Early Diagnosis of AD by Detecting Amyloid-Beta in the Retina of AD-Induced Mice Using IR Spectroscopy

Author

Zuha Waheed

Regn Number

329584

Supervisor

Dr. Aneeqa Noor

DEPARTMENT OF BIOMEDICAL ENGINEERING AND SCIENCES

SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY

ISLAMABAD

SEPTEMBER 2023

Early Diagnosis of AD by Detecting Amyloid-Beta in the Retina of AD-Induced Mice by Using IR Spectroscopy

Author

Zuha Waheed

Regn Number

329584

A thesis submitted in partial fulfillment of the requirements for the degree of

MS Biomedical Sciences

Thesis Supervisor:

Dr. Aneeqa Noor

Thesis Supervisor's Signature: _____

DEPARTMENT OF BIOMEDICAL ENGINEERING AND SCIENCES

SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY,

ISLAMABAD

SEPTEMBER, 2023

THESIS ACCEPTANCE CERTIFICATE

Certified that final copy of MS/MPhil thesis written by **Regn No. 00000329584 Zuha Waheed** of **School of Mechanical & Manufacturing Engineering (SMME)** has been vetted by undersigned, found complete in all respects as per NUST Statues/Regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfillment for award of MS/MPhil degree. It is further certified that necessary amendments as pointed out by GEC members of the scholar have also been incorporated in the said thesis titled, **Early Diagnosis of AD by Detecting Amyloid-beta in the Retina of AD Induced Mice Using IR Spectroscopy.**

Signature: 

Name (Supervisor): Aneeqa Noor

Date: 04 - Sep - 2023

Signature (HOD): 

Date: 04 - Sep - 2023

Signature (DEAN): 

Date: 04 - Sep - 2023

Declaration

I certify that this research work titled "*Early Diagnosis of AD by Detecting Amyloid-Beta in the Retina of AD-Induced Mice by Using IR Spectroscopy"* is my work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged/referred to.

Signature of Student

Zuha Waheed

Regn No. 00000329584

MS Biomedical Sciences

Proposed Certificate for Plagiarism

It is certified that the MS Thesis Titled **Early Diagnosis of AD by Detecting Amyloid-Beta in the Retina of AD-Induced Mice by Using IR Spectroscopy** by Zuha Waheed has been examined by us. We undertake the following:

a. Thesis has significant new work/knowledge as compared to already published or is under consideration to be published elsewhere. No sentence, equation, diagram, table, paragraph, or section has been copied verbatim from previous work unless it is placed under quotation marks and duly referenced.
b. The work presented is original and own work of the author (i.e., there is no plagiarism). No ideas, processes, results, or words of others have been presented as the Author's own work.
c. There is no fabrication of data or results which have been compiled/analyzed.
d. There is no falsification by manipulating research materials, equipment, or processes, or changing or omitting data or results such that the research is not accurately represented in the research record.
e. The thesis has been checked using TURNITIN (copy of originality report attached) and found within limits as per HEC plagiarism Policy and instructions issued from time to time.

Name & Signature of Supervisor

Dr. Aneeqa Noor

Signature: 

Plagiarism Certificate (Turnitin Report)

This thesis has been checked for Plagiarism. The Turnitin report endorsed by the Supervisor is attached.

Signature of Student

Zuha Waheed

Registration Number

329584

Signature of Supervisor

Copyright Statement

Acknowledgments

I am thankful to my Creator Allah Almighty for bestowing me the willpower as well as the direction to conduct this project.

I am profusely thankful to my beloved parents who are my ultimate support system.

I would like to express special thanks to my supervisor Dr. Aneeqa Noor for her help throughout my thesis. Her exceptional problem-solving skills, gentle teaching methods, and constant support have made this work possible. She is indeed an inspiration for all her students.

I am grateful to my seniors Hassan Nawazish and Sameen Mehtab for their tremendous support and cooperation. Each time I got stuck in something; they came up with a solution. Without their help, I wouldn't have been able to complete my thesis.

I would also like to thank Dr. Adeeb Shehzad and Dr. Asim Waris for being on my thesis guidance and evaluation committee. I am utterly grateful to Dr. Saima Zafar for motivating and guiding me throughout my thesis.

Finally, I would like to express my gratitude to all the individuals who have rendered valuable assistance to my study.

Dedicated to my exceptional parents and adored siblings whose tremendous support and cooperation led me to this wonderful accomplishment.

Table of Contents

Proposed Certificate for Plagiarism ..... **iii**

Plagiarism Certificate (Turnitin Report) ..... **iv**

Copyright Statement ..... **v**

Acknowledgments ..... **vi**

List of Figures ..... **x**

List of Abbreviations ..... **xi**

Abstract ..... **xiv**

1. INTRODUCTION **2**

1.1. Alzheimer's Disease 2

1.2. Epidemiology 3

1.3. Pathophysiology of AD 5

1.3.1. Cholinergic Hypothesis 5

1.3.2. Tau Hypothesis 6

1.3.3. Amyloid Hypothesis 7

1.4. Diagnosis of AD 8

1.5. Etiology 10

1.5.1. Aging 10

1.5.2. Genetic Fators 11

1.5.3. Environmental Factors 11

1.6. Pathophysiology Caused by Aluminum 12

1.7. Aims of the Current Research Study 13

1.7.1. Proposed Solution 13

2. METHODOLOGY **17**

2.1. Ethical Approval 17

2.2. IR-Based Device 17

2.2.1. Mechanical Design 17

2.2.2. Circuiting 17

2.2.3. Device Modification and Calibration 20

2.3. Preparation of AD-like Rodents 20

2.3.1. Animal Grouping and Caging 20

2.3.2. Chemical Dosage 20
2.4. Behavioral Testing 21
2.4.1. Y-Maze Test 21
2.4.2. Novel Object Recognition Test (NORT) 21
2.4.3. Morris Water Maze Test (MWM) 22
2.5. Mice Dissection 22
2.6. Histological Staining of Brain and Retina 22
2.6.1. Hematoxylin and Eosin Staining (H&E) 22
2.6.2. Thioflavin T (ThT) Dye Staining 23
2.7. Microscopy 23
2.8. Device Testing on Rodents 23
2.9. Statistical Analysis 24
3. RESULTS 26
3.1. Memory Retention and Learning Behaviors of $AlCl_3$-Treated Mice 26
3.1.1. Morris Water Maze Test 26
3.1.2. Y-maze Test 27
3.1.3. Novel Object Recognition Test (NORT) 27
3.2. Confirmation of the Presence of Aβ in the Retina and Brain 29
3.3. Validation of Protein Aggregations in the Retinal Tissue by IR Radiation 32
4. DISCUSSION 35
4.1. Challenges for Developing AD Diagnostic Tools 35
4.2. Eye Serves as a Window to the Brain 36
4.3. Aluminum-treated Rodents Show Signs of AD 37
4.4. Reflected IR Contributes to the Detection of Aβ 40
4.5. Conclusion 41
References 42

List of Figures

Figure 1: Comparison of a Healthy and AD Brain 3
Figure 2: Prevalence of AD Dementia in Different Countries 4
Figure 3: Acetylcholine Synthesis in the Synapses 6
Figure 4: Formation of Neurofibrillary Tangles 7
Figure 5: Formation of Amyloid Senile Plaques in the Brain 8
Figure 6: Aluminum Distribution in the Brain 13
Figure 7: Circuit Diagram 18
Figure 8: Hardware Device Design 19
Figure 9: Schematic of Ocular IR-based Device Testing on Mice 24
Figure 10: Morris Water Maze Test 26
Figure 11: Y-maze Test 27
Figure 12: Novel Object Recognition Test (NORT) 28
Figure 13: Hematoxylin and Eosin (H&E) Staining 30
Figure 14: Thioflavin T (ThT) Staining 31
Figure 15: Amyloid to Cell Ratio in Mice Cortex, Hippocampus and Retina 32
Figure 16: Reflected IR 33

List of Abbreviations

AD	Alzheimer's Disease
Aβ	Amyloid beta
NFTs	Neurofibrillary tangles
SPs	Senile plaques
IR	Infrared
ThT	Thioflavin T
H&E	Hematoxylin and eosin
LMIC	Lower middle-income countries
CVDs	Cardiovascular diseases
ChAT	Cholinesterase acetylcholine transferase
APP	Amyloid precursor protein
MMSE	Mini mental-state exam
ESR	Erythrocyte sedimentation rate
LFTs	Liver function tests
TFTs	Thyroid function tests
ECG	Electrocardiogram
CT	Computer tomography
MRI	Magnetic resonance imaging
PET	Positron emission tomography
EEG	Electroencephalogram
APOE	Apolipoprotein
CO	Carbon dioxide
NOx	Nitrogen oxides
SO_2	Sulfur dioxide
PM	Particulate matter
Al	Aluminum
PD	Parkinson's Disease
ROS	Reactive oxidative species

LD_{50}	Lethal dose of 50
BBB	Blood-brain barrier
BAF	Blue light autofluorescence
OCT	Optical coherence tomography
SLO	Scanning laser ophthalmoscopy
NIR	Near-infrared
CNS	Central nervous system
MS	Multiple sclerosis
OCD	Obsessive-compulsive disorder
LED	Light emitting diode
$AlCl_3$	Aluminum chloride
PLA	Polylactic acid
NORT	Novel object recognition test
MWM	Morris water maze
PBS	Phosphate buffer solution
PFA	Paraformaldehyde
ANOVA	Analysis of variance

Abstract

Accumulation of amyloid beta (Aβ) protein in the cerebral region of the brain is one of the hallmarks of AD and appears 15 to 20 years before the appearance of any symptoms of AD. This prolonged asymptomatic phase of AD gives a huge potential for the development of early detection and screening methods for AD. In addition to this, the fact that Aβ peptide also accumulates in the retina and leads to visual impairment, has shifted scientists' focus on the eye to be used as a diagnostic tool. Over the last decade, the use of infrared (IR) rays to detect and study amyloid plaques has opened new avenues in the field of neuroscience. The current study is designed to validate the diagnosis of AD by detecting Aβ in the retina using IR rays on $AlCl_3$-treated rodent models. Behavioral tests were conducted to confirm symptoms of AD such as impaired cognition and spatial learning induced in rodents, followed by mice dissection to perform histology including H&E and Thioflavin T (ThT) staining of their retinal and cerebral tissues for confirmation of the presence of amyloid aggregations. Rodents' eyes were exposed to IR light for the detection of amyloid plaques by the amount of IR radiation reflected. The presence of the plaques in the retina was confirmed by the reflectance percentage of IR light, the more the plaques in the retina, the lower the IR percentage reflectance as it would be absorbed by the aggregations encountered by IR radiation. When compared to healthy mice, AD mice showed three to five times less reflectance percentage of IR light. The findings of this study predict the utility of IR spectroscopy in the diagnosis of AD and other neurodegenerative diseases.

Key Words: *Amyloid-beta protein, Alzheimer's Disease, infrared spectroscopy, AD diagnosis, AD-mice models, amyloid aggregations*

CHAPTER 1
INTRODUCTION

1. INTRODUCTION

1.1. Alzheimer's Disease

Alzheimer’s disease (AD) is a complex neurodegenerative disorder that results in a decline in normal brain functions over time. It causes memory loss and affects the cognitive ability of the brain to the point that a person is unable to perform ordinary daily life functions such as moving around or feeding oneself. AD has been conceptualized as a disease continuum, meaning that it undergoes a long preclinical or asymptomatic phase, where the symptoms of AD do not appear for more than 10 to 15 years from the disease onset. Still, the neuronal changes at the molecular level start developing and by the time the signs of AD start appearing in the body, the condition at the molecular level is immensely damaged (Breijyeh et al., 2020).

In AD patients, cognition and memory impairment occur with advancement in age; however, the rate of this decline becomes double than that occurring in a normal individual and worsens with age. It is most common in older people aged 65 or above and less common in younger. The warning signals of AD include misplacing things more often and not being able to recall where to find them, memory loss to the extent that the individual is unable to recall familiar places or people, repeats questions and has trouble completing normal tasks.

An AD brain suffers from atrophy or shrinkage of the cerebral cortex, responsible for processing information and language, as well as shrinkage of the hippocampus, responsible for memory formation. The gyri, which are ridge-like elevated structures on the brain surface are narrowed, while the sulci, depression, or groove that separates the temporal lobe from the parietal and frontal lobes, is widened because of neuronal cell death. In addition to the neuronal loss, hindrance in neuronal communication occurs due to the deposition of aggregated and misfolded proteins in the synapses which further promotes cell death. The two proteins called tau forming neurofibrillary tangles (NFTs) and amyloid-beta (Aβ) forming senile plaques (SPs), that accumulate in the brain are the major molecular characteristic features of AD (Murphy et al., 2010).

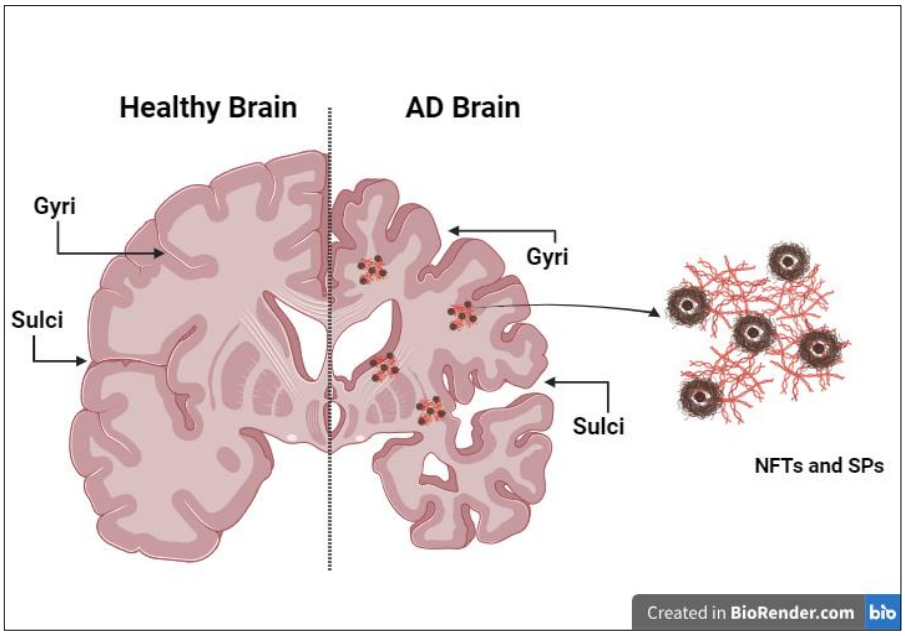

Figure 1: Comparison of a Healthy and AD Brain. Unlike a healthy brain, the AD brain undergoes excessive neurodegeneration along with the presence of aggregated senile plaques (SPs) and neurofibrillary tangles (NFTs).

1.2. Epidemiology

Dementia is one of the most significant social and health crises of the 21st century. At present, around 50 million people are living with AD, worldwide (Livingston et al., 2020). The present rate of AD patients suggests that the number of people suffering from AD becomes two-fold every 5 years (Monfared et al., 2022). The number of people with AD is predicted to exceed more than double what it is today by the year 2060. Globally, the prevalence of dementia and AD cases is increasing substantially, but this rate is dependent on the socioeconomic factors of a region as well. It has more than doubled since the year 1990 majorly because of bigger population size and increasing ages (Livingston et al., 2020).

In Europe, a study was conducted in 2017 that reported a higher occurrence of AD dementia in women. The meta-analysis that was carried out reported 3.31% prevalence in men, and 7.13% in women (Niu et al., 2017). Previously, a similar study conducted by China in 2013, also showed a higher prevalence in women (Chan et al., 2013). There might be an association of ethnicity as well with how AD dementia develops in individuals as reported by a population study conducted on clinically diagnosed AD patients from 1994 to 2012 in the US. The findings suggested twice the prevalence of

AD in African Americans than in European Americans (Rajan et al., 2019). The Project Alzheimer's Value Europe (PAVE) consortia reviewed the information available on the prevalence of AD and proposed best estimates on the overall number of people, globally across the AD continuum to give an idea of worldwide AD prevalence (Gustavsson et al., 2023).

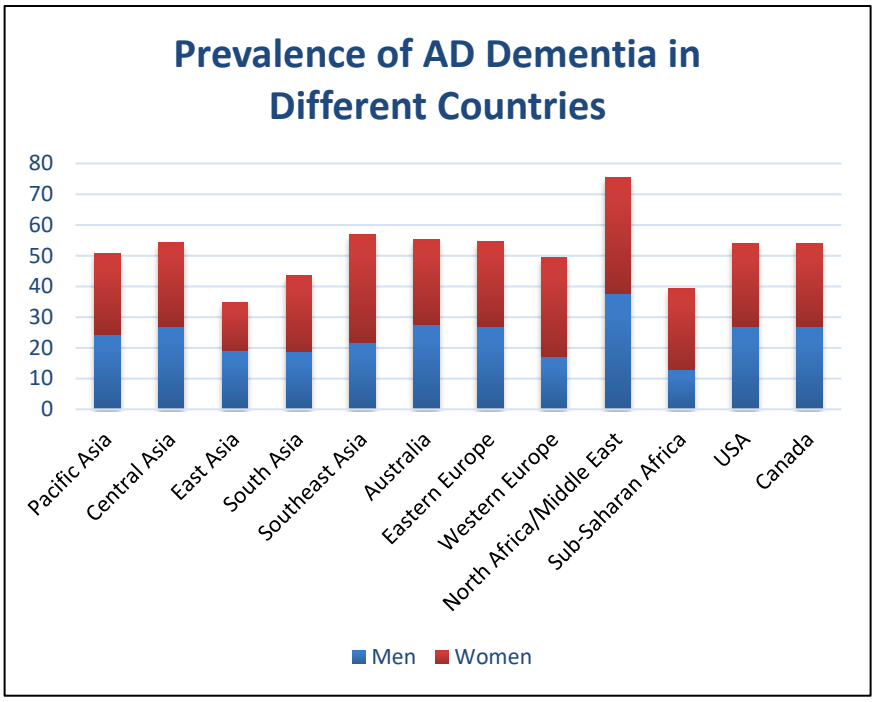

Figure 2: Prevalence of AD Dementia in Different Countries. This chart shows the mean of the percentage of prevalence of AD in men and women in different countries between age groups ranging from 65 to 89, assuming that 70% of the general dementia patients have clinical AD (Gustavsson et al., 2023).

In lower-middle-income countries (LMIC), including Pakistan, two-thirds of the individuals are living with dementia, presently. Less than 10 percent of people get AD diagnosis in such countries. AD symptoms are widely mistaken for aging due to a lack of awareness and a poor healthcare system, consequently increasing the number of people living with AD at an alarming rate (Mayeux et al., 2012). According to the Alzheimer's Association, this number was over 850,000 in 2015, while around 1 million people currently suffer from dementia, and may become more than 3 fold by the year 2050 if no preventive measure is carried out (Azhar, 2023).

People suffering from other health problems and trauma are at higher risk of developing AD, and unfortunately, a major proportion of the population in LMICs comes under

such category. There is a 7% higher risk of onset of AD with CVDs that result in stroke followed by destruction of cells in the thalamus, cortex, and hippocampal areas along with deposition of plaques leading to Alzheimer-type pathology (Pendlebury et al., 2009). Similarly, mid-life consistent hypertension has been reported to cause the development of late-life AD (Ruth et al., 2010). Type II Diabetes is reported to have a direct association with the accumulation of Aβ in the brain due to hyperinsulinemia that disrupts the clearance of Aβ protein (Profenno et al., 2010). Obesity directly impacts CVD development and diabetes which in turn are risk factors for AD (Atti et al., 2008). Smoking and drugs are common in LMICs as well that hinder the production of neurotransmitters, disrupt the cholinergic pathway, and ultimately lead to dementia (Mayeux et al., 2012).

1.3. Pathophysiology of AD

The rate of decline of the hippocampal as well as cortical regions of the brain have been used as a marker to predict the progression of AD, however, the pathways that lead to such atrophy are understudied. Because of complexity and limited access to the central nervous system (CNS), scientists are still struggling to get a full understanding of the mechanisms behind neurodegeneration in brain disorders. However, they have come up with several hypotheses that give logical reasoning to the occurrence of neuronal cell death (Finder et al., 2007).

1.3.1. Cholinergic Hypothesis

Acetylcholine is a chief neurotransmitter of the parasympathetic nervous system that is responsible for many important body functions including learning and memory (Sam et al., 2023). It is released into the synapse by a pre-synaptic neuron, acting as a mode of transmission of impulse from one neuron to another through the synapse. The cerebral cortex and hippocampus of the brain have cholinergic neurons, releasing an enzyme called choline acetyltransferase (ChAT) in their soma that travels to the axon terminal (Da Jeong et al., 2014; Maurer et al., 2017). ChAT in the axon terminal combines choline, a nutrient transported into pre-synaptic neurons by transporter protein in the membrane, with acetyl coA, to produce acetylcholine. The membrane of the post-synaptic neuron has cholinergic receptors to which the neurotransmitter, acetylcholine, binds and the impulse is transmitted. In the synapse, the excess acetylcholine is

hydrolyzed by the enzyme cholinesterase (Sam et al., 2023). In AD, there is less ChAT production, less choline, or an increased amount of cholinesterase enzyme, all resulting in decreased synthesis of acetylcholine. Experiments have shown that atrophy or injuries to the cortical regions of the brains of rodents and non-human primates lead to the introduction of attention deficit (Gonzalez et al., 2014).

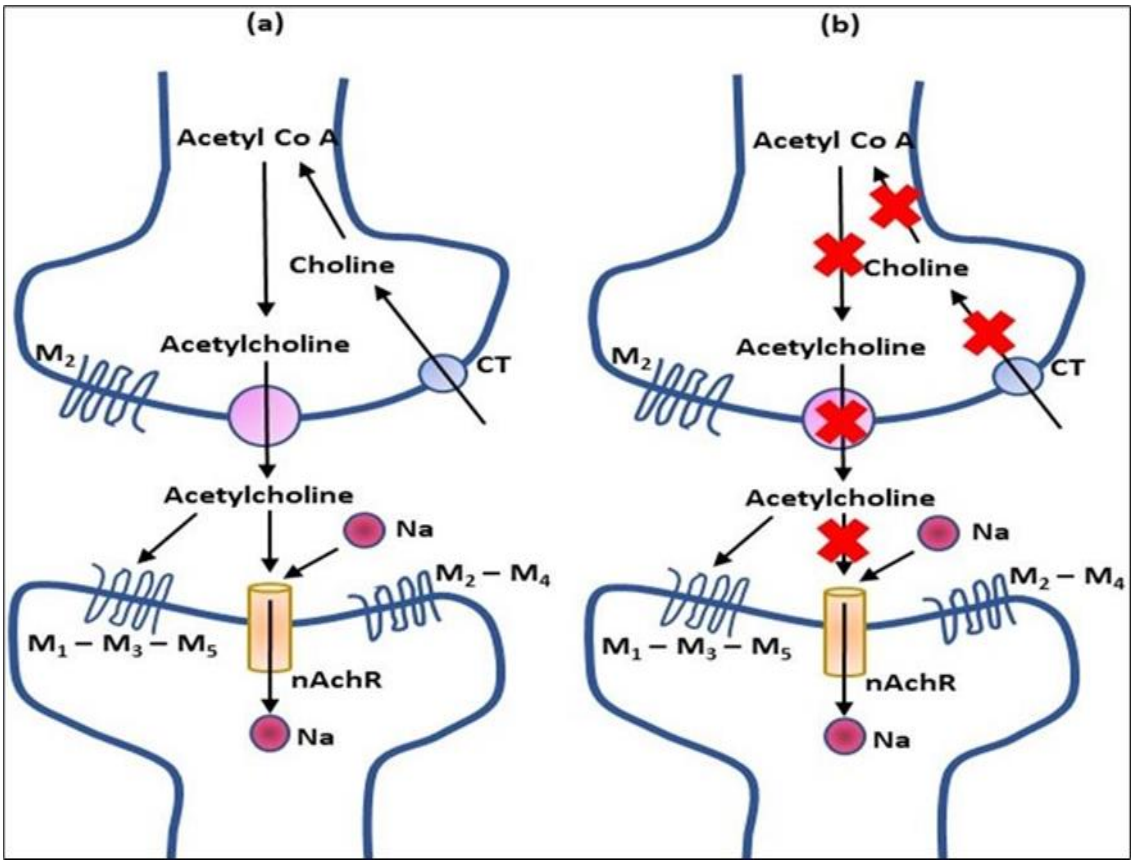

Figure 3: Acetylcholine Synthesis in the Synapses. (a). Healthy Synapse. In normal neurons, acetylcholine is produced which aids in neuronal communication. **(b). AD Synapse**. In AD, acetylcholine is released in less amount than normal which hinders neuronal communication.

1.3.2. Tau Hypothesis

Hyperphosphorylation of tau proteins that result in the formation of neurofibrillary tangles (NFTs) is considered an important hallmark of AD (Gong et al., 2008). Microtubules in the axons function as the cellular highway as they play an important role in the transmission of cellular products from the soma of neurons to terminals and vice versa. These microtubules have tau proteins on their surface to help them provide a stable structure. When, however, excess amyloid beta is formed, it hyper-

phosphorylates the tau proteins which break off, making the microtubule structure unstable and ultimately collapse (Hanseeuw et al., 2019). Hyper-phosphorylated tau proteins aggregate to form NFTs that hinder intracellular communication and promote neurodegeneration (Liu et al., 2020). Misfolded tau proteins promote further misfolding of the rest of the tau proteins, thus resulting in the spread of pathology across the brain.

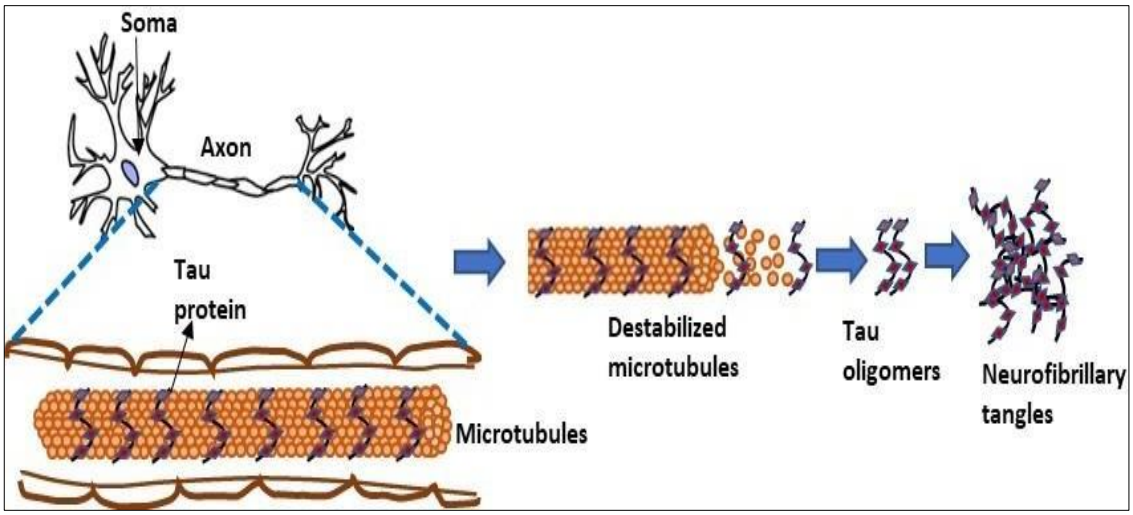

Figure 4: Formation of Neurofibrillary Tangles. Microtubules in the axons are stabilized by tau proteins. When tau is hyperphosphorylated, they break off from microtubules making them unstable and aggregating to form neurofibrillary tangles.

1.3.3. Amyloid Hypothesis

According to the amyloid hypothesis, there is a disruption in the amyloid cascade that produces extracellular senile plaques (SPs). These plaques are known to be produced due to the presence of amyloid beta protein fragments that should be degraded. The neuronal cell membranes have important surface proteins known as amyloid precursor proteins (APP) that are essential for cell growth and repair of neuronal cells after injury. Like all other proteins, APP should be degraded and disposed of after a certain time that occurs through two major pathways: the non-amyloidogenic and amyloidogenic pathways. In the non-amyloidogenic pathway, the membrane protein APP is cleaved sequentially by α-secretase and γ-secretase enzymes into certain regions, further broken down into smaller fragments that are disposed of easily by the body. In the amyloidogenic pathway, APP is degraded by β-secretase and γ-secretase into an Aβ monomer composed of 36 to 43 amino acids, which needs to be broken down through phagocytosis by microglial cells, and subsequently eliminated from the body. Receptor-

mediated internalization occurs by astrocytes and protease neprilysin also plays a crucial role in its degradation. In AD, Aβ starts accumulating due to its increased rate of production and decreased breakdown due to neurodegeneration of microglial cells and astrocytes along with inhibition of neprilysin. When excessive Aβ is produced, it aggregates together to form SPs that block the synapse, hindering the transmission of neurotransmitters from the pre-synaptic to the post-synaptic neuron. The lack of communication between the neurons makes them non-functional and the neuronal cells ultimately die. Microglial cells that help provide nutrition to neurons and aid in protecting cells by functioning as immune cells may malfunction. The aggregated Aβ is taken up by microglial cells, but this phagocytosis makes it abnormal, and it starts releasing lots of cytokines that damage the surrounding neurons causing cell lysis (Hampel et al., 2021).

Figure 5: Formation of Amyloid Senile Plaques in the Brain. Amyloid beta (Aβ) is produced by amyloid precursor protein (APP) by cleavage from β and γ-secretase that forms plaques and ultimately results in the formation of senile plaques.

1.4. Diagnosis of AD

Clinical diagnosis of AD is based on the signs and symptoms shown by the patient which are divided into two categories. First is a neuropsychological component consisting of aphasia, the inability to speak or communicate correctly, apraxia, the inability to carry out normal tasks although the sensory and motor nervous systems are intact, amnesia, the impairment of memory particularly the short-term memory, and

agnosia, the inability to recognize oneself or family members. The second component is neuropsychiatric which is associated with disturbances in the behavior and consists of misidentifications and paranoid ideation based on agnosia which is sometimes coupled with the belief that the objects or people are duplicated. It also includes hallucinations, most commonly auditory and rarely visual, along with aggression, and wandering (Burns, 2000; Bomasang et al., 2021).

Vascular dementia is memory loss due to damage in the blood vessels because of some injury or trauma and has overlapping signs and symptoms with AD. In AD patients, the condition progresses slowly over a long period, while in patients suffering from vascular dementia, the disease onset is often abrupt, and there may also be a history of strokes and hypertension. Unlike AD, in which the earliest signs are cognitive in nature, especially short-term memory impairment, dementia consists of changes in personality such as increased aggression and rigidness, diminished emotional control and responsiveness, and hyperactivity. Patients also have a greater tendency towards anxiety and depression in vascular dementia. However, all these signs overlap for both AD and dementia in the later stages (Bature et al., 2017).

Collecting comprehensive information regarding the patient's behavior is crucial to making a correct diagnosis. Investigating the patient is useful to get evidence regarding aphasia, the mental state of the patient, and the presence of any other anxieties. Doctors and other healthcare professionals commonly use a Mini-Mental State Examination (MMSE) of 11 questions and activities to check for cognitive impairment. It assesses 6 areas of mental abilities: attention and concentration, the orientation of time and place, short-term memory, speech ability, visual and spatial ability between the objects, and the ability to understand and follow instructions. The maximum score for MMSE is 30, where a score above 25 is considered normal, and below 25 indicates the presence of possible cognitive impairment. However, it is not a reliable test for every case, since the impairment related to memory, learning, and speech can also be due to any other abnormality (Gluhm et al, 2013).

Detailed physical examination tests including blood and urine screening, erythrocyte sedimentation rate (ESR), blood count, liver function tests (LFTs), thyroid function tests (TFTs), chest X-ray, electrocardiogram (ECG), etc., help rule out any other abnormality.

Indirectly the assessment of signs of the presence of any lesions and neurodegeneration can be identified using imaging techniques. Computed Tomography (CT) scanning aids in investigating intracranial lesions, and a further detailed picture of cerebral structures can be gathered from Magnetic Resonance Imaging (MRI) (Lombardi et al., 2020). Frontal lobe dementia and deficits in the blood flow throughout CNS can be diagnosed using a Positron Emission Tomography (PET) scan. In addition, Electroencephalography (EEG) is sensitive to the detection of delirium, which is a state of confusion, hallucination, and agitation (McKhann et al., 1984; Oostveen et al., 2021). Diagnosing AD as early as possible is important because there is no proper cure to revert this complex brain disorder. However, it is easier to manage AD at the initial stage and there are clinically approved medicines that can treat the initial symptoms of AD. If untreated, the condition of AD worsens with time, and it becomes irreversible. Therefore, there is a need to conduct studies on AD to help gain in-depth knowledge for better and more direct diagnostic approaches and accurate treatment methods.

1.5. Etiology

1.5.1. Aging

Aging is considered one of the major risk factors for AD. It is an irreversible, continuous, gradual process of natural changes in the body involving a decline in the overall potential of bodily functions due to a decrease in the rate of cell division and proliferation, telomere shortening (Hara et al., 2019), and an increase in oxidative stress. Like all other organs of the body, the brain also undergoes chemical and structural changes due to the process of aging (Peters et al., 2006).

At the cellular level, neurons slowly become less active. Myelin sheath starts deteriorating, dendrites become less branched, leading to smaller networks throughout the brain and the number of synapses between neurons also tails off over time. Brian shrinks in size, with some parts undergoing more atrophy than others, including the prefrontal cortex, cerebellum, and hippocampus. The cerebral cortex also becomes thinner with a more noticeable effect on frontal and temporal lobes.

Chemical changes include increased oxidative stress, and reduced release of acetylcholine, dopamine, serotonin, and other neurotransmitters, along with a decline in the receptors to which the neurotransmitters are supposed to bind. Consequently, these

structural and chemical changes affect the functions of the brain. Over time, gradual loss of memory, ability to process information, decline in focus due to distractions, and decrease in problem-solving capabilities start to appear. Since these signs of aging are like the symptoms of AD, it is one reason why the disease is difficult to distinguish. Unlike aging, these symptoms appear and worsen relatively faster with time in AD (Li et al., 2022).

1.5.2. Genetic Fators

A major impact on the increased risk of AD is the presence of apolipoprotein ε4 gene (APOE ε4) (Husain et al., 2021). It has 3 allele variants: ε2, ε3, ε4 that make 6 possible combinations i.e., ε2/ε2, ε2/ε3, ε2/e4, ε3/ε3, ε3/ε4, and ε4/ε4. Any combination having an ε2 variant lowers the risk of the onset of AD more than other combinations as it is known to be a protective allele, however, it is also a rare allele. On the other hand, when the ε4 allele is present in any combination, the risk of onset of AD is higher, while the risk due to the ε3 allele is lesser than that due to ε4 (Yamazaki et al, 2019; Husain et al., 2021). APOE is synthesized by microglial cells and astrocytes in the CNS and helps transport cholesterol to the neurons. Upon its lipidation by ATP binding cassette A1 (ABCA1) transporters, it binds to Aβ, facilitates its internalization by the glial cells, and disrupts Aβ clearance at the blood-brain barrier (BBB) (Liu et al., 2013). It is important to note that genetics is not the basis of AD, but rather one of the factors that could cause a higher probability of its development. Like other hereditary diseases, the chances are higher when AD is in siblings or parents than when it is in other relatives (Troutwine et al., 2022).

1.5.3. Environmental Factors

Environmental factors like infections, air pollution, diet, etc. cause oxidative stress and inflammation. Several studies on animal and cellular models showed signs and symptoms like AD when they were exposed to high levels of carbon monoxide (CO), nitrogen oxides (NOx), sulfur dioxide (SO_2), and particulate matter (PM) (Mir et al., 2020). Aggregation of Aβ protein has been observed to be enhanced due to a high-caloric diet and saturated fatty acids (Xu et al. 2015). Lead blocks the calcium-binding sites in the brain and is known to cross the BBB where it causes several damages. Infections due to bacteria such as Chlamydia pneumonia activate cytotoxic microglia

and astrocytes and result in the late onset of AD (Rahman et al., 2020; Ganz et al., 2022). Exposure to aluminum (Al) results in its accumulation in the cortical region of the brain and hippocampus where it causes protein misfolding (Mold et al., 2021).

1.6. Pathophysiology Caused by Aluminum

Al is a highly neurotoxic element and a major environmental risk factor for AD. It is not needed to perform any known physiological function in the human body and is regarded as a toxic element, especially a neurotoxin. Neuroscientists have established a strong association between Al and its toxic effects leading to brain disorders such as AD, Parkinson's Disease (PD), and other neurodegenerative diseases. It interferes with the cellular pathways by disrupting the redox status of cells and increasing oxidative stress. Al itself is non-redox in nature, but it can oxidize other compounds, and thus it can easily damage normal physiological mechanisms (Mujika et al., 2011). In a recent research study, balb-c mice were exposed to Al to learn the mechanism of the production of oxidative stress (Viezeliene, 2022). After 14 days of exposure, serum ferritin was measured which came out to be more than the normal case, with a lower amount of transferrin-bound iron. This suggested that Al replaced iron on an iron-binding protein, transferrin, causing a higher amount of free iron in serum which is directly associated with the production of reactive oxidative species (ROS). In addition to this, a very low dose of Al, such as a lethal dose of 50 (LD_{50}) can cause a lot of damage as well (Viezeliene, 2022).

Aluminum can cross the BBB and directly bind to the 1-40 chain of the Aβ sheet, changing its conformation and resulting in senile plaques. There is controversy over the exact mechanism by which the plaque formation occurs due to Al, however, there is evidence that Al does promote APP accumulation by suppressing the inhibiting domain in the brain essential for digesting the excess APP amount. It aids in the production of free radicals that result in oxidative stress and synthesizes inflammatory substances that damage the neurons (Mujika et al., 2011). Experimentation on animals has made it evident that Al promotes the accumulation of Aβ peptides in their brains. Al is, therefore, very commonly used to induce AD-like symptoms in rodent models (Viezeliene, 2022).

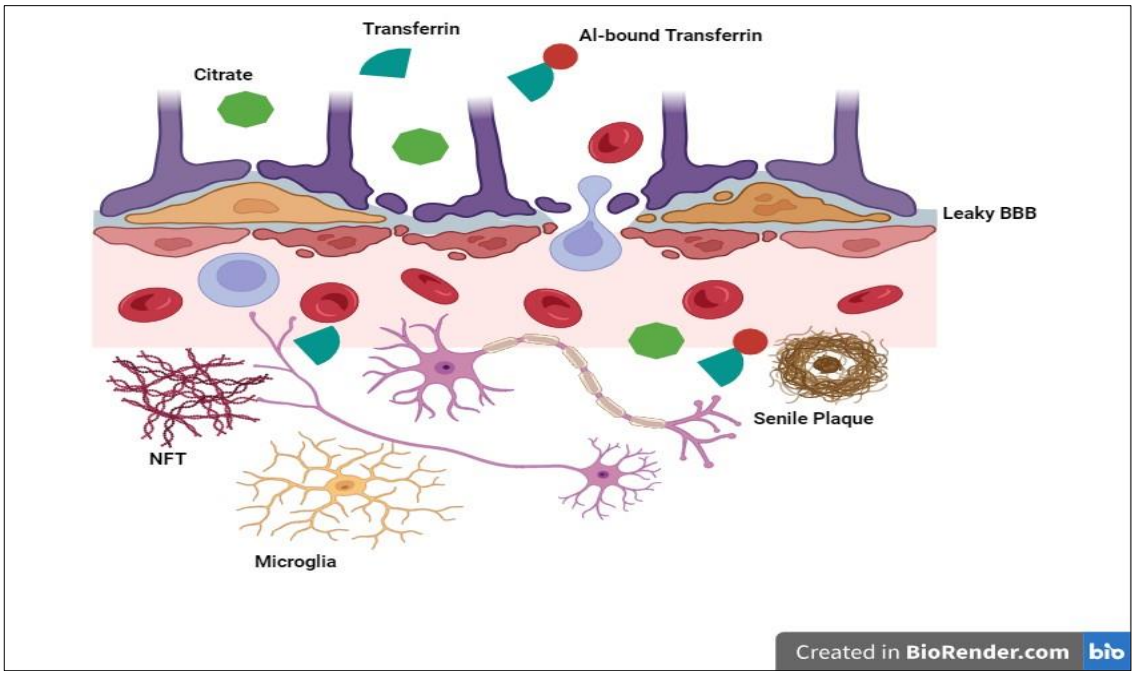

Figure 6: Aluminum Distribution in the Brain. Al crosses BBB and may accumulate in the NFTs, SPs, microglia, or astrocytes in the brain and interfere with the normal functionality of neurons.

1.7. Aims of the Current Research Study

AD is one of the major health as well as social crises of the 21st century. Despite arduous studies conducted on this complex disorder, there is still a lack of direct, early detection that aids in the mitigation of the physiological abnormalities at the initial stages. AD is indirectly diagnosed using imaging techniques such as magnetic resonance imaging (MRI), optical coherence tomography (OCT) positron emission tomography (PET), blue light autofluorescence (BAF), and scanning laser ophthalmoscopy (SLO). However, by the time diagnosis of AD is done through any means, most of the neurons are already irreversibly damaged (Oostveen et al., 2021).

1.7.1. Proposed Solution

Aβ and tau are two proteins whose presence is highly associated with AD. They start accumulating in the brain more than 10 years earlier than the appearance of any symptoms of AD, causing abnormalities at the molecular level before any changes in the behavior or personality of the person appear. This prolonged preclinical state of AD facilitates a huge potential for the development of early methods of AD diagnostics. Recently the use of infrared (IR) radiation has opened many new avenues for studying and diagnosing brain disorders. Near-infrared radiation (NIR) signal is reported to be

specific to the structure of the amyloid protein and has been used to study the mechanism of the self-assembly of Aβ in a label-free manner (Pansieri et al., 2019).

Over the last decade, the eye has captured scientists' attention to be used as a tool to detect the pathologies occurring in the brain. The function of the eyes and their movement is controlled by a diverse network of the brain stem and occipital lobe of the cerebral cortex. The pathology of these brain regions causes a decline in ocular function as well. Retina has been known as the extension of CNS, anatomically and developmentally. The axons of ganglion cells in the retina form an optic nerve, whose fibers further form CNS axons in effect. In fact, in recent neurological studies, structural changes in the retina of AD patients have been observed, including neurodegeneration of retinal tissue and plaque deposition in some cases (London et al., 2013).

Research on AD transgenic APP-rodents has shown that this accumulation of plaques in their retina occurs as cerebral protein deposits. The retina mirrors the effects of CNS in many important cases and therefore is a useful tool to detect the pathology occurring inside the brain. There is chronic intraocular inflammation in patients suffering from CNS lymphoma and composite ganglion thickness in Multiple Sclerosis (MS) patients. Retinal vasculature abnormalities including decreased arteriolar and venular fractal dimension in patients with AD. The task-evoked pupillary response is often observed in them as well. Patients with obsessive-compulsive disorder (OCD), autism, and schizophrenia have saccadic or smooth pursuit eye movement. Thus, the retina is an easily accessible organ, and visual impairment after neurological disorders should not be neglected (Onakpoya et al., 2010; London et al., 2013).

The current project is a continuation of a previous project that validated protein aggregation in the aging retina as an early retinal biomarker. An ocular IR-emitting device was engineered consisting of an Arduino circuiting board, with the camera connected to both the board and computer where the reflected IR values were displayed. Data from healthy volunteers was collected and correlated with their MMSE scores and ages. The device validated the presence of variations in the retinal proteins in the aging retinal and confirmed it as a promotor of AD diagnosis. In the current study, the ocular IR-based device was tested on AD-like rodent models, and using IR radiation, the protein accumulation in their retina was detected to see whether these plaques worked

as a biomarker of AD. The present work was designed to achieve the following objectives:

1. Preparation of $AlCl_3$-treated AD-like rodent models.
2. Conduction of rodent behavior to confirm induction of AD-like symptoms.
3. Validation of the presence of Aβ in the brain and retina by histological tests.
4. Modification of the IR device by downscaling its parameters to rodent eyes.
5. Testing the device on the retinas of AD-like rodents.

CHAPTER 2
METHODOLOGY

2. METHODOLOGY

The previously designed IR prototype for the detection of ocular protein was modified for the present study to test on AD-like rodent models. The current work consists of three major parts: the device remodeling phase, the AD-like rodents' preparation and testing phase during which the rodent behavioral and histological testing was conducted to confirm the presence of plaques in their brain and retinal tissues, and finally the device testing phase in which the hypothesis of the project was validated on rodents.

2.1. Ethical Approval

Before conducting the in vivo study, ethical approval with an IRB number 05-2023-ASAB-02/02 was acquired from the National University of Sciences and Technology – Internal Review Board (NUST-IRB) committee of NUST, Islamabad.

2.2. IR-Based Device

2.2.1. Mechanical Design

The materials used to fabricate the prototype were infrared – light emitting diode (IR LED) (B615, IMP1000, TH500, SA) with 5 mm diameter and wavelength of 940 nm, TSL 1401 linear CCD camera (Yahboom, China) with 128 pixels that was used as a sensor, resistors (220 ohm), Arduino Nano (model: ARDO05, Italy), battery (6SP 061225, China), 5V charging module, connecting wires, soldering iron, and glue gun. The prototype consisted of two parts, the eyepiece including the camera and LED, the mechanical design of which was 3D-printed using polylactic acid (PLA), and the Arduino circuitry board. The PLA used was green in color, so the internal side of the eyepiece was colored black to block the effects of any irrelevant external light. The circuit was designed on fritzing software and the mechanical design of eyepiece and circuitry were designed on SolidWorks 2021.

2.2.2. Circuiting

On the Arduino board, the positive arm of the LED was connected with a resistor which was connected to the digital pin D8, and the negative arm to the ground. The VCC pin of the sensor was connected to the 5V pin of Arduino, CLK, and SI pins were connected to D2 and D3 pins respectively, A0 was connected to A0, and GND was connected to

the ground of the Arduino board. A 3.7 V battery was connected to supply power and a reset button was connected for obtaining data values.

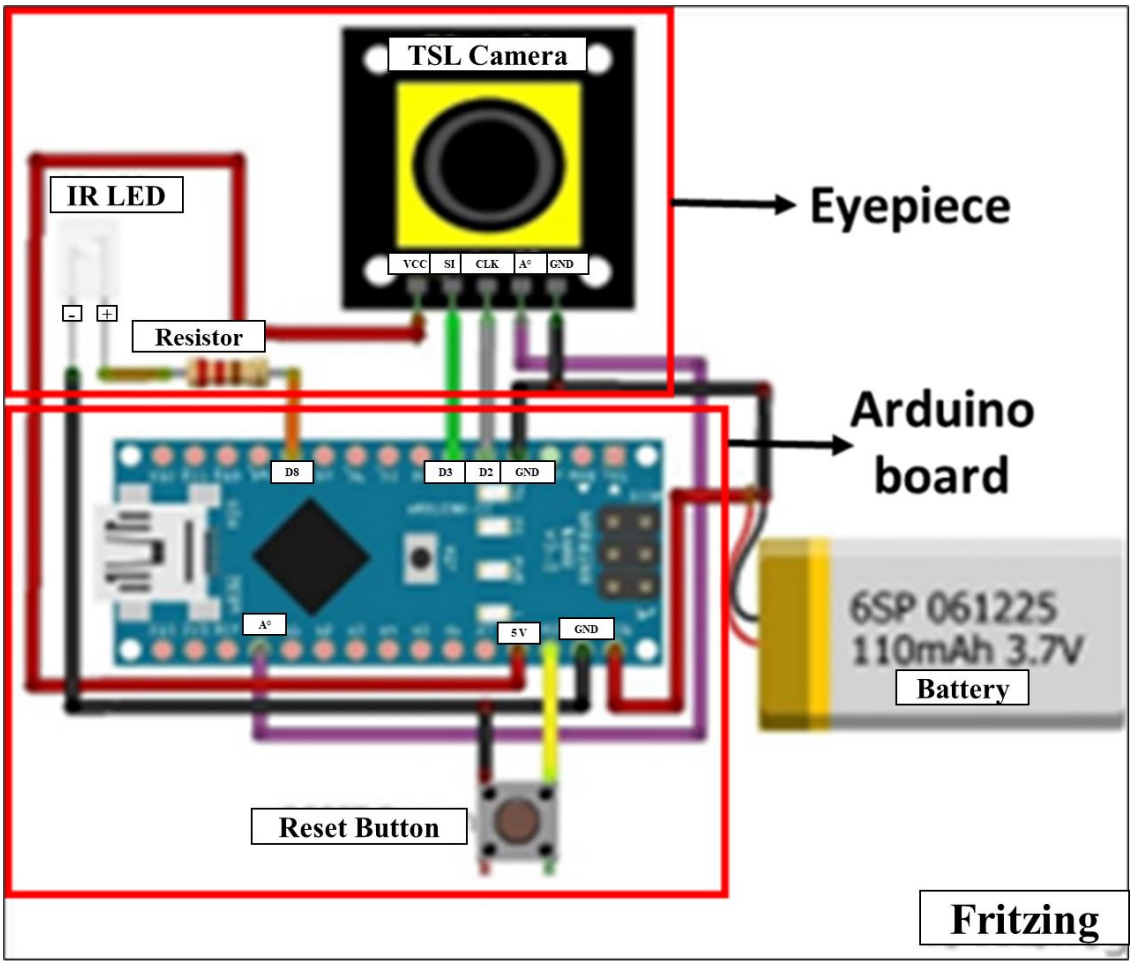

Figure 7: Circuit Diagram. The engineered prototype consists of an eyepiece and a circuit board. The eyepiece has an IR transmitter which is an LED and a sensor which is a camera connected at the same end. The wires of the LED and camera are connected to the circuit board.

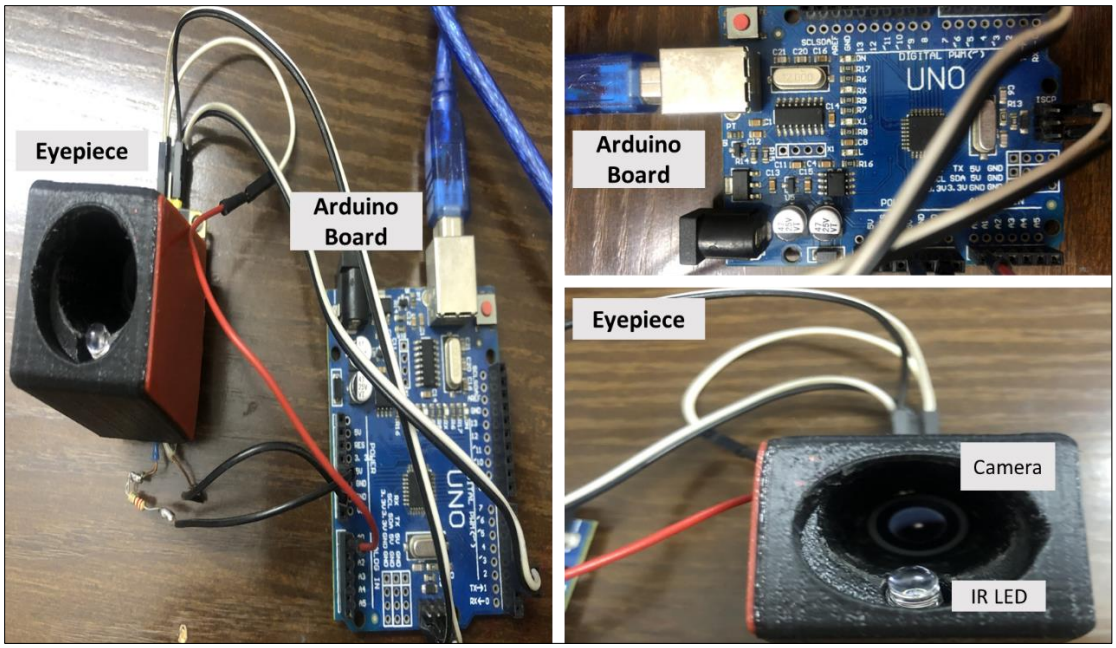

Figure 8: Hardware Device Design. It consists of mainly two parts: the eyepiece and the Arduino circuit board. The eyepiece further consists of an IR LED and a camera.

While designing the prototype, the light source and the sensor were placed at the same end of the eyepiece as per the maximum feasibility that the device design allowed us, and the data was collected based on the reflected values. It was made sure that nothing obstructed the mouse's eye while taking the reading and to remove any doubt, a mean of three readings was taken per mouse.

The camera attached to the eyepiece worked as a sensor of the reflected radiation and the value was displayed on Arduino (1.8.18) software installed in the computer. The camera sensor on receiving the light gave an analog reading that was a bell-shaped array of readings. This means that every time the power switch was turned on, the reading that the camera captured went from 0 to a full peak and fell back to 0. To exclude this hindrance so that the camera only captures the peak value, Arduino (1.8.18) software was coded to give only the stretch of the bell shape from each analog reading that would give a peak value. However, every peak value was different from one another once the reflected light hit the camera, the maximum being 1024 and the minimum value of 0 for an 8-bit analog-to-digital converter (ADC).

2.2.3. Device Modification and Calibration

Since the device was previously designed for the human eye, it was modified to be used on rodent eyes. The prototype was built in SolidWorks to check its dimensions and 3D-printed accordingly using PLA. For the human eye, the eyepiece diameter was 19 cm, while it was downscaled for the rat's eye to be around 6 cm. A small adjustable sheet of aluminum was attached to the eyepiece with a diameter measuring 3 cm for even smaller eyes of mice.

The prototype was calibrated using an already calibrated 3.2 mm UV silicon photo pin diode whose spectral range was 200-1100 nm. The IR LED was connected with the diode and different objects were first placed in the path of the LED and the readings were recorded. LED was held close to the mouse's eye while another IR LED was connected to the TSL 1401 camera, and the same procedure was repeated to record readings. The difference in values was corrected by adjusting the lens of the camera. Lens adjustment was carried out until both sensor readings were in accordance with each other.

2.3. Preparation of AD-like Rodents

2.3.1. Animal Grouping and Caging

Balb-c male mice (n = 14), and Albino male rats (n = 5), weighing on average 38 g and 220 g respectively, were supplied and housed in Animal House, NUST with temperature and humidity maintained around 25 ± 3 °C and 65 ± 5 % respectively. Animals were divided into two groups: the control group including the healthy rodents, and the diseased group which was being chemically treated to prepare AD-like rodent models. The current study was mainly focused on balb-c mice, however, to validate the functionality of the device on a wide range of animals, a small group of Albino rats was also tested.

2.3.2. Chemical Dosage

AD-like mice models were prepared by giving 20 mg/kg of $AlCl_3$ in distilled water orally for 42 days. To make the stock solution, 20 mg of $AlCl_3$ in 10 ml of distilled water was required, while 0.38 ml volume of the stock was used to administer $AlCl_3$ orally, using a mouth gauge, per mouse per day. For AD-like rats, intraperitoneal (IP)

injection of 150 mg/kg $AlCl_3$ and 300 mg/kg D-galactose was administered daily for 2 weeks (Yang et al., 2013; Xing et al., 2018).

2.4. Behavioral Testing

Once the AD-like mice models were prepared, behavioral testing was conducted on them that helped determine whether the mice showed any AD-like signs and symptoms, including slow learning, memory impairment, and affected spatial learning.

2.4.1. Y-Maze Test

The animal under study was placed in a Y-maze consisting of three arms: familiar, novel, and the start arm. Its ability to alternate between the different arms and the time spent in each arm was noted. Once its training phase was done, it was allowed to explore all three arms for 5 minutes and was recorded using a video camera to calculate the parameters that showed cognitive impairment. Percentage alteration was calculated by using the following formula:

$$\text{Percentage alterations} = \text{No. of}\frac{\text{alterations}}{\text{total entries}} \times 100$$

If the percentage alteration comes out to be less than 22%, it is considered cognitively impaired (Kraeuter et al., 2019).

2.4.2. Novel Object Recognition Test (NORT)

In NORT, the animals were exposed to a novel and a familiar object to assess whether they could recognize the already-interacted object and spend more time with the novel object. It helps evaluate the cognition of the rodents as well, particularly their recognition memory, as rodents tend to spend more time with objects they have not explored before. The following parameters were calculated to analyze the behavioral abnormalities:

Difference score = time exploring novel object - time exploring the familiar object

$$\text{Discrimination ratio} = \frac{\text{time exploring novel object}}{\text{total time spent with both objects}}$$

Animals show good cognition if they show a positive difference score, and a discrimination score greater than 0.5 (Lueptow, 2017).

2.4.3. Morris Water Maze Test (MWM)

The apparatus consisted of a circular stainless-steel tank with a diameter of around 2m and a height of walls of approximately 60 cm with four directions: North, South, East, and West, marked on it. It was half-filled with warm water (28 °C ± 3) and a circular platform of 10-12 cm diameter was submerged 1-2 cm below the surface in the northeast quarter of the tank. For the first 5 days mice were trained to locate the submerged platform from different directions and their escape latencies over the 5 days were noted. On the 6^{th} day, the platform was removed and the number of crossings into the target quadrant, the number of crossings over the platform spot, and the time spent in the target quadrant were noted (Bromley et al., 2011).

2.5. Mice Dissection

From the same cohort groups, 2-3 mice were dissected under general anesthesia to collect the brain and eyeball samples. An equal ratio of cold 4% paraformaldehyde and normal saline was used to fix the tissues. 4% PFA was freshly prepared before carrying out the dissection procedure. For its preparation, 800ml of 1x Phosphate Buffer Saline (PBS) was taken in a beaker, and 20g of PFA powder was added to it. The mixture was stirred at 60°C in the ventilation hood on a hot plate, but not boiled. A few drops of 5N NaOH were added to the PFA mixture until it became a clear solution. The solution was cooled to room temperature and filtered to remove any undissolved particles. The volume was adjusted to 500ml with 1x PBS, and the pH of the solution was checked and adjusted to 7 by adding a few drops of HCl. It was aliquoted into small volumes and stored at room temperature. Once the organs were fixed, the brain and eyeballs were isolated from the dissected animal, washed in PBS, and stored in 4% PFA at 4°C (Wu et al., 2021).

2.6. Histological Staining of Brain and Retina

2.6.1. Hematoxylin and Eosin Staining (H&E)

The fixed tissues were sliced into 4 µm thin slices and their microscopic slides were prepared. The slides were deparaffinized for which they were incubated at 63°C for 30 minutes, immersed in xylol for 2 minutes, and washed with different concentrations of ethanol (100%, 90%, 80%, 70%) for 2 minutes each, and then rehydrated with distilled water for 5 minutes. H&E staining of the sliced sections on the slides was carried out by

interaction with the following reagents: hematoxylin – 3 min, water wash – 1 min, differentiator (mild acid) – 1 min, water wash – 1 min, bluing – 1 min, water wash – 1 min, 95% ethanol – 1 min, eosin – 45 sec, 95% ethanol – 1 min, 100% ethanol – 1 min, xylene – 2 min; followed by a coverslip (Feldman et al., 2014; Alturkistani et al., 2015).

2.6.2. Thioflavin T (ThT) Dye Staining

After deparaffinization and rehydration of the slides as previously described, a drop of a working solution of 0.5% ThT dye in 0.1 N HCl was placed on the slides, which were then kept in a humidity chamber for 15 minutes. The slides were rinsed in deionized water and then cover-slipped (Biancalana et al., 2010).

2.7. Microscopy

H&E slides were viewed under a 4X – 100X Binocular Light Microscope (USA) and the images were photographed at 40x magnification, while ThT-stained slides were viewed under an XDY-2 Inverted Fluorescence Microscope (China) and the images were photographed at 10x magnification with a blue filter ranging between 420 to 490 nm wavelength. Image J (Java 8, WI, USA) software was used to count the cell numbers in H&E slides and amyloid plaque numbers in ThT-stained slides. The amyloid to cell count ratio was also calculated to observe the number of amyloids per cell for both the control and diseased tissues.

2.8. Device Testing on Rodents

Once the device was set up, the eyepiece was held close to the mouse's eye, and with the start button on the device, the readings showing the reflectance of IR radiation were taken into the computer. An average of three readings for both eyes of each mouse was taken. The raw readings were converted to reflectance percentage.

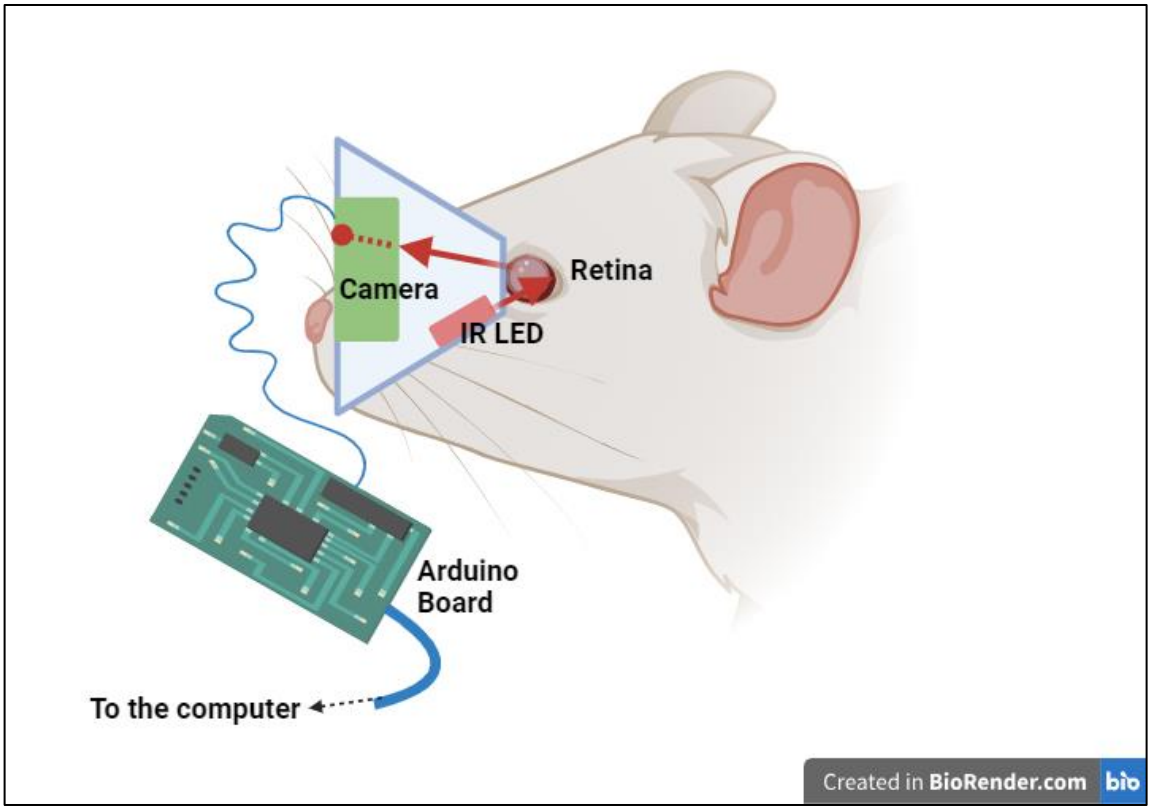

Figure 9: Schematic of Ocular IR-based Device Testing on Mice. The IR radiation from the LED in the eyepiece hit the mouse's retina, from where the reflected radiation was captured by the camera and it sent the values to the computer.

2.9. Statistical Analysis

Statistical analyses on the data obtained were carried out using GraphPad Prism (10.01, CA, USA). For the data sets consisting of two groups, an unpaired t-test was applied to compare the means of the two groups and to analyze whether there was a significant difference between them. For the data sets with more than two groups, involving two factors, a two-way analysis of variance (ANOVA) was conducted. While performing the statistical analyses, it was assumed that the data were independent, approximately normally distributed, and showed equal variance within each group to be compared. The differences with a p-value of < 0.05 were regarded as statistically significant.

CHAPTER 3
RESULTS

3. RESULTS

3.1. Memory Retention and Learning Behaviors of $AlCl_3$-Treated Mice

3.1.1. Morris Water Maze Test

Escape latency was observed over the five days of the training phase to assess the navigation skills of the mice. Their spatial retention memory is evaluated by the number of entries and time spent in the target quadrant and the number of times the mice crossed the platform area in the target quadrant during the 6th, probe trial day.

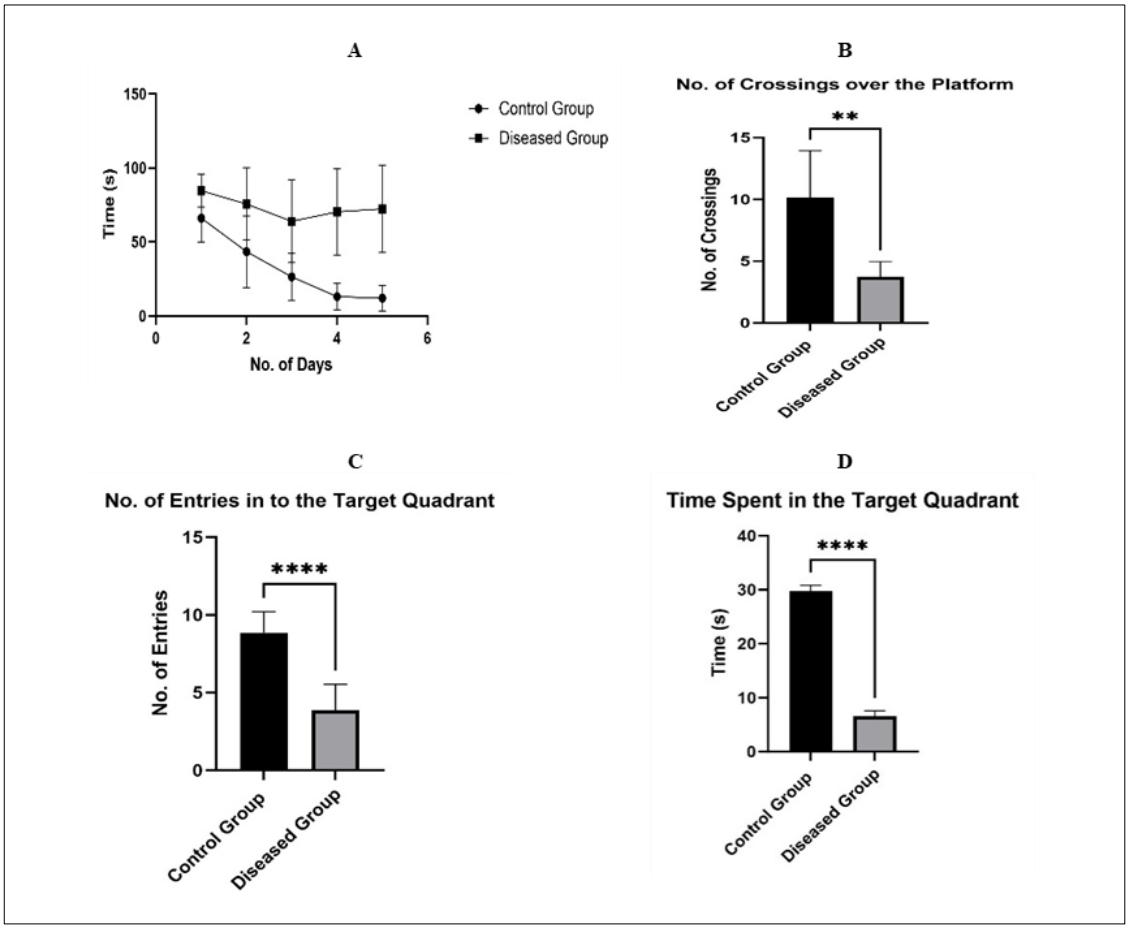

Figure 10: Morris Water Maze Test. A. Escape latency over the five days. **B.** No. of crossings **C.** no. of entries into the target quadrant, and **D.** the time spent in the target quadrant during the probe trial day. The data represents mean, and SD. Statistical analysis using a t-test was applied on no. of crossing, entries, and time spent by mice in the target quadrant. A significant difference of **$p < 0.01$, and ****$p < 0.0001$ was observed.

3.1.2. Y-maze Test

Rodents tend to alter their routes and explore areas they have not before. Percentage alterations were evaluated for both healthy and diseased groups to observe their ability to alter their routes and to assess their memory retention of previously explored areas. A significant difference between both groups is observed, with an increased number of alterations in the control group. When the time spent in the familiar and novel arm of the y-maze by both groups of mice is compared, $AlCl_3$–treated mice show a significant decrease.

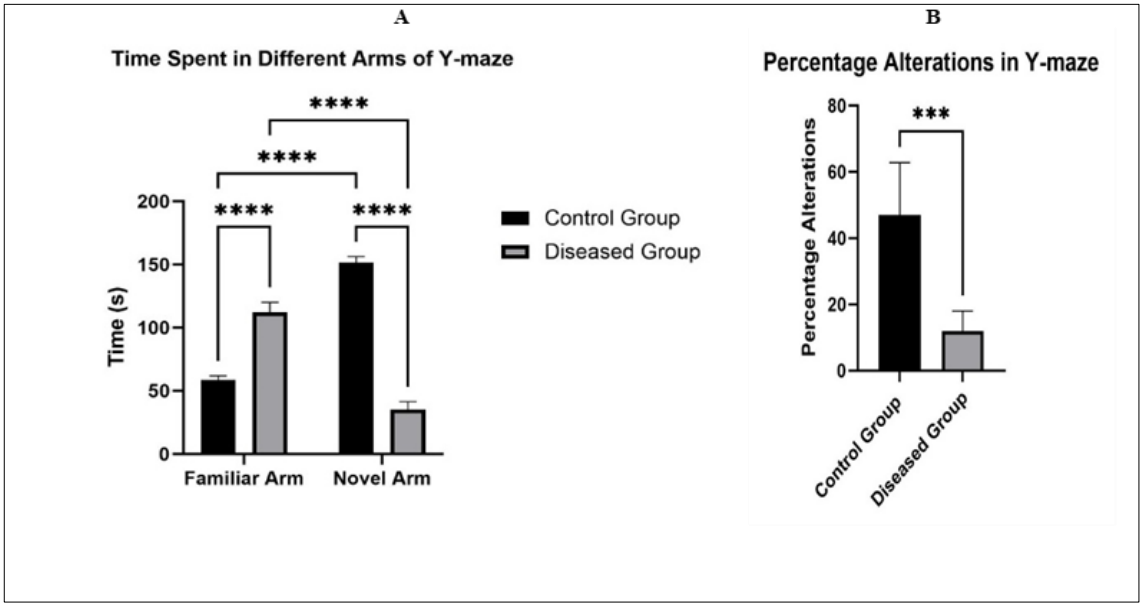

Figure 11: Y-maze Test. A. Amount of time spent by mice in the different arms of y-maze. **B.** Percentage of alterations by mice carried out in the y-maze. The data represents mean, and SD. T-tests on percentage alteration and ANOVA on time spent data were applied to carry out statistical analysis and a significant difference of ***$p < 0.001$ and ****$p < 0.0001$ was observed.

3.1.3. Novel Object Recognition Test (NORT)

Interestingly, in NORT, it can be seen from the results that the mice of both groups preferred spending most of the time in the box away from the object after spending some time exploring them. However, the control group still spent more time with the novel object than the diseased group, but the difference is not significant enough. The difference and discrimination scores for both groups were calculated. A positive difference score and a differentiation ratio greater than 0.5 indicates good cognition. It is evident from the graphs that the diseased group shows poor cognition, indicating that the mice are showing the symptoms of AD.

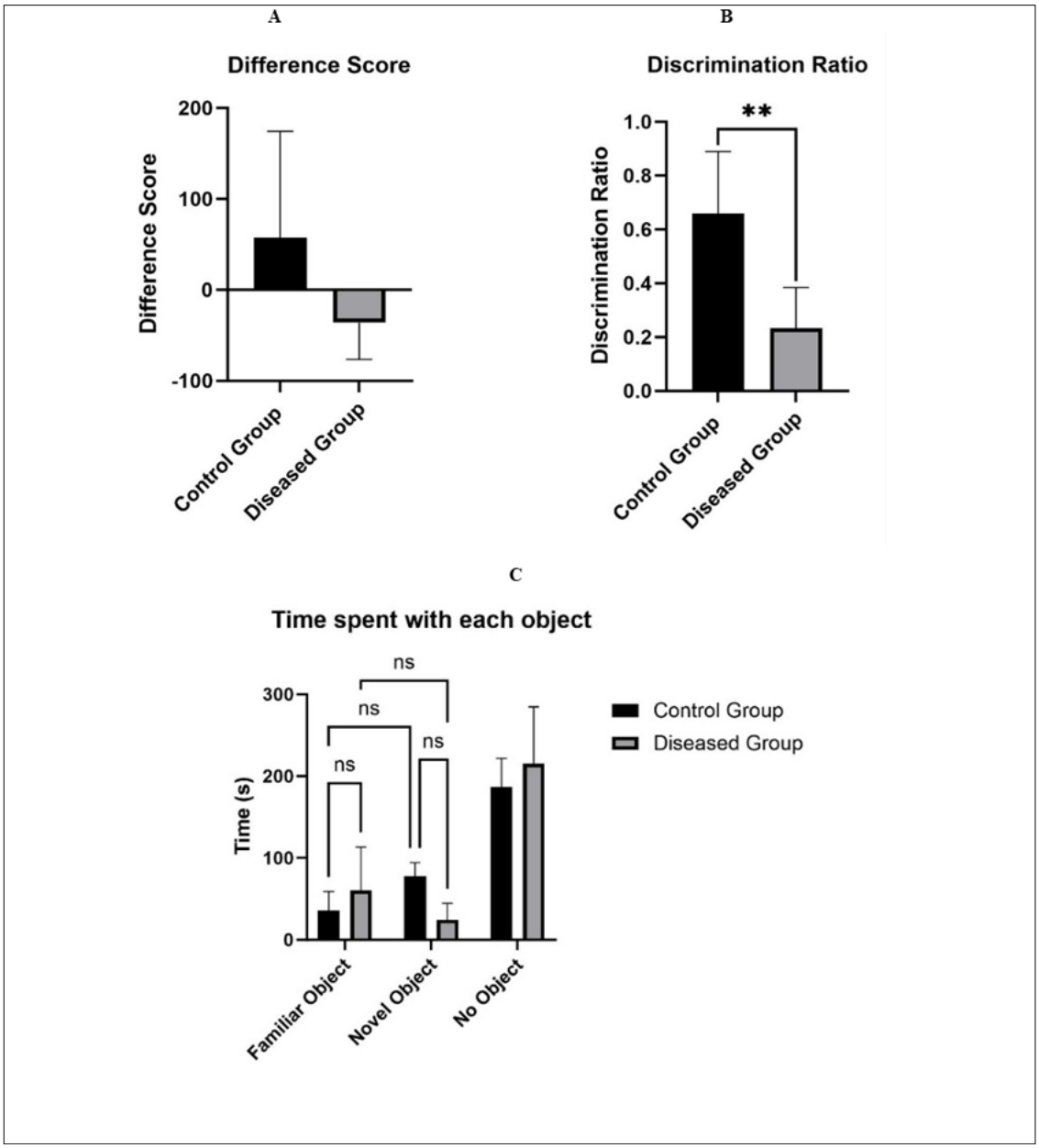

Figure 12: Novel Object Recognition Test (NORT). A. Difference-score showing the difference between the exploration time with familiar and novel objects by diseased and control mice groups. **B.** Discrimination Ratio showing the mice's ability to recognize between familiar and novel objects. **C.** Time spent by control and diseased groups of mice with the novel and familiar objects in an open filed box. The data represents mean, and SD. Statistical analysis using a t-test on discrimination ratio and ANOVA on time spent data were applied. The difference is not significant enough indicated on the time spent chart as 'ns' (ns, $p > 0.05$), while for the discrimination ratio, a significant difference of $^{**}p < 0.01$ was observed.

3.2. Confirmation of the Presence of Aβ in the Retina and Brain

Histopathological tests were performed to confirm the presence of Aβ in the cortex, hippocampus, and retina of mice. The H&E-stained slides were visualized under a light microscope at 40X magnification. Using Image J software, the cells under observation were counted for the tissues of both the control and diseased groups to compare the differences. Thioflavin T (ThT) staining of the sliced sections was also carried out to confirm the presence of Aβ protein and viewed under a fluorescent microscope. The bright yellow fluorescence indicates the presence of amyloid plaques in the tissues.

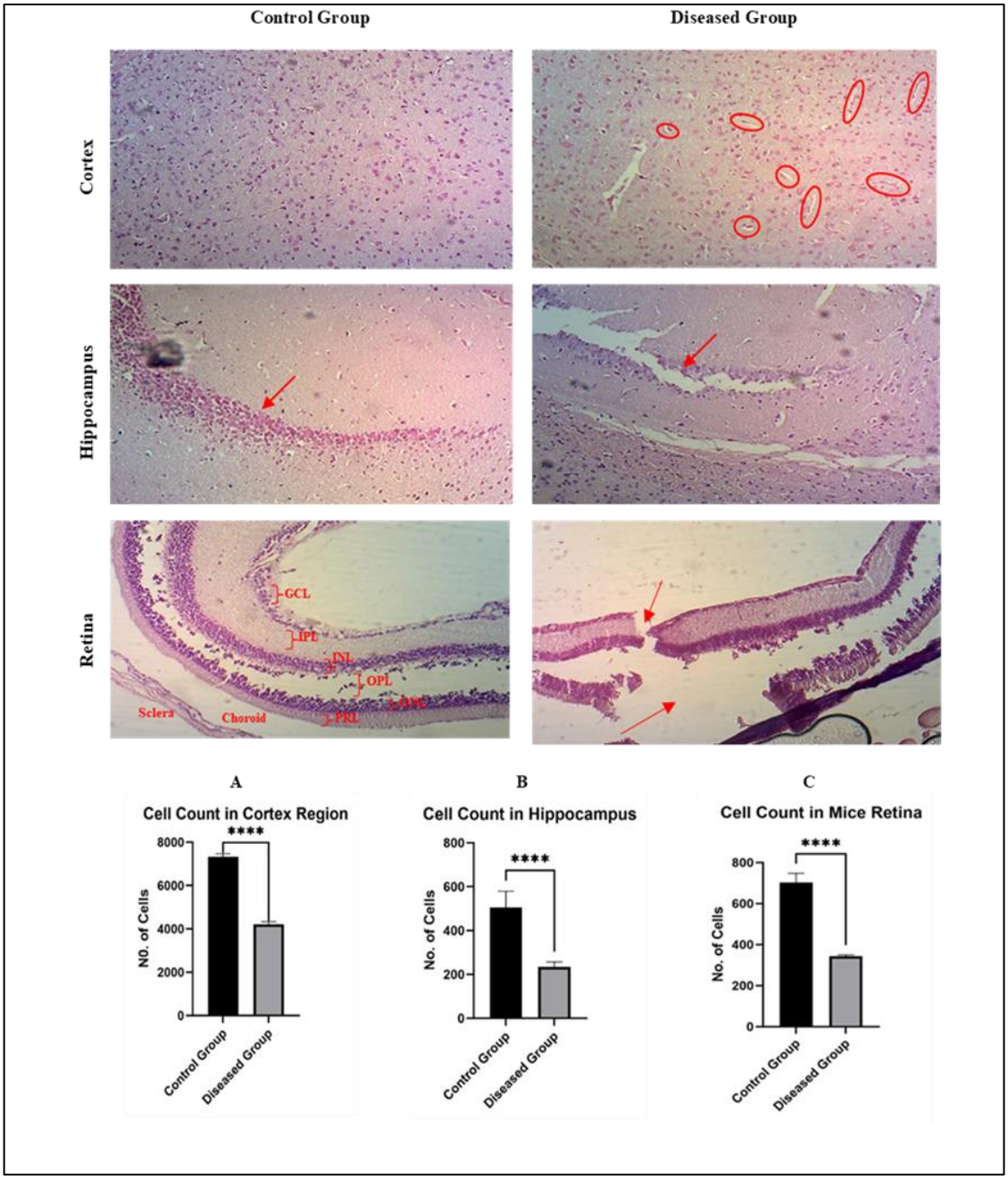

Figure 13: Hematoxylin and Eosin (H&E) Staining. H&E staining results of cortex, hippocampus, and retina of diseased mice are compared with those of healthy controls. Slides were viewed under a light microscope at 40X magnification. The diseased cortex shows excessive neurodegeneration and multiple nuclei. The arrow in the hippocampus compares the normal with the diseased one. The healthy retina shows visible layers: ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor layer (PRL) consisting of rods and cones, choroid layer, and sclera. However, in the diseased retina, the arrows point to the necrotic tissues. The graphs show cell counts in **A.** Cortex, **B.** Hippocampus, and **C.** Retina of mice. The data represents mean, and SD. T-test was

applied and a significant difference of ****p < 0.0001 in the number of cells in these regions of the control and diseased group was observed.

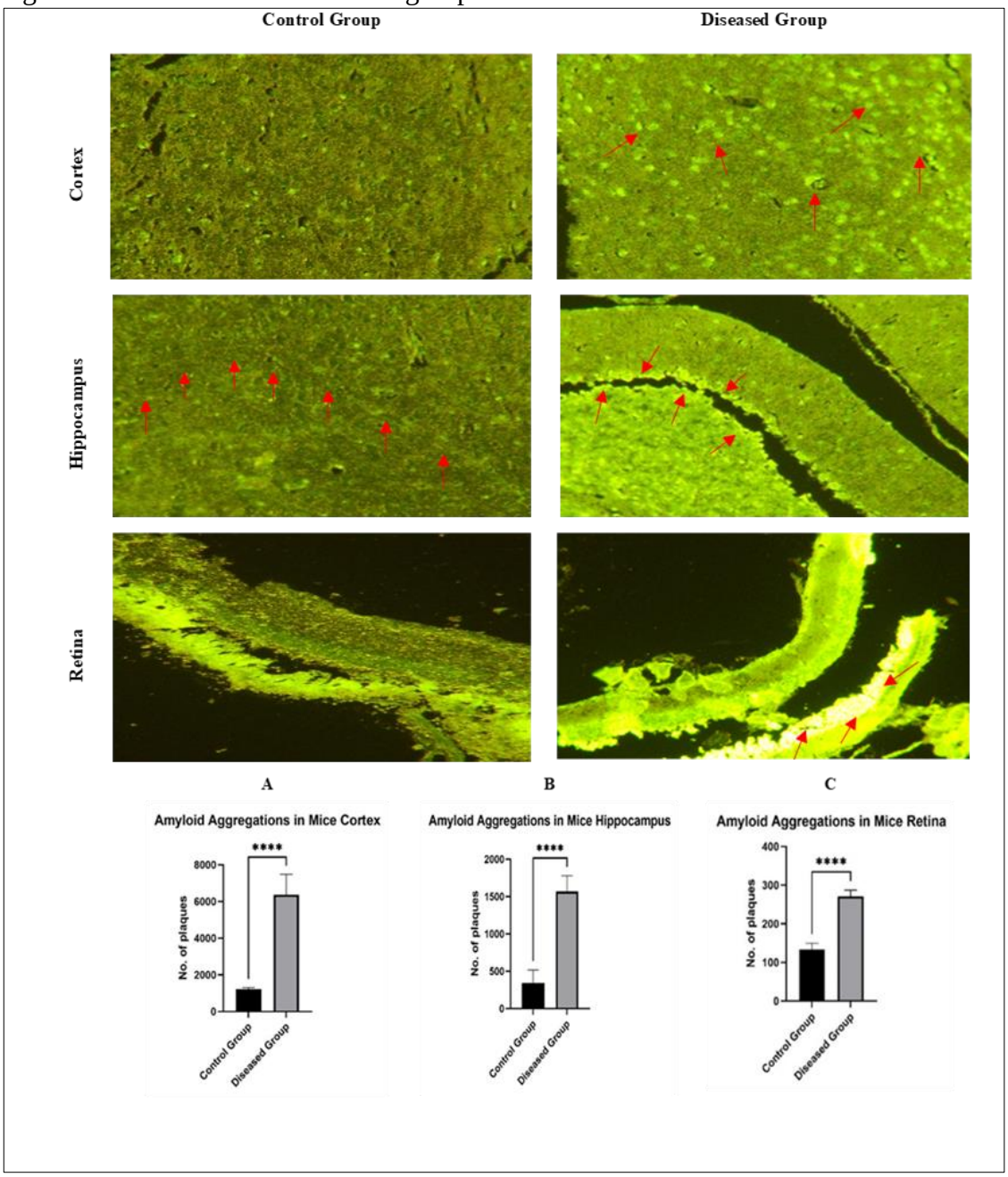

Figure 14: Thioflavin T (ThT) Staining. ThT staining of mice cortex, hippocampus, and retina indicates the presence of amyloid plaques in the regions that fluoresce bright yellow-green. The slides were photographed under a fluorescent microscope at 10X magnification. the arrows in the diseased cortex show aggregated Aβ deposits. The arrows in the control hippocampus show the hippocampus, while in the diseased hippocampus, fluorescent amyloid plaques are visible along the neurodegenerative region. The arrows in the diseased retina also point to the accumulated plaque deposits. The graphs show amyloid plaque count in **A.** Cortex, **B.** Hippocampus, and **C.** Retina of

mice. The data represents mean and SD. A T-test was applied, and a significant difference of ****$p < 0.0001$ was observed.

A ratio of amyloid aggregations to cell number was calculated to observe the number of amyloid aggregations per cell. The following formula was used to calculate the ratio:

$$\text{Amyloid to cell ratio} = \frac{\text{amyloid plaque number}}{\text{cell count}}$$

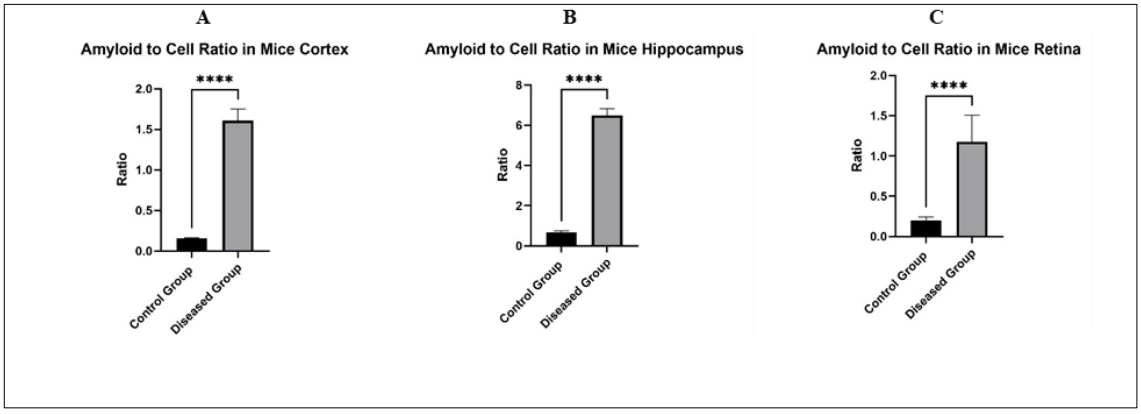

Figure 15: Amyloid to Cell Ratio in Mice Cortex, Hippocampus and Retina. The data represents the mean, and the error bars indicate SD. Statistical analysis was applied to the data using a t-test, and a significant difference of ****$p < 0.0001$ between the control and diseased groups was observed.

3.3. Validation of Protein Aggregations in the Retinal Tissue by IR Radiation

Control and diseased mice eyes were exposed to an IR beam, and the reflectance values were observed to validate the presence of proteins in the retinal tissue.

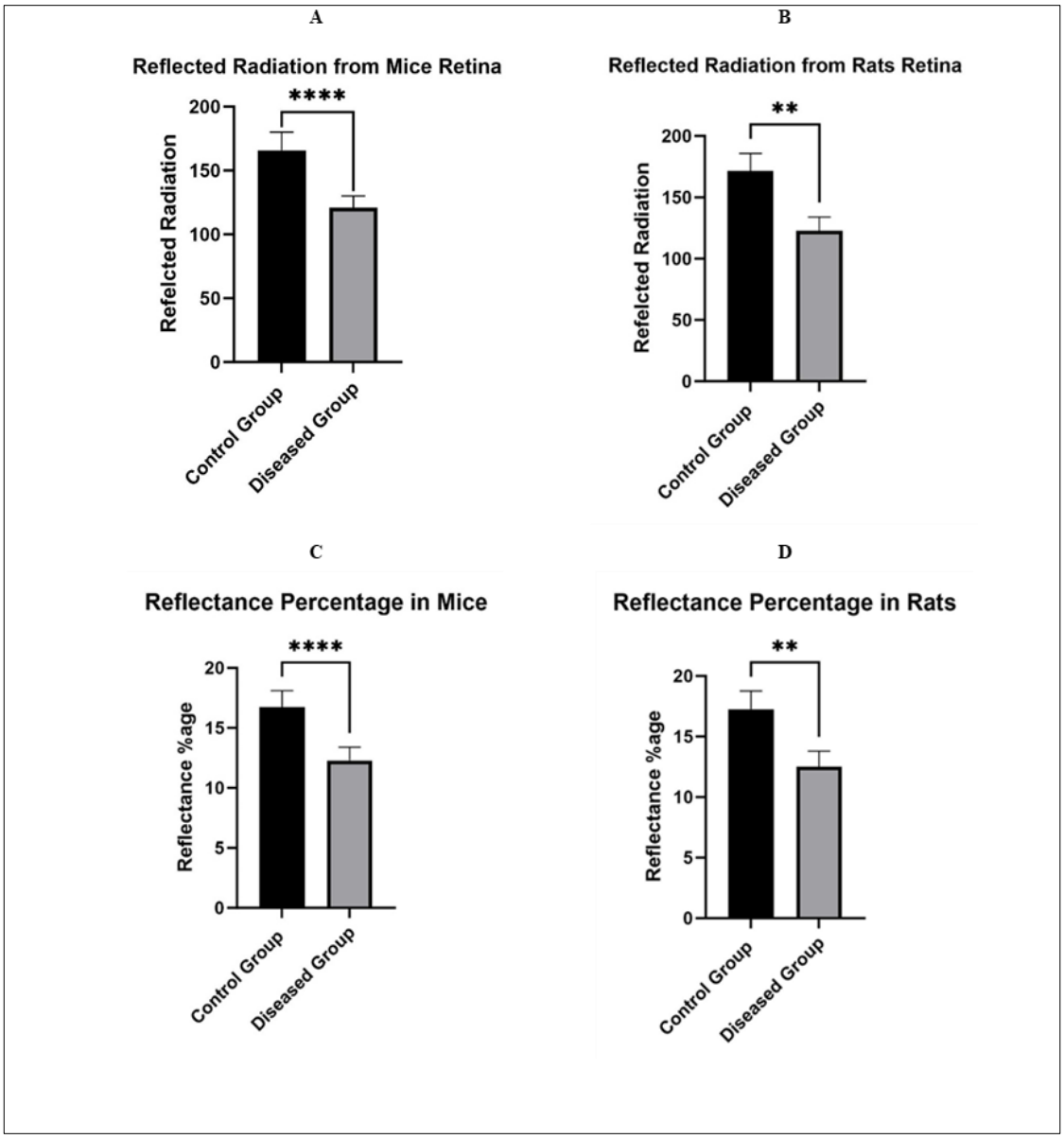

Figure 16: Reflected IR. A. Reflected IR radiations values from mice retina. **B.** Reflected IR radiation from rats' retinas. **C.** Reflectance percentage of IR in control and diseased groups of mice. **D.** Reflectance percentage of IR in control and diseased groups of rats. The data represents mean, and SD. A T-test was applied and a significant difference of ****$p < 0.0001$ and **$p < 0.01$ was observed.

CHAPTER 4
DISCUSSION

4. DISCUSSION

4.1. Challenges for Developing AD Diagnostic Tools

The current work focuses on detecting the presence of ocular Aβ peptides to formulate a non-invasive approach that can be a promising tool for asymptomatic AD diagnosis. IR light is used as a medium instead of white light because of its characteristic features of being penetrative up to the retina of the eye, and for its specificity with the structure of Aβ protein as described by Pansieri et al. (2019). The device includes simple circuiting on an Arduino board with an LED and camera attached to it, for the basic purpose of validating IR to detect amyloid plaques in the retina. The phenomenon used for detection follows Beer Lambert's Law of light reflectance and absorbance, according to which the amount of light absorbed is directly proportional to the molar absorptivity (ε), the path length, and the concentration of the constituent object that falls in its path. Animal experiments were conducted since the research idea was novel in its nature and confirmation of our hypothesis was essential before testing on human subjects.

With the lack of any curative method for AD and dementia, the major aim of neuroscientists is to look for suitable biomarkers that can be a promising tool for the early detection of AD and may help initiate early disease management and improve the quality of life. To date, scientists still face the challenge of understanding the root cause and factors causing brain pathologies due to the complex nervous system and limited access to the brain. Imaging techniques for an early screening method of brain disorders have been successful to a great extent, however, they are not regarded as stand-alone diagnostic tests for neurodegenerative disorders including AD, and still have limitations regarding their sensitivity and specificity.

Volume changes in the hippocampus are considered vital for detecting the progression of AD in patients with mild cognitive impairment (MCI) and can be measured by MRI studies as reported by Bomasang et al., 2021. A review of a combination of cohort studies that included patients with MCI of any age group concluded that MRI cannot be used as an independent test for the diagnosis of AD, and it was in accordance with the international guidelines for using MRI, according to which MRI can only be used to exclude non-degenerative cause of cognitive impairment only (Lombardi et al., 2020).

Robust research is being conducted on PET scans as well to prepare a promising diagnostic tool for AD. A radioactive tracer specific to the Aβ protein is introduced through a catheter in the patient's arm. A few radioactive tracers approved by the FDA are F-florbetapir, C-Pittsburgh Compound B (PiB), C-PiB, flutemetamol, and F-florbetaben (Hanseeuw et al., 2019). Serums such as cerebrospinal fluid (CSF) and blood have been used to track Aβ and tau traces as early detection biomarkers of AD progression. However, they are invasive as well and require additional tests to fully validate the presence of dementia in the asymptomatic phase (Bomasang et al., 2021).

4.2. Eye Serves as a Window to the Brain

For decades now, scientists have been using the eye, particularly the retinal tissue, as a platform to get insights from the CNS. The retina and brain both originate in the embryonic stages from the same layer of neurectoderm, giving rise to separate structures eventually (Graw, 2010; Sinn et al., 2013). Developmental studies have shown that by the 29th day of the embryo, the optic stalk and lens vesicle originate first from the ectoderm hollow ball, and then retinal layers and neural tube start developing, which remain connected throughout, making the eye as an extension of CNS in humans as described by Hoar, (1982).

The cells of both the brain and retina are similar in structure, with the exception that, unlike complex neuronal networks throughout the brain, the retinal cells work in linear, simple-layered manner (Boycott et al., 1991; Stiles et al., 2010). The output cells of the eye including the lens are connected to the ganglion cells, followed by bipolar cells that are connected to the photoreceptors that extend to the cortex of the brain. Masland, (2012) reported that retinal cells lack myelin on their axons, unlike neuronal cells. In addition, retinal tissue, and CNS also possess similar receptor classes such as excitatory including glutamate receptors, and inhibitory including Gama-aminobutyric acid (GABA) and glycine is reported by several studies (Araque et al., 2001; Watanabe et al., 2002; Eggers et al., 2011; Zhou et al., 2014). Other receptor classes that are common to both include receptors such as serotonin, dopamine, and melatonin (Kalloniatis et al., 2013).

Studies on the detailed structures of the eye and the brain have shown that both structures share the same vascular origin as well (Pournaras et al., 2008; Gkotsi et al., 2014). An internal carotid artery is the common blood vessel that supplies blood to both the brain and retina, with both organs having the highest metabolic activity and in turn the highest blood supply demand as well, as described by Ames, (2000) and Riley (2010). Diaz et al., (2017) report that like the BBB in CNS, the retina has a blood-retinal barrier (BRB), that regulates the transportation of ions, proteins, and other substances through blood into the retina.

In addition to the similarity in the structures, the retina mirrors the effects of pathologies in the brain as well (Yap et al., 2019). Many studies have reported retinopathy and problems associated with eye movement because of neurological disorders (Padungkiatsagul et al., 2020). For example, animal studies have shown that in PD, a decrease in dopaminergic neurons in the retina has been seen, slow functionality of retinal neurons due to depression, foveal thinning (140 vs. 151 μm) in the cases of anorexia, and neurodegeneration along with Aβ deposition in the cases of AD (Veys et al., 2019; Cosker et al, 2019; Zhang et al., 2022).

It has been studied that Aβ accumulation in the ocular tissues of AD patients initiates parallel to its deposition in the cortical tissues (Liang et al., 2021). Retina synthesizes Aβ protein by the same mechanism through which it is produced in the cortex of the brain. Research involving animal experimentation has suggested that the pathway involved in the aggregation of plaques in the retina may also be the same as that occurs in the AD brain (Prasad et al., 2017). Nguyen et al, (2017) report that the amyloid deposition occurs in all the retinal layers and the blood vessels as well. ThT staining results of this study are in accordance with the above-mentioned facts from the previous studies. In the present work, cortical and retinal tissues of diseased mice show a significant number of amyloid deposits, while the controls lack Aβ accumulation.

4.3. Aluminum-treated Rodents Show Signs of AD

Aluminum is a well-known neurotoxin that can easily cross the BBB and interfere with the normal physiological mechanisms of the human body (Linping, 2018). It can find a vast number of reservoirs in the brain for its storage such as glial cells, NFTs, and

amyloid plaques, and facilitates the deposition of Aβ in the cortex and hippocampus (Viezeliene, 2022). Since it is highly associated with the development of AD, it is commonly used by neuroscientists to induce AD symptoms in rodents (Ondreicka et al., 1996). For the current study, performing the behavioral tests was the first step to prove the presence of those symptoms in mice after treating them with $AlCl_3$ for 42 days. Further confirmation was done by conducting histopathological staining of mice's brain and retinal tissues since the behavioral tests show symptoms that overlap with many other brain disorders and may give false validations. The prime focus of the study was to investigate the working of the IR-based device to detect the presence of protein accumulations.

Rodents show alteration behavior, meaning that they tend to go to places they have not explored previously. Ideally, in the Y-maze, rodents should alternate between the three arms. If they have been in the “familiar arm”, they should go to the “novel arm” next, and then the “start arm”, and should remember that they have explored the previous arms. However, in diseased cases, rodents forget that they have already explored an arm, and do not alternate between the three arms of the Y-maze. The number of alterations shown by the control is higher than the diseased group, with the mean being higher than 22 percent, which indicates better cognition than diseased as experimented by Kraeuter et al., (2019) as well. The control group also spends a significant amount of time exploring the novel region of the Y-maze, unlike the diseased group which reflects their passive, non-curious behavior.

In NORT, the amount of exploration time spent on the novel object during the testing session is expected to be greater than the familiar object spent during the training session as implicated from the studies conducted by Sarkisyan et al., (2009); Oliveira et al., (2010), and Antunes et al., 2012). Compared to the animals in the control group, animals with impaired cognitive function may explore both objects equally without recognizing any difference between the familiar and novel objects. Animals show good cognition if they show a positive difference score, and a discrimination score greater than 0.5. While the difference and discrimination ratio turned out to be as expected for both the groups in the present work, the time spent by control and diseased mice with the familiar and novel objects does not show a significant difference in this case, and

instead spent a larger portion of the time around the box. Thus, behavioral tests should always be backed up by additional tests to validate the induction of a certain neurological abnormality under study.

The results of the escape latency in MWM show a significant decrease in the control group over the span of 5 days; however, the diseased group does not show any signs of learning and takes a relatively larger amount of time to reach the platform on the last day as well. Probe trial results indicate better memory retention of control mice since the number of crossings over the platform, the number of entries, and time spent in the target quadrant are significantly decreased in the diseased group, and fell in line with the expected results from studies conducted by Brandeis et al., (1989); Gallagher et al., (1997). Most of the mice in the diseased group would not move at all, once placed in the water, showing an even more decreased ability to recognize their surrounding environment.

The results of H&E staining indicated a clear difference in neurodegeneration in the brain and retina of $AlCl_3$–treated mice. The tissues of the diseased group visibly revealed fewer cells, indicating neuronal loss as reported by Eroglu et al., (2018) as well. In the ThT-stained slides of AlCl3–treated mice, there was an evident increase in protein aggregates as compared to the control. The above-mentioned results could be considered contradictory to each other, but it can be explained by the fact that protein deposition can occur regardless of cells being intact, such as in spaces between glial cells, around the membranes of the organelles, and deposits of liposome structures as reported by Iwamoto et al., (1997). From our results, the ThT staining image of the hippocampus of a diseased mouse brain shows an evident bright yellow fluorescence lined around the degenerated area (Themes, 2017). The H&E of the cortex shows neuronal loss at numerous spaces, however, in the ThT-stained slides, the same areas are covered with bright yellow-green fluorescents.

Further calculations such as amyloid to cell ratio in the diseased and control mice groups show a significantly increased number of amyloid deposits despite neurodegeneration in the diseased cortex and retina of mice. This observation is in accordance with the results from a study conducted on transgenic mice by Oakley et al., (2006) where amyloid plaque accumulation is higher regardless of necrosis. Our work

also presents $AlCl_3$ as a promising contributor to promoting Aβ in the brain and retina of rodents.

4.4. Reflected IR Contributes to the Detection of Aβ

The eyes of all the mice in the control and diseased groups were exposed to a low-power beam of IR light for less than 10 seconds. The value recorded in the computer was the reflectance value after it hit the retina of their eyes. Lomont et al., (2018) report that a lower reflectance value meant that the radiation was absorbed by amyloid protein accumulations and vice versa. IR radiation has been found to have specificity for the structure of Aβ sheets and gets absorbed by the protein once it encounters it as evidenced from Hadoux et al., (2019). This advancement has allowed scientists to study the Aβ structure in detail and has helped in label-free in vitro detection as well (Pansieri et al., 2019). Our data confirmed this assumption and showed a lower reflectance percentage for the diseased mice.

The in-built ADC in the Arduino board gave the raw values from 0 to 1024, where maximum meant that all the radiation was captured by the camera, while the lower values meant a decreased amount. These raw values from 0 to 1024 reflected the 5 V of the battery connected to the board divided into 8 bits. The raw values generated from the mice retina between the range of 150 to 200 indicate the deep penetrative power of IR radiation. The raw values above 700 indicate maximum reflected radiation. Upon hitting the camera with full IR light by covering the eyepiece with aluminum foil to ensure maximum reflectance, the value came out above 1000.

The reflectance percentage in both rats and mice is around 10% for the diseased group and 20% for the control group. This indicates that a major amount of IR light penetrated deep into their eyes. The difference in both the groups, however, means the presence of additional structures in the diseased group where more absorbance occurred. We predicted the presence of Aβ oligomer accumulation in the retina of $AlCl_3$–treated rodents that caused additional absorbance of IR radiation.

Comparing our predictions with the results of ThT staining, it is visible that the images show a vast number of yellow-green fluorescents that indicate Aβ aggregates in the retina, cortex, and hippocampus of diseased groups of mice. Amyloid plaque

aggregation count by Image J software gives a quantitative analysis of the difference in aggregates in both the groups, where it is significantly higher in the diseased group than the controls.

4.5. Conclusion

It is evident from the present study that IR plays a contributing role in detecting ocular Aβ load and can be a basis for developing a non-invasive AD diagnostic approach. A better diagnostic tool for AD dementia can help improve the quality life of patients by managing the disease symptoms at the initial stages. However, our hypothesis has only been tested on a small group of rodents, and the study is at its very initial stages of animal testing.

For future experimental work, the device design can be modified to include the absorbance spectrum as well, to help recognize the stage and extent of Aβ deposition in the retinal lining of the eye. It can be tested on transgenic APP AD mice to remove any ambiguity of the absence of the protein in the brain and eyes, and to shift complete focus on the device functionality to detect protein level. A detailed simulation of the functionality of the device design needs to be carried out to better understand the possible avenues that can enhance its working.

References

Alturkistani, H. A., Tashkandi, F. M., & Mohammedsaleh, Z. M. (2015). Histological Stains: A Literature Review and Case Study. *Global Journal of Health Science*, *8*(3), 72–79. https://doi.org/10.5539/gjhs.v8n3p72

Alturkistani, H. A., Tashkandi, F. M., & Mohammedsaleh, Z. M. (2015). Histological Stains: A Literature Review and Case Study. *Global Journal of Health Science*, *8*(3), 72–79. https://doi.org/10.5539/gjhs.v8n3p72

Ames, A. (2000). Cns energy metabolism as related to function. *Brain Research. Brain Research Reviews*, *34*(1-2), 42–68. https://doi.org/10.1016/S0165-0173(00)00038-2

Antunes, M., & Biala, G. (2012). The novel object recognition memory: Neurobiology, test procedure, and its modifications. *Cognitive Processing*, *13*(2), 93–110. https://doi.org/10.1007/s10339-011-0430-z

Araque, A., Carmignoto, G., & Haydon, P. G. (2001). Dynamic signaling between astrocytes and neurons. *Annual Review of Physiology*, *63*, 795–813. https://doi.org/10.1146/annurev.physiol.63.1.795

Atti, A. R., Palmer, K., Volpato, S., Winblad, B., Ronchi, D. de, & Fratiglioni, L. (2008). Late life body mass index and dementia incidence: Nine-year follow-up data from the Kungsholmen Project. *Journal of the American Geriatrics Society*, *56*(1), 111–116. https://doi.org/10.1111/j.1532-5415.2007.01458.x

Azhar, Z. (2023, April 26). Prevalence of Alzheimer’s disease in Pakistan. *TransparentHands*. Retrieved from https://www.transparenthands.org/prevalence-of-alzheimers-disease-in-pakistan/

Bature, F., Guinn, B.-A., Pang, D., & Pappas, Y. (2017). Signs and symptoms preceding the diagnosis of Alzheimer's disease: A systematic scoping review of literature from 1937 to 2016. *BMJ Open*, *7*(8), e015746. https://doi.org/10.1136/bmjopen-2016-015746

Biancalana, M., & Koide, S. (2010). Molecular mechanism of Thioflavin-T binding to amyloid fibrils. *Biochimica Et Biophysica Acta*, *1804*(7), 1405–1412. https://doi.org/10.1016/j.bbapap.2010.04.001

Bomasang-Layno, E., & Bronsther, R. (2021). Diagnosis and Treatment of Alzheimer's Disease:: An Update. *Delaware Journal of Public Health*, *7*(4), 74–85. https://doi.org/10.32481/djph.2021.09.009

Boycott, B. B., & Wässle, H. (1991). Morphological Classification of Bipolar Cells of the Primate Retina. *The European Journal of Neuroscience*, *3*(11), 1069–1088. https://doi.org/10.1111/j.1460-9568.1991.tb00043.x

Brandeis, R., Brandys, Y., & Yehuda, S. (1989). The use of the Morris Water Maze in the study of memory and learning. *The International Journal of Neuroscience*, *48*(1-2), 29–69. https://doi.org/10.3109/00207458909002151

Breijyeh, Z., & Karaman, R. (2020). Comprehensive Review on Alzheimer's Disease: Causes and Treatment. *Molecules (Basel, Switzerland)*, *25*(24). https://doi.org/10.3390/molecules25245789

Bromley-Brits, K., Deng, Y., & Song, W. (2011). Morris water maze test for learning and memory deficits in Alzheimer's disease model mice. *Journal of Visualized Experiments : JoVE*. Advance online publication. https://doi.org/10.3791/2920

Burns, A. [A.] (2000). Diagnosis and management of Alzheimer's disease. *Dialogues in Clinical Neuroscience*, *2*(2), 129–138. https://doi.org/10.31887/DCNS.2000.2.2/aburns Chan, K. Y.,

Wang, W., Wu, J. J., Liu, L., Theodoratou, E., Car, J., . . . Rudan, I. (2013). Epidemiology of Alzheimer's disease and other forms of dementia in China, 1990-2010: A systematic review and analysis. *Lancet (London, England)*, *381*(9882), 2016–2023. https://doi.org/10.1016/S0140-6736(13)60221-4

Christian Sam, & Bruno Bordoni (2023). Physiology, Acetylcholine. In C. Sam & B. Bordoni (Eds.), *StatPearls [Internet]*. StatPearls Publishing. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK557825/

Cosker, E., Moulard, M., Baumann, C., Luc, A., Angioi-Duprez, K., Laprévote, V., . . . Schwitzer, T. (2021). Complete evaluation of retinal function in Major Depressive Disorder: From central slowdown to hyperactive periphery. *Journal of Affective Disorders*, *295*, 453–462. https://doi.org/10.1016/j.jad.2021.08.054

Da Jeong, U., Lee, J. E., Lee, S. E., Chang, W. S., Kim, S. J., & Chang, J. W. (2014). Improvements in memory after medial septum stimulation are associated with changes in hippocampal cholinergic activity and neurogenesis. *BioMed Research International*, *2014*, 568587. https://doi.org/10.1155/2014/568587

Díaz-Coránguez, M., Ramos, C., & Antonetti, D. A. (2017). The inner blood-retinal barrier: Cellular basis and development. *Vision Research*, *139*, 123–137. https://doi.org/10.1016/j.visres.2017.05.009

Eggers, E. D., & Lukasiewicz, P. D. (2011). Multiple pathways of inhibition shape bipolar cell responses in the retina. *Visual Neuroscience*, *28*(1), 95–108. https://doi.org/10.1017/S0952523810000209

Feldman, A. T., & Wolfe, D. (2014). Tissue processing and hematoxylin and eosin staining. *Methods in Molecular Biology (Clifton, N.J.)*, *1180*, 31–43. https://doi.org/10.1007/978-1-4939 1050-2_3

Finder, V. H., & Glockshuber, R. (2007). Amyloid-beta aggregation. *Neuro-Degenerative Diseases*, *4*(1), 13–27. https://doi.org/10.1159/000100355

Gallagher, M., & Rapp, P. R. (1997). The use of animal models to study the effects of aging on cognition. *Annual Review of Psychology*, *48*, 339–370. https://doi.org/10.1146/annurev.psych.48.1.339

Ganz, T., Fainstein, N., & Ben-Hur, T. (2022). When the infectious environment meets the AD brain. *Molecular Neurodegeneration*, *17*(1), 53. https://doi.org/10.1186/s13024-022-00559-3

Gkotsi, D., Begum, R., Salt, T., Lascaratos, G., Hogg, C., Chau, K.-Y., . . . Jeffery, G. (2014). Recharging mitochondrial batteries in old eyes. Near infra-red increases ATP. *Experimental Eye Research*, *122*, 50–53. https://doi.org/10.1016/j.exer.2014.02.023

Gluhm, S., Goldstein, J., Loc, K., Colt, A., van Liew, C., & Corey-Bloom, J. (2013). Cognitive performance on the mini-mental state examination and the montreal cognitive assessment across the healthy adult lifespan. *Cognitive and Behavioral Neurology : Official Journal of the Society for Behavioral and Cognitive Neurology*, *26*(1), 1–5. https://doi.org/10.1097/WNN.0b013e31828b7d26

Gong, C.-X., & Iqbal, K. (2008). Hyperphosphorylation of microtubule-associated protein tau: A promising therapeutic target for Alzheimer disease. *Current Medicinal Chemistry*, *15*(23), 2321–2328. https://doi.org/10.2174/092986708785909111 Graw, J. (2010). Eye development. *Current Topics in Developmental Biology*, *90*, 343–386. https://doi.org/10.1016/S0070-2153(10)90010-0

Gustavsson, A., Norton, N., Fast, T., Frölich, L., Georges, J., Holzapfel, D., . . . van der Flier, W. M. (2023). Global estimates on the number of persons across the Alzheimer's disease continuum. *Alzheimer's & Dementia : The Journal of the Alzheimer's Association*, *19*(2), 658 670. https://doi.org/10.1002/alz.12694

Hadoux, X., Hui, F., Lim, J. K. H., Masters, C. L., Pébay, A., Chevalier, S., . . . van Wijngaarden, P. (2019). Non-invasive in vivo hyperspectral imaging of the retina for potential biomarker use in Alzheimer's disease. *Nature Communications*, *10*(1), 4227. https://doi.org/10.1038/s41467-019-12242-1

Hampel, H., Hardy, J., Blennow, K., Chen, C., Perry, G., Kim, S. H., . . . Vergallo, A. (2021). The Amyloid-β Pathway in Alzheimer's Disease. *Molecular Psychiatry*, *26*(10), 5481–5503. https://doi.org/10.1038/s41380-021-01249-0

Hanseeuw, B. J., Betensky, R. A., Jacobs, H. I. L., Schultz, A. P., Sepulcre, J., Becker, J. A., . . . Johnson, K. (2019). Association of Amyloid and Tau With Cognition in Preclinical Alzheimer Disease: A Longitudinal Study. *JAMA Neurology*, *76*(8), 915–924. https://doi.org/10.1001/jamaneurol.2019.1424

Hara, Y., McKeehan, N., & Fillit, H. M. (2019). Translating the biology of aging into novel therapeutics for Alzheimer disease. *Neurology*, *92*(2), 84–93. https://doi.org/10.1212/WNL.0000000000006745

Hoar, R. M. (1982). Embryology of the eye. *Environmental Health Perspectives*, *44*, 31–34. https://doi.org/10.1289/ehp.824431

Husain, M. A., Laurent, B., & Plourde, M. (2021). Apoe and Alzheimer's Disease: From Lipid Transport to Physiopathology and Therapeutics. *Frontiers in Neuroscience*, *15*, 630502. https://doi.org/10.3389/fnins.2021.630502

Inan-Eroglu, E., & Ayaz, A. (2018). Is aluminum exposure a risk factor for neurological disorders? *Journal of Research in Medical Sciences : The Official Journal of Isfahan University of Medical Sciences*, *23*, 51. https://doi.org/10.4103/jrms.JRMS_921_17

Iwamoto, N., Nishiyama, E., Ohwada, J., & Arai, H. (1997). Distribution of amyloid deposits in the cerebral white matter of the Alzheimer's disease brain: Relationship to blood vessels. *Acta Neuropathologica*, *93*(4), 334–340. https://doi.org/10.1007/s004010050624

Kalloniatis, M., Loh, C. S., Acosta, M. L., Tomisich, G., Zhu, Y., Nivison-Smith, L., . . . Arunthavasothy, N. (2013). Retinal amino acid neurochemistry in health and disease. *Clinical & Experimental Optometry*, *96*(3), 310–332. https://doi.org/10.1111/cxo.12015

Kraeuter, A.-K., Guest, P. C., & Sarnyai, Z. (2019). The Y-Maze for Assessment of Spatial Working and Reference Memory in Mice. *Methods in Molecular Biology (Clifton, N.J.)*, *1916*, 105–111. https://doi.org/10.1007/978-1-4939-8994-2_10

Li, L., Yu, X., Sheng, C., Jiang, X., Zhang, Q. [Qi], Han, Y., & Jiang, J. (2022). A review of brain imaging biomarker genomics in Alzheimer's disease: Implementation and perspectives. *Translational Neurodegeneration*, *11*(1), 42. https://doi.org/10.1186/s40035-022-00315-z

Liu, C.-C. [Chia-Chen], Liu, C.-C. [Chia-Chan], Kanekiyo, T., Xu, H., & Bu, G. (2013). Apolipoprotein E and Alzheimer disease: Risk, mechanisms and therapy. *Nature Reviews. Neurology*, *9*(2), 106–118. https://doi.org/10.1038/nrneurol.2012.263

Liu, M., Dexheimer, T., Sui, D., Hovde, S., Deng, X., Kwok, R., . . . Kuo, M.-H. (2020). Hyperphosphorylated tau aggregation and cytotoxicity modulators screen identified prescription drugs linked to Alzheimer's disease and cognitive functions. *Scientific Reports*, *10*(1), 16551. https://doi.org/10.1038/s41598-020-73680-2

Livingston, G., Huntley, J., Sommerlad, A., Ames, D., Ballard, C., Banerjee, S., . . . Mukadam, N. (2020). Dementia prevention, intervention, and care: 2020 report of the Lancet Commission. *Lancet (London, England)*, *396*(10248), 413–446. https://doi.org/10.1016/S0140 6736(20)30367-6

Lombardi, G., Crescioli, G., Cavedo, E., Lucenteforte, E., Casazza, G., Bellatorre, A.-G., . . . Filippini, G. (2020). Structural magnetic resonance imaging for the early diagnosis of dementia due to Alzheimer's disease in people with mild cognitive impairment. *The Cochrane Database of Systematic Reviews*, *3*(3), CD009628. https://doi.org/10.1002/14651858.CD009628.pub2

Lomont, J. P., Rich, K. L., Maj, M., Ho, J.-J., Ostrander, J. S., & Zanni, M. T. (2018). Spectroscopic Signature for Stable β-Amyloid Fibrils versus β-Sheet-Rich Oligomers. *The Journal of Physical Chemistry. B*, *122*(1), 144–153. https://doi.org/10.1021/acs.jpcb.7b10765

London, A., Benhar, I., & Schwartz, M. (2013). The retina as a window to the brain-from eye research to CNS disorders. *Nature Reviews. Neurology*, *9*(1), 44–53. https://doi.org/10.1038/nrneurol.2012.227

Lueptow, L. M. (2017). Novel Object Recognition Test for the Investigation of Learning and Memory in Mice. *Journal of Visualized Experiments : JoVE*. Advance online publication. https://doi.org/10.3791/55718

Masland, R. H. (2012). The neuronal organization of the retina. *Neuron*, *76*(2), 266–280. https://doi.org/10.1016/j.neuron.2012.10.002

Maurer, S. V., & Williams, C. L. (2017). The Cholinergic System Modulates Memory and Hippocampal Plasticity via Its Interactions with Non-Neuronal Cells. *Frontiers in Immunology*, *8*, 1489. https://doi.org/10.3389/fimmu.2017.01489 Mayeux, R., & Stern, Y. (2012). Epidemiology of Alzheimer disease. *Cold Spring Harbor Perspectives in Medicine*, *2*(8). https://doi.org/10.1101/cshperspect.a006239

Mayeux, R., & Stern, Y. (2012). Epidemiology of Alzheimer disease. *Cold Spring Harbor perspectives in medicine*, *2*(8), a006239. https://doi.org/10.1101/cshperspect.a006239

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., & Stadlan, E. M. (1984). Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology*, *34*(7), 939–944. https://doi.org/10.1212/wnl.34.7.939

Mir, R. H., Sawhney, G., Pottoo, F. H., Mohi-Ud-Din, R., Madishetti, S., Jachak, S. M., . . . Masoodi, M. H. (2020). Role of environmental pollutants in Alzheimer's disease: A review. *Environmental Science and Pollution Research International*, *27*(36), 44724–44742. https://doi.org/10.1007/s11356-020-09964-x

Mold, M. J., O'Farrell, A., Morris, B., & Exley, C. [Christopher] (2021). Aluminum and Tau in Neurofibrillary Tangles in Familial Alzheimer's Disease. *Journal of Alzheimer's Disease Reports*, *5*(1), 283–294. https://doi.org/10.3233/ADR-210011

Mujika, J. I., Ruipérez, F., Infante, I., Ugalde, J. M., Exley, C. [C.], & Lopez, X. (2011). Pro oxidant activity of aluminum: Stabilization of the aluminum superoxide radical ion. *The Journal of Physical Chemistry a*, *115*(24), 6717–6723. https://doi.org/10.1021/jp203290b

Murphy, M. P., & LeVine, H. (2010). Alzheimer's Disease and the Amyloid-β Peptide. *Journal of Alzheimer's Disease*, *19*(1), 311–323. https://doi.org/10.3233/JAD-2010-1221

Nguyen, C. T. O., Hui, F., Charng, J., Velaedan, S., van Koeverden, A. K., Lim, J. K. H., . . . Ivarsson, M. (2017). Retinal biomarkers provide "insight" into cortical pharmacology and disease. *Pharmacology & Therapeutics*, *175*, 151–177. https://doi.org/10.1016/j.pharmthera.2017.02.009

Niu, H., Álvarez-Álvarez, I., Guillén-Grima, F., & Aguinaga-Ontoso, I. (2017). Prevalencia e incidencia de la enfermedad de Alzheimer en Europa: Metaanálisis [Prevalence and incidence of Alzheimer's disease in Europe: A meta-analysis]. *Neurologia (Barcelona, Spain)*, *32*(8), 523 532. https://doi.org/10.1016/j.nrl.2016.02.016

Oakley, H., Cole, S. L., Logan, S., Maus, E., Shao, P., Craft, J., . . . Vassar, R. (2006). Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: Potential factors in amyloid plaque formation. *The Journal of Neuroscience : The Official Journal of the*

Society for Neuroscience, *26*(40), 10129–10140. https://doi.org/10.1523/JNEUROSCI.1202-06.2006

Oliveira, A. M. M., Hawk, J. D., Abel, T., & Havekes, R. (2010). Post-training reversible inactivation of the hippocampus enhances novel object recognition memory. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *17*(3), 155–160. https://doi.org/10.1101/lm.1625310

Onakpoya, O., Komolafe, E., Akintomide, F., Ajite, K., Komolafe, M., Adeolu, A., . . . Adegbehingbe, B. (2010). Ophthalmic manifestations in patients with intracranial tumours. *African Journal of Neurological Sciences*, *28*(1). https://doi.org/10.4314/ajns.v28i1.55137

Ondreicka, R., Ginter, E., & Kortus, J. (1966). Chronic toxicity of aluminium in rats and mice and its effects on phosphorus metabolism. *British Journal of Industrial Medicine*, *23*(4), 305 312. https://doi.org/10.1136/oem.23.4.305

Padungkiatsagul, T., Leung, L.-S., & Moss, H. E. (2020). Retinal Diseases that Can Masquerade as Neurological Causes of Vision Loss. *Current Neurology and Neuroscience Reports*, *20*(11), 51. https://doi.org/10.1007/s11910-020-01071-1

Paez-Gonzalez, P., Asrican, B., Rodriguez, E., & Kuo, C. T. (2014). Identification of distinct $ChAT^{+}$ neurons and activity-dependent control of postnatal SVZ neurogenesis. *Nature Neuroscience*, *17*(7), 934–942. https://doi.org/10.1038/nn.3734

Pansieri, J., Josserand, V., Lee, S.-J., Rongier, A., Imbert, D., Sallanon, M. M., . . . Forge, V. (2019). Ultraviolet–visible–near-infrared optical properties of amyloid fibrils shed light on amyloidogenesis. *Nature Photonics*, *13*(7), 473–479. https://doi.org/10.1038/s41566-019-0422 6

Pendlebury, S. T., & Rothwell, P. M. (2009). Prevalence, incidence, and factors associated with pre-stroke and post-stroke dementia: A systematic review and meta-analysis. *The Lancet Neurology*, *8*(11), 1006–1018. https://doi.org/10.1016/S1474-4422(09)70236-4

Peters, R. [R.] (2006). Ageing and the brain. *Postgraduate Medical Journal*, *82*(964), 84–88. https://doi.org/10.1136/pgmj.2005.036665

Peters, R. [Ruth], Pinto, E., Beckett, N., Swift, C., Potter, J., McCormack, T., . . . Bulpitt, C. (2010). Association of depression with subsequent mortality, cardiovascular morbidity and incident dementia in people aged 80 and over and suffering from hypertension. Data from the Hypertension in the Very Elderly Trial (HYVET). *Age and Ageing*, *39*(4), 439–445. https://doi.org/10.1093/ageing/afq042

Pournaras, C. J., Rungger-Brändle, E., Riva, C. E., Hardarson, S. H., & Stefansson, E. (2008). Regulation of retinal blood flow in health and disease. *Progress in Retinal and Eye Research*, *27*(3), 284–330. https://doi.org/10.1016/j.preteyeres.2008.02.002

Prasad, T., Zhu, P., Verma, A., Chakrabarty, P., Rosario, A. M., Golde, T. E., & Li, Q. (2017). Amyloid β peptides overexpression in retinal pigment epithelial cells via AAV-mediated gene transfer mimics AMD-like pathology in mice. *Scientific Reports*, *7*(1), 3222. https://doi.org/10.1038/s41598-017-03397-2

Profenno, L. A., Porsteinsson, A. P., & Faraone, S. V. (2010). Meta-Analysis of Alzheimer's Disease Risk with Obesity, Diabetes, and Related Disorders. *Biological Psychiatry*, *67*(6), 505 512. https://doi.org/10.1016/j.biopsych.2009.02.013

Rahman, M. A., Rahman, M. S., Uddin, M. J., Mamum-Or-Rashid, A. N. M., Pang, M.-G., & Rhim, H. (2020). Emerging risk of environmental factors: Insight mechanisms of Alzheimer's diseases. *Environmental Science and Pollution Research International*, *27*(36), 44659–44672. https://doi.org/10.1007/s11356-020-08243-z

Rajan, K. B., Weuve, J., Barnes, L. L., Wilson, R. S., & Evans, D. A. (2019). Prevalence and incidence of clinically diagnosed Alzheimer's disease dementia from 1994 to 2012 in a population study. *Alzheimer's & Dementia : The Journal of the Alzheimer's Association*, *15*(1), 1–7. https://doi.org/10.1016/j.jalz.2018.07.216

Sam, C., & Bordoni, B. (Eds.) (2023). *StatPearls [Internet]*. StatPearls Publishing.

Sarkisyan, G., & Hedlund, P. B. (2009). The 5-HT7 receptor is involved in allocentric spatial memory information processing. *Behavioural Brain Research*, *202*(1), 26–31. https://doi.org/10.1016/j.bbr.2009.03.011

Sinn, R., & Wittbrodt, J. (2013). An eye on eye development. *Mechanisms of Development*, *130*(6-8), 347–358. https://doi.org/10.1016/j.mod.2013.05.001

Stiles, J., & Jernigan, T. L. (2010). The basics of brain development. *Neuropsychology Review*, *20*(4), 327–348. https://doi.org/10.1007/s11065-010-9148-4

Tahami Monfared, A. A., Byrnes, M. J., White, L. A., & Zhang, Q. [Quanwu] (2022). Alzheimer's Disease: Epidemiology and Clinical Progression. *Neurology and Therapy*, *11*(2), 553–569. https://doi.org/10.1007/s40120-022-00338-8

Themes, U. F. (2017). Thioflavin T Stain: An Easier and More Sensitive Method for Amyloid Detection. Retrieved from https://basicmedicalkey.com/thioflavin-t-stain-an-easier-and-more sensitive-method-for-amyloid-detection/

Troutwine, B. R., Hamid, L., Lysaker, C. R., Strope, T. A., & Wilkins, H. M. (2022). Apolipoprotein E and Alzheimer's disease. *Acta Pharmaceutica Sinica B*, *12*(2), 496–510. https://doi.org/10.1016/j.apsb.2021.10.002

Van Oostveen, W. M., & Lange, E. C. M. de (2021). Imaging Techniques in Alzheimer's Disease: A Review of Applications in Early Diagnosis and Longitudinal Monitoring. *International Journal of Molecular Sciences*, *22*(4), 2110. https://doi.org/10.3390/ijms22042110

Veys, L., Vandenabeele, M., Ortuño-Lizarán, I., Baekelandt, V., Cuenca, N., Moons, L., & Groef, L. de (2019). Retinal α-synuclein deposits in Parkinson's disease patients and animal models. *Acta Neuropathologica*, *137*(3), 379–395. https://doi.org/10.1007/s00401-018-01956-z

Viezeliene, D. (2022). "Aluminium Induces Iron-Mediated Oxidative Stress in Brain Tissue". *Biomedical Journal of Scientific & Technical Research*, *42*(3). https://doi.org/10.26717/BJSTR.2022.42.006771

Wang, L. [Liang], & Mao, X. (2021). Role of Retinal Amyloid-β in Neurodegenerative Diseases: Overlapping Mechanisms and Emerging Clinical Applications. *International Journal of Molecular Sciences*, *22*(5). https://doi.org/10.3390/ijms22052360

Wang, L. [Linping] (2018). Entry and Deposit of Aluminum in the Brain. *Advances in Experimental Medicine and Biology*, *1091*, 39–51. https://doi.org/10.1007/978-981-13-1370 7_3

Watanabe, M., Maemura, K., Kanbara, K., Tamayama, T., & Hayasaki, H. (2002). Gaba and GABA receptors in the central nervous system and other organs. *International Review of Cytology*, *213*, 1–47. https://doi.org/10.1016/s0074-7696(02)13011-7

Wong-Riley, M. T. T. (2010). Energy metabolism of the visual system. *Eye and Brain*, *2*, 99 116. https://doi.org/10.2147/EB.S9078

Wu, J., Cai, Y., Wu, X., Ying, Y., Tai, Y., & He, M. (2021). Transcardiac Perfusion of the Mouse for Brain Tissue Dissection and Fixation. *Bio-Protocol*, *11*(5), e3988. https://doi.org/10.21769/BioProtoc.3988

Xing, Z., He, Z. [Zhongmei], Wang, S., Yan, Y., Zhu, H., Gao, Y., . . . Zhang, L. (2018). Ameliorative effects and possible molecular mechanisms of action of fibrauretine from Fibraurea recisa Pierre on d-galactose/AlCl3-mediated Alzheimer's disease. *RSC Advances*, *8*(55), 31646–31657. https://doi.org/10.1039/c8ra05356a

Yamazaki, Y., Zhao, N., Caulfield, T. R., Liu, C.-C. [Chia-Chen], & Bu, G. (2019). Apolipoprotein E and Alzheimer disease: Pathobiology and targeting strategies. *Nature Reviews Neurology*, *15*(9), 501–518. https://doi.org/10.1038/s41582-019-0228-7

Yang, W.-N., Han, H., Hu, X.-D., Feng, G.-F., & Qian, Y.-H. (2013). The effects of perindopril on cognitive impairment induced by d-galactose and aluminum trichloride via inhibition of acetylcholinesterase activity and oxidative stress. *Pharmacology, Biochemistry, and Behavior*, *114-115*, 31–36. https://doi.org/10.1016/j.pbb.2013.10.027

Yap, T. E., Balendra, S. I., Almonte, M. T., & Cordeiro, M. F. (2019). Retinal correlates of neurological disorders. *Therapeutic Advances in Chronic Disease*, *10*, 2040622319882205. https://doi.org/10.1177/2040622319882205

Zhang, J., Shi, L., & Shen, Y. (2022). The retina: A window in which to view the pathogenesis of Alzheimer's disease. *Ageing Research Reviews*, *77*, 101590. https://doi.org/10.1016/j.arr.2022.101590

Zhou, Y., & Danbolt, N. C. (2014). Glutamate as a neurotransmitter in the healthy brain. *Journal of Neural Transmission*, *121*(8), 799–817. https://doi.org/10.1007/s00702-014-1180-8