ASSOCIATION OF B-CELL LYMPHOMA 2 (*BCL2*) GENE SNP WITH SUSCEPTIBILITY TO ALZHEIMER'S DISEASE

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BAHRIA UNIVERSITY ISLAMABAD

PAKISTAN

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DR ALLAH BAKHSH

06-116212-002

Thesis submitted for fulfillment of the requirement for the degree of Master of Philosophy (MPhil) Biochemistry

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Program of study: MPhil Biochemistry

Thesis Title: " Association Of B-Cell Lymphoma 2 (*BCL2*) Gene SNP with susceptibility to Alzheimer's Disease "

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Dedicated to my Parents

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Dr Allah Bakhsh

ABSTRACT

Alzheimer's disease (AD) is neurodegenerative disorder characterized by a gradual and severe deterioration in cognitive abilities along with behavioral symptoms. Genes expression play a vital role in the development of Alzheimer's disease and can serve as specific diagnostic biomarkers. Recent studies showed that increased expression of *BCL2* protein promotes axon regeneration and provides protection by regulating cellular resilience and apoptosis pathways. On the basis of such studies, *BCL2* gene is suggested to be a target for early detection and therapeutic intervention in AD. Therefore, this study was planned to evaluate the association of B-cell lymphoma 2 (*BCL2*) gene single nucleotide polymorphism (SNP) rs921884063 with susceptibility to Alzheimer's disease. The case control-based study divided the subjects into two groups 1) Age and Gender matched controls (n=21), 2) AD Patients (n=20). These patients were taken after assessment of cognitive abilities and behavioral symptoms and were further investigated for gene single nucleotide polymorphism of *BCL2*. This study has explored the association of B-cell lymphoma 2 (*BCL2*) gene single nucleotide polymorphism (SNP) rs921884063 in AD for making prognosis and early diagnosis. The Chi square test was used for analysis which showed a significant association of *BCL2* SNP rs921884063 with progression of Alzheimer's disease ($p= 0.001$). Odds ratio revealed that targeted SNP has significant association with the increased risk of Alzheimer's disease [3.9947, CI 95% (2.0774-7.6812 $p<0.001$)]. The association of genotype was analyzed by applying genotype models which confirmed that the heterozygous G/A genotype showed significant protective role against the development of disease in codominant [OR CI95%=0.21, (0.05-0.83, $p<0.01$)], dominant [OR CI95%=0.71, (0.04-0.65, $p<0.01$)], and over dominant models [OR CI95%=0.3, (0.09-1.19, $p<0.05$)]. In this study the genotype G/A showed a significant protective role against the disease. The study concluded detection of a genetic marker for progression of AD will help in developing therapeutic strategies for treatment and management of disease.

Key words: Alzheimer's disease, B-cell lymphoma 2, *BCL2*, Single Nucleotide Polymorphism, SNP, Genotype

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LIST OF ABBREVATIONS

AD	Alzhiemers Disease
MCI	Mild Cognitive Impatiment
BCL2	B-Cell Lymphoma 2
APP	Amyloid Precursor Protien
DNA	Deoxyribonucleic Acid
APOE	Apolipoprotein E
ROS	Reactive Oxygen Specie
AL	Alimonium
BBB	Blood Brain Barrier
DM	Diabetes Militus
LPO	Lactate Dehydrogenase Phosphate
NDMA	N- Methyl D- Aspartate
SNP	Single Nucletide Polymorphism
T-ARMS-PCR	Tetra-Primer Amplification Refractory Mutation system-PCR
PET	Positron Emission Tomography
CSF	Cerebro Spinal Fluid
FDA	Food and Drug Administraion
CRH	Corticotropin Releasing Hormone
AMPA	α-Amino-3-hydroxy-5-Methyl-4-isoxazolePropionic Acid
GABA	Gamma-Amino Butyric acid
NE	Nor- Epinephrine
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
CRP	C- Reactive Protien
VEGF	Vascular Endothelial Growth Factor

ERC	Ethical Review Committee
EDTA	Ethyline Diamine Tetra Acetic Acid
MRI	Magnetic Resonance Imaging
TE	Tris EDTA
BMI	Body Mass Index
RCLB	Red Cell Lysis Buffer
UV	Ultravoilet
RNA	Ribonucleic Acid
MAF	Minor Allelic Frequency
NCBI	National Center for Biotechnolohy Information
PCR	Polymerase Chain Reaction
SPSS	Statistical Programm for Social Sciences
SDS	Sodium Dodecyl Sulfate

ANNEXURES

A	BUMDC- FRC Approval Letter
B	BUMDC- ERC Approval Letter
C	Consent Form
D	Subject Evaluation Form
E	Hospital / Institute Card
F	Turnitin Plagiarism Check Report

SECTION 1

INTRODUCTION

1.1 BACKGROUND

Alzheimer's disease (AD) is a disorder that affects the brain cells and causes neurodegenerative changes. AD is neuropathologically defined by atrophy of brain cortex over time (Ju & Tam, 2022). These changes in the cortex are progressive in nature. It is caused by the abnormal processing and aggregation of normally soluble proteins in the neurons. These altered conformations of soluble protein cause increasing neuronal cell death and brain tissue atrophy ultimately leading to memory loss and abnormal neural functions. There is a correlation between the accumulation of insoluble protein in the brain and the onset of symptoms resembling AD(Kim et al., 2020). Neurodegenerative condition is responsible for the development of dementia. It is the most prevalent condition that is associated with dementia. It is a frequent cause of dementia in adults (Mintum et al., 2021).

AD is characterized by a gradual and severe deterioration in cognitive abilities along with behavioral symptoms that leads to gradual memory loss along with impairment of language. Characteristic neurobehavioral and neuropathological features, such as the loss of neurons, brain shrinkage, and significant cognitive impairment are characteristics of the disease (Weller & Budson, 2018).

Individuals suffering from AD usually present with multitude of symptoms including memory loss and disorientation. The most prevalent symptom of AD is difficulty in learning . Memory loss, depression, difficulty in concentration and difficulties in performing daily life activities are hallmarks of AD. It greatly affects the mental and psychological abilities of a person causing diminished cognitive skills. Apart

from mental disabilities , it is also responsible for the diminished level of problem-solving skills over the time (Atri, 2019).

It is the most common form of dementia around the world. Globally a large number of people are being affected by this disorder every day. The number of individuals that are being affected by this progressive neurogenerative disorder is growing. It accounts for 70-80 percent of cases of dementia. The number of individuals suffering from AD is projected to have doubled by the year 2040, from the current level. Over 50 million individuals worldwide are reported to be suffering from AD-related dementia (Kshirsagar et al., 2022). It accounts for around two-thirds of all dementia diagnosis around the globe (Rasmussen & Langerman, 2019). More than 2% of the global population in highly developed nations has this disorder (Mintum et al., 2021). This disorder is responsible for sixty to seventy percent of all dementia cases in older adults globally. It is anticipated that by the year 2025, there will be over 22 million dementia sufferers worldwide and without appropriate treatments, the population is projected to reach 152 million by the year 2050 (Kshirsagar et al., 2022). Currently, 58% of the world's ageing population with dementia resides in low to medium income nations; by 2050, this proportion will increase to 68%. China, Pakistan, and their south Asian and western Pacific neighbors are seeing the greatest increase in the old population (Adamson et al., 2020). Prevalence of AD patients in Pakistan is reported to be 1.72 percent (Aurooj & Mahmood, 2022) . According to the latest WHO statistics released in 2020, Alzheimer's & Dementia associated fatalities account for 1.04 percent of total deaths in Pakistan and it rank 121 in world in age adjusted mortality rate.

The progression of AD includes asymptomatic preclinical AD, mild cognitive impairment (MCI), mild, moderate, and severe AD associated dementia (Swanson et al., 2021). AD can be classified into two forms i.e. Early-onset AD and Late-onset AD, based on the onset of symptoms associated with this condition. Early-onset AD is the less dominant form of the disease, and it affects two to ten percent of the population. It often develops in 20s or 30s of age. Late-onset AD is predominant in adults beyond the age of 65. Since the pathogenic mechanisms of AD are so similar to those observed in older brains, it was long believed that the disease was caused by an accelerated ageing process (Cuyvers & Sleegers, 2016).

Apart from Early-onset AD and Late-onset AD, there is a rare form of AD. It is a familial disorder and typically manifests with autosomal dominant inheritance. It

accounts for less than 1 % of all patients suffering from it. In contrast, there is a sporadic type of AD that is believed to be caused by a mix of environmental exposures and genetic predisposition. AD is a familial disease that is inherited by autosomal dominant trait. Many individuals who lack autosomal pattern of inheritance can get the disorder at an early age. On the other hand, individuals with late-onset AD have also been discovered with autosomal dominant mutations(Armstrong, 2019).

There are 3 genes mutations that have been identified that have the potential to cause autosomal dominant AD. These gene mutations include APP, PSEN1, and PSEN2.6. They are responsible for between 5 and 10 percent of cases of AD that manifest in early adulthood. Observational study reveals that 30–48 percent of AD patients had a first-degree relative with the condition. This percentage compared to 13–19 percent of normal people (Arber et al., 2021).

Studies have reported that there is a 3.5-fold increased chance of getting the AD among first-degree relatives compared to the general population. Numerous instances of AD with an early start indicate an autosomal dominant pattern of inheritance. Additionally, there is a strong correlation between AD and Down syndrome. Genetics has a very complex role in the development of AD (Fortea et al., 2021).

From an etiological standpoint, AD is acknowledged as a complicated disorder. Although it is yet unknown what causes AD, certain factors have been recognized to be responsible for development and progression of the disorder. These include a wide variety of factors ranging from age, genetic susceptibility , environmental factors, neurotoxins as well as the dietary habits of an individual (Armstrong, 2019; Silva et al., 2019). The pathophysiology of AD also includes several neurotransmitter system abnormalities and pathophysiological mechanisms. It is also observed that metabolic disorders, loss of synapses, synaptic dysfunction, cholinergic insufficiency, Inflammatory responses , oxidative stress nay also be responsible for development of AD (Hampel et al., 2018). The underlying pathogenic mechanisms and neurotransmitter systems that contribute to the development of AD (AD) are complex. Abnormal cell functioning like microRNA dysregulation, mitochondrial structural and functional abnormalities lead to neuronal death and ultimately leads to development of AD (Johnson et al., 2021).

Several environmental, genetic, and physiological variables, including environmental , traumatic brain injury, cardiovascular, hypertension, renal illness, diabetes, individual with high body mass index and occupational exposures, and lifestyle

conditions have also reported to be associated with disease progression (Kshirsagar et al., 2022). AD is considered as a polygenetic neurodegenerative brain condition, and the neuropathological hallmarks of the atrophy process (Ju & Tam, 2022).

The probability of an individual acquiring AD increases with the increasing age. Age is strongly associated with the development of disorder. Studies have shown that oxidized lipids, proteins, and DNA exponentially increase with age in people with AD. Ageing along with other risk factors like oxidative stress, metabolic abnormalities and changes in neurochemical pathways are observed to have combine effects in causing neurodegenerative process leading to development of AD (Breijyeh & Karaman, 2020).

Genetics is a potential risk for AD. Recent advances in genomic research have uncovered evidence that the genetic mutations are responsible for the underline complexity of AD. Studies based on Next Generation Sequencing (NGS) have already established genetics as the vital factor for development and progression of disorders. The biggest genetic risk factor identified for AD is APOE gene polymorphism. More than 20 loci and genes for AD have been discovered. AD compromises unusual and structural variants as well as functional variants. The changes have caused a significant paradigm to change in the conventional understanding of the genetic risk factors for AD (Martens et al., 2022).

A functionally relevant genetic variation has the potential to give insight into molecular pathways responsible for developing AD. Various techniques are available to study the functional assessment of genetic risk variations. Findings from these assessments have greater potential for creation of techniques for disease modification and prevention which will help in early diagnosis and forecasting an individual's likelihood of developing AD.

Apoptosis is an intrinsic biochemical process. It is process at cellular level that governs cell death. It is crucial for the survival of the cell. It is a process that is responsible for maintaining constant internal environment of cell. It is a predetermined process that is responsible for the regulation of several molecular events. The molecular events include cell proliferation, development of fetus and normal functioning of the immune system. Consequently, in the development of neurogenerative manifestations and progression of AD, apoptosis appears to be an exclusive contributor. The unusual neuronal loss is the ultimate result of an apoptotic cascade that has been dysregulated. The event precedes the development of AD and correlates well with the degree of

dementia (Yetirajam & Kanneganti, 2022). Increase level of amyloid b peptide (Ab) is responsible for apoptosis. There is also a correlation between elevated calcium levels and the process of apoptosis, and it has been hypothesized that *BCL2* plays a role in the regulation of calcium homeostasis inside cells (Kim et al., 2020). The potential explanation for the abnormal control of intracellular calcium levels by A lies in the downregulation of *BCL2* in human neurons (Cascella & Cecchi, 2021).

The B-cell lymphoma-2 (*BCL2*) protein family is essential for cellular health. Primarily, it controls the commencement of mitochondrial apoptosis. It also modulates the key process for the functioning of neurons by altering intracellular Ca2+ signaling. Studies have reported that numerous members of *BCL2* family have been identified to have significant influence in the normal functioning of the neurons (Callens et al., 2021).

It has been reported that *BCL2* gene prevents brain degeneration by modulating cellular resilience and apoptotic pathways. It stimulates axon regrowth and has anti neuroinflammatory effects. Healthy populations with *BCL2* AA genotype had decreased *BCL2* levels and grey matter volume which is further associated with altered cognitive and motor functions (Chang et al., 2018). A single nucleotide polymorphism in (*BCL2*) gene alter *BCL2* protein expression in the brain. Age significantly affects the expression levels of neuronal *BCL2* proteins (Callens et al., 2021). For that reason, pharmacological modalities that upregulates *BCL2* protein expression and work against -amyloid cascades and could be used for the treatment of AD (Chang et al., 2018).

In a healthy individual, mitochondria and nuclear membrane produce *BCL2* protein. It inhibits expression of capase-9 by interacting with Apaf-1.The processing of APP and tau may be slowed down by overexpressing *BCL2*, which also lowered the quantity of neurofibrillary tangles and extracellular deposits of amyloid (L. Wang et al., 2020).

In individuals suffering from rare form early onset AD, it was reported to have mutation in amyloid precursor protein (APP). In some cases, genetic mutation on chromosome 14 has also been reported and it is considered as an important risk factor for development of AD. The apolipoprotein E gene allele 4 (ApoE4) plays a crucial role in APP processing. Those who have allele 4 of the apolipoprotein E gene are more likely to

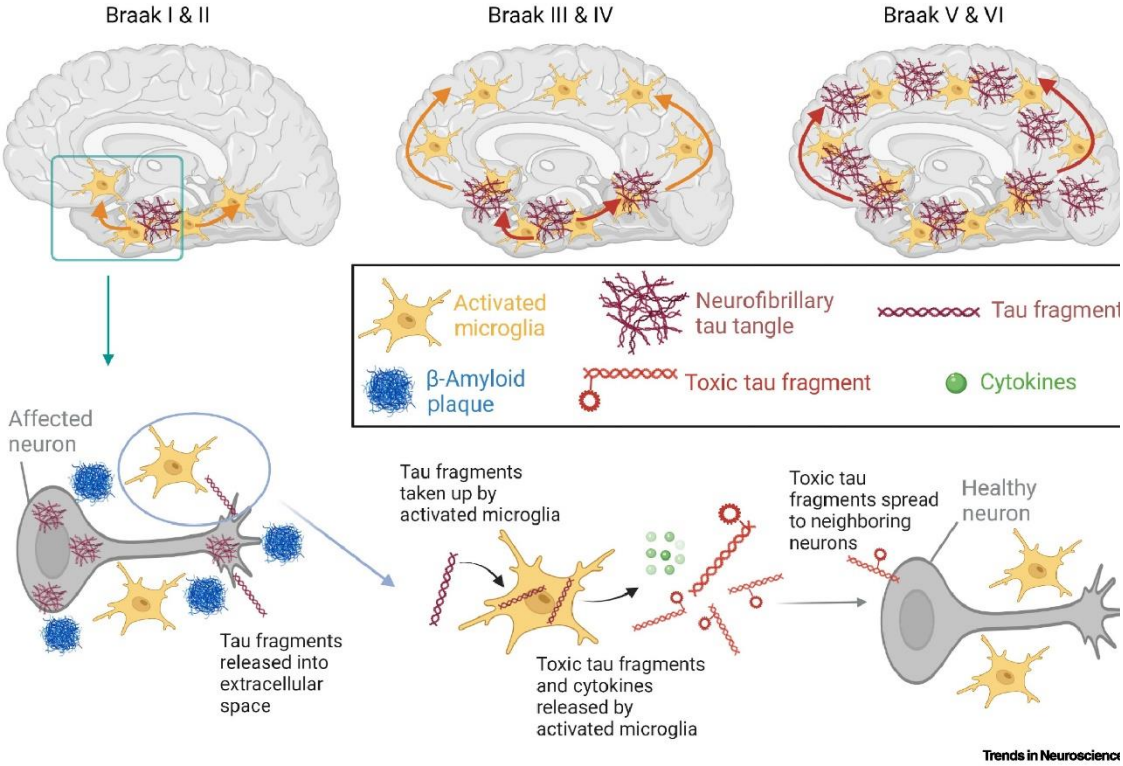

Figure 1.1: β- Amyloid Plaque and Tau protein in Alzheimer's Disease (Hopp S.C.et al. 2018)

acquire AD as this genetic variation encourages the production of amyloid plaques (Liu et al., 2022).

The amyloid hypothesis suggests that there are certain external factors which prevent degradation of APP. AD may be directly caused by variations in the genes encoding the amyloid precursor proteins, presenilin-1 and presenilin-2. Conditions of oxidative stress promote the cross-linking and aggregation of cytoskeletal proteins such as α peptide and tau protein. This leads to accumulation of APP intracellularly which ultimately aggregates to form oligomeric lethal amyloid-beta (Aβ) plaques, and accumulation of phosphorylated tau protein in the form of neurofibrillary tangles (p-tau). The plaques are caused by the enzyme's elastase and secretase. These plaques and tangles are then responsible for disruption of neural cell network and ultimately cell death of a neuron (Karran & De Strooper, 2022).

Accumulation of abnormal proteins in the brain cells in the form of neurofibrillary tangles leads to synaptic loss in cortex and hippocampus. As the condition progresses, these protein deposits substantially disrupt normal brain functions. Consequently, this leads to impaired functioning of neuron resulting in memory loss and psycho-emotional dysfunction and ultimately neuronal death. Reduced synaptic density and neuronal loss in certain brain areas, such as the cerebral cortex and hippocampus, as well as a persistent reduction in cognitive function, are crucial diagnostic criteria for AD (Kshirsagar et al., 2022).

The abnormal accumulation of these plaques is also responsible for alteration of metabolic and structural composition of pyramidal neurons in the cortex and hippocampus. It is also observed that when α aggregates come in contact with components of nerve cells, it causes a chain of reaction that ultimately results in production of intracellular reactive oxygen species (ROS) (Cheignon et al., 2018).

There are certain triggers which cause alteration in APP cleavage system by producing oligomeric toxic species of α plaques that have not yet been identified. The overproduction of these toxic species results in disarrangement of neural network which leads to neural death. This causes impairment of mechanism involved in synaptic plasticity. There are studies which suggest that in pyramidal neurons of the cortex and hippocampus , certain physiological and anatomical changes cause imbalance in the production of toxic oligomeric species (Arber et al., 2021).

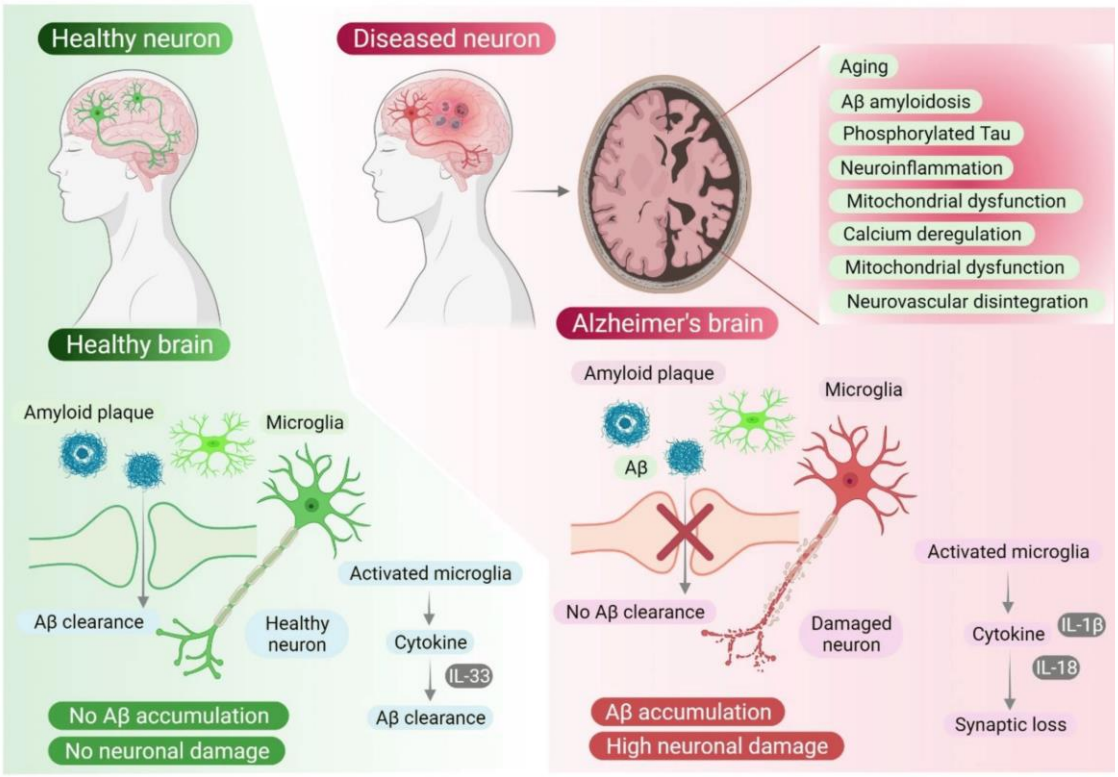

Figure 1.2: Pathophysiology of Alzheimer's Disease (Akash .et al. 2022)

For the formation of aggregates of cytoskeletal protein i.e., α peptide, tau and others as well as for the cross-linkage formation, oxidative condition of the cell provides a favorable condition. In addition, the interaction of Aβ aggregates with the membranes of nerve cells triggers a chain of events that ultimately leads to a buildup of ROS within the cell (Nam et al., 2018). The microtubule-associated tau protein in the brain of a person with Alzheimer's disease oxidizes and becomes hyperphosphorylated. It is also possible for it to create proteinaceous deposits inside of cells in the form of neurofibrillary tangles (NFTs), which are of diagnostic value as well (Wegmann et al., 2021).

NFTs are one of the most important neuropathological hallmarks of Alzheimer's disease (AD). Neurons that carry NFTs have been shown to lose tubulin related proteins in addition to other cytoskeletal microtubules. It is generally agreed that signal transduction pathways that connect the phosphorylation and dephosphorylation of proteins are the primary contributors to the development of NFTs. These microtubules related tau proteins go through an aberrant process called hyperphosphorylation throughout the pathogenic phase, which eventually leads to tau polymerization and aggregation, which then leads to the creation of NFTs in different parts of the cortex of brain. The quantity of NFTs correlates with the severity of the disorder. NFTs predominantly manifest in areas of the brain involved for the regulation of many cognitive domains. These areas are impacted by the AD degenerative process, and the density of NFTs is positively linked with many features of cognitive decline. It is believed that NFTs play a significant role in the course of AD (Naseri et al., 2019).

Environmental toxins stimulate the release of reactive oxygen species(ROS). Neurons in the brain are highly susceptible to it as they are responsible for damaging the neurons. ROS has high oxygen demand for energy production. They also halt the activity of antioxidant enzymes. They are easily peroxidized by lipids because their composition contains high concentrations of polyunsaturated fatty acids (PUFAs). Studies suggest that any environmental factor that has the capability to increase the production of ROS has a potential to damage neurons and cause premature ageing and neurodegenerative disorders (Bai et al., 2022).

Recent studies suggest that chemical substances and air pollutants neurotoxin in nature and contribute to the progression of AD. It is observed that since the discovery of Aluminum (AL) in the late 70s , there has been a sudden increase in the reported cases of AD. There is a certain way in which AL gains access to brain cells. The most common

ways include gastric medication like antacids, cooking utensils and deodorants. Acid rain frequently contains substantial levels of soluble Al salts. With the increasing age, the blood-brain barrier (BBB) gradually weakens. When an individual with weakened BBB, has prolong exposure to high quantities of AL, it accumulates in the brain and accelerates the aging process via oxidative modification and ROS production. This results in activation of glial cells which results in multiple neurodegenerative disorders including AD (Das et al., 2021).

Metabolic activities produce free radicals that contribute to the ageing process. An increase in production of ROS is associated with mitochondrial failure. Metabolic dysfunction is associated with development and progression of several neurodegenerative diseases, including AD. The neurodegenerative processes of AD have recently been hypothesized to be accelerated by any reduction in metabolic activity (Takeishi et al., 2021).

Individuals with high body mass index and impaired glucose metabolism are at higher risk of developing AD because they have impaired metabolic activities that are responsible for the production of high levels of free radicals. The inhibition of crucial enzymes by α plaques that leads to the impairment of energy metabolism by causing a disturbance in the mitochondrial membrane is an example of such metabolic activities (Butterfield & Halliwell, 2019).

There is a high concentration of insulin receptors in key brain areas like the hippocampus. Multiple epidemiological studies have established an association between type-2 diabetes mellitus. Hyperglycemia is also responsible for the induction of apoptosis and breakdown of tau protein. The chance of acquiring AD is elevated by a factor of two in those with type-2 diabetes because it is associated with brain abnormalities, accelerated aging and cognitive abnormalities. In addition to DM, hypertension, hypercholesterolemia and atherosclerosis are also significant risk factors for development and progression of AD (Iadecola & Gottesman, 2019).

Specific diet which contains vitamins, antioxidants and polyphenols associated with reduced chance of developing AD (Leri et al., 2021). On the contrary, unhealthy diets like junk food, alcohol and saturated fat increase the risk of AD (G. Wang et al., 2023).

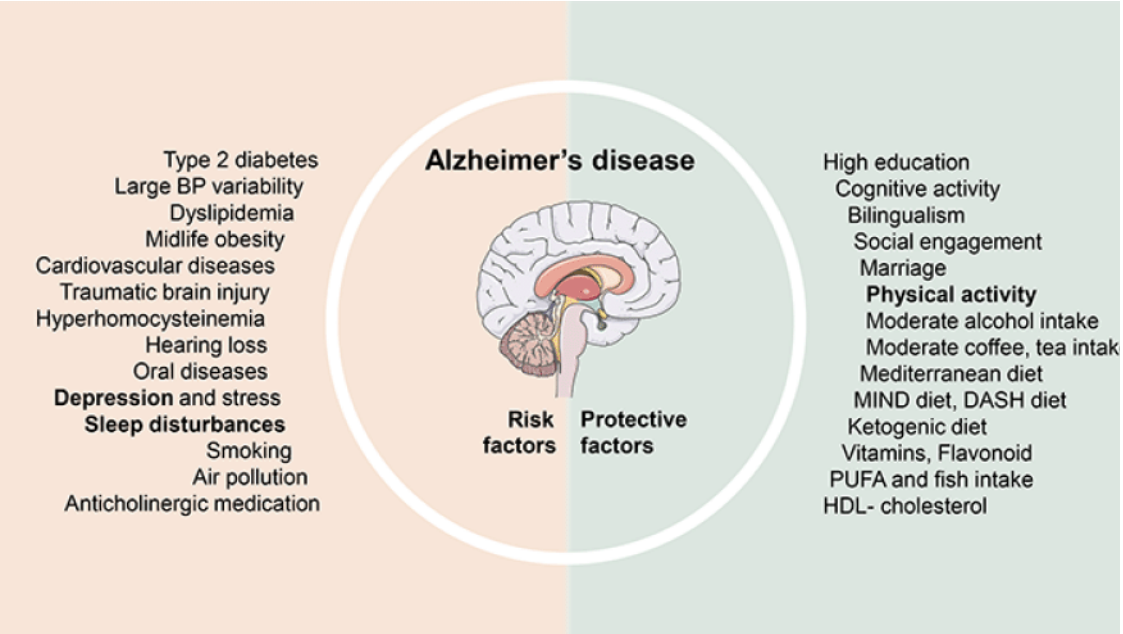

Figure 1.3: Risk and Protective Factors of Alzheimer's Disease (Zhang,X.X. el. al 2021)

Studies suggest that imbalance of heavy metals can lead to production of metal amyloid complexes and free radicals which may contribute to degenerative process of AD. It is observed in certain studies that a diet heavy in copper and saturated fatty acids can be a potential risk factor for AD (Singh et al., 2022).

It is observed that early onset AD may be caused by trans fatty acids as they speed up amyloidogenic process of APP. On contrary to it, long chain unsaturated fatty acids have been associated with a lower risk of dementia and AD (Nwaru et al., 2022).

Oxidative stress has a harmful effect on normal biological systems. These effects include cell death, raised intracellular calcium levels, and change in protein structure. They initiate inflammatory responses as well as disrupt normal functionality of essential macromolecules. Neurotoxicity caused by these oxidative stress factors is responsible for neurodegeneration. An individual whose eating habits include a healthy diet like fresh fruits and vegetables is less likely to develop dementia and AD as this diet contains high concentrations of antioxidants. They prevent not only the development of the disorder but also help to slow down the progression of disease (Singh et al., 2019).

In the autopsies of brain of AD patients, high concentrations of lactate dehydrogenase phosphatase (LPO) were found. In the same study it was also observed that brain cells in these autopsies had low levels of polyunsaturated fatty acids (PUFAs). The presence of these chemical substances in neurons makes it susceptible to injury related to free radicals. It is also observed that the highest alteration in the pathophysiological site by oxidative compounds was temporal lobe which also has highest activity of LPO (Acharige & Pflum, 2021).

DNA strand breaks, DNA-protein cross-links, DNA-DNA cross-links, sister chromatid exchange, and base changes are all examples of structural damage caused by oxidative stress. These harmful occurrences follow the production of free radicals (Pao et al., 2020).

Dietary variables are rapidly being recognised as important in improving cognitive health and lowering the risk of age-related cognitive decline, including AD, in the study of nutritional epidemiology as a whole. More investigation into the underlying processes and appropriate nutritional strategies for preserving brain function is required (Śliwińska & Jeziorek, 2021).

There are extremely limited treatment choices available for the management of AD. Keeping in mind the burden of disease and growing expenses attached with it , there is a need for early detection of patients in early stage of disease.

Currently there is no definite treatment modality available. Presently available pharmacological treatment options for dementia and AD are very limited and they include NDMA receptor antagonist and cholinesterase inhibitors (Marucci et al., 2021). These drugs have been demonstrated to improve quality of life when provided at the proper time during the disease's development; nevertheless, they do not influence the disease's progression or pace of decline.

Although these treatment options are limited , they are also expensive. So, the focus should be on detecting susceptible individuals far earlier in the disease progression so that urgent action can be taken. This will significantly help in health care cost as well as burden of disease. Even though there is no preventative or curative medication available for this condition, early sickness or disease risk diagnosis is essential so that the individual and his or her care givers have time to make choices and plan for the future (Rasmussen & Langerman, 2019).

There is need for development of targeted therapeutic approaches that are efficacious against AD. For this purpose, there is need to look at cellular factors responsible for pathogenesis of disease so that targeted therapeutic approaches can be used to improve quality of life for individuals suffering from AD. Early diagnosis of the individual at risk is necessary so that appropriate action can be taken to slow or stop future deterioration. Early disease or risk diagnosis will help the person and their caregivers to make decisions in time and prepare for the future (Porsteinsson et al., 2021).

The pharmacological drugs that are currently approved for modulating cholinergic transmission encompass donepezil, rivastigmine, and galantamine. These interventions have demonstrated efficacy in enhancing cognitive function among individuals and alleviating the societal and economic challenges associated with their condition. The efficacy of these interventions has been established in individuals who have received a diagnosis of mild to moderate AD (Marucci et al., 2021).

Memantine is a pharmacological compound that functions as an antagonist of the NMDA receptor. The prescription of this medication is a common practice for the management of AD during its moderate to severe stages. Pharmacological compounds

that impede the production of Aβ and function as antagonists for the 5HT6 receptor have exhibited promise in augmenting cognitive faculties in individuals suffering from AD (Guo et al., 2020).

Gene therapy has emerged as a prospective intervention with the potential to enhance the management of AD, owing to the well-established and validated association between the disorder and genetic mutations and variations (W. Chen et al., 2020).

Theranostics is an innovative technological advancement that has the potential to greatly transform the current therapeutic options available for AD. Moreover, it can be employed for diagnostic purposes in individuals suffering from AD. The utilization of gold nanorods, in conjunction with Aβ15-20 and polyoxometalates, has been found to exhibit the capacity to detect, inhibit, and eliminate amyloid aggregates by employing near infra-red radiation (Sarabia-Vallejo et al., 2023).

Ongoing research is being conducted on immunotherapy with the aim of developing anti-amyloid and anti-tau agents that can selectively target misfolded tau proteins and facilitate neuronal regeneration. Immunotherapy encompasses the utilization of antibodies to neutralize and solubilize Aβ. The mechanism of phagocytosis relies on the process of opsonization of Aβ plaque, which in turn triggers phagocytosis by microglia. In the early stages, antibodies exhibit the ability to bind to Amyloid seed and effectively hinder its propagation (Song et al., 2022).

Peptidomimetics refers to synthetic compounds that mimic the structure and function of natural proteins, enabling them to interact with biological targets and demonstrate comparable or enhanced biological activity. Peptide inhibitors can be obtained through the derivation of the Aβ sequence. In addition, the phosphorylated tau protein and Human β-Secretase could also be potential targets for intervention (Kalita et al., 2020).

The interplay between metals and Aβ leads to the generation of ROS and perturbation of the physiological equilibrium of metal ions, thereby contributing to the pathogenesis of AD. Metal chelators are chemical compounds that interfere with the formation of complexes between Aβ peptides and metal ions, thereby reinstating the equilibrium of metal ions and mitigating neurotoxic effects (L. L. Chen et al., 2023).

AD is characterized by a notable increase in the concentration of metal ions, namely copper, iron, and zinc, which is approximately three times greater than the typical

levels. As a result, the main metallic elements that were the subject of chelation were determined. Deferoxamine and Deferiprone are extensively acknowledged as primary metal chelators that have been specifically developed for the purpose of binding iron. The treatment of memory impairment induced by iron involves the prevention of ROS production and the mitigation of metal-induced toxicity (Fasae et al., 2021).

Extensive research has been conducted on animal models and human subjects to examine the effects of prebiotics and probiotics on AD. Previous studies utilizing animal models have demonstrated a correlation between an increase in Actinobacteria and Bacteroidetes and a reduction in memory impairment, neuronal inflammation, and immune responses. The results of the study suggest that regular participation in physical exercise and the consumption of probiotic supplements have demonstrated the ability to hinder the progression of AD and alleviate its related symptoms (Jemimah et al., 2023).

So, the main object of this study is to identify the genetic biomarkers associated with AD for early detection of Alzheimer's disease and progressive neurodegeneration of the brain so that effective strategy against the neurodegenerative process associated with AD can be developed. Early identification of persons at risk and prompt diagnosis would aid in the recommendation of mid-adult lifestyle adjustments that will prevent or decrease the course of illness systems.

1.2 RESEARCH GAP/ RATIONALE OF THE STUDY

The study will help in expanding the understanding the complex nature of Alzheimer disease especially in Pakistan, where a limited data available regarding expression of *BCL2* gene in Alzheimer's disease. The study will evaluate the association of B-cell lymphoma 2 (*BCL2*) gene single nucleotide polymorphism (SNP) rs921884063 with susceptibility to Alzheimer's disease in Pakistani population and its association with clinical features of the disease. This will facilitate identifying a reliable genetic maker for early diagnosis of disease which will pave the way for early intervention. The study will explore the genetic marker for the prognosis of disease and to develop therapeutic strategy to manage the disease. This will eventually alleviate the devastating impact of disease not only on the patients but also on their families. The study will also contribute to informed decision making, appropriate resource allocation and advocacy for increased funding in the future , benefiting individuals suffering from Alzheimer's disease and society as a whole.

1.2.1 Theoretical Gap

Despite advances in understanding the pathophysiology of Alzheimer's Disease, researchers have not yet identified early detectable signs, medicines, or therapies that might prevent or reduce its course. Current therapies for Alzheimer's Disease may only give symptomatic relief with a range of adverse effects. These drugs are effective only on intact neurons and cannot prevent the ongoing neurodegenerative process. Therefore, there is an urgent need to explore treatment modalities that not only restore brain functioning but also prevent neurodegeneration. The diagnosis of AD using a specific genetic marker and current technological breakthroughs would aid in a better knowledge of disease prediction, monitoring, progression, and therapy effects in clinical practice and clinical trials. The combination of noninvasive biomarker identification techniques with current advances in novel diagnostic tests would be a breakthrough in the medical world. Utilizing a minimally invasive test based on using T-ARMS-PCR (tetra-primer amplification refractory mutation system-polymerase chain) analysis of specific *BCL2* gene polymorphism would give improved choices for diagnosing Alzheimer's Disease with cognitive issues.

1.2.2 Contextual Gap

PET imaging and tests that use cerebrospinal fluid (CSF) can be used to identify Alzheimer's before dementia develops however researchers on identifying most sensitive and reliable blood based diagnostic test to detect Alzheimer’s disease is in progress and need more attention so that early diagnostic tools can be available for the symptomatic patients.

1.2.3 Methodological Gap

Several studies have demonstrated that blood tests utilizing the detection of beta-amyloid protein in plasma can serve as a screening tool for diagnosing Alzheimer's disease in patients with clinically significant features. However, this technique requires Mass Spectroscopy, which is not easily accessible in pathological laboratories. As a result, there is a need to investigate alternative minimally invasive, cost-effective, convenient, and readily available screening tests for diagnosing Alzheimer's disease.

1.3 PROBLEM STATEMENT

Despite breakthroughs in the understanding of the pathogenesis of AD, researchers have yet to identify early detectable markers, drugs, or interventions that might prevent or slow the progression of AD. Current AD therapies may only provide symptomatic relief with a variety of side effects. This study is designed to determine an effective convenient method for early detection and identification of genetic markers associated with AD and to monitor the therapeutic strategy for the AD. Early identification of individuals at risk and quick diagnosis can assist in proposing midlife lifestyle modifications that will prevent or minimize the severity of disease. The optimal strategy is early intervention since the patient's level of function is preserved for a longer period. Delaying the onset of dementia by early detection and treatment is helpful for people and may result in large cost savings for healthcare systems.

1.4 RESEARCH QUESTION/ HYPOTHESIS OF STUDY

A) Null Hypothesis

There is no significant association of B-cell lymphoma 2 (*BCL2*) gene single nucleotide polymorphism (SNP) rs921884063 with susceptibility to Alzheimer's disease.

B) Alternate Hypothesis

There is a significant association of B-cell lymphoma 2 (*BCL2*) gene single nucleotide polymorphism (SNP) rs921884063 with susceptibility to Alzheimer's disease.

11.5 OBJECTIVES

1. To evaluate the association of *B-cell lymphoma 2* (*BCL2*) gene single nucleotide polymorphism (SNP) rs921884063 with susceptibility to Alzheimer's disease.

2. To determine the relationship between *BCL2* gene single nucleotide polymorphism (SNP) with biochemical and clinical parameters

1.6 SIGNIFICANCE OF THE STUDY

Alzheimer's disease is a frequent cause of dementia in adults globally as well as in Pakistan. Alzheimer's & Dementia associated fatalities account for 1.04 percent of total deaths in Pakistan and it rank 121 in world in age adjusted mortality rate. Studies are required to identify effective markers to help in early diagnosis and prognosis of AD. This study will be conducted to evaluate the association of *B-cell lymphoma 2* (*BCL2*) gene single nucleotide polymorphism (SNP) rs921884063 with susceptibility to Alzheimer's disease. The polymorphism of this gene with clinical features of the disease will also be evaluated. This study will aim to help early diagnosis of Alzheimer's disease patients by blood-based screening tests of selected bio and genetic markers.

SECTION 2

LITERATURE REVIEW

Dementia is the predominant neurodegenerative disorder that affects a significant portion of the global population. There is a growing incidence of individuals that are affected by this disorder. It is characterized by a progressive and observable decline in cognitive functioning. This results in diminished ability of an individual to autonomously carry out everyday activities (Livingston et al., 2020). Dementia can accurately be classified as a syndrome rather than a singular pathological condition. Dementia arises from a diverse range of factors, encompassing primary neurological, neuropsychiatric, and medical conditions. The concurrent presence of multiple diseases has frequently been observed to have an impact on the presentation of dementia syndrome in individuals. In elderly population, Alzheimer's disease and dementia with Lewy bodies are highly prevalent. In contrast, it is widely noted that traumatic brain injury and brain tumors play a substantial role in the etiology of health issues among the younger adult population (Hendriks et al., 2021).

Notable advancements have been achieved in molecular neuroimaging, the comprehension of clinico-pathologic correlation, and the creation of innovative biomarkers. However, there is a strong anticipation among medical professionals for the emergence of therapeutic interventions that possess the capacity to modify the course of neurodegenerative dementias. In the preceding era, healthcare professionals with diverse expertise and specialization in various medical domains possessed the advantageous capacity to mitigate emotional distress, provide comprehensive treatment for underlying illnesses, administer medications to enhance cognitive, neuropsychiatric, and motor functions, advocate evidence-based strategies that promote brain health, and enhance the overall well-being of patients and their families (Wendrich-van Dael et al., 2020).

The treatment of dementia is an important but often overlooked part of brain health care for older people. The use of personalized therapeutic interventions with people

who have been diagnosed with dementia could lead to small improvements in their general quality of life. Still, when these interventions are used together, they have a synergistic effect that leads to a significant and useful result. Also, because dementia care is such a broad condition, the effects of these treatments on both economics and sociology are very important (Sinha et al., 2020).

Alzheimer's disease (AD) is a neurogenerative disorder that affects elderly individuals. It is defined neuropathologically as progressive atrophy of brain cells. It is caused by the accumulation of insoluble proteins in the brain cells over the time which cause the death of neuronal cells. The gradual death of brain cell results in the brain tissue atrophy which leads to development of classical symptoms of AD i.e., memory loss and abnormal cognitive functions in an individual. Studies have shown a positive relation between the onset of symptoms of AD and accumulation of insoluble protein in the brain cells. All these neuropathological changes are responsible for the development of dementia. That is why AD is the most prevalent condition that is associated with dementia in adults (Scheltens et al., 2021).

AD is characterized by two main histopathological features, the presence of extracellular amyloid plaques made of hydrophobic beta-amyloid peptides that form insoluble aggregates, and the presence of intracellular neurofibrillary tangles made of hyperphosphorylated microtubule-associated Tau proteins. The buildup of two main types of clusters causes neurodegeneration to get worse over time. This process is marked by the slow spread of these aggregates throughout the brain, which causes memory loss, cognitive decline, serious dementia, and eventually death (Dubois et al., 2021).

Classical symptoms of AD include gradual and severe deterioration of cognitive functions that is accompanied by behavioral changes. These symptoms ultimately lead to loss of memory and impairment of spoken language in individuals suffering from AD. The condition is also responsible for neuropathological changes in individuals which include brain shrinkage and loss of neurons which are responsible for significant cognitive impairment which is the hallmark feature of AD. Apart from mental disabilities , AD is also responsible for the diminished level of problem-solving skills over the time (Porsteinsson et al., 2021).

AD is the most common form of dementia around the world. There is a growing number of individuals that are being affected by this disorder. AD is responsible for at least 70-80 percent of cases presented with dementia. It is estimated that the number of

individuals suffering from AD will double in the next 15 years (Trevisan et al., 2019). One study reported that Over 50 million individuals worldwide are reported to be suffering from AD-related dementia. It is estimated that by 2025, diagnosed cases will be around 22 million and in the absence of proper medical treatment that number will grow to reach 152 million in the next 25 years (Kshirsagar et al., 2022). There is not only a growing number in developing countries, but it is also reported that more than 2% of the population in highly developed nations are also suffering from this disorder (Mintum et al., 2021). Currently, 58% of the world's ageing population with dementia resides in low to medium income nations; by 2050, this proportion will increase to 68%. Asian countries especially China and Pakistan are reporting a great number of individuals that are suffering from AD every year (Adamson et al., 2020). In Pakistan, AD is reported to affect 1.7 % of total population (Aurooj & Mahmood, 2022) . According to Word Health Organization Dementia and AD associated death account for 1.04 % of total deaths in Pakistan.

According to the World Health Organization's update on epidemiology of AD, it is anticipated that by the year 2050, global incidence of dementia will increase threefold. The estimated prevalence of dementia in 2010 was approximately 35.6 million individuals. There is a positive correlation between advancing age and the prevalence of dementia. In particular, the demographic of individuals aged 65 and older exhibits an estimated incidence rate of approximately 5-8%, whereas those aged 85 and older encounter a higher prevalence rate ranging from 25-50%. The prevalence of AD in males was observed to be 19-29% lower in comparison to females. According to data from 2010, the countries with the highest prevalence of individuals affected by dementia, were China, the United States, India, Japan, Germany, Russia, France, and Brazil. FDA reported in 2013 that the number of individuals impacted in each of these countries surpassed one million (M. Rahman et al., 2021).

The spectrum of AD comprises a range of stages. These stages include asymptomatic preclinical AD, advancing to mild cognitive impairment (MCI), and culminating in mild, moderate, and severe AD-associated dementia. AD can be classified into two distinct forms, known as Early-onset AD and Late-onset AD, which are distinguished by the timing of symptom onset associated with this specific condition. Early-onset AD is regarded as a relatively less common form of the disorder, affecting an estimated two to ten percent of the overall population. The onset of this condition often

occurs in an individual's third or fourth decade of life. Late-onset AD predominantly affects individuals who have surpassed the age of 65. The observed similarities in the pathogenic mechanisms of AD and those found in aged brains have led to a longstanding hypothesis that the etiology of this disease is linked to an accelerated ageing process (Parnetti et al., 2019).

In addition to the well-documented forms of Early-onset AD and Late-onset AD, there exists a less prevalent variant of AD. It exhibits familial patterns and is typically characterised by the manifestation of autosomal dominant inheritance. It represents a minority, accounting for less than 1% of the overall patient population affected by this condition. Conversely, there is a distinct manifestation of AD that is proposed to emerge as a result of genetic susceptibility and interaction with environmental elements (O'Connor et al., 2020).

The discipline of genetics offers a viable susceptibility factor for the onset of AD. Recent developments in genomic research have provided compelling evidence suggesting that genetic mutations play a substantial role in the complex etiology of AD. The utilization of Next Generation Sequencing (NGS) in research has definitively established genetics as the primary factor influencing the onset and advancement of diverse disorders (Giau et al., 2019).

A number of cases of AD can be linked to genetic mutation. Three genetic mutations have been identified that have the capacity to induce autosomal dominant AD. The gene mutations involve the genes APP, PSEN1, and PSEN2. A form of Alzheimer's disease that is passed down through families is caused by these genes. They assume accountability for a percentage that varies between 5 and 10 percent of instances of early-onset AD. Symptoms of family Alzheimer's disease (FAD) tend to show up earlier than those of sporadic AD, usually between the ages of 30 and 50. The results of an observational study suggest that a notable percentage, ranging from 30 to 48 percent, of individuals diagnosed with AD had a familial connection in which a first-degree relative also exhibited the same disorder. This percentage stands in stark contrast to the range of 13-19 percent observed in individuals who do not exhibit any abnormalities. It has been noted that individuals who experience the onset of AD at a later stage in life have also been found to possess autosomal dominant mutations (Jia et al., 2020).

According to research findings, individuals who are first-degree relatives have a significantly increased likelihood, approximately 3.5 times higher, of developing the illness compared to the general population. AD exhibits multiple instances of early onset, indicating a hereditary pattern of autosomal dominant inheritance. Furthermore, a strong correlation has been established between AD and Down syndrome. The intricate nature of the relationship between genetics and the development of AD is evident (Cannon-Albright et al., 2019).

It is thought that late-onset AD is caused by a complicated interaction between genetic and environmental factors. It is believed that genetic factors account for about 70% of the risk of getting AD. Most people agree that the APOE gene is the most important risk factor for sporadic AD. People who have the 4 variants are about 3 times more likely to get AD than people who do not have the 4 variations. People with a homozygous genotype for the 4 variants have a chance that is about 12 times higher than people with a heterozygous genotype. Studies have found more than 20 genetic risk factors by studying different cases (Raulin et al., 2022).

It is hypothesized that inflammatory processes, cholesterol metabolism, and endosomal-vesicle recycling pathways are all linked to the development of certain diseases. Most people agree that activation of microglia in reaction to amyloid deposition is the main reason for development of AD. Focused genetic methods and next generation sequencing have also shown that some fewer common genes are linked to a higher risk of AD. Epidemiological data suggests that education and exercise may help prevent AD. On the other hand, people who have high blood pressure and diabetes in middle age are more likely to be at a higher risk of getting AD. Scientists have been looking into the association between fat deposition and the risk of developing AD. But it is not fully understood how arterial risk factors may affect the development of AD, mostly because epidemiological studies have not confirmed the diagnosis through pathological analysis (Feringa & van der Kant, 2021; Sinyor et al., 2020).

Numerous studies have documented a correlation between the advancement of diseases and a range of environmental, genetic, and physiological factors. The factors encompassed in this list comprise environmental conditions, traumatic brain injury, cardiovascular issues, hypertension, renal illness, diabetes, individuals with high body mass index, occupational exposures, and lifestyle conditions. AD is a well-established neurodegenerative disorder that is known to have a polygenetic basis. It primarily affects

the brain and is characterised by specific neuropathological features that are associated with the process of atrophy (Xu et al., 2021).

There exists a positive correlation between advancing age and the probability of an individual developing Alzheimer's disease AD. A notable association has been observed between age and the initiation of disorder. According to research findings, there is a exponential increase in the levels of oxidized lipids, proteins, and DNA in individuals affected by Alzheimer's disease as they progress in age. The phenomenon of ageing, when combined with multiple risk factors including oxidative stress, metabolic abnormalities, and changes in neurochemical pathways, has been observed to have a cumulative effect on the progression of neurodegenerative processes that ultimately result in the initiation of AD (Zhang et al., 2021).

Apoptosis is a naturally occurring biochemical process within an organism. Cell death is a biological process that is intricately controlled within the cellular level. The preservation of cellular viability is of paramount significance. Homeostasis refers to the intricate physiological process that is accountable for the maintenance of a stable internal environment within a cell. The coordination of numerous molecular events is controlled by a pre-established mechanism. The molecular mechanisms are involved in cellular proliferation, embryonic development, and the optimal operation of the immune system. Hence, within the framework of the neurodegenerative manifestations and progression of AD, apoptosis emerges as a prominent factor that contributes significantly. The aberrant reduction of neurons is the ultimate outcome of an uncontrolled apoptotic cascade. The event in question precedes the initiation of AD and demonstrates a significant correlation with the extent of cognitive deterioration. The increase in amyloid beta peptide (Aβ) concentrations has been recognised as a contributing factor in the initiation of apoptosis. A positive correlation has been observed between elevated levels of calcium and the occurrence of apoptosis. There exists a hypothesis suggesting that the protein *BCL2* potentially plays a role in regulating intracellular calcium homeostasis. The atypical regulation of intracellular calcium concentrations by factor A may be explained by the downregulation of *BCL2* in human neurons (Sharma et al., 2021).

The B-cell lymphoma-2 (*BCL2*) protein family is of paramount importance in the maintenance of cellular homeostasis. The primary purpose of this process is to govern the initiation of mitochondrial apoptosis. Moreover, it regulates the primary mechanism implicated in the operation of neurons by modifying intracellular calcium signaling.

Numerous studies have provided evidence that a significant proportion of constituents belonging to the *BCL2* family have been recognised for their notable influence on the effective operation of neuronal cells (Kim et al., 2020).

Based on available studies, it is widely postulated that the *BCL2* gene exerts a protective effect against brain degeneration by modulating cellular resilience and apoptotic pathways. This substance induces axonal growth and demonstrates anti-neuroinflammatory properties. Individuals of healthy population and the *BCL2* AA genotype demonstrate diminished levels of *BCL2* and grey matter volume, which is further associated with altered cognitive and motor capabilities. The expression of the *BCL2* protein within the brain has been observed to be modified by a particular genetic variation, referred to as a single nucleotide polymorphism (SNP), located within the *BCL2* gene. The age of an individual has a significant impact on the expression levels of neuronal *BCL2* proteins. Hence, it is plausible that pharmacological interventions aimed at augmenting the expression of *BCL2* protein and mitigating the -amyloid cascades could offer promising prospects for the therapeutic management of Alzheimer's disease AD (Erdal et al., 2020).

The synthesis of the *BCL2* protein is facilitated by both the mitochondria and the nuclear membrane in a physiologically intact individual. The interaction between Apaf-1 and the substance under investigation results in the downregulation of caspase-9 expression. The phenomenon of increased expression of *BCL2* has been observed to potentially slow down the processing of amyloid precursor protein (APP) and tau, resulting in a simultaneous decrease in the presence of neurofibrillary tangles and extracellular amyloid deposits (Czabotar & Garcia-Saez, 2023).

Tau proteins are a group of neuronal proteins that have a significant impact on the polymerization and stabilization of microtubule assembly. As a result, they contribute to the preservation of cytoskeletal integrity in cells. The process of phosphorylation, involving the modification of serine/threonine residues, functions as a regulatory mechanism. The regulation of the binding process involves a wide range of kinases that play a vital role in the development of neurofibrillary tangles (NFTs). The dysregulation of p35, a well-established activator of CDK5, is caused by the activation of calpain by Aβ. The excessive activation of cyclin-dependent kinase-5 (CDK5) is a direct result of an overload of cytosolic calcium. Following this, the kinase takes on the role of hyper phosphorylating the tau protein, resulting in a decrease in its binding affinity to

microtubules. Non-fungible tokens (NFTs), also known as neurofibrillary tangles (NFTs), arise as a result of the excessive addition of phosphate groups to tau protein, resulting in their buildup within the cytosol of cells and subsequent interference with cellular processes. Moreover, it exerts influence on customary cellular mechanisms such as synaptic transmission, axonal transport, signal transduction, and ultimately culminates in progressive cellular degeneration. Hyperphosphorylation may occur as a result of either genetic mutations or dysregulation of kinase activity (Wegmann et al., 2021a).

Researchers generally agree that signal transduction pathways, which mediate the process of protein phosphorylation and dephosphorylation, are of significant importance in the development of NFTs. During the pathogenic phase, there is an abnormal process called hyperphosphorylation that occurs in microtubule-associated tau proteins. The aforementioned procedure ultimately culminates in the polymerization and aggregation of tau, resulting in the formation of NFTs in diverse cortical regions of the brain. A positive association has been observed between the number of NFTs and the severity of the disorder. NFTs predominantly manifest within cerebral regions associated with the regulation of diverse cognitive domains (LO VASCO, 2023).

According to the hypothesis, the process of Aβ plaque formation and deposition takes place in multiple regions of the brain. The brain perceives these plaques as exogenous entities, leading to an inflammatory and immune response mediated by the activation of microglia and the secretion of cytokines. This enzymatic activity leads to the generation of two isoforms, namely Aβ1-40 and Aβ1-42. Presenilin 1 (PS1) or presenilin 2 (PS2) are the principal components of γ-secretase. Under normal circumstances, the soluble isoform commonly observed is Aβ1-40. Nevertheless, modifications in the process of cleavage can result in the generation of Aβ1-42, a peptide that exhibits a greater inclination to aggregate and form plaques. The heightened aggregation observed can be ascribed to the inclusion of two supplementary amino acids, namely isoleucine and alanine, in the Aβ1-42 peptide. The modification in the cleavage pattern arises due to genetic mutations in the APP gene, presenelin 1, presenelin 2 genes, or the apolipoprotein E (APOE4) gene (M. M. Rahman & Lendel, 2021).

In conjunction with genetic mutations, it is plausible that diverse neuropeptides contribute to the process of plaque formation. In particular, it is possible that reduced levels of corticotrophin-releasing hormone (CRH), somatostatin, and neuropeptide Y are involved, while increased levels of Angiotensin II may play a role in either the abnormal

breakdown of the amyloid precursor protein (APP) or the impaired removal of the Aβ1-42 fragment. The presence of amyloid-beta (Aβ) and tau aggregates has been observed to lead to impaired synaptic plasticity and neuronal cell death. However, the hypothesis has generated significant debate, and a recent study indicates that pharmaceutical interventions aimed at inhibiting the formation of amyloid plaques do not exhibit any noticeable effect in terms of reversing or slowing down cognitive decline. Hence, it is crucial to give great significance to the investigation of therapeutic interventions that specifically address non-amyloid factors, including tau proteins, inflammation, oxidative stress, and other associated mechanisms (Ishii et al., 2019).

The cognitive process involves the operation of the cholinergic system. The system's malfunction is implicated in the pathogenesis of neurodegenerative disorders, including AD . The cholinergic neurons situated in the nucleus basalis of Meynert demonstrate a propensity for the aggregation of amyloid plaque and NFTs. The degeneration of these pathological deposits occurs as a result of pro-inflammatory mechanisms, ultimately resulting in a deterioration of cognitive function. Insufficient cholinergic activity gives rise to changes in the blood-brain barrier's permeability, which disrupts the proper transportation of metabolites and impedes the removal of amyloid plaques, thereby worsening the pathological state. The potential negative impact on synapse integrity can arise from the existence of nicotinic acetylcholine receptors (nAChRs) that are permeable to Ca2+. Aβ demonstrates the greatest binding affinity towards α7- and α4β2-nAChRs located in the synaptic regions of the hippocampus and cortex. The observation of increased levels of acetylcholinesterase (AChE) and decreased levels of choline acetyl transferase can indicate the depletion of acetylcholine and the worsening of dementia. The contribution of AchE and amyloid-beta (Aβ) peptide to the progression of plaque formation has been identified. A correlation has been observed between the degeneration of noradrenergic neurons in the locus coeruleus and the occurrence of cognitive impairment and neurodegeneration. Astrocytes are equipped with a diverse array of noradrenergic receptors, which play a significant role in augmenting synaptic plasticity and promoting the facilitation of cognitive processes such as learning and memory (Bekdash & Matsukawa, 2021).

Serotonin is known to have a substantial impact on the pathophysiological mechanisms underlying AD . A considerable proportion of individuals who have been diagnosed with AD encounter a decline in brainstem serotonergic neurons and

demonstrate diminished levels of neurotransmitters. The serotonergic input originating from the midbrain raphe nuclei exerts an influence on the modulation of cortical plasticity and memory. Cognitive impairment occurs as a consequence of the disruption of this particular pathway. The facilitation of synaptic plasticity maintenance is achieved through the activation of NMDA and AMPA receptors via the binding of glutamate. Synaptic damage occurs due to neuronal depolarization and excitotoxicity resulting from imbalances in the metabolism of glutamate and glutamine. The induction of hypersensitivity in NMDA receptors and the disruption of NMDA activity regulation by Aβ have been observed, leading to the manifestation of excitotoxicity. The interplay between gamma-aminobutyric acid (GABA) and serotonin is evident in the dorsal raphae nuclei, a brainstem region housing a significant population of serotonergic neurons. The administration of a 5HT6 receptor antagonist result in an increase in serotonin levels by modulating GABAergic neurons, thereby improving cognitive decline. It demonstrates a decrease in the activity of gamma-secretase, resulting in a reduction in the formation of amyloid plaques. The observed synaptic damage in individuals diagnosed with AD is ascribed to the decrease in GABAergic inhibition on cholinergic and glutamatergic neurons. Therefore, the cognitive process requires an intricate interaction among neurotransmitters. The potential exacerbation of symptoms associated with AD can be attributed to the presence of imbalanced neurotransmitters (Aaldijk & Vermeiren, 2022).

Individuals affected by a less common form of early-onset Alzheimer's disease AD have been observed to exhibit a genetic mutation in the amyloid precursor protein (APP). There have been documented instances of genetic mutation on chromosome 14, which are considered to be a significant risk factor in the progression of AD in certain cases. The ApoE4 allele, which is a variant of the apolipoprotein E gene, plays a crucial role in the metabolism of amyloid precursor protein (APP). Individuals who carry allele 4 of the apolipoprotein E gene have a higher vulnerability to developing AD as a result of the genetic variant's inclination to facilitate the formation of amyloid plaques (Abyadeh et al., 2019; Savoy et al., 2021).

The amyloid hypothesis postulates that certain extrinsic factors hinder the degradation of amyloid precursor protein (APP). AD can potentially manifest due to genetic variations within the genes encoding amyloid precursor protein, specifically presenilin-1 and presenilin-2. The presence of oxidative stress conditions promotes the phenomenon of cross-linking and aggregation of cytoskeletal proteins, particularly α

peptide and tau protein. As a result, there is an accumulation of amyloid precursor protein (APP) within the cells, leading to the formation of oligomeric lethal amyloid-beta (Aβ) plaques. Furthermore, there exists a buildup of phosphorylated tau protein in the structure of neurofibrillary tangles, commonly referred to as p-tau. The enzymatic activities of elastase and secretase are considered to be responsible for the formation of plaques. The disruption of the neural cell network, ultimately resulting in the death of a neuron, can be attributed to the presence of plaques and tangles (Frisoni et al., 2021).

The degradation of synaptic connections in the cortex and hippocampus is caused by the existence of abnormal proteins, referred to as neurofibrillary tangles, within brain cells. As the progression of the disease ensues, the substantial buildup of these protein deposits profoundly hampers normal cognitive functions within the brain. Consequently, this occurrence leads to compromised neuronal functioning, resulting in the manifestation of memory impairment and psycho-emotional dysfunction, ultimately culminating in neuronal demise. The critical diagnostic criteria for AD involve the presence of reduced synaptic density and neuronal loss in particular regions of the brain, such as the cerebral cortex and hippocampus, accompanied by a persistent deterioration in cognitive abilities (Padmanabhan et al., 2021).

The aberrant accumulation of these plaques is also responsible for altering the metabolic and structural composition of pyramidal neurons in the cortex and hippocampus. Moreover, it has been noted that the interplay between α aggregates and cellular constituents of nerve cells triggers a series of sequential responses, ultimately resulting in the production of reactive oxygen species (ROS) within the cell (Bhatt et al., 2021).

Specific stimuli have the ability to elicit alterations in the APP cleavage system, leading to the creation of oligomeric toxic forms of α plaques that have yet to be identified. The overproduction of these toxic species leads to the disturbance of the neural network, ultimately culminating in the demise of neuronal cells. This phenomenon results in the disturbance of the mechanisms linked to synaptic plasticity. Multiple research studies have provided evidence suggesting that physiological and anatomical changes occurring in pyramidal neurons situated in the cortex and hippocampus can result in an uneven production of harmful oligomeric species (X. Q. Chen & Mobley, 2019).

Under conditions of oxidative stress, the cell provides a favorable environment for the aggregation of cytoskeletal proteins, including α peptide and tau, as well as the

formation of cross-links. In addition, the interaction between Aβ aggregates and nerve cell membranes triggers a cascade of events that ultimately lead to the buildup of reactive oxygen species (ROS) within the cellular milieu (Nam et al., 2018). Moreover, it possesses the capacity to produce proteinaceous aggregations within cells referred to as neurofibrillary tangles (NFTs), which hold diagnostic importance (Wegmann et al., 2021).

Environmental factors also play a vital role in the development of AD. Certain types of environmental toxins are responsible for stimulation that results in the production of reactive oxygen species (ROS). These reactive species are highly toxic for the integrity of the cell specially for neurons and are responsible for damaging them. These reactive species also have the ability to disrupt normal enzymatic activity. They contain high concentration of polyunsaturated fatty acids (PUFAs); therefore they are easily peroxidized by lipids. Studies suggest that any environmental factor that has the capability to increase the production of ROS has a potential to damage neurons and cause premature ageing and neurodegenerative disorders (Bai et al., 2022).

Recent studies have provided evidence suggesting that chemical substances and air pollutants exhibit neurotoxic properties and are associated with the progression of AD . A significant association has been observed between the identification of Aluminium (AL) in the late 1970s and a subsequent increase in documented instances of AD . There is a distinct mechanism through which artificial intelligence (AI) can penetrate brain cells. The most commonly employed approaches involve the use of gastric medications, including antacids, culinary utensils, and deodorizers. Significant quantities of soluble aluminium salts are frequently found in acid rain. The blood-brain barrier (BBB) undergoes a gradual decline in strength as individuals advance in age. When an individual who has a compromised blood-brain barrier (BBB) is exposed to substantial quantities of AL over a prolonged duration, AL tends to accumulate in the brain and accelerate the ageing process by means of oxidative modification and the generation of reactive oxygen species (ROS). The initiation of glial cell activation contributes to the onset and progression of diverse neurodegenerative conditions, including AD (Dey & Singh, 2022).

Neuroinflammation is also a risk factor for the development of AD. The presence of amyloid-beta (Aβ) plaque has been shown to be associated with brain injury, and acute inflammation has been identified as a critical mechanism for providing protection against these injuries. However, the persistent activation of microglia hinders their ability to

efficiently eliminate the plaque. They disrupt the balance between pro and anti-inflammatory cytokines as they retain their ability to secrete pro-inflammatory cytokines. Accumulation of Aβ deposits in microglia triggers the activation of Toll like Receptors and their co receptors. Proinflammatory cytokines have the potential to induce impairment of spines of dendrite of neurons and alters the clearance of Aβ from microglia. The activation of cyclin-dependent kinases (CDKs) is initiated by the influence exerted by these cytokines, resulting in the hyperphosphorylation of tau and an augmentation in the formation of Aβ plaque. Neuroinflammation can also be elicited by diverse cellular entities, including endothelial cells, oligodendrocytes, and neurons. The neuron comprises a diverse array of molecules that exhibit anti-inflammatory properties. Brain endothelial cells are capable of synthesizing immune molecules as a result of their exposure to Aβ plaque (Onyango et al., 2021).

Aβ has been associated with the possession of antimicrobial properties. A proposal has been put forth suggesting that neurons infected by pathogens exhibit a heightened prevalence of Aβ deposition and NFTs. Therefore, it can be postulated that the presence of an untreated infection that persists for an extended duration could be considered a plausible etiological factor that contributes to the initiation of AD. The immune system becomes activated upon the detection of an infection. The activation of Toll-like receptors is initiated by it (Vigasova et al., 2021).

The brain and gut of an individual has a communication that is bidirectional. There is a positive correlation between AD and gut microbiota alteration. Gut alteration results in increased production of bile acid which has the ability to cross the brain barrier and accumulate within the brain. This results in apoptosis, the production of reactive oxygen species, inflammation, and ultimately neurodegeneration .

The release of norepinephrine (NE) occurs following the activation of N-Methyl-D-aspartate (NMDA) receptors through the binding of the neurotransmitter glutamate. Norepinephrine (NE) functions as a primary neurotransmitter in the noradrenergic, noncholinergic, and enteric nervous systems. Several specific strains of gut microorganisms, including Bifidobacteria and Lactobacilli, possess the capacity to enzymatically convert nitrite and nitrate compounds into nitric oxide (NO). Both gut bacteria and Streptomyces possess the capacity to biosynthesize nitric oxide (NO) via the enzymatic activity of their nitric oxide synthase (NOS). The potential for excessive production of nitric oxide (NO) can arise from alterations in the activity of any of these

gut microbes, in conjunction with an increased intake of nitrate. The excessive production of nitric oxide (NO) can lead to subsequent axonal degeneration, neuroinflammation, and ultimately, the development of neurodegenerative disorders (Sun et al., 2020).

Individuals with higher body mass index and impaired glucose metabolism are at an increased risk of developing AD as a consequence of compromised metabolic processes leading to heightened production of free radicals. An instance of metabolic activities can be observed in the disruption of energy metabolism resulting from the perturbation of the mitochondrial membrane, which occurs as a consequence of the inhibition of vital enzymes by α plaques (Majoka & Schimming, 2021).

Prominent regions of the brain, such as the hippocampus, demonstrate a significant presence of insulin receptors. Numerous epidemiological studies have established a significant correlation between the occurrence of type-2 diabetes mellitus. Hyperglycemia is also responsible for the initiation of apoptosis and degradation of tau protein. There exists a positive association between type-2 diabetes and the development of AD , with individuals affected by the former having a doubled risk of developing the latter. This increased likelihood can be attributed to the presence of cerebral abnormalities, accelerated ageing processes, and cognitive impairments commonly observed in individuals with type-2 diabetes. In addition, it is important to note that hypertension, hypercholesterolemia, and atherosclerosis are significant risk factors that play a role in the initiation and progression of AD (Chakrabarty et al., 2022).

There is a correlation between the adoption of a dietary regimen that includes vitamins, antioxidants, and polyphenols and a reduced risk of developing AD . In contrast, research has indicated that the consumption of diets that are deemed unhealthy, characterized by a high intake of junk food, alcohol, and saturated fat, is associated with an increased risk of developing Alzheimer's disease (Arslan et al., 2020).

According to existing research, there is evidence suggesting a correlation between an uneven distribution of heavy metals and the development of metal amyloid complexes, as well as the production of free radicals. These factors are believed to contribute to the degenerative advancement of AD . Numerous studies have documented a positive association between a dietary intake high in copper and saturated fatty acids and an elevated susceptibility to AD (Bakulski et al., 2020).

There exists evidence indicating a potential association between the emergence of early onset AD and the presence of trans fatty acids, which have been observed to expedite the amyloidogenic process of amyloid precursor protein (APP). On the contrary, there exists empirical evidence indicating a correlation between long-chain unsaturated fatty acids and a diminished susceptibility to dementia and Alzheimer's disease (Hirata, 2021).

Extensive evidence has been gathered regarding the adverse effects of oxidative stress on various physiological systems. The effects that have been observed include cellular apoptosis, increased levels of intracellular calcium, and changes in protein conformation. These entities provoke inflammatory responses and disrupt the regular operation of essential macromolecules. The occurrence of neurodegeneration can be attributed to the neurotoxic effects caused by factors associated with oxidative stress. Individuals who maintain a diet that includes fresh fruits and vegetables demonstrate a decreased susceptibility to the onset of dementia and AD , as these dietary selections are abundant in antioxidants. In addition to hindering the initiation of the disorder, these factors also play a role in slowing down the progression of the ailment (Veurink et al., 2020).

AD patients' brain autopsies revealed heightened concentrations of lactate dehydrogenase phosphatase (LPO). The study also presented a significant finding concerning the reduced concentrations of polyunsaturated fatty acids (PUFAs) in the brain cells of the individual’s examined postmortem. The vulnerability of neurons to injury caused by free radicals is ascribed to the existence of chemical compounds within their structure. Moreover, it has been observed that oxidative compounds elicit the most substantial alterations at the pathophysiological site, specifically in the temporal lobe, which also demonstrates the greatest degree of lipid peroxidation activity (Acharige & Pflum, 2021).

Oxidative stress is responsible for causing a range of structural damage, which includes but is not limited to DNA strand breaks, DNA-protein cross-links, DNA-DNA cross-links, sister chromatid exchange, and base changes. The generation of free radicals is subsequently followed by these harmful occurrences (Ionescu-Tucker & Cotman, 2021).

The field of nutritional epidemiology is progressively recognizing the importance of dietary factors in promoting cognitive health and mitigating the risk of age-related

cognitive decline, specifically AD . Additional investigation is required to explore the underlying mechanisms and ascertain appropriate dietary strategies for the maintenance of cognitive function.

PET scan and CSF protein analysis are the main diagnostic test for AD. P-tau181 biomarker has the ability to confirm and predict the presence of AD. PET scan exhibits a specificity of 100% and a sensitivity of 96% in the detection of disorder. The expenses related to conducting CSF p-tau, Aβ42, and total tau protein analysis are comparatively more cost-effective. The diagnostic accuracy CSF ranges from 85% to 90%. Studies have provided evidence that pathological abnormalities can be identified as early as two decades prior to the appearance of observable clinical symptoms. It is advisable to utilize biomarkers as a means to expedite the prompt detection and intervention of AD (Janelidze et al., 2020).

C Reactive Protein is also identified as the potential risk factor for development of AD. In Individuals suffering from AD, the presence of amyloid tangles accelerates the dissociation of CRP into its monomeric form which serves as a mediator that links vascular trauma and inflammation. It is also associated with neuronal damage, and the progression of dementia. There is a positive association between the ApoE genotype and the levels of peripheral CRP. Individuals possessing the ApoE4 genotype demonstrate a decrease in plasma concentrations of CRP in comparison to individuals having the ApoE3 genotype. A positive association has been observed between high concentrations of CRP and reduced cognitive functions (McFadyen et al., 2020).

There exists a correlation between the levels of certain biomarkers, namely vascular endothelial growth factor (VEGF) in CSF and the pathological activity observed in the brains of elderly individuals (Mahoney et al., 2019).

In the realm of transgenic mouse models, it has been noted that the concentrations of Aβ42 and Aβ40 in both plasma and cerebrospinal fluid (CSF) demonstrate an initial elevation as age progresses. Nevertheless, as the buildup of Aβ in the brain advances, there is a subsequent decrease in these levels, ultimately leading to the initiation of

cognitive impairment. Throughout the duration of the observational study, it was noted that individuals who demonstrated an upward trend in Aβ42 levels had a significantly greater probability, three times higher, of developing AD compared to those whose levels decreased (Leuzy et al., 2021).

Brain lipids are of significant importance in various biological and physiological processes, particularly in the transmission of nerve impulses and the signaling mechanisms within the CNS. Individuals suffering from AD have significantly low levels of sphingolipid (Crivelli et al., 2020).

In light of the fundamental tenets of Mendelian genetics, one can deduce that AD exhibits patterns of inheritance. The APOE gene's ε4 allele, with a prevalence of 14%, is a notable risk factor in the pathogenesis of AD. The occurrence of early onset AD is frequently associated with an increased presence of Aβ pathology (Husain et al., 2021).

There are extremely limited treatment choices available for the management of AD. Keeping in mind the burden of disease and growing expenses attached with it , there is a need for early detection of patients in early stage of disease.

Although these treatment options are limited , they are also expensive. So, the focus should be on detecting susceptible individuals far earlier in the disease progression so that urgent action can be taken. There is need for development of targeted therapeutic approaches that are efficacious against AD. For this purpose, there is need to look at cellular factors responsible for pathogenesis of disease so that targeted therapeutic approaches can be used to improve quality of life for individuals suffering from AD. Early diagnosis of the individual at risk is necessary so that appropriate action can be taken to slow or stop future deterioration.

OPERATIONAL DEFINITIONS

***BCL2* Polymorphism:** is an antiapoptotic factor that is important in normal B-cell development and differentiation. The t(14;18) (q32;q21) brings the *BCL2* gene under the control of immunoglobulin heavy-chain gene (IgH) enhancers and leads to overexpression of *BCL2* protein. *BCL2* overexpression has prognostic importance.

Cognitive Decline: It is a kind of cognitive impairment. It is one of the first obvious signs of Alzheimer's disease and other associated dementias. Cognitive decline includes consistently missing important appointments and dates, difficulty in remembering recent events or talk, unable to make rational decision, feeling more and more stressed and frustrated, trouble following a command and comprehending directions or instructions, trouble finding way back home, reduced capacity plan and coordinate activities and development of impetuous nature.

Cognitive Score: A typical score is regarded to be 26 or above, at the very least. People without cognitive impairment had an average score of 27.4, whereas those with moderate cognitive impairment (MCI) had an average score of 22.1 and those with Alzheimer's disease had an average score of 16.2.

Neurodegeneration: Refers to the gradual wasting away of neurons and the accompanying loss of function.

Neuronal Loss: Neuronal death is normal throughout the development of the nervous system, but it is abnormal when it occurs because of illness or damage to the brain or spinal cord. Cell death may occur in a variety of ways, including apoptosis and necrosis. It is commonly accepted that these two processes represent separate types of cell death.

Amyloid beta plaque: According to the amyloid hypothesis, the creation of oligomeric toxic species A plaques is caused by APP going through a malfunction of the

physiological cleavage system because of certain unknown stimuli. This dysfunction leads to the production of amyloid plaques. This, in turn, may produce impairment of mechanism involved in synaptic plasticity, which leads to disarrangement of neural network and cell death. Moreover, this may induce impairment of mechanisms involved in synaptic plasticity. APP suffers from a malfunction of the physiological cleavage mechanism, which leads to the generation of oligomeric hazardous products.

SECTION 3

RESEARCH METHODOLOGY

3.1 STUDY DESIGN

Case Control Study

3.2 SUBJECTS

The study was conducted on Male and Female participants with age range 50 to 80 years fulfilling the inclusion criteria were included in the study. Age-gender matched controls were collected from the healthy individuals for the comparison.

3.3 PLACE OF SAMPLE COLLECTION / SETTING

The study was conducted in Bahria University Health Sciences, Karachi in collaboration with Jinnah Postgraduate Medical Center, Karachi and Dr. A. Q. Khan Institute of Biotechnology and Genetic Engineering (KIBGE) University of Karachi.

3.4 PATIENTS RECRUITMENT CRITERIA

3.4.1. Inclusion Criteria for Patients

- Individuals with diagnosed case of Alzheimer's disease
- Individuals currently on treatment of Alzheimer's disease
- Individuals with diagnosis of probable Alzheimer's disease according to NINCDS-ADRDA criteria (with or without white matter lesion)

- Family history of Alzheimer Disease

3.4.2 Inclusion Criteria for Controls

Age and Gender matched healthy individuals between the age 50-80 years.

3.4.3 Exclusion Criteria for Patients

- History of schizophrenia
- Bipolar disorder
- Recurrent Psychotic disorders
- Any somatic, Psychotic, or neurologic disorder that may have caused cognitive impairment.
- Previous brain injury

3.4.4 Exclusion Criteria for Controls

Age and Gender matched healthy individuals between the age 50-80 years who do not have present or previous history of any trauma or neurological disorder.

3.6 DURATION OF STUDY

3.6.1 Individual study period**:** 06 Months

3.6.2 Total study period**:** 01 Year

3.7 SAMPLE SIZE ESTIMATION

Calculated by OpenEpi, Version 3. 0% confidence interval and 5% confidence limit. Sample size was drawn by taking in consideration the parameters used in article (Aurooj & Mahmood, 2022)

Sample Size for Frequency in a Population	
Population size(for finite population correction factor or fpc)(*N*):	1000000
Hypothesized % frequency of outcome factor in the population (*p*):	1.72%+/-5
Confidence limits as % of 100(absolute +/- %)(*d*):	
Design effect (for cluster surveys-*DEFF*):	1

Sample Size(*n*) for Various Confidence Levels		
Confidence Level(%)	**Sample Size**	
95%	26	

Equation
Sample size *n* = [DEFF*Np(1-p)]/ [(d^2/Z$^2_{1-\alpha/2}$*(N-1)+p*(1-p)]

3.8 SAMPLING TECHNIQUE

Non-Probability Convenient Sampling

3.9 HUMAN SUBJECT AND CONSENT

This study received approval from the research ethics committee of Bahria University Health Science Campus, Karachi (FRC-BUHS-50/2022-513), with the letter approval number (ERC 113/2022).

Permission was acquired from the director and head of the medical institute (Jinnah Postgraduate Medical Center, Karachi & Dr. A. Q. Khan Institute of Biotechnology and Genetic Engineering (KIBGE) University of Karachi) to carry out the research at the respective hospital and institute. Blood samples of patients (n=20) and controls (n=21) were collected through venipuncture after taking their informed consent. Blood samples were collected in vacutainers containing EDTA (Ethylenediaminetetraacetic acid). Specific code was provided to the samples to maintain the confidentiality of the patient's data. All the samples were stored at -80°C for the molecular analysis.

3.10 MATERIALS

3.10.1 Questionnaire

Attached as annex.

3.10.2 Consent Form

Attached as annex. Participants who were able to accept the study procedures were enrolled upon signing informed consent forms in Urdu and English

3.10.3 Drugs

N/A

3.10.4 Equipments and Reagents

- SYBR™ Safe DNA Gel Stain
- DNA Gel Loading Dye (6X)
- Agarose I (Molecular Biology Grade)
- Agarose
- 50 bp DNA Ladder
- 100 bp DNA Ladder

3.11 PARAMETERS OF STUDY

This study evaluated the role of *B-cell lymphoma 2* (*BCL2)* gene single nucleotide polymorphism (SNP) rs921884063 with susceptibility to the Alzheimer's disease using T-ARMS-PCR (tetra-primer amplification refractory mutation system-polymerase chain).

3.11.1 Genetic Marker

BCL2 Gene

3.11.3 Clinical Parameters

- **Mental Status Testing**

To evaluate the degree of cognitive impairment and memory skills based on scores on these tests.

- **Brain Imaging Test**

Brain imaging used to rule out other causes, such as hemorrhages, brain tumors or strokes or to distinguish between different types of degenerative brain disease and to establish a baseline about the degree of degeneration. The brain-imaging technologies included:

- Magnetic resonance imaging (MRI)
- Computerized Tomography (CT)

3.12 PROTOCOL OF STUDY

3.12.1 Sample Collection

- Performa and informed consent were filled by the 41 subjects including 21 Age matched controls and 20 subjects diagnosed with Alzheimer's Disease according to National Institute of Neurological and Communicative Diseases and Stroke & Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for the diagnosis of Alzheimer's disease.

- Five (05) ml blood samples were collected in EDTA vacutainers which were coded to ensure confidentiality of the subjects. These samples were used for isolation of DNA using salting-out method.

3.12.2 Extraction of DNA

Isolation of DNA was performed via inorganic method (salting-out) as per reported by (Miller et al., 1988). The procedure was modified for the 1mL blood in two days. The preparation of reagents was mentioned in table 3.1 to 3.6. The performed DNA extraction protocol is given below:

- To a 2ml microcentrifuge containing 0.9ml RCLB (Red Cell Lysis Buffer), 0.5ml blood was added followed by 10 seconds vortex mixing and centrifuge at 13000 rpm for 1.5 minutes. The supernatant was discarded. Repeat the process again by adding the same volume of RCLB and supernatant was discarded.

- To the similar tube 0.5ml blood was added followed by 10 seconds vortex mixing and centrifuge at 13000 rpm for 1.5 minutes. The supernatant was discarded.

- To the obtained palette autoclaved deionized water (2ml) was added and incubated at room temperature for 15 minutes followed by centrifuge at 13000 rpm for 1 minute and supernatant was discarded.

- To the palette autoclaved deionized water (480 µl), 10 SDS (100µl), 5x proteinase K buffer (160µl) and Proteinase K enzyme (30µl) was added. The mixture was incubated overnight at 56°C in shaking water bath.

- Next day samples were brought to room temperature and 6M NaCl (100 µl) was added followed by 15 seconds vortex and centrifuge for 5 minutes at 13000rpm. The supernatant was transferred to a separate microcentrifuge (1ml) and a similar step was performed twice.

- The obtained supernatant was collected in separate microcentrifuge (2ml) and chilled 100% ethanol (2ml) was added followed by gentle mixing till the DNA threads were obtained shown in figure 3.1. The tubes were than centrifuge at 13000rpm and supernatant was discarded.

- The obtained palette was washed with 70% ethanol (1ml) and centrifuge at 13000 rpm.

- To the palette TE buffer (60 µl) and DNA pellet was dissolved at 56°C for 3 hours.

The RCLB solution was prepared for the lysis of red blood cells. In the solution molar solutions for Tris and $MgCl_2$ were separately prepared. Sucrose was gradually added to the prepared mixture. To the mixture triton 100X was added and pH was maintained to 7.4. The solution was filtered for the removal of undissolved particles.

The solutions of Ethylenediamine tetra acetic acid (EDTA) and NaCl were prepared and mixed in the mentioned amount. The volume of solution was makeup up to 50ml.

3.12.3 Quantitative Analysis Of DNA

DNA was quantified using NanoDrop spectrophotometer. This spectrophotometer works on the principle of light measurements absorbed by the test sample to examine the quantity (concentration) of test sample. In this procedure microliter (0.5-2µl) of sample was utilized to identify the concentration of DNA in nanogram. The NanoDrop utilized dual-beam system, in which the light crosses the reference and test sample mixture. The reference is considered as the corrected sample to minimize the background noise such as protein and chemical impurities. Moreover, the instrument measure concentration in the presence of UV (ultraviolet) range in between 220nm to 320nm. However, the DNA was observed at wavelength 260nm, and concentration of sample was calculated by formula given by Beer-Lambert law. According to this law the light absorbed by the sample is directly proportional to the sample concentration and total length of the distance covered by the light. The quantity of sample provided by the instrument is based on the comparison of concentration of reference to the test sample in ratio of A260/A280 (Figure 3.2) which indicates ~1.8 as pure, >1.8 as RNA contamination and < 1.8 as chemical contamination.

3.12.4 Qualitative Analysis Of DNA

The agarose gel electrophoresis was performed to analyze the DNA integrity. Agarose gel electrophoresis is the procedure worked on the principle of separation of DNA bands based on their charge and sizes. This procedure allows the travelling of changed molecules using gel as matrix via electric filed. The quality of genomic DNA was observed using 0.8% gel. Agarose (0.8gm) was mixed in 100ml TBE (Tris-Borate-EDTA), the mixture was boiled to dissolve agarose residuals and bring it to the room temperature. In the mixture 3µl of sybersafe dye was added and comb was applied and set at room temperature to solidify the gel. Once gel solidified, the comb was removed and placed in the electrophoresis chamber containing enough of the 1X TBE. Samples (4µl) were mixed with the 6X loading dye (2µl) and loaded in the wells. The setup was run at 100V voltage, 300A current for 50 minutes. The gel was observed under Gel Doc system (Figure 3.3).

3.12.5 Dilution of DNA Samples

To perform PCR (polymerase chain reaction) the samples concentration 50ng/µl is required. The DNA samples were diluted in TE buffer using the following formula.

$$\text{Stock volume} = (\textit{Required final concentration} \div \textit{Total stock concentration}) \times \text{Final volume of diluted sample}$$

3.12.6 Selection of Targeted Single Nucleotide Polymorphism (SNP)

The obtained DNA samples were utilized to identify the frequency of SNP (rs921884063) in *BCL2* (*B-cell lymphoma 2*) gene among the patients and controls. The SNP with MAF (minor allelic frequency) >0.05 was selected and information regarding mentioned SNP was obtained from ensembl genome browser 92 (https://asia.ensembl.org/index.html) and NCBI (National Center for Biotechnology Information) dbSNP (https://www.ncbi.nlm.nih.gov/snp/). As this database carries data of most of the population from the European, African American, and Sub-Saharan African.

3.12.7 Designing of Primers for the *BCL2* gene

The targeted region of DNA was amplified by T-ARMS PCR (Tetra Primers Amplification Refractory Mutation System Polymerase Chain Reaction). Primers were designed for SNP rs921884063 of *BCL2* gene by online available software Primer1 (http://primer1.soton.ac.uk/primer1.html). In *BCL2* gene SNP rs921884063 is responsible for the replacement of single nucleotide from G to C (G>A) which results in change in codon from TGG/TGA. This change in codon results in stop gain mutation where it TGA codes for Ter (Termination opal or umber) instead of W (tryptophan). The primer sequences are mentioned in table 3.7 and the principle of T-ARMS PCR is mentioned in figure 3.4.

The optimization of T-ARMS PCR was done initially by applying temperature gradient in thermocycler machine from temperature 59.7 °C to 66.5°C to identify best temperature where maximum annealing of targeted primers was achieved. The obtained PCR product was observed by agarose gel (3%) electrophoresis (Figure 3.5). The reaction of PCR was performed on Bio-Rad PCR Machine.

The reaction was carried out by providing initial denaturation (95°C) for 5 minutes followed by another cycle of denaturation (95°C) for 30 seconds. The annealing temperature 64.2°C was provided for 45 seconds so that primers were bound to the targeted region of the amplification of desired polymorphism. The sequence was extended further by providing an extension temperature of 72°C for 45 seconds. This cycle was further proceeded for 35 times followed by additional elongation at 72°C for 8 minutes. The amplified product is stored at 4°C and product was observed by agarose gel (3%) electrophoresis followed by gel visualization under the Gel document system (Figure 3.6).

3.12.8 Preparation of Reaction Mixture and Cycle

The single nucleotide polymorphism (SNP) rs921884063 (G>A) in *BCL2* gene was amplified by tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS PCR). T-ARMS PCR was performed in the single reaction tube containing two sets of outer and inner primers. The reaction mixture was prepared by adding 2µL (50ng/µL) of DNA, 0.8µL of outer (10µM) and inner (12µM) primers

followed by 1.5µL of $MgCl_2$ (5 mM) and master mix Dream green^{TM} 12µL. The mixture was adjusted to the volume 25µL by adding nuclease free water (Table 3.9).

Genotypic and demographic data were analyzed by various software which includes primer blast was used to get desired product size. Insilco polymerase chain reaction was performed using NCBI web browser (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) for the confirmation of PCR product sizes. MedCal software was used to calculate the odds ratio and SPSS was used to calculate the Chi-square. The association of genotype was identified by SNPstats software.

3.12.7 Conditions Optimization of Annealing Temperature

The optimization of T-ARMS PCR was done initially by applying temperature gradient in thermocycler machine from temperature 59.7 °C to 66.5°C to identify best temperature where maximum annealing of targeted primers was achieved. The obtained PCR product was observed by agarose gel (3%) electrophoresis (Figure 3.5). The reaction of PCR was performed on Bio-Rad PCR Machine.

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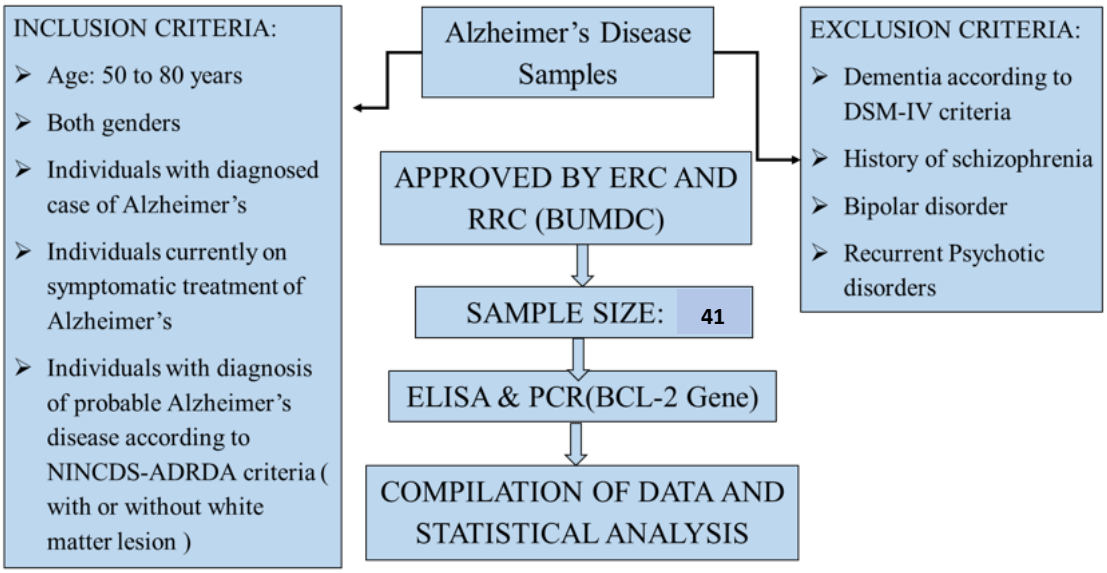
3.13 ALGORITHM OF STUDY



3.14 STATISTICAL ANALYSIS

3.14.1 Statistical and Bioinformatic Analysis

Genotypic and demographic data were analyzed by using various analytical software. Primer Blast was used to get the desired product size. Insilco polymerase chain reaction was performed using NCBI web browser (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) for the confirmation of PCR product sizes. MedCal software was used to calculate the odds ratio. SPSS version 23 was used to calculate the Chi-square. The association of genotype was identified by SNPstats software.

Table 3.1: Red Cell Lysis Buffer (RCLB)

Reagents	100ml
Sucrose	10.95 g
1M Tris	1.2ml (1M= 1.211 g in 10 ml)
1M $MgCl_2$	0.5 ml (1M= 2.033 g in 10 ml)
Triton	1 ml

Table 3.2: Proteinase K Buffer (5X)

Reagents	50ml
0.5M EDTA	12 ml (0.5M= 9.3g in 50ml/pH 8.0)
4M NaCl	4.69 ml (4M=11.7g in 50ml)

Table 3.3: SDS (10%)

Reagents	50ml
SDS (Sodium dodecyl sulfate)	10g

Table 3.4: NaCl (6M)

Reagents	50ml
NaCl	35ml

Table 3.5: Ethanol (70%)

Reagents	50ml
Absolute Ethanol	35ml
H_2O	15ml

Table 3.6: TE (Tris EDTA) Buffer

Reagents	100ml
1M Tris Base (pH 8.0)	1 ml (10mM)
0.5M EDTA (pH 8.0)	200µl (1mM)

Table 3.7. Sequences of Primers of SNP rs921884063

Primers	Sequences	Product Size
Outer Forward	´5ACTTCGCCGAGATGTCCAGCCAGCTG3´	270bp
Outer Reverse	´5CACCAAGTGCACCTACCCAGCCTCCGTTA3´	
Inner Forward (G)	´5AGCTCTTCAGGGACGGGGTGAACGGA3´	130bp
Inner Reverse (A)	´5CCGAACTCAAAGAAGGCCACAATCCTCACC3´	196bp

Table 3.8: Reaction Mixture (25µL)

Reagents	Reaction Quantity 1X
Water	6.3 µL
Forward Outer Primer	0.8 µL
Reverse Outer Primer	0.8 µL
Forward Inner Primer	0.8 µL
Reverse Inner Primer	0.8 µL
$MgCl_2$	1.5 µL
DNA	2 µL
Master Mix	12 µL
Total Volume	25 µL

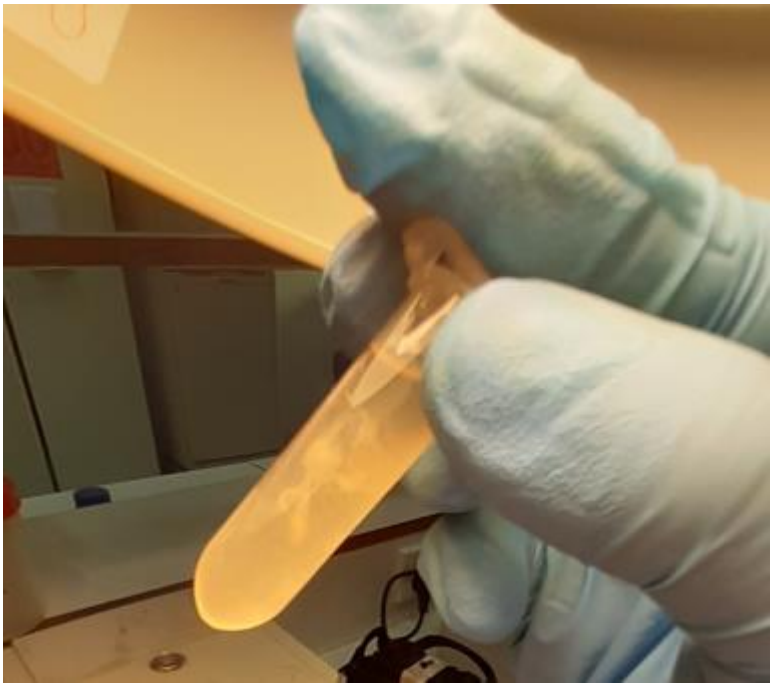


Figure 3.1: DNA Precipitation

The representation of DNA precipitation in to threads in the presence of absolute ethanol

Figure 3.2: DNA Concentration

Representation of DNA concentration at absorbance ratio A260/A280 in NanoDrop spectrophotometer

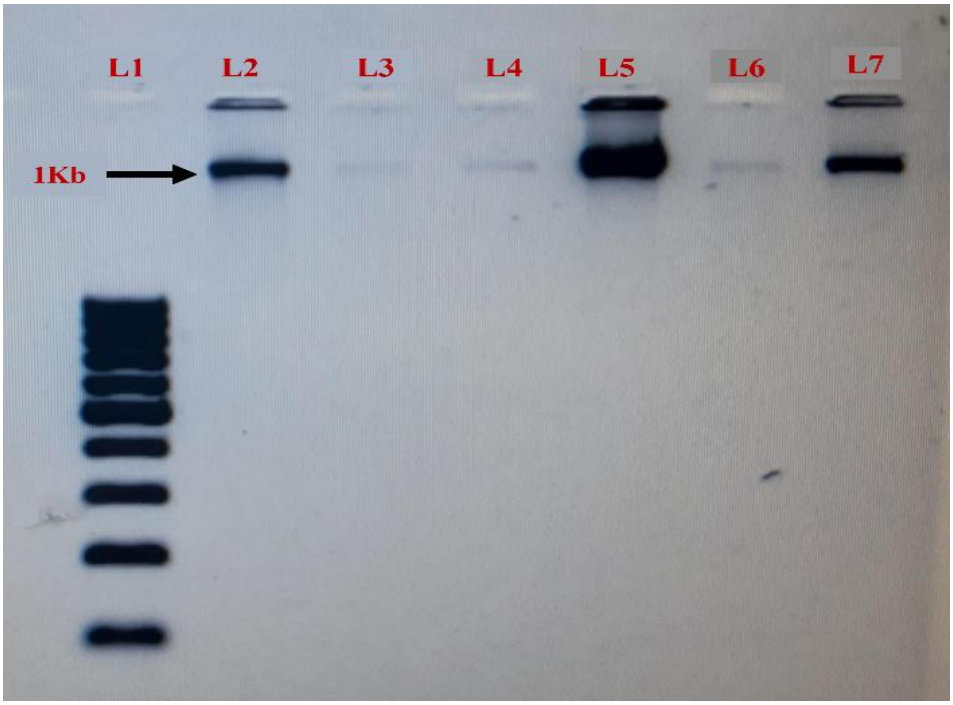


Figure 3.3: DNA Quantity

Representation of DNA quality on 0.8% agarose gel. L1 to L7 indicates lanes, where L1 contains ladder and L2-7 contains different samples at 1000bp (1kb). The picture depicts DNA as pure and intact.

Figure 3.4: T-ARMS PCR

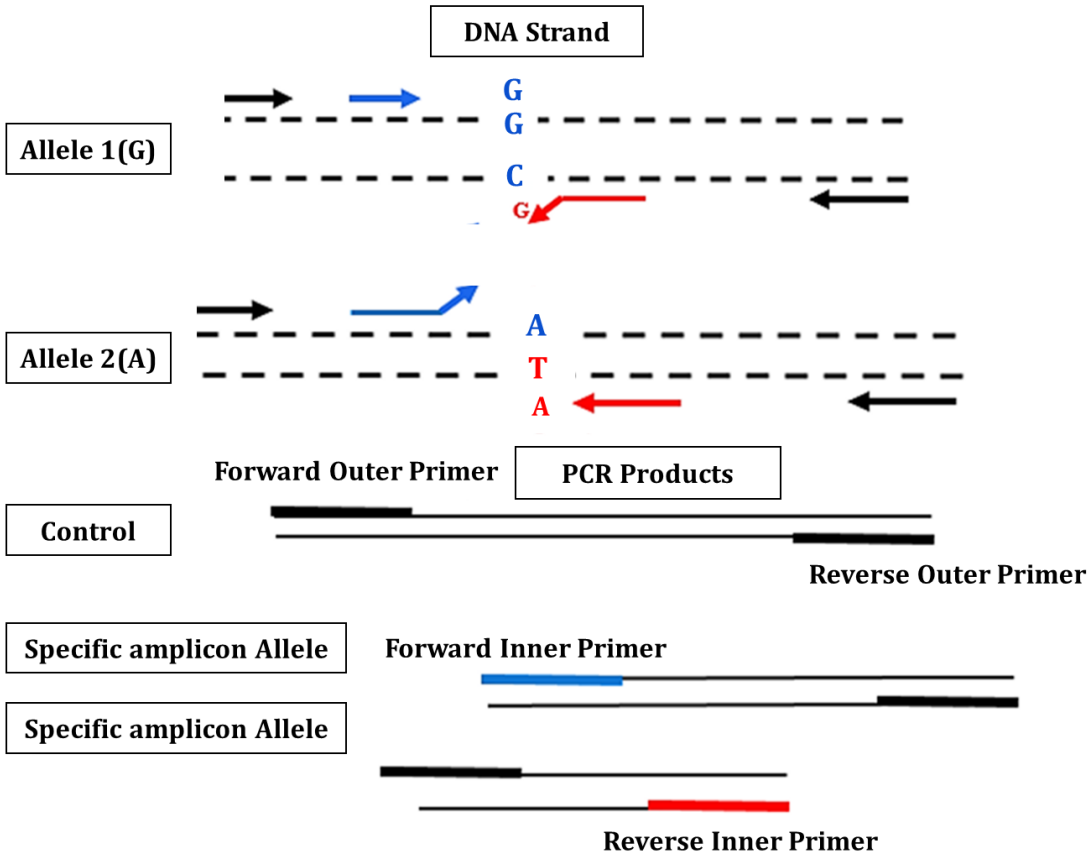


Figure 3.4: T-ARMS PCR

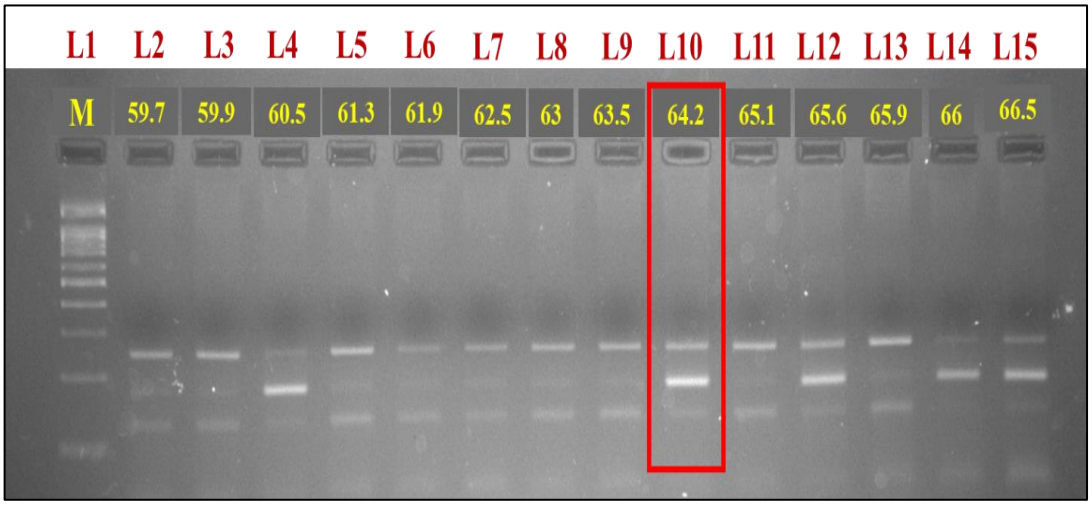


Figure 3.5: Temperature Optimization for Annealing Temperature

Representation of temperature optimization providing annealing temperature 59.7°C to 66.5°C. Lane 1 (L1) contains 100bp ladder, whereas L2 to L15 contain amplified PCR product on 3% agarose gel. Among all temperature 64.2°C was selected as an annealing temperature due to clear and sharp band after the optimization.

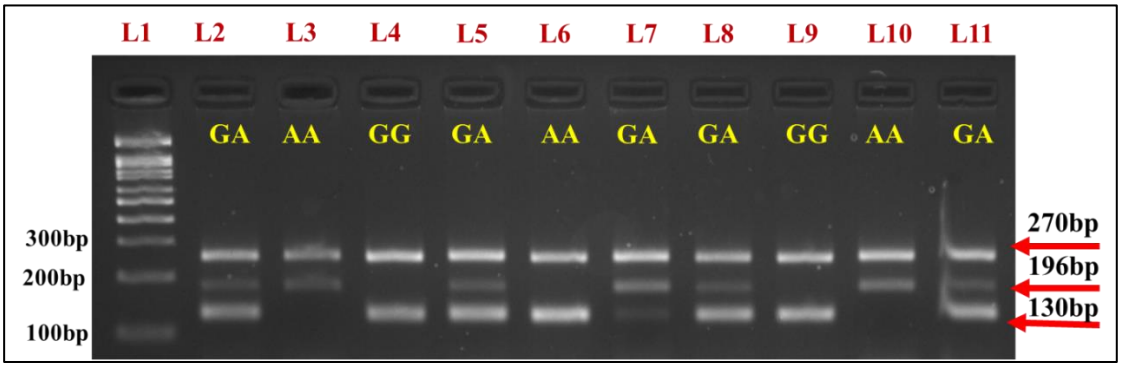


Figure 3.6: SNP rs92188406359 Genotype

Representation of obtained genotypes of SNP rs92188406359. Lane 1 (L1) contains a 100bp ladder, whereas L2 to L11 contain amplified PCR product of targeted polymorphism on 3% agarose gel. The presence of two bands showed homozygous genotype, whereas three bands indicate the heterozygous genotype. The homozygous wildtype genotype appeared as two bands 270bp and 130bp, homozygous mutant genotype appeared as two bands at 270bp and 196bp. The heterozygous genotype appeared as three bands at 270bp, 196bp, and 130bp. The band at 270bp showed inner control band from outer primer which is common for all genotypes.

CHAPTER 4

RESULTS

4.1 DEMOGRAPHIC PROFILE OF THE CONTROLS AND CASES

4.1.1 Gender

The study was conducted on 41 age and gender matched control (n=21) and diagnosed cases of Alzheimer disease (n=20). The frequency of females was 7 (33.3%) and 7 (35%) in Control and Case Group respectively. The frequency of males was 14 (66.6) and 13 (65%) in Control and Case Group respectively.

4.1.2 Mean Age

The mean age of patients along with the standard deviation was 75.86 ± 5.102 years and 74.65 ± 7.450 years in in Control and Case Group respectively.

4.1.3 Mean BMI

The mean BMI of patients along with the standard deviation was 25.74 ± 2.36 and 26.48 ± 2.61 in Control and Case Group respectively.

4.1.4 Ethnicity

Major portion of the individuals were Urdu speaking 19 (46.3%), followed by Sindhi 10 (24.4%), Pathan 8 (19.5%), Baloch 02 (4.9%) and Punjabi 2 (4.9%).

4.2 *BCL2* GENE SNP rs92188406359 ANALYSIS

4.2.1 Allelic Frequencies

The allelic frequency of mutant A allele was found to be frequent among cases (45%) than controls (17%). However, the allelic frequency of wild type allele G is found to be frequent among controls (83%) than cases (55%).

4.2.2 Genotype Frequencies

The genotype frequency revealed that the wild type of homozygous genotype GG was predominantly appeared among controls (67%) in comparison to cases (25%). On the other hand, the homozygous mutant genotype AA was found in cases (0.8%), whereas in controls mutant genotype is not appeared among the obtained samples. The heterozygous GA genotype was found to be frequent among the cases (60%) in comparison to the controls (33%).

4.2.3 Association of *BCL2* SNP rs921884063 with Alzheimer's Disease

It was observed that mutant genotype AA is not found in controls, whereas the frequency in cases was 0.8%. The association analyses were done by applying Chi-square analysis which showed that SNP rs921884063 is significantly associated with the progression of Alzheimer's disease with Chi-sq value 8.56 and p-value 0.001. The strength of association was analyzed by odds ratio which revealed that targeted SNP is significantly associated with the increased risk [3.9947, CI 95% (2.0774-7.6812 $p<0.001$)] of Alzheimer's disease.

4.2.4 Association of *BCL2* SNP rs921884063 Genotypes

The association of genotype was analyzed by applying genotype models which confirms that the heterozygous G/A genotype showed significant protective role against the development of disease in codominant [OR CI95%=0.21, (0.05-0.83, $p<0.01$)], dominant [OR CI95%=0.71, (0.04-0.65, $p<0.01$)], and over dominant models [OR CI95%=0.3, (0.09-1.19, $p<0.05$)]. The odds ration <1 revealed protective role of SNP,

odds ratio >1 revealed risk association with the progression of disease, whereas the odds ratio 1 indicates that there is no relation among the SNP and disease. In this study the genotype G/A showed a significant protective role against the disease.

Table 4.1.1: Proportion of Male and Female in Control and Case Groups

Variable		Controls n (%)	Cases n (%)
Gender	Female	7 (33.3)	7 (35)
	Male	14 (66.6)	13(65)

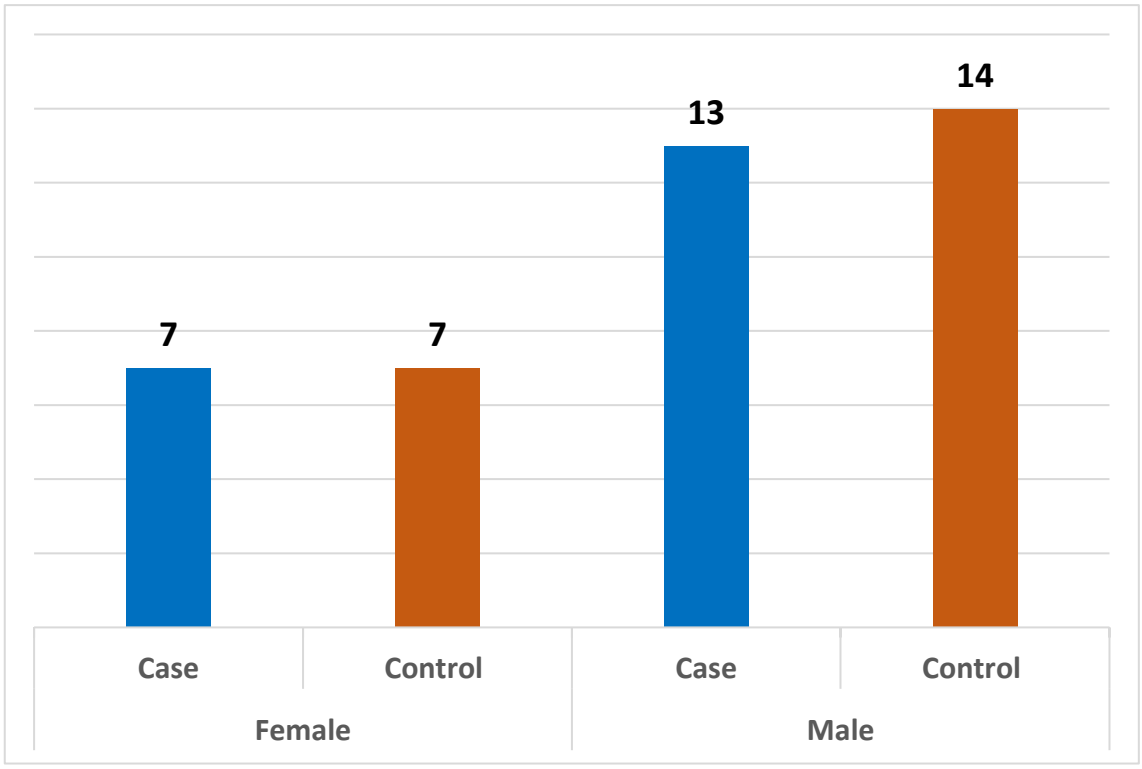


Figure 4.1.1: Proportion of Male and Female in Control and Case Groups

Table 4.1.2: Mean Age of Control and Case Groups

Variable	Control Mean ± SD	Case Mean ± SD
Age (Years)	75.86 ± 5.102	74.65 ± 7.450

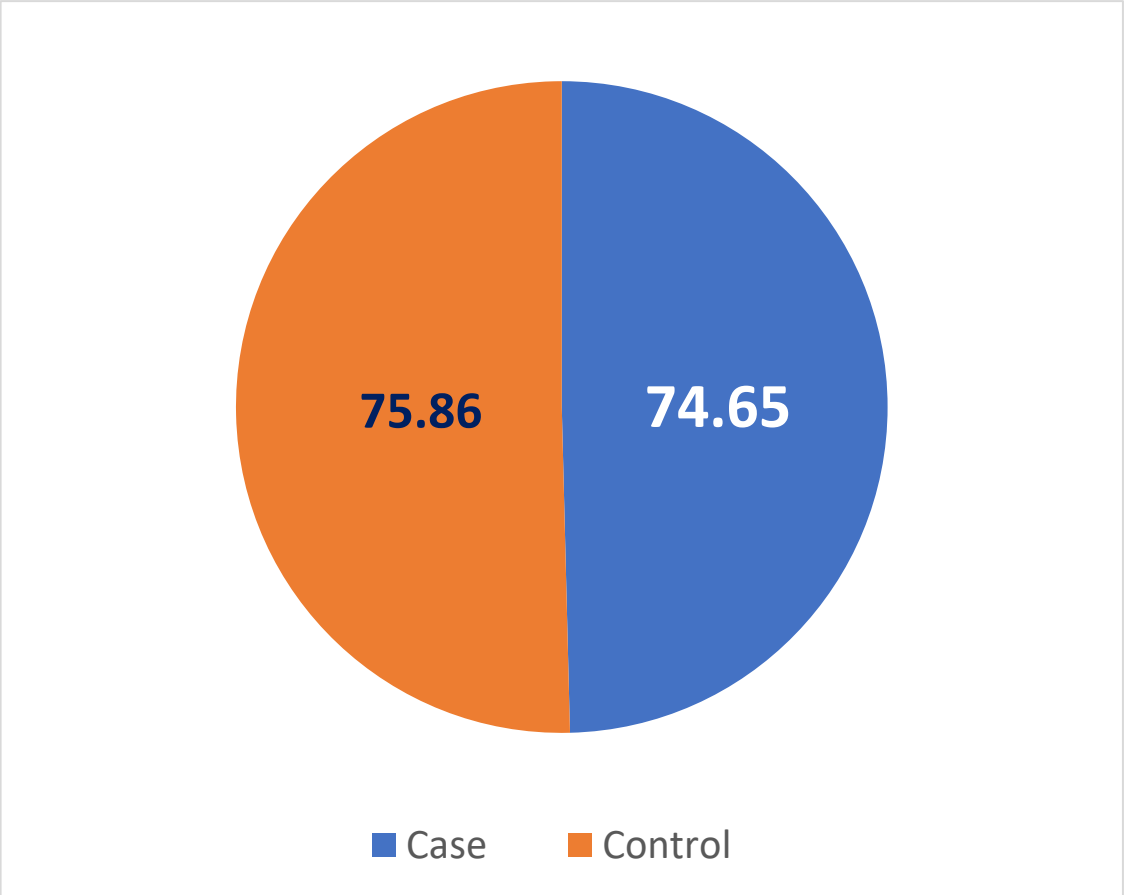


Figure 4.1.2: Mean Age of Control and Case Groups

Table 4.1.3: Mean BMI in Control and Case Groups

Variable	Control Mean ± SD	Case Mean ± SD
BMI	25.74 ± 2.36	28.48±3.77

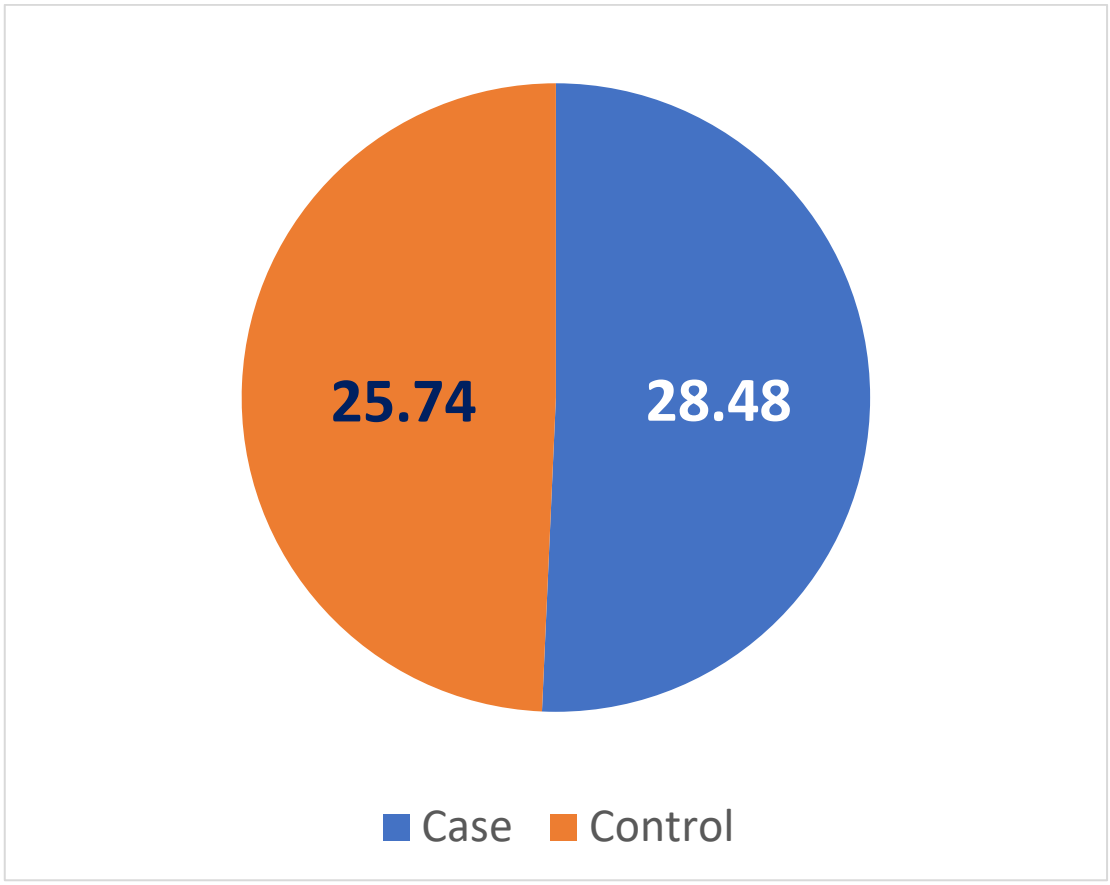


Figure 4.1.3: Mean BMI in Control and Case Groups

Table 4.1.4: Ethnicity of individuals in Control and Case Groups

Variable		Frequency (n)	Percentage (%)
Ethnicity	Baloch	2	4.9%
	Sindhi	10	24.4%
	Urdu	19	46.3%
	Pathan	8	19.5%
	Punjabi	2	4.9%

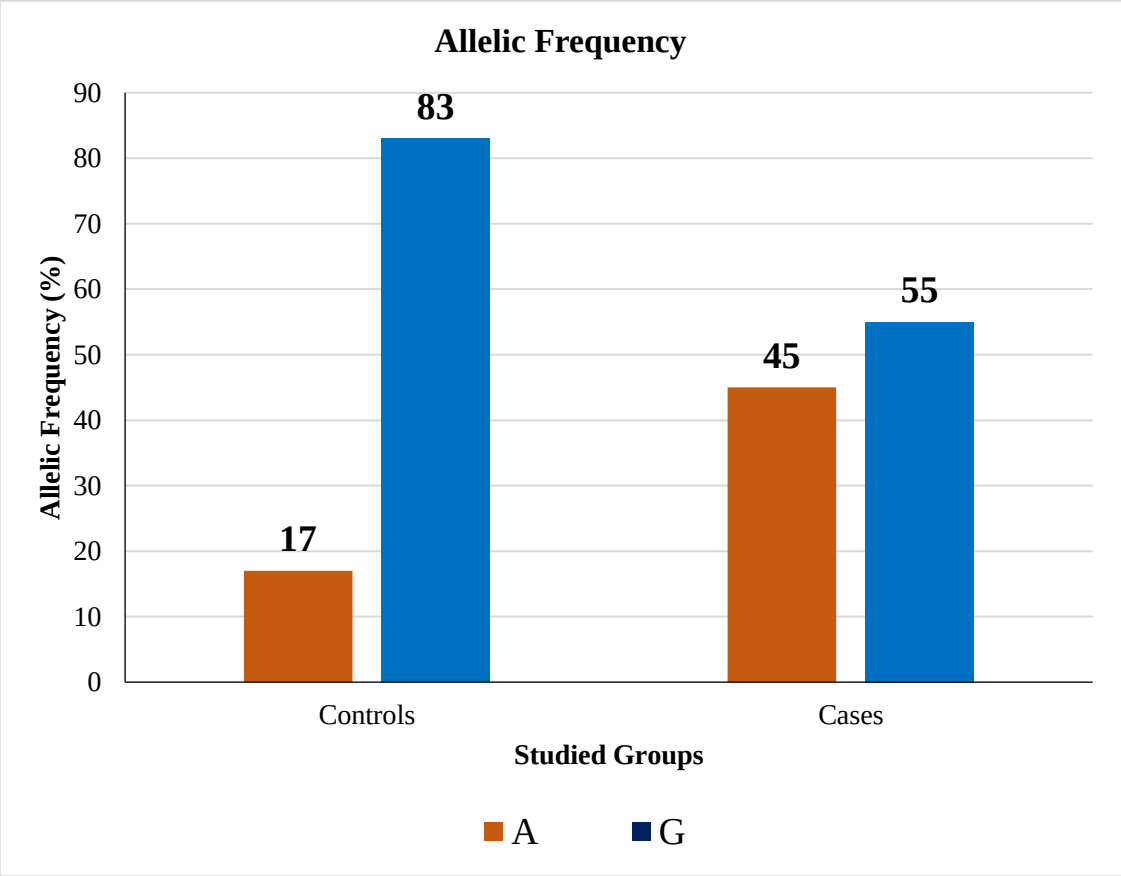


Figure 4.2.1: Allelic Frequencies among Control and Case Groups

Representation of Allelic frequency among cases (purple) and controls (Blue). The frequency is mentioned in percentages.

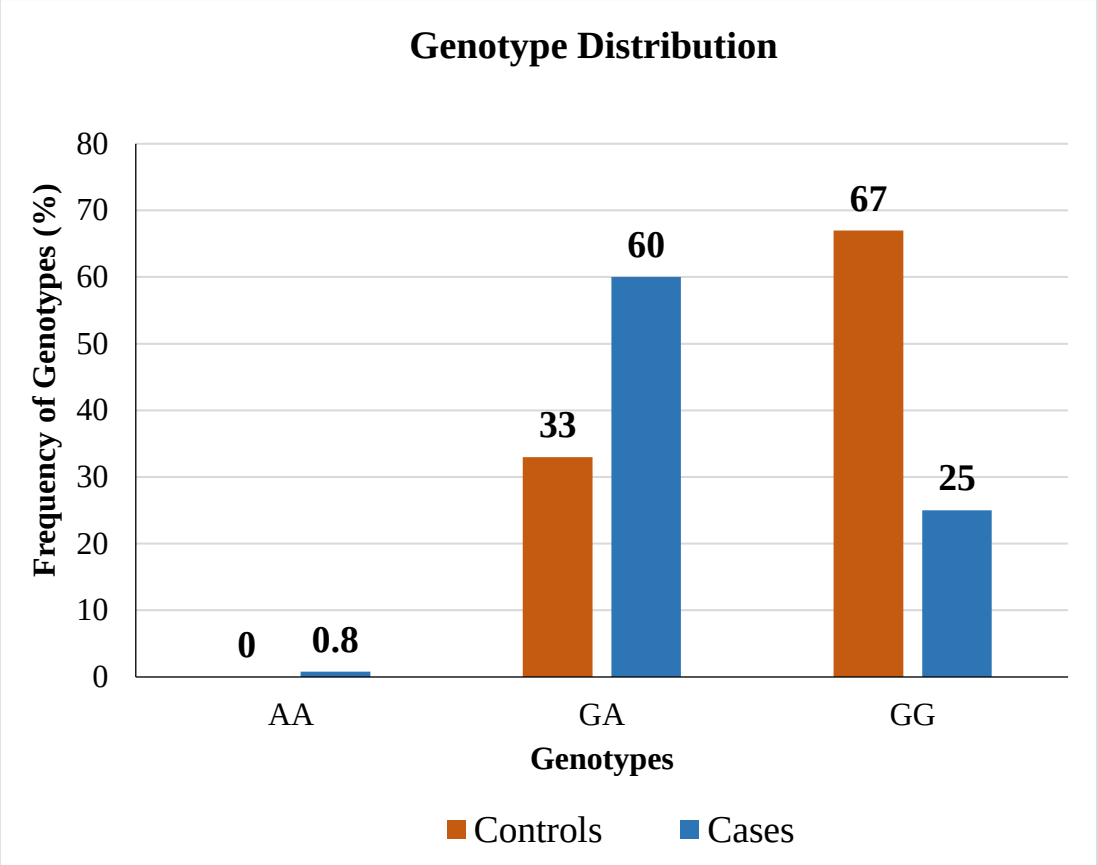


Figure 4.2.2: Genotype Frequencies among Control and Case Groups

Representation of genotype frequency in percentage among the cases (Orange) and controls (Green).

Table 4.2.3: Association of *BCL2* gene SNP (rs921884063) with Alzheimer's Disease

Genotypes	Controls (n = 21)		Cases (n = 20)		Chi-Square (X^2) (p-Value)	Odds Ratio (p-value)	CI (95%)
Genotype Frequency (%)	GG	67	GG	60	**8.56** (p<0.01)**	**3.9947*** (p<0.001)**	**2.0774 - 7.6812**
	GA	33	GA	25			
	AA	0	AA	0.8			
Allelic Frequency (%)	G	83	G	55			
	A	17	A	45			

n= number of samples, p-values represented as p<0.01(**), and p<0.001 (***)

Table 4.2.4: Genotype Association of SNP (rs921884063) with Alzheimer's Disease

Model	Genotype	Controls n (%)	Patients n (%)	OR (95% CI)	AIC	BIC
Codominant	G/G	14 (66.7)	5 (25)	1.00	52.9	58
	G/A	7 (33.3)	12 (60)	**0.21 (0.05-0.83)** **		
	A/A	0 (0)	3 (15)	0.00 (0.00-NA)		
Dominant	G/G	14 (66.7)	5 (25)	1.00	53.4	56.8
	G/A-A/A	7 (33.3)	15 (75)	**0.71 (0.04-0.65)** **		
Over dominant	G/G-A/A	14 (66.7)	8 (40)	1.00	51.5	54.9
	G/A	7 (33.3)	12 (60)	**0.3 (0.09-1.19)** *		

OR=odds ratio, **p<0.01, *p<0.5, Bold values indicates significant results

CHAPTER 5

DISCUSSION

Alzheimer's disease (AD) is the most prevalent disease that is progressively increasing form of dementia globally. AD manifests primarily through memory loss and language impairment, impacting cognitive skills and problem-solving abilities. The disease's neuropathological basis involves the progressive atrophy of the brain cortex due to the accumulation of insoluble proteins, including tau and amyloid proteins, leading to neuronal dysfunction and death. It constitutes around 70-80 % of cases of dementia. It is projected to affect millions with estimates suggesting the number of sufferings from it could double by 2040. AD encompasses not only the well-known early-onset and late-onset forms but also a rare familial variant that showcases autosomal dominant inheritance and constitutes less than 1% of cases. Observational studies have shown a higher occurrence of AD in first-degree relatives compared to the general population, highlighting the genetic underpinnings of the disease.

While recent advancements in genetic research have shed light on the underlying genetic architecture of AD, but still effective diagnostic, preventive, or treatment approaches are lacking due to complex etiology of disease and lack of effective interventions. The ongoing advancements in genetic research, including extensive next-generation sequencing (NGS) and genome-wide association studies (GWAS), are continually unraveling the intricate genetic underpinnings of multifactorial AD. Among the numerous genetic risk factors, the most prominent is the *BCL2* gene single nucleotide polymorphism (SNP). Over 20 risk genes and loci linked to AD have been identified, altering the conventional understanding of its genetic risk factors.

These discoveries highlight the complex interplay of structural and functional genetic variants in AD's pathogenesis, prompting a paradigm shift. Functional assessment of these genetic variations employs diverse techniques, offering insights into the molecular mechanisms behind AD and potentially expediting the development of disease-

modifying and preventive strategies. This underscores the need for establishing a robust foundation for long-term population health benefits. Immediate applications include the diagnosis and prediction of an individual's AD risk, facilitating access to genetic testing for hazardous mutations. Despite the limited current therapeutic options for AD, genetic research has crucial implications for patients and families, paving the way for future interventions that could significantly impact disease management.

In our research, Alzheimer's was found to be more prevalent in males than females as out of 20 cases, 13 (65%) were male and 7 (35%) were female. It was consistent with the study conducted to evaluate the sex-specific genetic association of AD. It was found that sex-specific neurofibrillary tangles were more prevalent in males (49%) as compared to females (46%). These results link to a novel locus on chromosome 17 that confers male-specific protection from tau pathology and highlight the value of assessing genetic associations in a sex-specific manner (Dumitrescu et al., 2019).

According to a study, there is a perception that AD is more frequently observed in females compared to males. However, the actual prevalence of AD is higher among males. The prevalence of underdiagnosis in males can be attributed to the tendency for misdiagnosis in a significant proportion of cases. (Murray et al., 2016).

On contrary to our results, the meta-analysis showed that prevalence of AD was 0.4% in women and 0.2% in men (RR = 1.54, CI = 0.69–3.44, I 2 = 38%). The incidence of the disease was 0.02% in women and 0.01% in men (RR = 1.50, CI = 0.91-2.48, I 2 = 0%). The incidence rates of AD per 100,000 person-years ranged from 0 to 132 in women and from 0 to 42 in men (Peeters et al., 2022).

Another longitudinal study found a higher female prevalence in AD as compared to male. The study used postmortem data of 1453 individuals and investigated sex differences in AD and other brain pathologies. The majority were women (67%), showing higher levels tau tangle density ($p < 0.001$) and a potential difference in amyloid-β load ($p = 0.056$) (Oveisgharan et al., 2018) .

Another study found that women had a higher likelihood of being diagnosed with dementia AD, compared to men, while non-Alzheimer's dementia was more evenly distributed between genders. The incidence rates of dementia were initially similar for both men and women, but they diverged after the age of 80, with women showing higher rates beyond 90 years of age. The study also noted that women's survival rates were higher

than men's, especially beyond age 90, suggesting that longevity might contribute to the higher incidence of dementia in women at older ages (Beam et al., 2018).

2/3 of all the individuals suffering from AD are females (Andrew & Tierney, 2018). The exact cause of higher prevalence in women is unknown but a study suggest that it may be due to follicle stimulating hormone (FSH) that increases dramatically in women after menopause and this could be the possible trigger for development of AD in females (Ye,K. 2022).

According to our research findings, it was observed that the mean age of individuals affected by AD was 74.65 years. This suggests a notable prevalence of AD within the older population. The typical age of onset for Alzheimer's disease is 65 years or older . It is reported that AD affects approximately 6% of people over the age of 65 years (Guest, 2019). The projected population of individuals in the United States aged 65 and above diagnosed with Alzheimer's dementia is anticipated to reach 13.8 million by the middle of the 21st century. A positive correlation has been observed between increasing age and the likelihood of an individual developing AD. The process of ageing, in conjunction with various risk factors such as oxidative stress, metabolic abnormalities, and alterations in neurochemical pathways, has been observed to have a cumulative impact on the advancement of neurodegenerative mechanisms, ultimately leading to the onset of AD (Zhang et al., 2021).

In a recent clinical cohort study conducted within a community setting, the association between age and AD along with other prevalent neuropathologies was examined in a sample of 1420 individuals. The study findings indicated that there exists a positive correlation between age and the likelihood of developing Alzheimer's dementia at the time of death. Specifically, the results revealed that with each additional year of age, the logarithm of the odds of Alzheimer's dementia increased by 0.067 units, corresponding to an odds ratio of 1.070. The findings exhibited consistency in relation to cognitive impairment and the degree of cognitive functioning. Nevertheless, there exists a non-linear association between age and pathology of AD. The study revealed that the prevalence of pathologic AD exhibited a notable increase until approximately 95 years of age, after which it stabilized. The quantitative indicators of Alzheimer's disease pathology exhibited a notable decrease in individuals aged 95 and above. The correlation between age and other neuropathologies exhibited notable differences compared to Alzheimer's

disease, as the majority of these pathologies showed an upward trend with increasing age (Farfel et al., 2019).

The primary role of *BCL2* gene is the regulation of cellular homeostasis, including the precise regulation of cell proliferation and programmed cell death. It regulates the initiation of mitochondrial apoptosis and governs the fundamental mechanism involved in the functioning of neurons through the alteration of intracellular calcium signaling. The gene also act as protection against cerebral degeneration through the regulation of cellular resilience and apoptotic pathways. Individuals who possess the *BCL2* AA genotype exhibit reduced levels of *BCL2* and grey matter volume, which is additionally linked to changes in cognitive and motor abilities (Erdal et al., 2020).

Recently it was reported that SNPs of *BCL2* gene were found to be associated with development and progression of AD as it is responsible for slowing the processing of APP and tau protein, resulting in a simultaneous decrease cognitive function in the presence of neurofibrillary tangles and extracellular amyloid deposits (Chang et al., 2018).

The current study was aimed to target *BCL2* gene single nucleotide polymorphism (SNP) rs921884063 with susceptibility to Alzheimer's disease. It was found that the allelic frequency of mutant A allele was found to be frequent among cases (45%) than controls (17%). However, the allelic frequency of wild type allele G is found to be frequent among controls (83%) than cases (55%).

The genotype frequency revealed that the wild type of homozygous genotype GG was predominantly appeared among controls (67%) in comparison to cases (25%). On the other hand, the homozygous mutant genotype AA was found in cases (0.8%), whereas in controls mutant genotype is not appeared among the obtained samples. The heterozygous GA genotype was found to be frequent among the cases (60%) in comparison to the controls (33%).

To check the genetic evolution within nature throughout the generations, Hardy-Weinberg equilibrium as calculated using SNPStats. It was observed that SNP rs921884063 was not found to be in Hardy-Weinberg equilibrium in subjects from Karachi.

The strength of association between *BCL2* gene single nucleotide polymorphism (SNP) rs921884063 and risk of development of AD was analyzed by odds ratio. It was

concluded that targeted SNP might be associated with the increased risk of Alzheimer's disease [3.9947, CI 95% (2.0774-7.6812 p<0.001)].

The risk association of separate genotype was determined by applying dominance codominance and over dominant model using SNPStats software. It was observed that the heterozygous G/A genotype showed significant protective role against the development of disease in codominant [OR CI95%=0.21, (0.05-0.83, p<0.01)], dominant [OR CI95%=0.71, (0.04-0.65, p<0.01)], and over dominant models [OR CI95%=0.3, (0.09-1.19, p<0.05)].

Given that this was the first study to demonstrate a connection between a BCL2 gene polymorphism and AD, no fitted model odds ratio was used.

The study satisfies that BCL2 gene single nucleotide polymorphism (SNP) rs921884063 is associated with AD. The findings indicate that while genotype risks are statistically significant inside the model, a larger sample size may eliminate any significant relationships with AD susceptibility.

5.2 IMPLICATION

5.2.1 Theoretical Implication

Evaluating BCL2 gene polymorphisms holds promise for clear understanding of molecular pathways which are involved in the development of AD. Insights into influence of BCL2 gene polymorphisms on disease's progression might help to strategies that modulate BCL2 expression, supporting cell survival and slowing down disease advancement. The identification of BCL2 gene polymorphisms associated with AD could pave the way for the creation of early diagnostic biomarkers. While focused on Alzheimer's, these findings may also extend their relevance to other neurodegenerative disorders, potentially unraveling the role of *BCL2* in various contexts. This multifaceted exploration not only deepens our understanding of Alzheimer's complexities but also contributes to the broader scientific understanding of the complex association between genetics and neurodegeneration.

5.2.2 Practical Implication

The study highlights the potential significance of genetic variations within the *BCL2* gene. It has the potential to guide the development of tailored therapeutic approaches. Individuals harboring specific genetic variants could potentially benefit more from interventions targeting pathways linked to BCL2. It will empower clinicians to identify individuals at risk even before clinical symptoms emerge, facilitating proactive and timely interventions.

5.2.3 Policy Implication

N/A

5.3 LIMITATION AND STRENGTH OF THE STUDY

5.3.1 Limitations

Following are the limitations of the study:

- Single center study
- Small sample size

5.3.2 Strengths

Following are the strengths of the study:

- First time study in Pakistan that will provide new diagnostic modality for Alzheimer's disease.

5.4 FUTURE RESERCH DIRECTION/ RECOMMENDATIONS

- Further studies should be carried out on the neighboring SNPs of *BCL2* gene to check for linked SNPs.

- Large-Scale Genetic Studies should be conducted on diverse population to identify specific *BCL2* gene polymorphisms associated with Alzheimer's disease and to understand the effects of broader range of genetic variations and their potential impact on AD risk.

- Future studies should be done on the functional consequences of *BCL2* gene polymorphisms on protein expression, localization, and function to determine whether specific variants influence BCL2's anti-apoptotic properties or its interactions with other proteins involved in AD-related processes.

- Association between *BCL2* gene polymorphisms and established AD biomarkers should be evaluated so that insights into the relationship between *BCL2* variants and disease progression can be comprehended.

- Future studies on evaluation of epigenetic modifications associated with *BCL2* gene polymorphisms and their potential impact on AD susceptibility.

- Use of animal models to further investigate the relationship between *BCL2* variants and AD-like pathology. These models can provide valuable insights into the molecular mechanisms underlying AD development.

5.5 CONCLUSION

- All the participants of the study were recruited following the National Institute of Neurological and Communicative Diseases and Stroke & Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for the diagnosis of Alzheimer's disease.

- Patients suffering from Alzheimer's disease with have impaired cognitive function and loss of memory with a mean age of (74.65±7.450) years.

- B-cell lymphoma 2 (BCL2) gene single nucleotide polymorphism (SNP) rs921884063 might be associated with risk of Alzheimer's disease [3.9947, CI 95% (2.0774-7.6812 $p<0.001$)].

- There is a strong association of B-cell lymphoma 2 (BCL2) gene single nucleotide polymorphism (SNP) rs921884063 with Alzheimer's disease. This B-cell lymphoma 2 (BCL2) gene single nucleotide polymorphism (SNP) rs921884063 should be used as genetic marker for early diagnosis of Alzheimer disease

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ANNEXURES

A) BUMDC- FRC Approval Letter

B) BUMDC- ERC Approval Letter

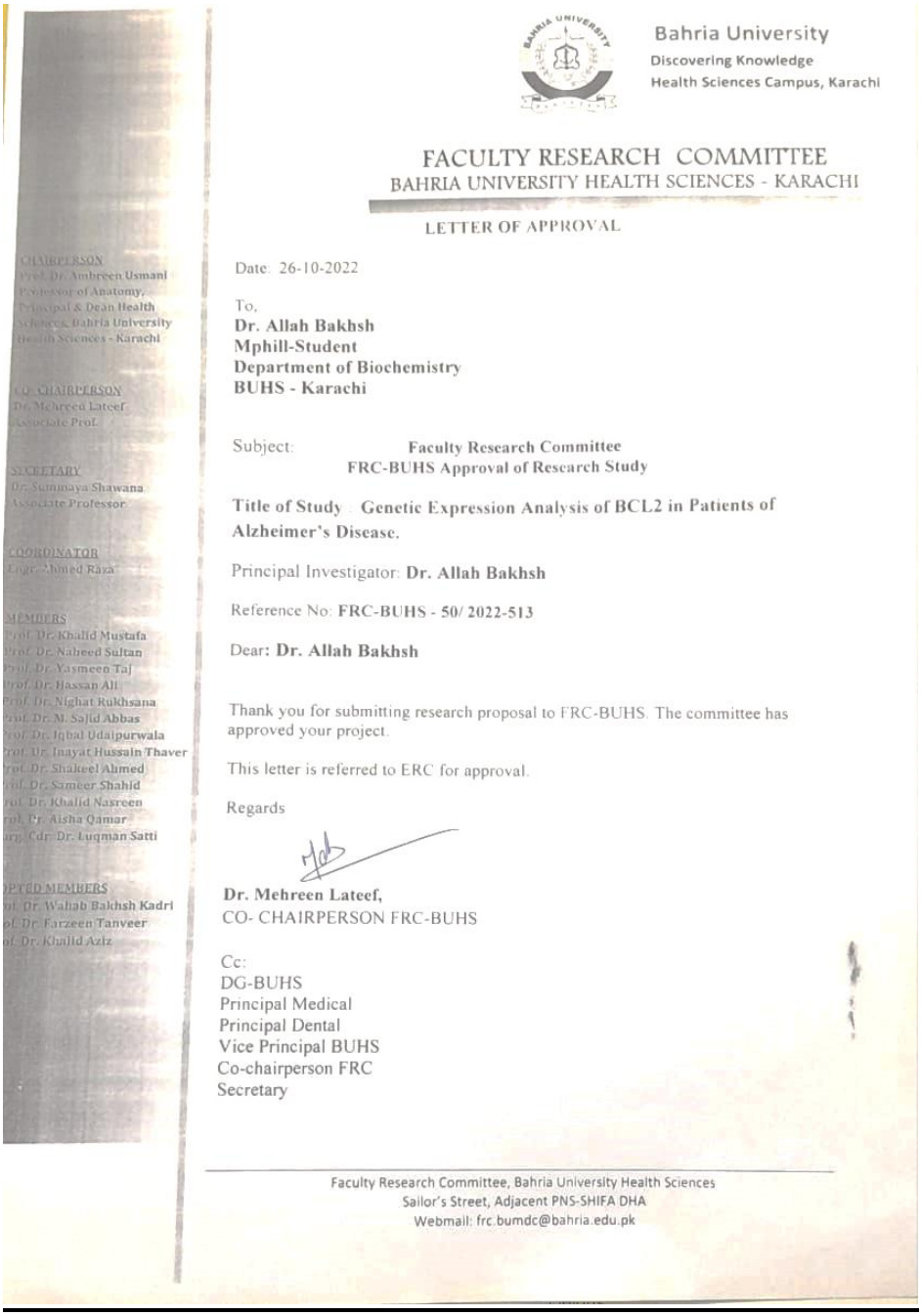
C) Consent Form

D) Subject Evaluation Form

E) Hospital / Institute Card

F) Turnitin Plagiarism Check Report

A



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Principal & Dean Health
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Health Sciences - Karachi

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Bahria University
Discovering Knowledge
Health Sciences Campus, Karachi

FACULTY RESEARCH COMMITTEE
BAHRIA UNIVERSITY HEALTH SCIENCES - KARACHI

LETTER OF APPROVAL

Date: 26-10-2022

To,
Dr. Allah Bakhsh
Mphill-Student
Department of Biochemistry
BUHS - Karachi

Subject: **Faculty Research Committee**
FRC-BUHS Approval of Research Study

Title of Study : Genetic Expression Analysis of BCL2 in Patients of Alzheimer's Disease.

Principal Investigator: **Dr. Allah Bakhsh**

Reference No: **FRC-BUHS - 50/ 2022-513**

Dear**: Dr. Allah Bakhsh**

Thank you for submitting research proposal to FRC-BUHS. The committee has approved your project.

This letter is referred to ERC for approval.

Regards

Dr. Mehreen Lateef,
CO- CHAIRPERSON FRC-BUHS

Cc:
DG-BUHS
Principal Medical
Principal Dental
Vice Principal BUHS
Co-chairperson FRC
Secretary

B

Bahria University
Discovering Knowledge
Health Sciences Campus Karachi

ETHICAL REVIEW COMMITTEE
LETTER OF APPROVAL

Date: 19-Dec-22

Reference:
FRC-BUHS-50/2022-513

PATRON
Prof. Ambreen Usmani
Principal & Dean
Health Sciences(BU)

CHAIRPERSON
Dr. Quratulain Javaid

SECTRETARY
Dr. Ambreen Surti

MEMBERS
Prof M Alamgir
Prof Anis Jafarey
Prof Aisha Qamar
Ms Nighat Huda
Surg Cdre Amir Ejaz
Prof Reza H Syed
Ms Shabina Arif
Mr M Amir Sultan
Prof Dr Rafat Murad
Ms NajmusSahar Ilyas

Dr. Allah Bakhsh
MPhil Candidate
Department of Biochemistry
BUHS-Karachi

Subject: Institutional approval of research study

Title of Study: genetic expression analysis of BCL2 in patients of Alzheimer's disease

Principal Investigator: Dr. Allah Bakhsh

Reference No: ERC 113/2022

Dear Dr. Allah Bakhsh,

Thank you for submitting the above mentioned study proposal. ERC Bahria University Health Sciences Campus has reviewed this project in the meeting held on 16-Dec-2022 and gives approval. Kindly notify us when the research is complete.

Regards,

19/12/2022
DR. AMBREEN SURTI
Secretary, ERC
BUHS

19/12/2022
DR. QURATULAIN JAVAID
Chairperson, ERC
BUHS

Cc:
Principal BUHS

B

Bahria University
Health Sciences Campus, Karachi

IRB — No one left behind for research.

Institutional Review Board

IRB Members Profile

Maj. Gen (Retd). Prof. Dr. Shehla Baqai - Patron
Principal, Medical & Dean, HS
deanhs@bahria.edu.pk

Prof. Dr. Inayat Hussain Thaver - Chairperson
HOD, CHS
inayat.bumdc@bahria.edu.pk

Prof. Dr. Shazia Shakoor, - Vice Chairperson
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Associate Professor Anatomy
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Ms. Abida Razzaq - Member
Associate Professor
PN. Navy Nursing College
razzaq.bumdc@bahria.edu.pk

Dr. Najmus Sahar - Member
Asst Prof of DPT
nsahar.bumdc@bahria.edu.pk

BUHS-IRB # 020/23
Date: October 5, 20233
Name of PI: Dr. Allah Bakhsh
Affiliation & Department: Biochemistry
Address: BUHS

Subject: ENDORSEMENT & APPROVAL OF YOUR RESEARCH PROPOSAL:
"Association of B-Cell Lymphoma 2 (*BCL2*) gene SNP with susceptibility to Alzheimer's disease"

Dear Dr. Allah Bakhsh,

I am writing this letter at the request of Dr. Allah Bakhsh to confirm that we support the research project, "Association of B-Cell Lymphoma 2 (*BCL2*) gene SNP with susceptibility to Alzheimer's disease."

This research proposal was previously approved by the Faculty Research Committee (FRC) with a slightly different title, "**Genetic expression analysis of *BCL2* in patients of Alzheimer's disease," issued on BUHSC.FRC-32/2023. This followed the approval by the then ERC Ref ERC 113/2022.**

The research will assess the association of B-cell lymphoma 2 (*BCL2*) gene single nucleotide polymorphism (SNP) rs921884063 with susceptibility to Alzheimer's disease in the Pakistani population and its association with clinical features.

I have been provided with the study information and assured that minor changes have been made to the new title. Therefore, I will support the project under the proposed guidelines in the IRB application for six months. If any unanticipated problems or adverse advents occur, it is up to the Principal Investigator, Dr. Allah Bakhsh, to report these events to the IRB as prompely as possible. This research will be a valuable contribution to the area of study/goal of study, and we will be happy tosupport this endeavor.

Sincerely,

Prof Dr. Inayat H. Thaver-
FCPS (Community Medicine); PhD (Public Health)
Chairperson, Institutional Review Board (IRB)
Bahria University Health Sciences Campus,
Karachi

Name: Prof. Dr. Inayat H. Thaver
Chair,
Institutional Review Board.

IRB Office, BUHSC(K) Adjacent PNS SHIFA, Sailor Street, DHA Phase – II Karachi
Office No. +92-21-35319491-6 Ext: 1080 | Fax: +92-21-99332689

C

WRITTEN INFORMED CONSENT FORM OF PATIENT

You are giving your consent to participate voluntarily and at your own will in this research clinical trial project that aims to evaluate the association of B-cell lymphoma 2 (BCL2) gene single nucleotide polymorphism (SNP) rs921884063 with susceptibility to Alzheimer's disease

You have been explained in detail the nature and significance of participating in the project and you understand the provided explanation.

You have been told that findings of your disease and your data will be kept strictly confidential and will be used only for the benefit of community, publications and paper presentations.

You have been explained that laboratory investigations will be conducted for the diagnosis of disease. For this purpose, you fully agree to give your blood samples at the beginning and end of study.

You also agree to give all relevant information needed, in full and to the best of your knowledge to the researcher. It is clarified to you that no incentive will be provided to you for participating in the study except the cost of lab investigations and drugs, whereas you do have the right to withdraw from the study at any time.

You are advised to contact Dr. Allah Bakhsh on mobile number: 0333-3374415 or visit Jinnah Post Graduate Medical Center, Karachi in case of any query/ emergency related to your disease.

Name of Patient: _____

S/D/ W/o _____

Signature / Thumb impression of patient: _____

Name of Researcher: _____

Signature of Researcher: _____

C

مریض کے لئے اجازت فارم

آپ اس تحقیقی کلینکل ٹرائل پروجیکٹ میں رضاکارانہ طور پر اور اپنی مرضی سے حصہ لینے کے لیے اپنی رضامندی دے رہے ہیں جس کا مقصد (BCL2) B-cell lymphoma 2 جین سنگل نیوکلیوٹائڈ پولیمورفیزم rs921884063 (SNP) کے ساتھ الزائمر کی بیماری کے لیے حساسیت کا جائزہ لینا ہے

آپ کو اس تحقیقاتی عمل میں حصہ لینے کے فطرت اور اہمیت کے بارے میں تفصیل سے وضاحت کی گئی ہے اور آپ فراہم کردہ وضاحت کو سمجھتے ہیں.

آپ کو بتایا گیا ہے کہ آپ کی بیماری اور آپ کے اعداد و شمار کو سختی سے خفیہ رکھا جائے گا اور صرف کمیونٹی کے فائدے، اشاعتوں اور آرٹیکل اشاعت کے لئے استعمال کیا جائے گا.

آپ کو وضاحت کی گئی ہے کہ لیبارٹری کی تحقیقات, تشخیص کے لئے کئے جائیں گے. اس مقصد کے لئے آپ اپنے خون کے نمونے کو، مطالعہ کے آغاز اور اختتام پر مکمل طور پر دینے کے لئے اتفاق کرتے ہیں اور جب بھی اس کے درمیان ضروری ہو۔

آپ پوری طرح سے اور بہتر علم کے ساتھ محقق کو تمام متعلقہ معلومات دینے پر اتفاق کرتے ہیں.

یہ آپ کو واضح کیا گیا ہے کہ لیبارٹری کی تحقیقات اور ادویات کی قیمت کے علاوہ مطالعہ میں حصہ لینے کے لئے آپ کو کوئی ادائیگی نہیں کی جائے گی، جبکہ آپ کو کسی بھی وقت تحقیقاتی عمل کو چھوڑنے کا حق حاصلہ ہے.

آپ کو مشورہ دیا جاتا ہے کہ **ڈاکٹر اللہ بخش** کے موبائل نمبر: **0333-3374415** پر رابطہ کریں اور آپ کی بیماری سے متعلق کسی بھی سوال یا ہنگامی صورت حال کے معاملے میں **جناح پوسٹ گریجویٹ میڈیکل سینٹر اسپتال کراچی** سے رابطہ کریں.

مریض کا نام: _____

والد / شوہر کا نام: _____

مریض کا علاج: _____

مریض کے دستخط / انگوٹھے تاثر: _____

محقق کا نام: _____

محقق کا دستخط: _____

تاریخ: _____

D

SUBJECT EVALUATION FORM

S.NO: _____

Name _____ Father/ Husband Name _____

Age _____Years Gender: Male / Female

Ethnicity _____ Marital status Married / Unmarried

Weight _____ kg Height _____ m BMI _____

Co-Morbid

Hypertension Yes / No Diabetes Yes / No

Resident of Industrial Area Yes / No

Year Alzheimer's was diagnosed _____

Most Common Symptoms _____

Frequency of forgetting home address _____

Name of Relatives you can remember _____

Associated Symptoms _____

Any psychological issues _____

Any family member having same illness _____

Currently taking any medicine _____

Other co-existing medical conditions _____

MEDICATION HISTORY: Please list any medications you are currently taking

MEDICATION	**DOSE**	**REASON**

D

QUESTIONNAIRE

Name _____ Father/ Husband Name _____

Age _____Years Gender: Male / Female

Address _____

Contact _____

Weight _____ kg Height _____ m BMI _____

Hypertension Yes / No Diabetes Miletus Yes / No

Stroke Yes / No Myocardial Infarction Yes / No

1. Probable Alzheimer's disease according to NINCDS-ADRDA criteria Yes / No
2. Diagnosed case of Alzheimer's Disease? Yes / No
3. Currently on symptomatic treatment of Alzheimer's Disease? Yes / No
4. Family history of Alzheimer Disease? Yes / No
5. Are you suffering from Dementia (according to DSM-IV criteria) ? Yes / No
6. Do you have a history of schizophrenia? Yes / No
7. Have you ever been diagnosed with bipolar disorder? Yes / No
8. Have you ever been diagnosed with Recurrent Psychotic disorders? Yes / No
9. Are you suffering from any somatic disorder that may have caused cognitive impairment? Yes / No
10. Are you suffering from any psychotic disorder that may have caused cognitive impairment? Yes / No
11. Are you suffering from any neurologic disorder that may have caused cognitive impairment? Yes / No
12. Any previous history of brain injury? Yes / No

13. Do you have trouble making decisions about what to eat?

☐ Never ☐ Sometimes ☐ Always

14. Do you have trouble making decisions about which cloth to wear?

☐ Never ☐ Sometimes ☐ Always

15. Do you have trouble making decisions about what to read?

☐ Never ☐ Sometimes ☐ Always

16. Do you have trouble making plans with family/friends?

☐ Never ☐ Sometimes ☐ Always

17. Do you have trouble focusing while watching TV?

☐ Never ☐ Sometimes ☐ Always

18. Do you have trouble concentrating while watching TV?

☐ Never ☐ Sometimes ☐ Always

19. Do you have trouble concentrating while playing on the phone/tablet?

☐ Never ☐ Sometimes ☐ Always

20. Do you have trouble focusing/ concentrating while listening to music?

☐ Never ☐ Sometimes ☐ Always

21. Do you forget the names of familiar objects?

☐ Never ☐ Sometimes ☐ Always

22. Do you forget to use general phrases?

☐ Never ☐ Sometimes ☐ Always

23. Do you get easily confused while driving?

☐ Never ☐ Sometimes ☐ Always

24. Do you get easily confused while using tools?

☐ Never ☐ Sometimes ☐ Always

25. Do you easily get lost in places that are familiar to you?

☐ Never ☐ Sometimes ☐ Always

26. Do you easily get lost in your neighborhood ?

☐ Never ☐ Sometimes ☐ Always

27. Do you easily get lost in grocery stores?

☐ Never ☐ Sometimes ☐ Always

28. Do you easily forget the way back home?

☐ Never ☐ Sometimes ☐ Always

29. Do you miss social cues, which may lead to not understanding what others are saying?

☐ Never ☐ Sometimes ☐ Always

30. Do you miss social cues, which may lead to not understanding what others are laughing at inappropriate times?

☐ Never ☐ Sometimes ☐ Always

31. Do you miss social cues, which may lead to not understanding what others are saying that is viewed as offensive?

☐ Never ☐ Sometimes ☐ Always

32. Do you miss social cues to staying on a topic despite a lack of interest by others?

☐ Never ☐ Sometimes ☐ Always

33. Do you get confused with recalling the day of the week, month, or year?

☐ Never ☐ Sometimes ☐ Always

34. Do you get confused with recalling important dates?

☐ Never ☐ Sometimes ☐ Always

35. Do you repeat yourself in conversations?

☐ Never ☐ Sometimes ☐ Always

36. Do you need help getting dressed?

☐ Never ☐ Sometimes ☐ Always

37. Do you need help remembering to take medication?

☐ Never ☐ Sometimes ☐ Always

38. Do you need help handling your finances?

☐ Never ☐ Sometimes ☐ Always

39. Are these symptoms getting worse? Yes / No

40. Do these difficulties reflect changes from how you were functioning a few years ago? Yes / No

E

IRB

NO.F.2-81/2022-GENL/241/JPMC
JINNAH POSTGRADUATE MEDICAL CENTRE
KARACHI.75510.

Dated the 29-08-2022

Dr. Allah Bakhsh
M. Phil Scholar
Department of Biochemistry
Bahria University Health Sciences Karachi campus
Karachi.

Subject: Association Of B-Cell Lymphoma 2 (BCL2) Gene Single Nucleotide Polymorphism (SNP) rs921884063 with susceptibility to Alzheimer's Disease.

With reference to your application / letter dated 15th June, 2022, on the subject noted above and to say that the Institutional Review Board has approved your subject proposal.

Prof. Khalid Sher, Head Department of Neurology W-28, JPMC, Karachi is the co-supervisor of this study.

Prof. Syed Mehboob Alam
Chairman, Institutional Review Board Committee
JPMC, Karachi.

Copy forwarded for information and necessary action to:

- Dr. Mehreen Lateef, Head Department of Multidisciplinary Research Lab & Medical Lab Technology Quality Assurance Manager, Bahria University Health Sciences Karachi Campus.
- Prof. Khalid Sher, Head Department of Neurology W-28, JPMC, Karachi.

F

PLAGIARISM REPORT

MPhil Thesis Dr Allah Bakhsh

ORIGINALITY REPORT

7%	2%	7%	1%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Muhs, A.. "O1-06-06", Alzheimer's & Dementia: The Journal of the Alzheimer's Association, 200607 Publication	4%
2	Sahil Khan, Kalyani H. Barve, Maushmi S. Kumar. "Recent Advancements in Pathogenesis, Diagnostics and Treatment of Alzheimer's Disease", Current Neuropharmacology, 2020 Publication	1%
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