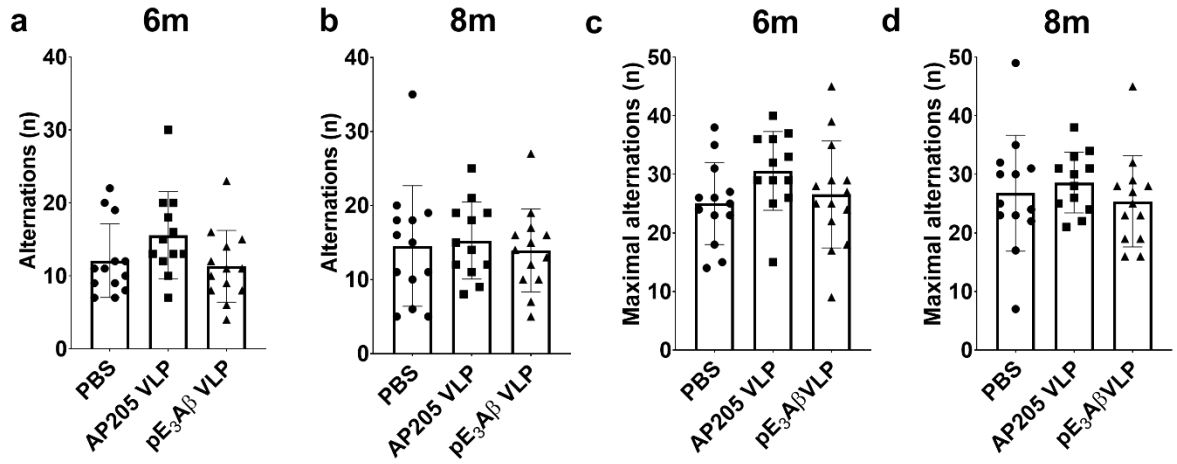

Figure 3.1.3.1: **Explorative behavior (alternations (a, b) and maximal alternations (c, d)) in six- and eight-month-old female 5xFAD mice in YM test.**

Table 3.3: **Treatment effects on spontaneous alternations in six- and eight-month-old female 5xFAD mice in the YM test.**

Treatment group	Alternations (n)	Maximal Alternations (n)
PBS 6m	12.08 ± 5.02	25.00 ± 7.01
PBS 8m	14.54 ± 8.14	26.77 ± 9.90
AP205 VLP 6m	15.58 ± 5.96	30.56 ± 6.73
AP205 VLP 8m	15.25 ± 5.19	28.58 ± 5.16
pE$_3$Aβ VLP 6m	11.86 ± 5.16	26.57 ± 9.14
pE$_3$Aβ VLP 8m	13.92 ± 5.62	25.38 ± 7.78

Mean ± SD of explorative behavior (alternations and maximal alternations) in six- and eight-month-old female 5xFAD mice in the YM test.

III.2. Immunofluorescence analysis of biochemical changes in 5xFAD mice

3.2.1. Differences in Aβ42 plaque number in the cerebral tissues of 5xFAD mice

As displayed in *Fig. 3.2.1.1a* and *c,* and *Tab. 3.4* the numbers of extracellular Aβ42-positive plaques in cerebral tissues of the neocortex (NCTX) of the $pE_3Aβ$ VLP-treated group mice were statistically significantly lower compared to those of the PBS group (132.69±15.97 vs. 170.55±32.98, p=0.0038). The number of $Aβ42^+$ plaques counted in the AP205 VLP-treated group mice had a trend to be lower than in the PBS group (151.71±31.42 vs. 170.55±32.98), but the difference was not statistically significant (*Fig. 3.2.1.1c* and *Tab. 3.4*).

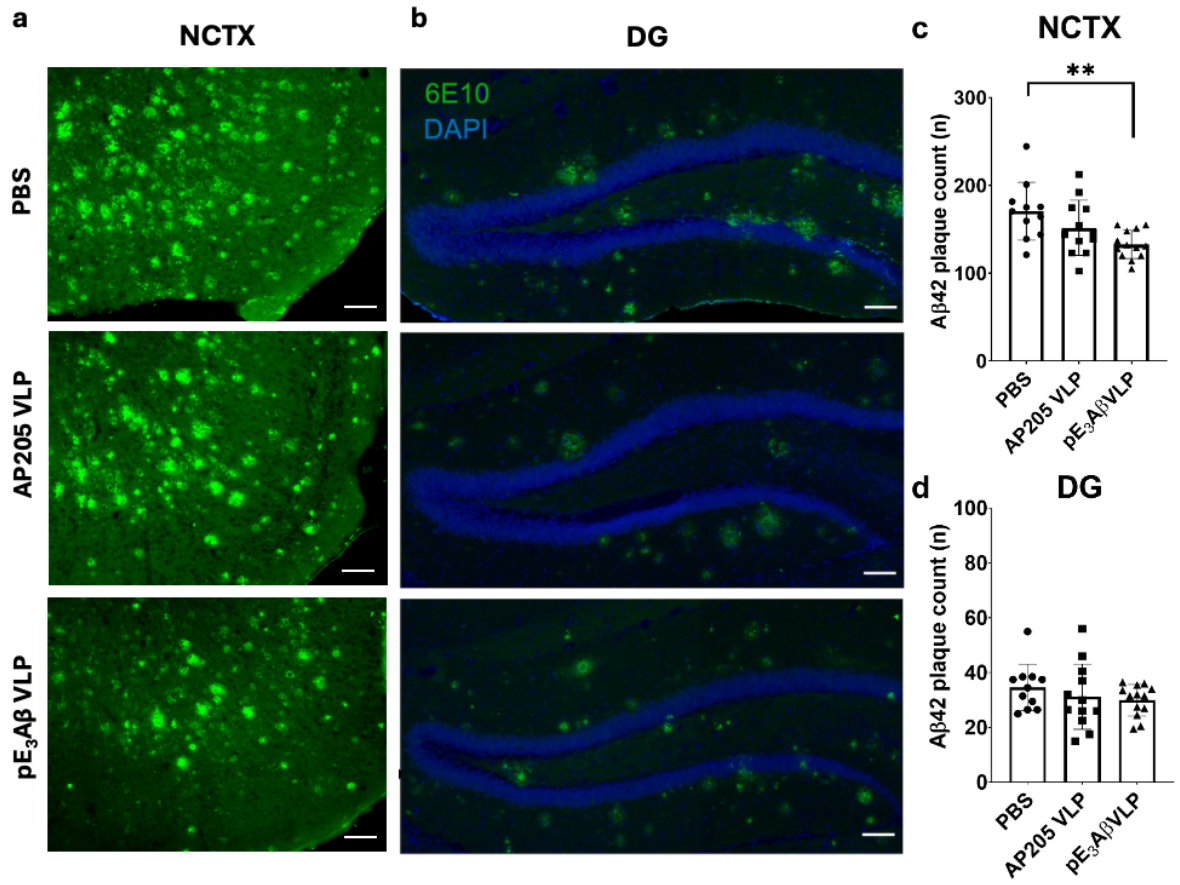

Figure 3.2.1.1: **Immunization with $pE_3Aβ$ VLP vaccine candidate reduces $Aβ42^+$plaques in the NCTX of eight-month-old 5xFAD mice.** *Immunofluorescence microscopy representative images show $Aβ42^+$ plaques (green) and cellular nuclein (blue) in the NCTX (a) and DG (b). Statistical analyses for $Aβ42^+$ plaque counts are displayed for the NCTX (c) and DG (d). Scale bar = 200 µm. **p<0.01 vs. PBS.*

Table 3.4: **Results of Aβ42$^+$ plaque count analysis in the NCTX of eight-month-old 5xFAD mice following treatment.**

Treatment group	Aß42$^+$ plaques (n)
PBS	170.55 ± 32.98
AP205 VLP	151.71 ± 31.42
pE$_3$Aβ VLP	132.69 ± 15.97

Quantification of extracellular Aβ42$^+$ plaques in the NCTX of eight-month-old 5xFAD mice (mean ± SD).

In comparison to the statistically significant reduction of Aβ42$^+$ plaque numbers counted in the NCTX of 8-month-old female 5xFAD, pE$_3$Aβ VLP treatment did not cause statistically significantly lower numbers of Aβ42$^+$ plaques in the DG (*Fig. 3.2.1.1d* and *Tab. 3.5).*

Table 3.5: **Results of Aβ42$^+$ plaque count analysis in the DG of eight-month-old 5xFAD mice following treatment.**

Treatment group	Aß42$^+$ plaques (n)
PBS	34.59 ± 8.50
AP205 VLP	31.25 ± 11.90
pE$_3$Aβ VLP	29.96 ± 5.71

Quantification of extracellular Aβ42$^+$ plaques in the DG of eight-month-old 5xFAD mice (mean ± SD).

3.2.2. Effects on GFAP$^+$ astrocyte density in 5xFAD mice following immunization

Densitometry analysis of GFAP in the NCTX revealed only slight changes between the different treatment groups of animals (*Fig, 3.2.2.1a,* and *c*). Changes in GFAP OD in the NCTX between the AP205 VLP-treated group and the PBS control group (253.32 ± 0.62 vs. 253.25 ± 0.7) were not statistically significant. In contrast, the pE$_3$Aβ VLP-treated group showed a statistically significantly reduced GFAP OD in the NCTX of 5xFAD mice in comparison to the PBS control group (252.74 ± 0.51 vs. 253.25 ± 0.7; p=0.048), as displayed in *Fig, 3.2.2.1a,* and *c,* and *Tab. 3.6.*

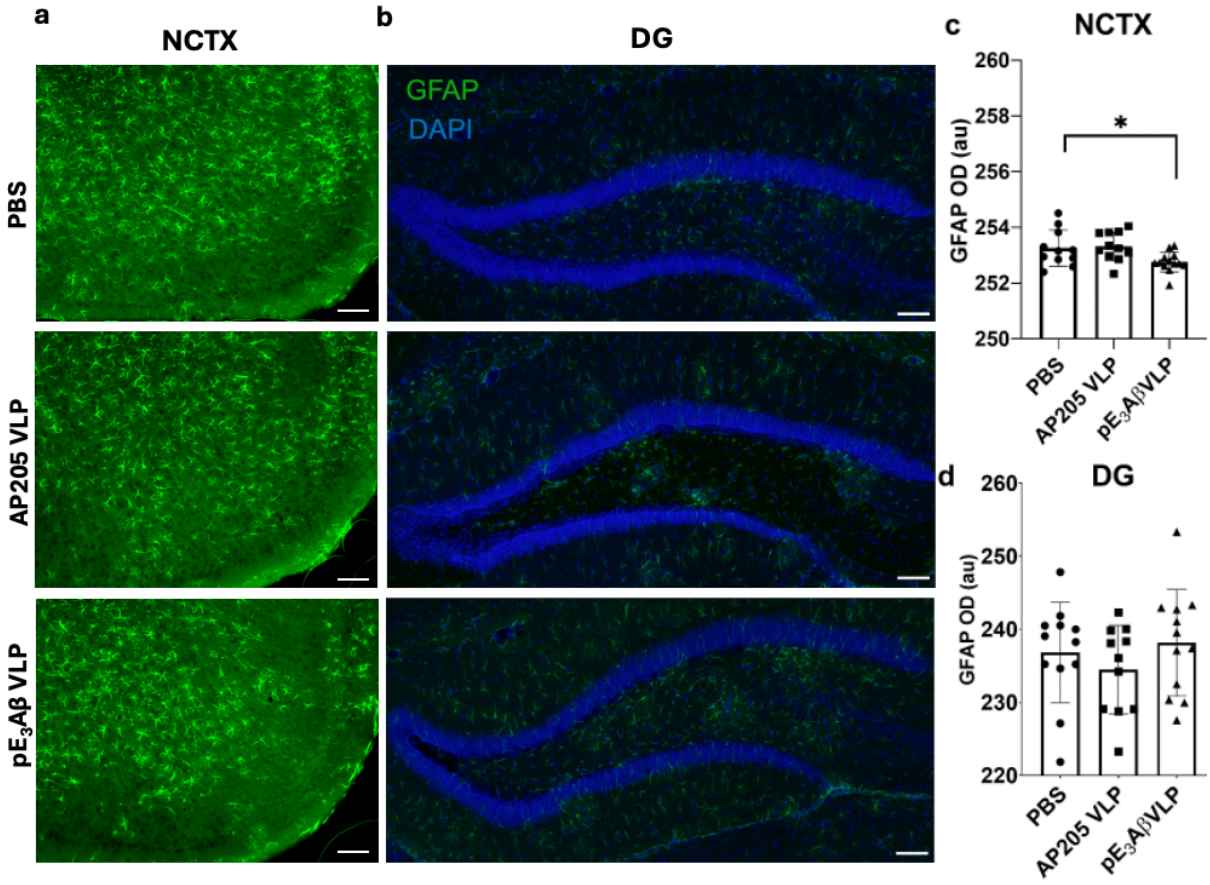

Figure 3.2.2.1: **pE$_3$Aβ VLP treatment reduces the number of GFAP$^+$ astrocytes in the NCTX of eight-month-old 5xFAD mice.** *Immunofluorescence microscopy representative images show GFAP$^+$- astroglial cells (green) and cellular nuclein (blue) in the NCTX (a) and DG (b). Statistical analysis of GFAP$^+$ astrocyte analysis of the NCTX and DG are shown in (c) and (d), respectively. Scale bar = 200 µm. *p<0.05 vs. PBS.*

Table 3.6: **Results of GFAP densitometry analysis in the NCTX of eight-month-old 5xFAD mice following treatment.**

Treatment group	GFAP OD (au)
PBS	253.25 ± 0.7
AP205 VLP	253.32 ± 0.62
pE$_3$Aβ VLP	252.74 ± 0.51

Quantification of activated astrocytes (GFAP-positive) in the NCTX of eight-month-old 5xFAD mice (mean ± SD).

The pE$_3$Aβ VLP treatment did not show a statistically significant reduction of GFAP$^+$ astrocytes in the DG tissues of 8-month-old female 5xFAD mice (*Fig. 3.2.2.1b, d,* and *Tab. 3.7).*

Table 3.7: **Results of GFAP densitometry analysis in the DG of eight-month-old 5xFAD mice following treatment.**

Treatment group	GFAP OD (au)
PBS	236.85 ± 6.87
AP205 VLP	234.47 ± 6.07
$pE_3A\beta$ VLP	238.17 ± 7.30

Quantification of activated astrocytes (GFAP-positive) in the DG of eight-month-old 5xFAD mice (mean ± SD).

3.2.3. Effects of active immunization on the number of Iba-1^+ cells in 5xFAD mice

The comparison of ionized calcium binding adaptor molecule 1-positive (Iba-$^{1+}$) activated microglial cells near Aβ plaques in the NCTX and DG showed a general trend of reduced microglial activation in the AP205 VLP group compared to the PBS control. In the NCTX, the AP205 VLP group exhibited fewer activated microglial cells (68.55 ± 15.15) than the PBS group (81.73 ± 25.78). The $pE_3A\beta$ VLP treatment was associated only with a slight increase of Iba-$^{1+}$ microglial cells in the NCTX (84.42 ± 23.77) (see *Fig. 3.2.3.1a* and *c, Tab. 3.8*). The ANOVA analysis, however, indicated no statistically significant differences among the groups in the NCTX of female 5xFAD mice.

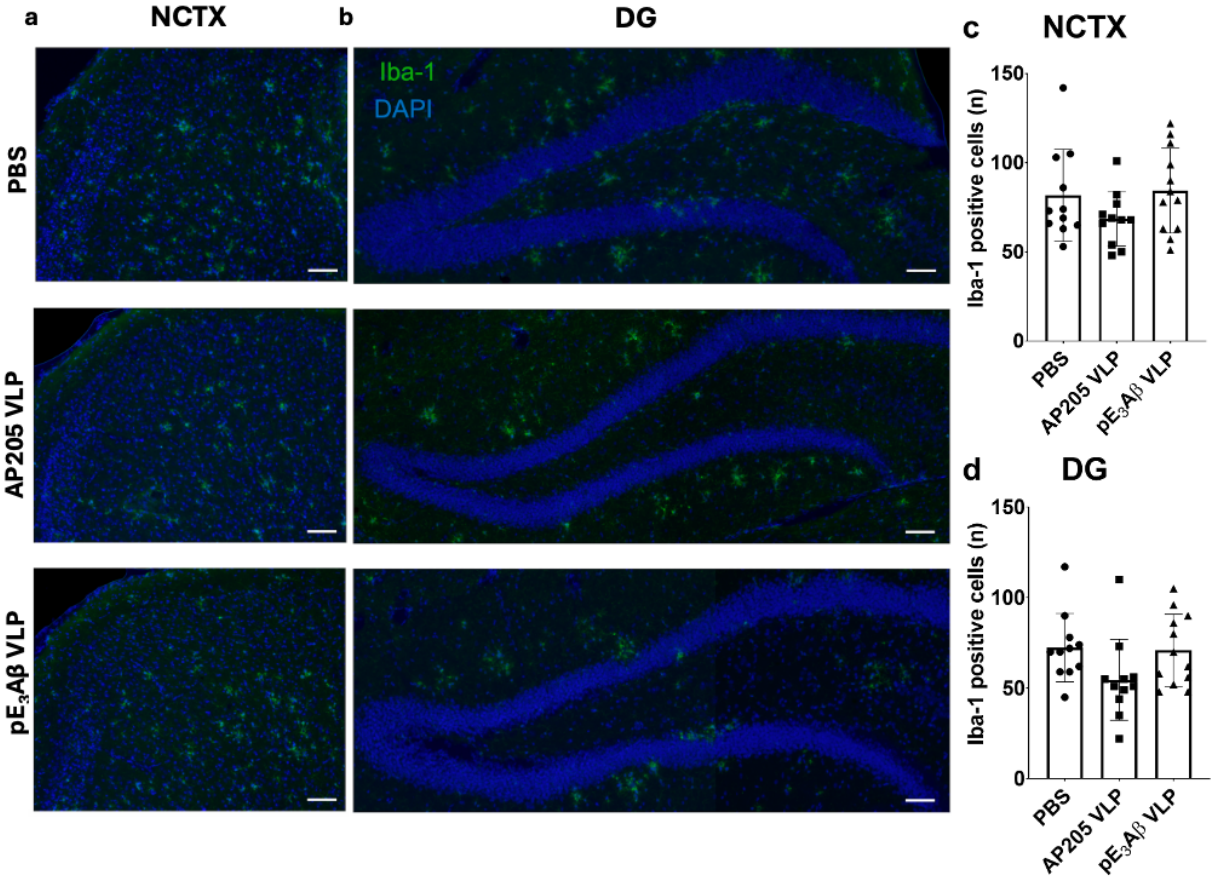

Figure 3.2.3.1: **The number of activated microglial (Iba-1^+) cells in the NCTX and DG of eight-month-old 5xFAD mice was not statistically significantly changed.**

Immunofluorescence microscopy representative images show activated microglial cells (green) and cellular nuclei (blue) in the NCTX (a) and DG (b). Statistical analyses show the count of Iba-1$^+$ cells in the NCTX (c) and DG (d).

Table 3.8: **Results of quantification of Iba-1$^+$ cells in the NCTX of eight-month-old 5xFAD mice following treatment.**

Treatment group	Iba-1$^+$ cells (n)
PBS	81.73 ± 25.78
AP205 VLP	68.55 ± 15.15
pE$_3$Aβ VLP	84.42 ± 23.77

Quantification of activated Iba-1$^+$ microglial cells in proximity to Aβ42 plaque accumulations in the NCTX of eight-month-old 5xFAD mice (mean ± SD).

Similarly, in the DG, the AP205 VLP group showed a reduced number of activated microglial cells (54.64 ± 22.42) compared to the PBS control (72.36 ± 18.87), while the pE$_3$Aβ VLP group demonstrated only a minimal reduction (70.92 ± 19.87) (see *Fig. 3.2.3.1b* and *d*, *Tab. 3.9*). The changes were also not statistically significant.

Table 3.9: **Results of quantification of Iba-1$^+$ cells in the DG of eight-month-old 5xFAD mice following treatment.**

Treatment group	Iba-1$^+$ cells (n)
PBS	72.36 ± 18.87
AP205 VLP	54.64 ± 22.42
pE$_3$Aβ VLP	70.92 ± 19.87

Quantification of activated Iba-1$^+$ microglial cells in proximity to Aβ42 plaque accumulations in the NCTX of eight-month-old 5xFAD mice (mean ± SD).

IV. DISCUSSION

AD is a neurodegenerative disease that leads to progressive memory impairments and cognitive decline. Over 55 million individuals are currently affected by dementia. It is estimated that AD contributes to around 60-70% of all dementia cases worldwide (Greenblat, 2025).

This study aimed to investigate and evaluate novel active immunization based on the VLP AP205 platform in a preclinical model of FAD. The idea was based on creating an effective treatment modality directly influencing the "root of the problem". The administration of VLP-based vaccines is considered safe and effective, eliciting a strong humoral immune response that facilitates the efficient removal of pathogenic formations in the host organisms (Kheirvari, Liu and Tumban, 2023). Compared to passive immunotherapy, which requires repeated and costly invasive administrations, these active immune responses with targeted antibody generation offer more effective and sustainable therapeutic outcomes (Zagorski *et al.*, 2023).

Within this study, we discovered that active immunization of adult female 5xFAD mice with either AP205 VLP carrier or the pE$_3$Aβ VLP vaccine candidate did not produce improvement in behavioral impairments associated with FAD at the age of six and eight months. These findings did not correspond to findings of previous studies, which reported that active immunization against the Aβ plaque burden resulted in significant reductions of cerebral Aβ accumulations and was simultaneously connected with improved AD-related behavioral impairments in 5xFAD mice (Bach *et al.*, 2009; Fu *et al.*, 2017; Bakrania *et al.*, 2022). One possible explanation for the lack of behavioral improvement might be the age of the mice at the time when the behavioral tests were conducted. The ages of six and eight months might have been too early to detect AD-characteristic behavioral changes. Other authors have also reported that six-month-old 5xFAD mice exhibit rather mild memory impairments at that age (Oblak *et al.*, 2021; O'Leary and Brown, 2022; Faisal *et al.*, 2023). Oblak and colleagues further reported that the 5xFAD mice they studied showed only mild and inconsistent cognitive impairments even at the age of twelve months (Oblak *et al.*, 2021). In their study, Forner *et al*. observed that even when cognitive impairments began to appear between the age of four and five months in their 5xFAD mice, a reduced motor performance, such as in maximal speed and total distance travelled in the OF only became apparent at an age of 18 months (Forner *et al.*, 2021). Similarly, O'Leary and Brown reported that impaired learning was detected in 5xFAD mice between five to nine months of age, whereas motor impairments became apparent between 12 and 15 months of age (O'Leary and Brown, 2022).

Next, we assessed the biochemical effects of active anti-Aβ immunization. Using the immunofluorescent examination of the cerebral tissues of female 5xFAD mice, we discovered

that $pE_3Aβ$ VLP-treatment significantly reduced Aβ burden in the NCTX of adult female 5xFAD mice. **By effectively reducing — and thereby delaying — the main underlying pathological process of AD, the $pE_3Aβ$ VLP-vaccine candidate demonstrated disease-modifying effects** (EMA, 2016; Cummings and Fox, 2017)**.**

A significant reduction in astrogliosis in the NCTX was also observed following treatment with $pE_3Aβ$ VLP. Considering that Aβ42 stimulates astrocytic activation (Frost and Li, 2017; Lennol *et al.*, 2021; Mun, Park and Choi, 2024), it might be possible that a reduction of Aβ42 was associated with reduced numbers of activated astrocytes.

Although the number of activated microglial cells in the NCTX and DG of all mice were not significantly different, microglial cells were predominantly localized near $Aβ42^+$ plaques in $pE_3Aβ$ VLP-treated 5xFAD mice. In this group, the count of activated microglial cells was slightly, but not significantly, higher in the NCTX compared to the sham-treated group. **The fact that in that group a statistically significantly lower Aβ42 plaque count was observed in the NCTX suggests that enhanced microglial-mediated Aβ clearance resulted from the active immunization.** Microglial activation has been associated with cerebral Aβ removal in mouse models (Wilcock *et al.*, 2004; Guan *et al.*, 2022) and in humans (van Olst *et al.*, 2025). In a study by Zamora *et al.*, similar results were reported: decreased amyloid load and increased numbers of microglial cells following anti-Aβ-VLP vaccine administration in APP/PSI transgenic mice (Zamora *et al.*, 2006). Also, multiple other groups reported increased microglial activation following Aβ immunotherapies, and this activation resulted in decreased numbers of Aβ plaques in cerebral tissues (Su *et al.*, 2022; Cummings *et al.*, 2023; Loeffler, 2023). **Overall, we and other authors show that microglia are co-localized with Aβ plaques in the NCTX and DG regions, highlighting the key role of microglia in amyloid clearance.**

This study highlights both the importance and effectiveness of the active VLP-based approach to Alzheimer's treatment. It demonstrates the efficacy of active anti-Aβ immunization in reducing $Aβ42^+$ plaques in one cerebral region of adult female 5xFAD mice. At the same time, this study shows that although a significant reduction of Aβ burden is not associated with improved cognitive outcomes in 5xFAD mice.

This study has potential limitations.

The chosen sample size in this study was n=12-13 individuals per group. Curtis *et al*. define group sizes of n<20 as small (Curtis *et al.*, 2015). Smaller sample sizes may tend to not create statistically significant results due to the lack of statistical power (Sullivan, Weinberg, and Keaney, 2016).

Within our study, only adult female 5xFAD mice were used, so it remains unknown how male individuals would have reacted toward the different treatments. In several studies, it was found that female 5xFAD mice presented higher amounts of Aβ plaques within brain tissues compared to same-age male 5xFAD subjects (Forner *et al.*, 2021; Sil *et al.*, 2021; Hu *et al.*, 2023; Poon *et al.*, 2023). No completely healthy age-matched adult female wild-type control subjects were included in this study, which could have provided more clarity on the severity of the disease in 5xFAD mice treated with PBS only.

The animals were examined until eight months of age to assess early and mid-stage treatment effects. Until then, changes within brain tissues were seen, but no significant changes in behavioral patterns occurred. Extending the observation period beyond eight months, as done in other studies (Ismeurt, Giannoni and Claeysen, 2020; Szu et al., 2020; Forner et al., 2021; Jullienne et al., 2023; O'Leary and Brown, 2024), could provide a better understanding of potential treatment effects throughout the disease progression in older 5xFAD mice. **Longer investigation periods allow further evaluation of long-term outcomes, such as the progression of behavioral changes, reduction of Aβ plaques, and late-onset treatment effects.**

Despite the identified limitations of our study, the obtained results remain valid for addressing the central research questions. We used established protocols and the well-accepted transgenic 5xFAD mice model to assess the Aβ42 plaque burden, astrocytic, and microglial activation.

All animals in the three groups were handled under identical conditions, ensuring observed differences can be connected to the administered treatment. Standardized procedures were followed to minimize bias. The obtained results align with findings from similar research studies, supporting their consistency and relevance.

Our obtained results suggest that future studies could benefit from including both sexes of 5xFAD mice to provide a better understanding of how sex-specific factors may influence treatment outcomes. An extended research duration would allow the investigation of whether notable tissue-level changes observed in early stages of the disease correlate with enhanced performance in behavioral assessments as the animals age. Including healthy wild-type controls in future studies is crucial to enable a clearer distinction between the regular disease progression in sham-treated 5xFAD mice and completely healthy littermates.

Based on the findings of this study, future research in the field of AD should focus on exploring VLP-based immunization as a potential disease-modifying treatment. This approach could hold promise in halting the disease progression of AD pathology.

V. CONCLUSIONS

1. The $pE_3A\beta$ VLP vaccine candidate significantly reduced Aβ plaque formation, as well as astroglial activation in the NCTX of 5xFAD mice.
2. Microglial activation was not ameliorated by the $pE_3A\beta$ VLP treatment, either in the NCTX or in the DG.
3. The absence of significant cognitive improvements following $pE_3A\beta$ VLP treatment suggests that the vaccine candidate only provided mild therapeutic efficiency in 5xFAD mice.
4. Our findings emphasize the need for further, longitudinal research of active immunization using VLP-based vaccines in 5xFAD mice of both sexes to further understand the potential of this therapeutic approach.

ACKNOWLEDGMENTS

At this point, I would like to sincerely thank all those people who have joined and supported me throughout my journey of medical studies.

I am particularly grateful to my supervisor and lecturer, Dr. med. Vladimirs Piļipenko, for his exceptional guidance and support throughout the entire process of conducting and completing my diploma thesis.

Additionally, I would like to express my gratitude to the Faculty of Medicine and Life Sciences of the University of Latvia, as well as all my professors and lecturers, who enabled me to fulfill my dream of studying human medicine and for sharing their personal knowledge and experiences with me during my academic pathway.

I would like to express my sincere gratitude to the State Education Development Agency (Valsts izglītības attīstības aģentūra) for their financial assistance during my last year of medical studies provided through the Latvian State Scholarship.

I would also want to thank the laboratory mice, without whom this study would not have been possible.

Finally, I would like to express my deepest gratitude to my parents, my partner, and my friends for their endless support, encouragement, and patience during every stage of my academic journey.

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DOCUMENTATION PAGE

This Diploma Thesis, "**Investigation of a Novel Active Immunization Approach in a Transgenic Mouse Model of Alzheimer´s Disease**" was developed at the Faculty of Medicine and Life Sciences of the University of Latvia.

With my signature, I attest, that this research has been carried out without aid or assistance. Used information was obtained only from indicated sources and the electronically submitted copy of this diploma work complies with printout.

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The diploma thesis is presented at the meeting of the State Examination Commission of Higher Professional Study Program "Medicine" on May 19th, 2025.

Protocol No. _____

Secretary of Commission: _____ _____

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