Prevalence of APP-associated novel polymorphism in Pakistani Population.

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This work is dedicated to my parents and adored siblings whose tremendous support and cooperation led me to this wonderful accomplishment.

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APP	Amyloid precursor protein
Aβ	Amyloid beta
AD	Alzheimer Disease
BACE1	Beta-secretase
ADAM10	alpha-secretase
NFT	neurofibrillary tangles
PCR	polymerase chain reaction
PSEN1	presenilin-1
APOE	Apolipoprotein E
LOAD	Late onset Alzheimer's disease
PDB	Protein Data Bank
WHO	World Health Organization
IDE	Insulin Degrading Enzyme
PSEN2	presenilin-2

ABSTRACT

Alzheimer's disease is a prevalent neurodegenerative disease characterized by dementia occurs in aged individuals. The report of prevalence of AD showed that AD and dementia patients are estimated as 35.6 million in 2010 and this number can reach up to 66 million by 2030.

The present study is the first one to assess the presence of APP genotype in the Pakistani population with relevance to AD, and structural analysis of wild type APP, 710 (V>G) and 718 (I>L), and 720 (L>S) mutated APP with ligands (BACE1, ADAM10, and Nicastrin). No study of this sort has been carried out in Pakistan before. The *in silico* results showed that mutated APP at 718 codon interacts with serine residue of BACE1, and tyrosine residue with Nicastrin. Change in these residues cause protein to undergo aberrant denaturing and results in generation of amyloids due to differential binding of mutated APP protein with ligands in AD. SNP analysis showed the prevalence of 718 (I>L) mutation in 4% of studied population. Symptoms of 718 (I>L), mutation in APP are Hippocampus atrophy, dementia with remarkable oral tendency, and Amnesia. It is imperative to establish organizations that can create awareness among the masses regarding AD which is exponentially increasing in Pakistan.

Key Words: PCR, Polymorphism, APP, Alzheimer's disease, BACE1, Nicastrin, ADAM1

CHAPTER 1: INTRODUCTION

1.1 Alzheimer's disease (AD):

Alzheimer's disease (AD) is a prevalent neurodegenerative disease characterized by dementia occurs in aged individuals. The world Alzheimer report showed that AD and dementia patients are estimated as 35.6 million in 2010 and this number can reach up to 66 million by 2030 (Shen et al., 2018). AD is characterized by memory loss due to the degeneration and loss of neurons in the cortical regions of brain. Besides cognitive decline in AD, various other disease such as cardiovascular disease, tumor, and dysfunction of sensory system also occurs in AD (Masters et al., 2015). AD can be hereditary and can also occurs due to external factors that are not genetic such as head trauma, injury, and environmental factors.

Many researches have been done to understand the genetics of neurodegenerative disease such as AD lately. Apolipoprotein e4 allele present on chromosome 19 is one of the major cause of familial AD. (Jarmolowicz et al., 2015). Various other genes are also related with Alzheimer's disease via point mutations. Mutation in genes such as presenilin-1 (PSEN1) present on chromosome 14, presenilin-2 (PSEN2) present on chromosome, and Amyloid precursor protein (APP) gene on chromosome 21 are causative of familial Alzheimer disease (Goate et al., 1991). There are many evidences that support that pathogenesis of AD is due to the amyloid beta (Aβ) accumulation in the brain. Aβ is generated when proteolytic enzymes β-secretase (BACE1), and γ-secretase cleave the amyloid precursor protein sequentially. This results in the generation of Aβ peptides of various lengths. The most prevalent peptide is $A\beta_{40}$ that is produced. Another species $A\beta_{42}$ isoform is also generated which is the key pathogenic and abundant deposited in the subcortical region of brain (Tiwari et al., 2019). This condition significantly contributes to the disability burden among the elderly population worldwide. With an expected annual expenditure of $1 trillion US dollars worldwide, AD adversely affects patients, their families, and the economies of nations (Chowdhary et al., 2022).

Although no definitive cure for AD patients currently exists, there are therapeutic interventions available that enhance the overall quality of life for patients and mitigate the frequency and severity of symptoms. Alzheimer's disease (AD) can be triggered due to Aβ and tau protein aggregation

within and surrounding the cerebral cells. According to (Serrano-Pozo et al., 2011), Alzheimer disease occurs when there are abundant levels of senile plaques and neurofibrillary tangles (NFTs), which manifest when tau and Aβ protein are deposited in diverse regions of the brain as shown in Figure 1.1. These enduring alterations possess the potential to induce cognitive deterioration, synaptic and neuronal loss within the brain's memory-controlling domain, memory impairments, and ultimately, dementia (Chowdhary et al., 2022).

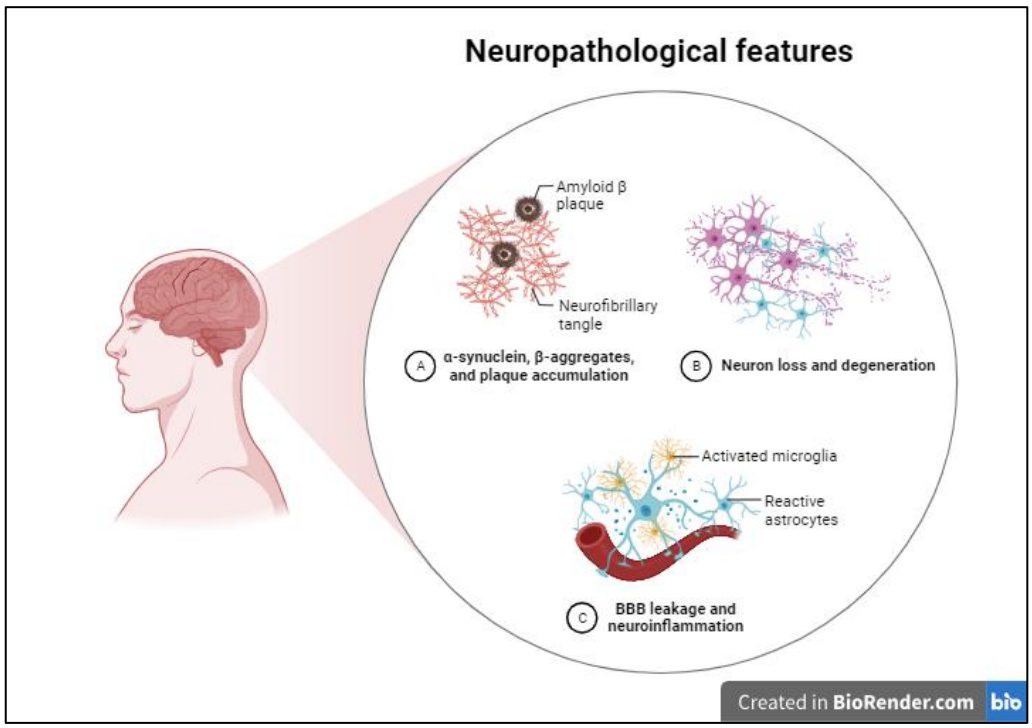


Figure 1.1 Neuropathological features of AD patient. The main pathological features that are the hallmark of AD are accumulation of α-synuclein, Aβ, and formation of plaque in the cortical region of brain. Other features include loss or degeneration of neurons and neuro-inflammation.

1.2 Neurodegeneration

Neurodegeneration, a prevailing characteristic of neurodegenerative diseases, corresponds to the gradual deterioration of neuronal structure and function, ultimately resulting in neuronal death.
In patients with AD, symmetric cortical atrophy represents a commonly observed manifestation of neurodegeneration. During the initial stages of AD, the cortex and hippocampus experience impairment, while the posterior cingulate gyrus and adjacent precuneus are also affected (Sabo et al., 2001). The atrophy subsequently spreads throughout the brain in a temporal-parietal-frontal trajectory, reflecting the sequence in which the accumulation of neurofibrillary tangles (NFTs)

occurs. Until the advanced phases of the disease, cortices responsible for vision, movement, and sensation typically remain unaffected by atrophy. The process of atrophy is further intensified by neuronal death (Masters et al., 2015).

Similar to NFTs, neuronal loss targets the same areas and cortical layers, particularly in regions associated with the MTL. However, the fact that there is a higher degree of neuronal loss compared to the presence of NFTs suggests that neurons tangles die through a distinct mechanism, as opposed to tangle-free neurons. Additionally, damage and synaptic loss also contribute to atrophy, affecting the same areas and cortical layers as NFTs and neuronal loss. It is postulated that synapse loss precedes neuronal loss, as indicated by the surpassing of synaptic loss over neuronal loss (Levy et al., 1990). Consequently, in AD dementia, synapse loss serves as a prominent morphological indicator of cognitive function.

1.3 Neuroinflammation

The reaction of the immune system to alterations in the central nervous system (CNS), involving the responses of astrocytes and microglia, is commonly known as neuroinflammation. Neuroinflammation is an infrequent characteristic of several neurodegenerative diseases, such as AD, FTD, and PD. The inflammation can accelerate the progression of these disorders and lead to neurodegeneration. It is now comprehended that responsive astrocytes and stimulated microglia surround amyloid plaques in the brains of AD patients. The initial discovery of glial cells in amyloid plaques was made by Alois Alzheimer (Shen et al., 2018). Furthermore, various investigations using mRNA measurements, immunohistochemistry, and other techniques have identified abnormal expression of several inflammatory mediators, like cytokines, in the AD brain. Furthermore, activated microglia have not been observed in patients with AD in regions such as the cingulate cortex, striatum, frontal, temporal, parietal, and occipital association cortical regions, all of which align with areas of heightened amyloid load, as shown by in vivo studies of neuroinflammation utilizing PET ligands that bind to activated microglia (DiSabato et al., 2016).

1.4 AD biomarkers

The term "biomarker" refers to an objectively quantifiable indicator of a biological process, whether it is within the normal range or indicative of a pathological condition, or even a response to a pharmaceutical therapy (Lewczuk et al., 2018). This biomarker can be measured using

physiological, biochemical, or anatomical parameters. The use of biomarkers as a diagnostic tool may provide some advantages, like the capacity to detect pathology at an earlier stage with increased accuracy, anticipate the progression of diseases, gain deeper understanding of pathogenesis, choose patients for treatment trials who display signs of disease pathologies, and use biomarkers as surrogate endpoints in clinical trials. Amongst the most widely recognized indicators for Alzheimer's disease are imaging biomarkers and biochemical cerebrospinal fluid (CSF) biomarkers.

1.4.1 Molecular biomarkers

Blood and cerebrospinal fluid have served as the primary sources of biochemical biomarkers for research on Alzheimer's disease. In the search of identifying biomarkers for AD in biofluids, researchers have identified three distinct waves. The initial wave focused on discovering and validating key biomarkers for AD in cerebrospinal fluid, namely $A\beta_{42}$ and total tau (T-tau). Additionally, the specific CSF profile associated with AD, characterized by high levels of T-tau and phosphorylated tau (P-tau), and low levels of $A\beta_{42}$, was credited to P-tau. In the second wave of AI detection tool research, the search for CSF biomarkers expanded to include proteins such as YKL-40, which mimics chitinase-3-like injury to neurons, neurofilament light (NFL), an indicator of neuroinflammation, and fatty acid-binding protein 3 (FABP3), a marker for neuronal damage (Lewczuk et al., 2018).

CSF, a bodily fluid, has an intracranial volume ranging from 140 to 270 mL. About 25% of this volume fills the brain's ventricles, whereas the rest of the volume envelops the brain and spinal cord. The choroid plexus, located in the ventricles, serves as the primary site of CSF production. From there, CSF diffuses into the spinal cord, subarachnoid space, and basal cisterns. CSF turnover is significant, with an average replenishment rate of four times per day and a flow rate of approximately 0.4 mL/min (Wang-Dietrich et al., 2013). CSF does not play any role in removing metabolic waste products generated by neurons and glial cells. Additionally, it facilitates the movement of physiologically active substances throughout the brain.

1.4.2 Blood Biomarkers

Previous studies show that many researches have been done to investigate blood-based biomarkers related to AD. In primary care assessments and regular sample collection for longitudinal

evaluations, blood is a more readily available biofluid than CSF, thus making blood biomarkers the preferable option. Unfortunately, the discovery of blood biomarkers for AD has proven to be challenging for various reasons. One such reason is that biomarkers with very low concentrations can permeate the blood-brain barrier and become further diluted in the blood, resulting in even lower concentrations (Wojsiat et al., 2017). Also, the expression of biomarkers in peripheral tissues may complicate the identification of the biomarker's contribution to the central nervous system.

Despite these challenges, a small number of intriguing blood-based biomarkers have been identified, including NFL, which exhibits a strong correlation with CSF NFL. As for tau biomarkers and Aβ proteins, the situation has been less clear. However, recent advancements in ultrasensitive tests have led to the evolution of this field, and new research on tau and amyloid biomarkers in blood has also yielded promising results. The future development of blood-based biomarkers is likely to be driven by methods such as microRNA, neuron-enriched exosome preparations, and ultrasensitive measuring techniques.

1.5 APP Mutations as causative agent of familial AD

The manifestation of familial Alzheimer's disease (FAD) as a result of APP mutations tends to concentrate primarily at specific locations where the enzymes β-secretase and γ-secretase process proteins. This process ultimately leads to the release of Aβ peptides into the luminal/extracellular compartment. In order to investigate the KM670/671NL Swedish APP mutation, researchers examined skin fibroblasts from carriers of the mutation and transfected Swedish APP cells (Suzuki et al., 1994). The findings consistently indicated an increase in the overall release of Aβ. This discovery marked a significant breakthrough. Following the cloning of the enzyme, it was revealed that this particular mutant, whose mechanism is well understood, enhances the APP sequence as a substrate for BACE1. This heightened affinity for the substrate results in an elevated generation of Aβ, which impacts the cellular compartment where the cleavage occurs. In contrast to wild-type (WT) APP, Swedish APP may already undergo processing in the trans-Golgi network compartment, as indicated by data from non-neuronal cell lines. On the other hand, WT APP must be transported to the cell surface and recycled into early endosomes through BACE1 processing (Hampel et al., 2021).

These distinct characteristics of Swedish APP carry therapeutic implications. Given that all BACE1 inhibitors currently under clinical development target the active site, it can be inferred that

they compete with the substrate. However, compound affinities are diminished in Swedish APP-expressing systems, which has implications for their pharmacological effectiveness. Consequently, BACE1 inhibitor medications may have limited efficacy in inhibiting BACE1 among carriers of the Swedish APP mutation. Furthermore, it is improbable that antibodies blocking BACE1 would reach the early intracellular regions where Swedish APP is cleaved in a clinical setting. Recent research has shown that BACE1 antibodies were unable to inhibit the enzyme in a Swedish APP transgenic mice model, unlike in WT animals, thus providing supporting evidence for this notion (Zhou et al., 2023). The remaining FAD mutations typically occur away from the region where γ-secretase cleaves. Mechanistically, the majority of these mutations cause an elevation in the $A\beta_{42}/A\beta_{40}$ ratio, with the V717 FAD mutants presenting the strongest evidence.

This finding significantly supports the causal role of the longer $A\beta_{42}$ peptide, which appears to be necessary for the development of senile plaques in animal models. However, the discovery of ε-cleavage, which leads to the release of the APP intracellular domain (AICD), has raised the possibility that abnormal APP/AICD signaling could provide an alternative explanation for how AD is induced by APP FAD mutations. The ε-cleavage occurs near the cytosolic face of the membrane and is analogous to the S3 cleavage of the Notch receptor. Additionally, it is mediated by γ-secretase, releasing an intracellular domain that can recruit auxiliary proteins and potentially alter gene expression in the nucleus (Giaccone et al., 2010).

The unresolved question that persists pertains to the precise mechanisms through which these FAD mutations stimulate the increase in the ratio of $A\beta_{42}$ to $A\beta_{40}$. One potential solution to this predicament may lie within the operational method of γ-secretase and its process of dividing substrates. The existence of various types of Aβ peptides, including $A\beta_{33, 34, 37, 38, 39, 40, 42,}$ and $_{43}$, has been observed in both cellular supernatants and cellular lysates as a result of γ-Secretase cleavage taking place at multiple sites within the APP. Recent evidence suggests that this cleavage process occurs in a sequential manner, starting at the ε-cleavage site, with subsequent synthesis of tripeptides reflecting the periodicity of the α-helix. This process advances from the ε-cleavage site to the γ-cleavage sites (Turner et al., 2003).

According to this hypothesis, the initiation sites for $A\beta_{42}$ and $A\beta_{40}$ would not be located at positions 48 (APP T719) and 49 (APP L720), respectively, within the Aβ domain. The increase in the ratio of $A\beta_{42}$ to $A\beta_{40}$ would occur if there is reduced efficiency in initiating the $A\beta_{40}$ lineage at L720, or increased efficiency in initiating the $A\beta_{42}$ lineage of peptides at T719. In this context, it is crucial

that the region spanning residues T714 through V717 contains essential structural elements that regulate the binding of the enzyme and the orientation of the enzyme for the initiation of a lineage. From a mechanistic perspective, these mutations could be seen as variants that resemble partial loss-of-function.

1.6 Alzheimer's disease pathogenesis:

According to Van Der Flier and Scheltens, the extracellular senile plaques of Aβ can exhibit the accumulation of various morphological configurations, encompassing compact, classic, disseminated, and neurotic plaques in the brain. In the presence of the proteolytic enzymes γ-secretase and β-secretase, the transmembrane amyloid precursor protein (APP) functions as a precursor for the generation of Aβ deposits (Breijyeh & Karaman, 2020a). These proteolytic enzymes cleave APP to generate a range of amino acid fragments, including $A\beta_{40}$ and $A\beta_{42}$ (Yiannopoulou & Papageorgiou, 2020). There exist multiple forms of Aβ monomers, one of which manifests as amyloid fibrils that are both large and soluble, consequently triggering the deposition and accumulation of amyloid plaques in the brain. These plaques are soluble in nature and have the potential to diffuse extensively across the brain (Armstrong, 2019).

According to Cras et al. (1991), the accumulation of Aβ leads to neurotoxicity, dysfunction in neurons, and activation of astrocytes and microglia, all of which contribute to visible shrinkage of the brain, loss of synapses, and an accelerated decline in cognitive function (Tabaton & Piccini, 2005). NFTs, which are abnormal clusters of tau protein that have undergone hyperphosphorylation, disrupting the microtubules that make up the cytoskeleton (Perl, 2010). Under normal circumstances, tau binds to microtubules and stabilizes them, but in Alzheimer's disease, tau molecules detach from the microtubules and attach to other tau molecules, resulting in the formation of tangled structures within the neurons. The presence of NFTs impairs the neural transport mechanism, eventually compromising the connection between synapses (González et al., 2018).

1.7 Clinical features of AD and Diagnosis

1.8 Symptoms of AD

The initial signs of Alzheimer's disease (AD) consist of memory loss and recurring instances of forgetfulness, which ultimately progress to dementia as shown in Figure 1.2 (Metaxas &

Kempf, 2016). After the inability to recall and retrieve words, there is a subsequent development of anomia (Bäckman et al., 2004). Anomia is characterized by the difficulty in finding and articulating the appropriate words due to cognitive impairment within the brain region responsible for verbal expression and comprehension (Serrano-Pozo et al., 2011). Furthermore, AD patients encounter symptoms such as semantic impairment, challenges in problem-solving and concentration, as well as frequent feelings of disorientation. In addition to cognitive symptoms, neuropsychiatric manifestations linked to AD encompass psychosis, apathy, auditory or visual hallucinations, delusions, irritability, and sadness (Cummings et al., 1985). In the final stages of AD, the patient suffers from ataxia and eventually loses mobility completely (Li et al., 2014).

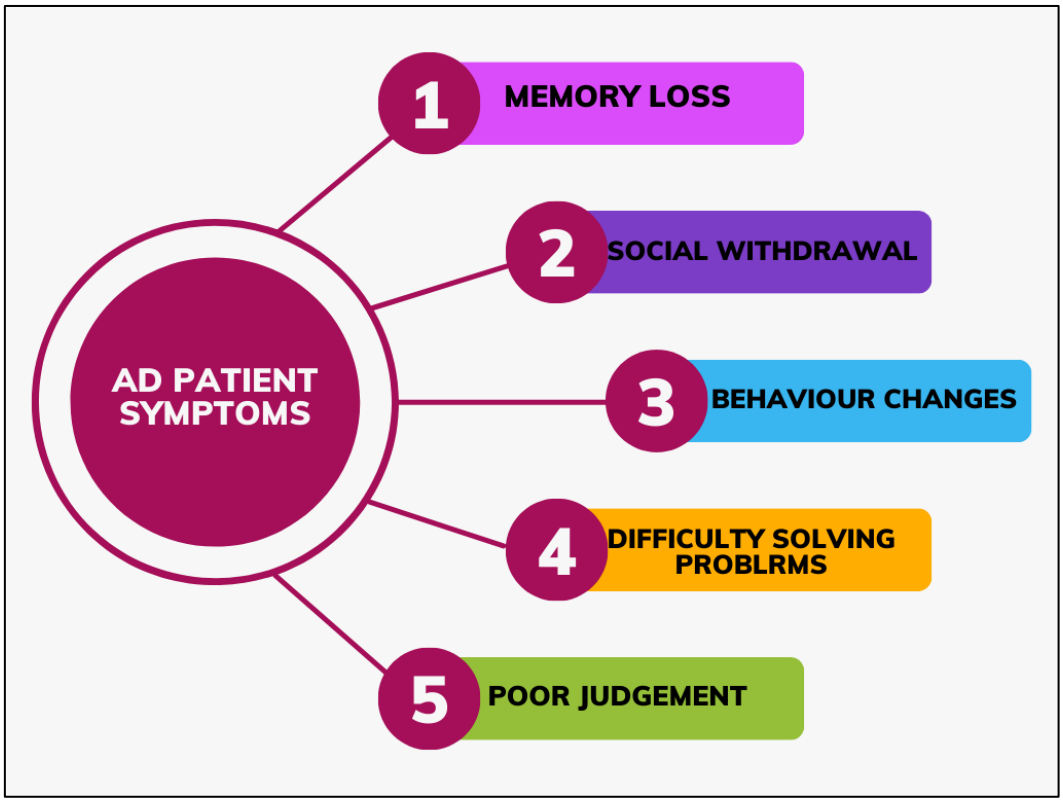


Figure 1.2 Symptoms of AD patient. The early signs and symptoms of AD include memory loss, social withdrawal, changes in personality and behavior, difficulty in problem solving, and unable to perform technical tasks.

1.9 Etiology of AD

The etiology and risk factors of Alzheimer's disease are believed to be numerous and complex. The significant risk factor for AD is age advancement, which is an irreversible condition. It

leads to various complications such as reduced brain volume, synaptic losses, and the accumulation of senile plaques, and NFTs in the cortical regions of brain (Bäckman et al., 2004). These age-related factors significantly contribute to the manifestation of AD) (González et al., 2018). Consequently, individuals who are older than 60 years of age are mostly affected this disease (Cummings et al., 1985). Environmental factors, including inadequate nutrition, exposure to harmful metabolites, and infections, are noteworthy examples of external risk factors (Guerreiro & Bras, 2015). Additionally, genetic factors play a pivotal role in the development of AD. Mutations in genes such as APP, presenilin-1 (PSEN1), presenilin-2 (PSEN-2), and Apolipoprotein E (APOE) are also the risk factors for AD. These risk factors increase the risk of developing AD by 20-40% (Wainaina et al., 2014).

1.10 Subtypes of AD

According to neuroimaging and neuropathology research, Alzheimer's disease is a multifaceted condition that has been categorized into various subgroups based on factors such as age at onset, cognitive decline, hereditary risk, and pathological changes (Whitmer et al., 2005). Approximately 95% of cases manifest after the age of 65 and are attributed to this disease. The mutations in APP gene is implicated in the onset of AD, as stated by (Ferreira et al., 2020). Recent investigations have demonstrated that the composition of $A\beta_{42}$ filaments are different between SAD and familial AD. The quantity of Type I filaments in the brains of individuals diagnosed with sporadic Affective Disorder (SAD) was found to be greater compared to those without the condition, as documented by Dorszewska et al. (2016). Merely 5% of familial cases of Alzheimer's disease (AD), a hereditary disorder, are annually identified, as reported by Yang et al. in 2022. Due to the relatively young age at which it typically manifests, this particular form of AD is often referred to as early onset AD (EOAD), with the majority of cases demonstrating autosomal dominant inheritance. Consequently, if one biological parent possesses the condition, there is a 50% likelihood of their offspring would have familial Alzheimer's disease (FAD) if they inherit the gene that is responsible for causing fAD as indicated by Wolfe in 2015. The occurrence of FAD can be ascribed to genetic mutations in various genes such as APP, PSEN1, PSEN-2, and BACE1 (Whitmer et al., 2005).

1.11 APP Function in Brain

Amyloid precursor protein APP is a transmembrane protein that plays important functions in the brain and nervous system such as it plays its role in synaptic plasticity and synaptogenesis.
The roles of APP are much more clearly understood, and many of the functions played by contact receptors and released substances on the tissue level instead of the cellular level. One transmembrane region, a tiny cytoplasmic tail, and a sizable extracellular domain make up the entirety of full-length APP, which makes up 90% of the protein mass present in the brain region. Because of this kind of structure, scientists have hypothesized that APP might serve as a receptor that shows interaction with ligands (Kang et al., 1987). While many cellular receptors investigated in vitro have well-established functions, ligands, and intracellular pathways, the same is not necessarily true of APP.

1.11.1 Relation of APP with glial cells

For instance, an early study that used a preserved neuronal cell line showed that adhesion is promoted by the APP to the substrate and other cells as glial cells of the brain (Kibbey et al., 1993). The glial cells of the brain, which direct future neurons to the proper places in the embryonic cortex, express APP at high levels showing that the attachment of neurons to glial cells, stimulated by APP, plays a significant role in brain development (Trapp & Hauer, 1994). According to previous studies, it is justified to classify APP as a contact receptor. Several high-specificity APP ligands have been found in the extracellular matrix of the neuron in the brain, although it is yet unknown how these interactions cause signal transduction. It was observed in various studies that was done both in-vivo and in-vitro that neurogenic activity in stationary, immature neurons promotes the adhesion action of APP in migratory cells of brain (Allinquant et al., 1995).

1.11.2 Molecular role of APP in axonal development

Recently, the molecular role of APP's contribution to axonal development has been clarified: regulatory proteins known as Fe6 and Mena are bound to the cytoplasmic tail of APP. This complex of Fe65, Mena, and APP becomes a ternary complex which is located at the dynamic adhesion sites in the growth cone that play a role in the migration of non-neuronal cells. Co-expression of APP and Fe65 significantly speed up migration in non-neuronal cells (Sabo et al., 2001). While research using cultured cells alone is unable to fully explain the temporal significance

of APP participation in brain development, in vivo investigations have provided some insight into the locations and times throughout brain development where APP is particularly required. The role of APP in making longer connections is supported by the fact that during the prenatal period, the APP levels are increased which indicates its participation in the development of the brain (Clarris et al., 1995).

Additionally, the role of APP in axon was proven in Drosophila, where a lack of the APP homolog results in a failure of arborization and, as a result, a decrease in the reproduction ability of the organism (Leyssen et al., 2005). The neuronal morphogenesis and the development of useful synapses, also depend on APP. In neurons of the hippocampus when cultured revealed that APP expression enhances glutamate responsiveness, and other research has shown electrophysiological properties of APP deficiency. On the other hand, in vivo research has focused on the significance of APP for the electrical and morphological maturation of certain synapses. APP appears to be essential for the maturation of particular neuronal subtypes (Tominaga-Yoshino et al., 2001).

1.12 Processing of Aβ precursor protein

Partial protein sequence data of Aβ was used in 1987 to discover the human APP gene to find the target cDNA. It was revealed through western blotting technique that on chromosome 21 (21q21.2-3), the gene of APP was located. The APP protein present inside the brain has a large extracellular domain and a condensed cytoplasmic portion (Levy-Lahad et al., 1995). Aβ is released from APP protein by two enzymes which cleave it from APP through the amyloidogenic pathway. Enzymes that cleave the Aβ from APP are called β-secretase and γ-secretase as shown in Figure 1.3. APP695 is the predominant splice form in neurons through cleavage. Two different proteolytic processes are used i.e. non-amyloidogenic and amyloidogenic to break the precursor proteins.

In the non-amyloidogenic processing route, an enzyme known as α-secretase cleaves APP inside the Aβ domain, which results in the accumulation of a large soluble ectodomain (sAPPα) and a C-terminal fragment (C83) with 83 residues. Several of the enzymes showing protease activity in the ADAM family exhibit α-secretase activity. The α-secretase cleaves C83 later, that results in the development of the P3 molecule and the amyloid precursor protein intracellular domain (AICD) (Roberts et al., 1994).

In the amyloidogenic process, enzyme called β-secretase or also known as BACE1, a membrane-tethered protease, cleaves APP at the N-terminus of the Aβ domain, producing a (sAPPβ) and a

99-residue, which is fragment of C- terminal known as C99. Then, PSEN presenilin, a catalytic component of the membrane-embedded complex known as γ-secretase, cuts and cleaves C99 to release Aβ peptides and AICD. The γ-secretase cleavage site is uninhibited, resulting in the production peptides of Amyloid beta such as fragment of 40 peptides known as $A\beta_{40}$ and $A\beta_{42}$ fragment peptide known as $A\beta_{42}$ which accumulated in the brain and lead to AD (Chow et al., 2010). $A\beta_{40}$ is the most prevalent species produced as a result of the amyloidogenic pathway cleavage under physiologically normal circumstances, with $A\beta_{42}$ accounting for just 10% of all Aβ. $A\beta_{42}$ is regarded as the harmful peptide, though, as it is more likely to form fibrils and encourages the creation of Aβ aggregates, which are the main contributors to neurotoxicity and dementia such as AD (Sherrington et al., 1995).

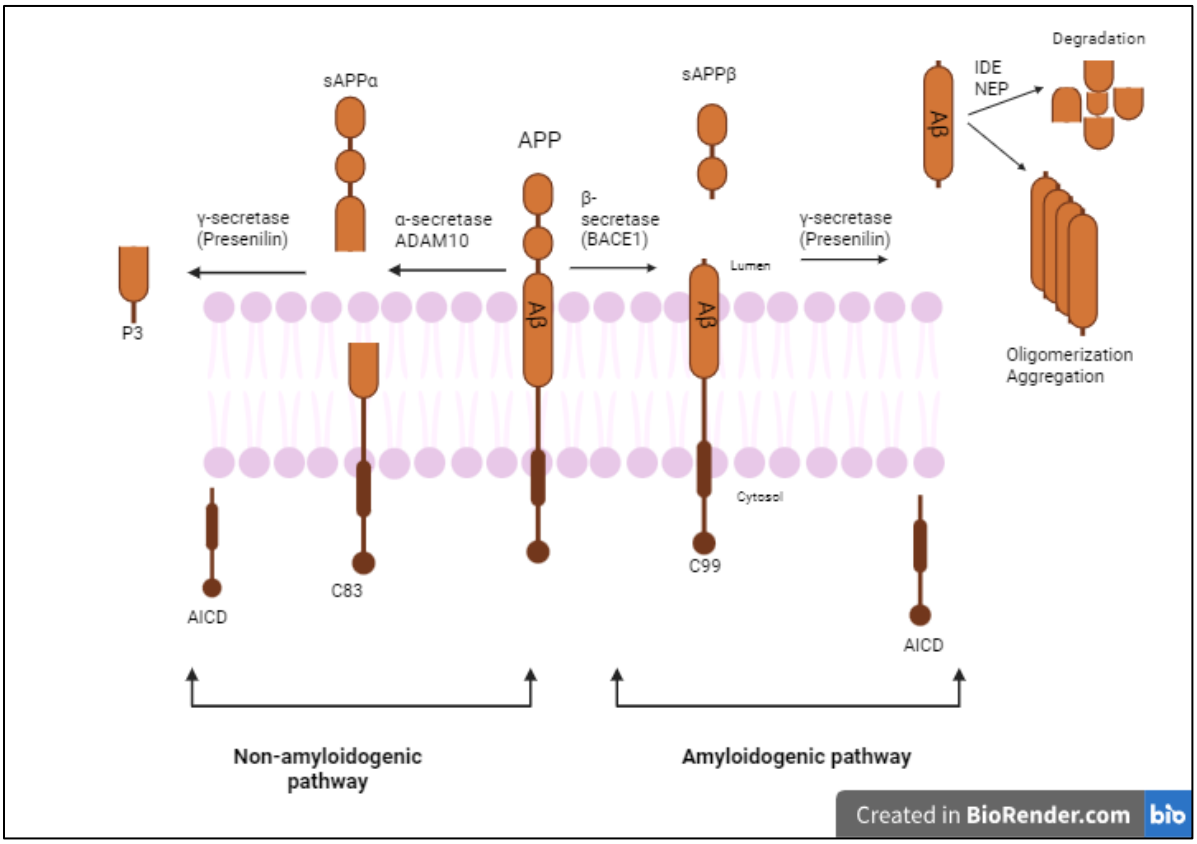


Figure 1.3: APP processing in AD. APP is processed in brain by two pathways i.e. amyloidogenic and non-amyloidogenic. In the non-amyloidogenic processing route, an enzyme known as α-secretase cleaves APP inside the Aβ domain, which results in the accumulation of a large soluble ectodomain (sAPPα) and a C-terminal fragment (C83) with 83 residues. In amyloidogenic pathway the transmemnbrane APP that is consist of Aβ domain is cleaved through β-secretase and further cleaved by γ-secretase. Aβ domain is released and either it is degraded by IDE (insulin degrading enzyme) or its oligomerization occurs in the brain that leads to progression of AD.

1.13 APP in Alzheimer Disease

Initially APP was identified due to its deposition of clumps such as Aβ in the brain regions of Alzheimer disease AD and dementia patients, but not by its morphoregulatory functions in brain of normal individuals. The deposition of clumps or plaques in dementia patients consists of Aβ peptides such as $A\beta_{40}$ and $A\beta_{42}$, a peptide that is cleaved from APP through proteases. The Aβ peptide is produced by a cleavage of APP that is quantitatively less significant than the cleavage by α-secretase. When present in low physiological concentrations, Aβ can perform normal neurotrophic and signaling actions in the brain of individuals. However, amyloid beta aggregates abundantly that build in vivo in the brains of AD patients are strong neurotoxins (Zhang et al., 2011).

1.13.1 Synaptic dysfunction in AD patients due to APP mutations

Although there are senile plaques in the brains of people who are older and, in particular, those with Alzheimer's disease, however the amount of soluble Aβ in the brain correlates more strongly with dementia severity than does the amount of Aβ aggregated in plaques. This significant neuropathological finding supports the hypothesis that soluble oligomers of Aβ may be the species causing synaptic dysfunction in AD patients as these species impair the synaptic plasticity, instead of fibrillar amyloid aggregates, which were first thought to be the major neurotoxic species (Greenfield et al., 1999). In comparison to normal brains, AD brains consist of larger amounts of soluble Aβ oligomers, and these oligomers are specifically bound to a subset of dendritic spines. The amyloidogenic pathway in which α -secretase plays role, which then cleaves APP at one of many possible peptide bonds in its transmembrane region producing the C-terminus of the Aβ-peptide, in a process known as proteolytic synthesis. Aspartic protease was discovered to be component of β-Secretase (Turner et al., 2003).

1.13.2 APP interaction with its ligands relevant to AD

On the other hand, the activity of enzyme called β-secretase is linked to a high molecular weight protein complex with four critical components that cleave numerous substrates in addition to APP, Presenilin 1 or 2, and Nicastrin (Edbauer et al., 2003). Studies in Drosophila suggested the existence of other, auxiliary variables that might control the activity of α-secretase and hence perhaps affect the production of Aβ. CD147 and TMP21 are two of these auxiliary factors that

have lately come to light in these processes. α-Secretase cleaves APP after it has been initially broken down by other two enzyme called β - or γ -secretase because it does not act on entire APP but only on fragments lacking the extracellular domain (Loewer et al., 2004). After the cleavage of APP, Aβ domain is released and degraded by IDE enzyme. After their degradation, Aβ fragments start accumulating in brain which leads to the progression of AD.

1.14 Missense mutations in APP

The APP gene consists of total 18 exons. Alternative splicing of these exons of APP leads to the formation of several proteins that range from 695 to 770 amino acids. Aβ peptide is encoded when the APP exon 16 and 17 is spliced (Giaccone et al., 2010). The majority of APP missense variations are linked to Alzheimer Disease AD in autosomal-dominant manner that mostly start appear at the age of 60 or above in humans. Recently, it was shown that a mutation in the APP codon 673 was linked to AD. So far up till now, scientists have reported more than 26 missense polymorphisms or mutations that are present in the gene of APP. Aβ sequence of the APP gene contains most of these missense mutations, while some occurs at the side region of it (Goate et al., 1991).

1.14.1 Mutant Aβ production due to APP mutations associated with AD

The toxic peptide, Aβ peptide, is cleaved by two enzymes known as β-secretase and γ-secretase from the APP protein. A study was done on Swedish pedigree that revealed that Aβ mutation are present at 670 and 671 codon of exon 16 of APP. As the result of these mutations, at codon 670 the amino acid of APP protein replaces lysine with asparagine, and at 671 position the amino acid transformed to leucine from methionine. Due to these changes in the amino acid sequences, levels of $A\beta_{40}$ and $A\beta_{42}$ are amplified which causing the large production of Aβ and causes neurotoxicity (Levy et al., 1990). Due to mutations in the Aβ Domain and how they affect Aβ formation within the Aβ sequence, ten harmful polymorphisms have been identified. These polymorphisms are D678V, E682K, A692G, D694N, L705V, and A713T that are present in the APP sequence. These mutations appeared in families of the ethic region of Leuven, Flemish, Dutch, Artic, and Italian pedigrees. When the mutations were studied in vitro, it was found out that peptides of Aβ that were mutant aggravating faster and quickly in the patients' brains as compared to wild type peptides of Aβ. Large amount of Aβ aggregation into amyloid fibrils is facilitated by the decreased cleavage of α-secretase and secreted Aβ species' high hydrophobicity.

1.14.2 Consequences of APP mutations

Mutations within the Aβ domain can have complex consequences on APP processing through various pathways. For instance, E693G mutations in the APP sequence promotes the development of Aβ protofibrils (Wisniewski et al., 1991). Hemorrhagic stroke is also linked to mutations that eventually cause the enhanced levels of $A\beta_{40}$ species. The Impact of C-Terminal Aβ-Domain Mutations on Aβ-Formation the leading mutation that was linked to fAD was V717l in APP exon 17 in a family of British descent, with a mid-50s onset age (Goate et al., 1991). Since this study, several other families have been found that contained this mutation as well as several others (V717G, V717L, and V717F) at the same amino acid.

The early onset polymorphisms are present in the transmembrane domain of APP at the site where β-secretase cleave at codon 714, 715, 716 and 717 of APP. Other mutations at codon 723 and 724 where ε-secretase performed cleavage and mutations occurred. In the C-terminal Aβ domain, fourteen mutations are identified, at codons 714 to 724 of APP gene sequence. These mutations affect how each β-secretase functions, leading to abnormal APP processing. In fact, these APP mutations close to the Aβ sequence's C-terminus boosting the generation of longer Aβ peptides, particularly those that finish at residue 42 ($A\beta_{42}$), which is more likely to aggregating quickly (Hardy, 1997).

1.14.3 Autosomal dominant APP mutation

Autosomal-Dominant Mutation Profile in the Neuropathological Profile Autosomal-dominant APP mutations that surround the Aβ sequence and near the cleavage sites done by two important enzymes β and α-secretase. This eventually end in the resulting of either increased levels of overall Aβ production or a particular neurotoxic peptide, which are hydrophobic in nature and hence more likely to trigger fibrillogenic pathway (Suzuki et al., 1994). On the other hand, aggregation and fibrillation is occurring faster due to the mutations that are present in the amino acid sequences of Aβ domain. It is the hallmark of Alzheimer Disease and dementia that production of $A\beta_{42}$ peptides, senile plagues and other neurotoxic fibrils are increased. Thus mutations in APP gene cause the change in amino acid sequences at various region of Aβ domain and leading to Aβ deposition the brain leading to AD and dementia (Suzuki et al., 1994). Following table 1 consists of number of mutations that are present in APP gene.

Table 1.1 Mutations of APP. Missense mutations of APP with their respective pathogenesis.

Sr #	Mutation	Change in DNA	Pathogenicity
1.	A201V	Substitution	Benign AD
2.	A235V	Substitution	Benign AD
3.	D244G	Substitution	AD: not classified
4.	D243N	Substitution	Benign AD
5.	E296K	Substitution	AD: not classified
6.	E246K	Substitution	Likely Benign AD
7.	P299L	Substitution	AD: not classified
8.	D332G	Substitution	AD: not classified
9.	V340M	Substitution	Uncertain significant AD
10.	G342S	Substitution	Uncertain significant AD
11.	E380K	Substitution	Uncertain significant AD
12.	E665D	Substitution	Benign AD
13.	V669L	Substitution	AD: not classified
14.	A673T	Substitution	AD: protective
15.	D678H	Substitution	Pathogenic
16.	E682K	Substitution	AD: not classified
17.	K687Q	Substitution	Pathogenic
18.	L688V	Substitution	Pathogenic
19.	A692G	Substitution	Pathogenic
20.	E693K	Substitution	Pathogenic
21.	L705V	Substitution	Pathogenic
22.	V710G	Substitution	Pathogenic
23.	L714A	Substitution	Pathogenic
24.	V715M	Substitution	Pathogenic
25.	I716F	Substitution	Pathogenic
26.	V717L	Substitution	Pathogenic
27.	I718L	Substitution	Pathogenic
28.	L720S	Substitution	Pathogenic

1.15 Aim of study

AD cases are rising significantly in Pakistan. It has reported nearly 2 lac cases of dementia up till now. As dementia progress in later age, the number of people age above 65 will increase from 8 to 27 million by 2050. This indicates that chance of dementia cases will also increase in Pakistan. Pakistan is a lower middle country where not much of research has been done on dementia and its genetic causes. So, the aim of current study is to do the SNP analysis of prevalence of 718 (I>L) APP mutations associated with AD in Pakistani population. We used the computational tools to evaluate the structural variations of wild-type APP and mutated APP 718 (I>L) with ligands (ADAM10, BACE1, and Nicastrin). Afterwards, it was proceeded by SNP analysis of APP 718 (I>L) through genotyping. No sort of this study has been done before.

In our current study we did the:

- Evaluation of structural variations associated with 718 (I>L), APP polymorphisms.
- SNP analysis of prevalence of APP-associated 718 (I>L) polymorphism.

CHAPTER 2: MATERIALS AND METHODOLOGY

2.1 *In silico* Tools

In silico tools are used to understand the protein -protein or protein ligand interactions. These tools are helpful in drug discovery process. These computational tools are used for investigating the protein homology, data mining, and identification of compounds. Following shown in Table 3.1 *in silico-based* Software and online tools have been used in this study for the molecule interaction and their docking.

Table 2.1 Software used in this study. This table shows the software that are used in our study for the computational analysis.

Software	Application	Reference
Alphafold	Tool used to download the protein structures.	(Jumper et al., 2021)
Protein Data Bank	Tool to download ligands structures.	(Berman, 2000)
HDock	To perform Molecular docking pf molecules.	(Yan et al., 2017)
PyMol	To create mutagenesis in protein.	(Rigsby & Parker, 2016)

2.1.1 Selection of ligands and proteins

APP serves as a receptor for many ligands and interact with them. In our study, we have taken APP protein wild type sequence uniprot ID P05067 from Alphafold in PDB format. The protein was further cleaned in Discovery Studio software. During cleaning all water molecules and Hetatoms were removed, and active sites were selected as shown in Figure 2.1 This protein structure was saved as .pdb format for further analysis. This molecule has four chains A, B, C and D shown in blue color in Figure 2.1. The function of this molecule is to perform peptidase activity, metal ion binding and metalloprotease activity.

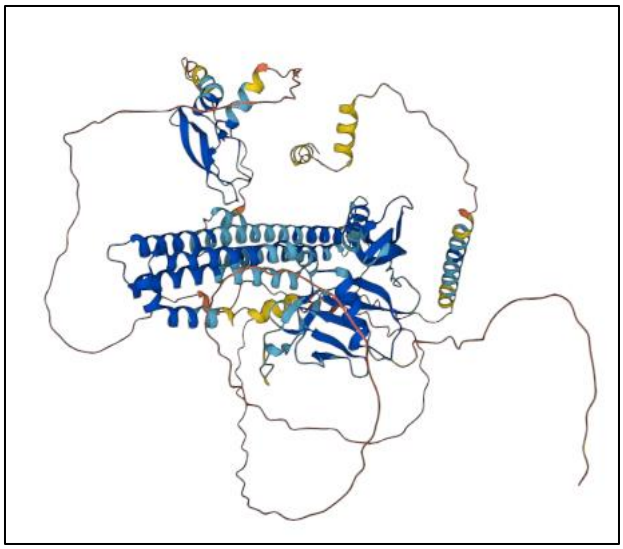


Figure 2.1 APP 3D Protein Structure retrieve from Alpha Fold. This figure represents the 3D protein structure of APP structure.

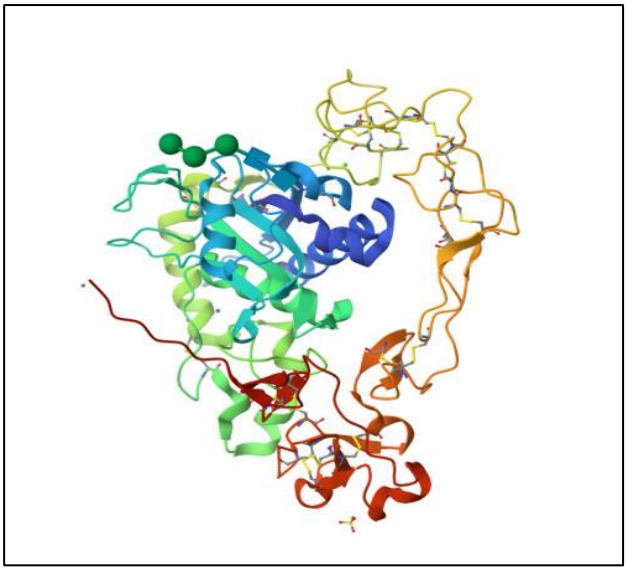


Figure 2.2 ADAM10 protein structure retrieved from PDB. This is the extracellular domain of disintegrin and metalloproteinase domain containing protein 10 (ADAM10) with sequence length of 449.

In this study three ligands were selected i.e., BACE1, ADAM10, and Nicastrin shown in Figures. The protein structures of ADAM10 were retrieved from PDB software. While protein structures of BACE1 and Nicastrin were downloaded from alpha fold in .pdb format files. The ligands selected in our study are shown in figures below. In Figure 2.3, the 3D structure of gamma secretase is shown. The protein was further cleaned in Discovery Studio software. During cleaning all water molecules and Hetatoms were removed, and active sites were selected as shown in Figure 2.3. This protein structure was saved as .pdb format for further analysis. In Figure 2.4, the 3D

structure of BACE1 is shown. This structure is downloaded from Alpha fold and saved in.pdb format. This structure was also cleaned in discovery studio and its hetatoms were and water molecules were removed before the further analysis on Hdock. After cleaning, the structure was uploaded on Hdock for its docking with macromolecule i.e. APP.

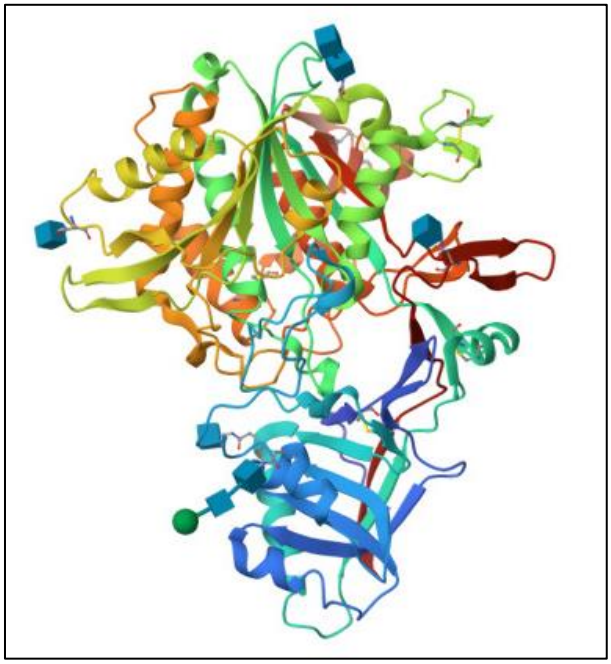


Figure 2.3 3D structure of gamma secretase (Nicastrin). This 3D structure shows the domains of Nicastrin. This was used as one of ligands in our study.

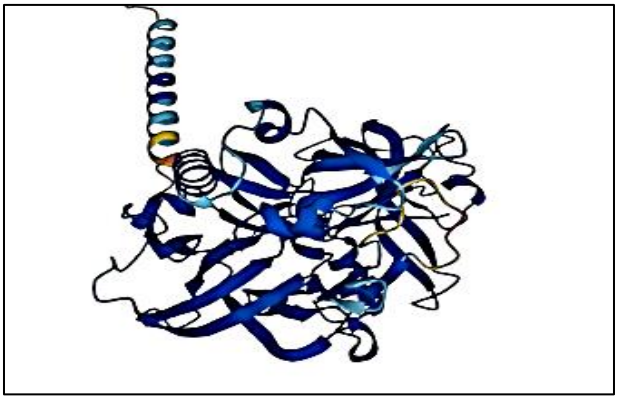


Figure 2.4 3D structure of beta secretase BACE1. This 3D structure shows the domains of BACE1. This was used as one of ligands in our study.

2.1.2 Mutagenesis

Using Pymol software mutagenesis was performed to create mutated APP protein. In our study, mutations in APP protein sequences at three different positions were created. The first mutation

that was created was at 718 codons in which isoleucine was replaced with leucine. In the second mutation leucine was substituted with serine at 720 codon of APP protein sequence. In 3rd mutated APP protein valine was replaced with glycine at its 710 codons. After creating mutations, these proteins were further used for molecular docking with ligands.

2.1.3 Molecular Docking

Molecular docking of Wild-type APP type and mutated APP at 710, 718 and 720 codons were performed through using HDock server. In Hdock our protein file in .PDB format was uploaded. Hdock performs the protein-protein docking to predict the docking score of two molecules. After docking of molecules is done, the docking score is generated. The more negative the score is; the stronger model is interacted.

2.1.4 2D visualization

After the docking has been done and docking score is generated, then these complexes are visualized to see the interaction. In our study we used Ligplot+ for 2D visualization of our complexes.

2.2 Genotyping

2.2.1 Blood Sample Collection

In total, 50 blood samples of the general population were collected from the Islamabad Diagnostic Center with informed consent. The blood samples were collected in EDTA tubes and were stored in the refrigerator at -20ºC.

2.2.2 DNA extraction

Commercially available DNA Extraction kit was used in the extraction of genomic DNA. Each kit is designed to extract 50 DNA samples; hence one kit was used for the extraction of 50 DNA samples. In the initial phase, a total of 200 µl of blood and 600 µl of lysis buffer was added into the centrifuge tube to initiate the lysis of red blood cells. Afterwards, this solution was centrifuged at 12000 rpm, resulting in the formation of a pellet. The supernatant was then discarded. Following the removal of the supernatant, 200 µl solution A added to the pellet and thoroughly mixed through vortex. 20 µl of RNase was then added to the Eppendorf tube and placed within the incubator for

approximately 10 minutes. Following this, Proteinase K was added into the tube, and allowed to incubate at a temperature of 60^{o}C for a duration of 45 minutes. Then solution B is added and centrifuged for 2 minutes at 12000 rpm. Discard the supernatant and repeat this three times. Transfer the solution into spin column and add elution buffer into this and centrifuge again. Store the DNA at -20^{o}C. The quantity and quality of the DNA was determined by Colibri NanoDrop (Titertek-Berthold, Germany).

2.2.3 Primers

To facilitate DNA extension, two distinct sets of oligo-deoxyribonucleotide primers were chosen based on previously published research findings shown in Table 3.2. The first primer set consisted of a 20-nucleotide (nt) forward and reverse primer pair, denoted as F1 and R1, respectively. These primers were designed to specifically target and align with the DNA sequence of the healthy APP gene. In order to confirm the site of the APP mutation, a supplementary set of primers was used. The predicted portions of the APP gene, measuring 305 bp and 510 bp, were identified using 1% agarose gel electrophoresis. Multiple cycles of template denaturation, primer annealing, and extension were performed using a polymerase chain reaction (PCR) equipment.

Table 2.2 Table of Primers. List of primers used in this study with their product length and temperature.

Name	Primer sequence	Length bp	Temp
APP Healthy (Forward) F1	GACCAACCAGTTGGGCAGAG	20	54
APP Healthy (Reverse) R1	CATGGAAGCACACTGATTCG	20	53
APP Mutation (Forward) F2	CAAATGTTCCACCTGTCAAAGGG	23	56
APP Mutation (Reverse) R2	TCAAGTTTACCTACCTCCACCACAC	25	57

2.2.4 Reaction Mixture

Reaction mixture for PCR product was made using commercially available master mix. A total of 25µl of reaction mixture wasmade (Table 3.3).

Table 2.3 List of PCR ingredients. List of all the ingredients along with their quantities used to make 25µl PCR mix.

	Ingredients	**Quantity (ul)**
1)	DNA template	2.00
2)	Nuclease free water	8.5
3)	Forward primer	1.00
4)	Reverse primer	1.00
5)	PCR master mix	12.5
	Total	**=25 µl**

2.2.5 Reaction Conditions

A total of 12.5 µl of PCR master mix 8.5µl of Nuclease free water, 1µl of forward primer, 1µl of reverse primer and 2µl of DNAtemplate were added in the PCR tube to make 25 µl of total volume. The PCR cycling conditions included initial denaturation at 94°C for 3 minutes followed by 35 cycles at 94°C for 30 seconds. For optimization purposes, gradient PCR was set at different annealingtemperatures of 52°C, 53°C, 54°C, 55°C and 56°C at 35 seconds (the temperature of the primers was calculated by adding the temperatures of forward and reverse primers and thendividing the answer by 2 to take average). Gradient temperatures were followed by extension step at 72 °C for 45 seconds and a final extension at 72°C for 7 minutes. The product obtained after the PCR was examined under gel electrophoresis to see the bands ofthe respective alleles.

2.2.6 Agarose Gel Electrophoresis

Gel electrophoresis was carried out to confirm the presence of DNA and to determine whether annealing has taken place at desired temperatures or not by checking the locationof the bands against the DNA ladder. DNA ladder of 100-1500 base pairs was used.
For DNA, 1% of agarose gel was made. 50X TBE buffer (Solarbio, Catalogue# T1060, China) was used. For PCR product, 2% of agarose gel was made. 10X TAE buffer, was used.

2.2.7 Gel analysis

The gels were visualized using benchtop 2UV Trans-illuminator (catalog no.95-0449-02, Cambridge, UK).

CHAPTER 3: RESULTS

3.1 *In silico* results

Molecular Docking was performed on HDock software. The PDB files of both proteins (macromolecule, ligands) were uploaded on the Hdock server. It started the docking of macromolecule APP and mutated APP with ligands that were BACE1, Nicastrin and ADAM10. Hdock server predicted the following docking score of our desired protein and ligands. It can be seen in Table 4.1 that docking score of Wild-type APP with ligands and mutated APP with ligands have differences. Wild-type APP with BACE1 gave -175.45 score, but due to mutation at 718 codons the docking was changed to -176.26. It occurred because the mutated APP at codon 718 bounded with Serine residue. Changes in the binding of protein with serine residue causes the polymerization of protein which eventually causes the aggregation of protein and hence neurotoxicity.

Similarly, when we compared the wild APP and 718 mutated APP with Nicastrin it can be seen that docking score has been changed from -227.91 to -238.09 that is due to presence of extra hydrogen bonding. Hydrogen bonding interaction causes the formation of protein folding and denaturing. Hence the protein binds with different targets with different intensity that eventually ends up causing the fibrillation of protein. It can also be seen in Table 4.1 when we compare the Wild-type APP and 710 mutated APP with ligands BACE1, ADAM10 and Nicastrin. The docking score has changed from -175.45 to -158.4, -215.28 to -206.36 and -227.91 to -244.6 respectively. This is due to the extra interaction of mutated 710 APP with ligands that cause aberrant cleavage and denaturing of protein. Table 5 shows docking score from HDock. Table 4.1 shows the docking score of wild-type APP and mutated APP I718L, L720G, V710G with its ligands retrieved from Hdock. From Table 4.1 it is derived that APP I718L has enhanced binding with Nicastrin as compared to wild type APP binding with Nicastrin.

Table 3.1 Docking score of lignds with wild type and mutated APP. Table shows the docking score of wild-type APP and mutated APP I718L, L720G, V710G with its ligands retrieved from Hdock.

	BACE1	**ADAM10**	**Nicastrin**
Wild-type APP	-175.45	-215.28	-227.91
I718L	-176.26	-215.28	-238.09
L720G	-176.26	-215.28	-247.25
V710G	-158.4	-206.36	-244.6

3.1.1 Ligplot 2D visualization results

By using Ligplot+ software, the 2D interaction of proteins was seen. In the following Figure 3.1 it was shown comparison of Wild-type APP and 718 mutated APP with BACE1. From the Figure 3.1 it was observed that 718 mutated APP has an interaction with serine residue highlighted with red circle. Also, APP 718 has interacted with BACE1 at Tyrosine residue as well. The interaction with serine residue causes the folding and denaturing of protein which was seen in APP 718 mutation. A comparison of Wild-type APP and 718 mutated APP with Nicastrin was made after 2D visualization on Ligplot+ software. After the comparison was made, it was observed in Figure 8 that 718 APP has interacted with Tyrosine 565 residue of Nicastrin which was not present in Wild APP with Nicastrin. It was also observed that 718 APP has an extra Hydrogen bonding with Nicastrin. These changes such as Tyrosine residue change, and extra hydrogen bonding cause the polymerization of protein which aggregates in the brain and cause neurotoxicity. This also caused the difference of docking scores of both APP with Nicastrin that was -227.91 and with 718 APP with Nicastrin it was -244.60.

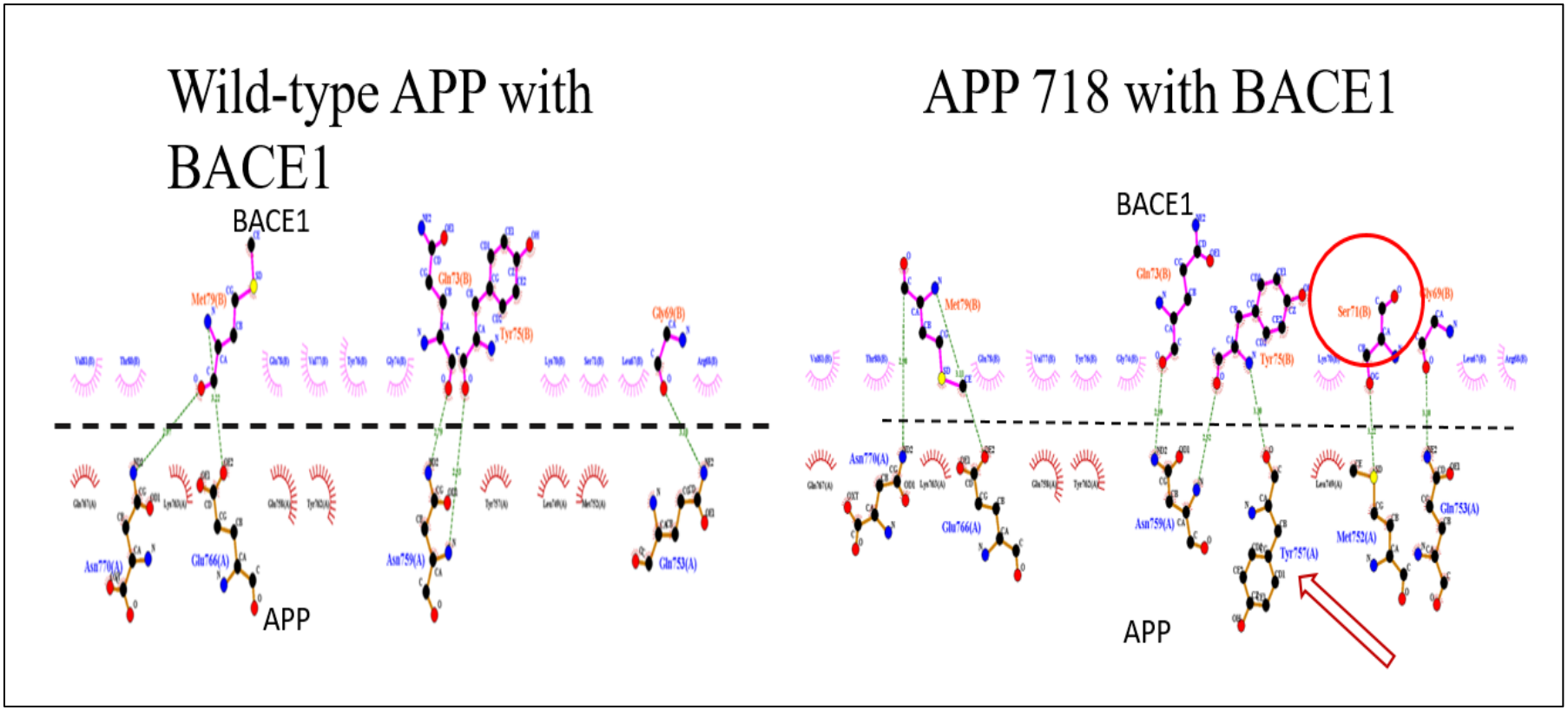


Figure 3.1 Wild-type APP and 718 mutated APP with BACE1. In this Figure wild-type APP and mutated I718L APP interaction with BACE1 is shown.

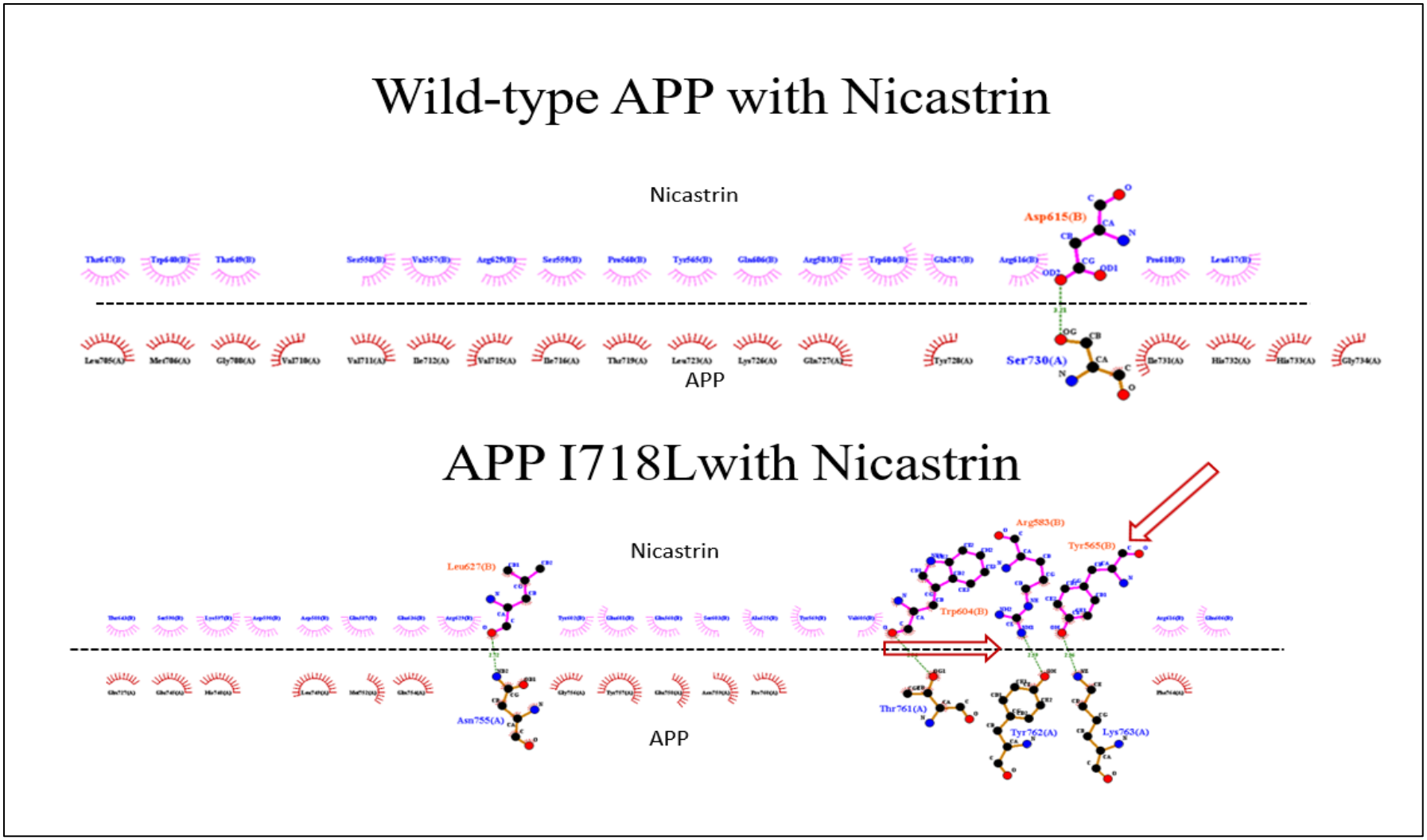


Figure 3.2 Wild-type APP and mutated 718 APP with Nicastrin. In this Figure wild-type APP and mutated I718L APP interaction with Nicastrin is shown.

When the Wild-type APP and 718 APP docked with ADAM10, it was observed through 2D interaction that no change was found between both, therefore the docking score also remained same i.e., -215.28 which can be seen in Figure 3.3.

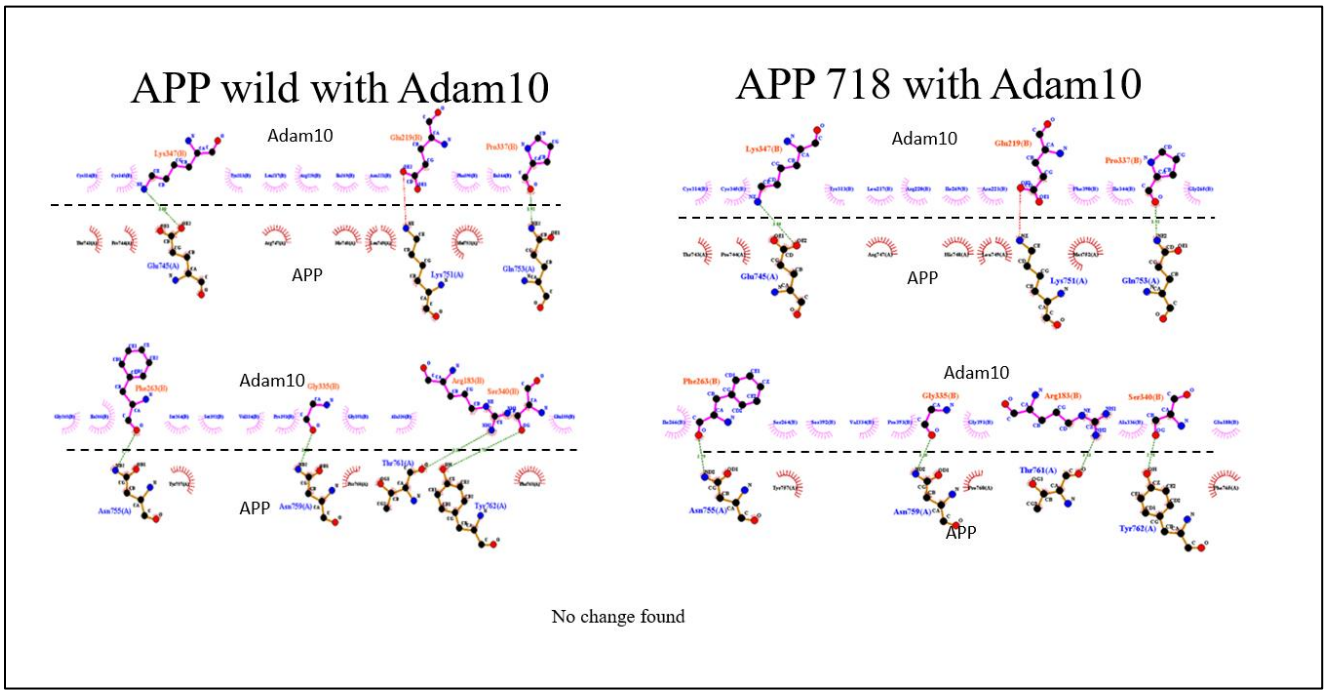


Figure 3.3 Wild-type APP and mutated 718 APP with ADAM10. In this Figure wild-type APP and mutated I718L APP interaction with ADAM10 is shown.

A comparison of Wild-type APP and 20 mutated APP with BACE1 was made after 2D visualization on ligplot+ software. After the comparison was made, it was observed in Figure 3.4 that no change was found, therefore the docking score of both also remained same.

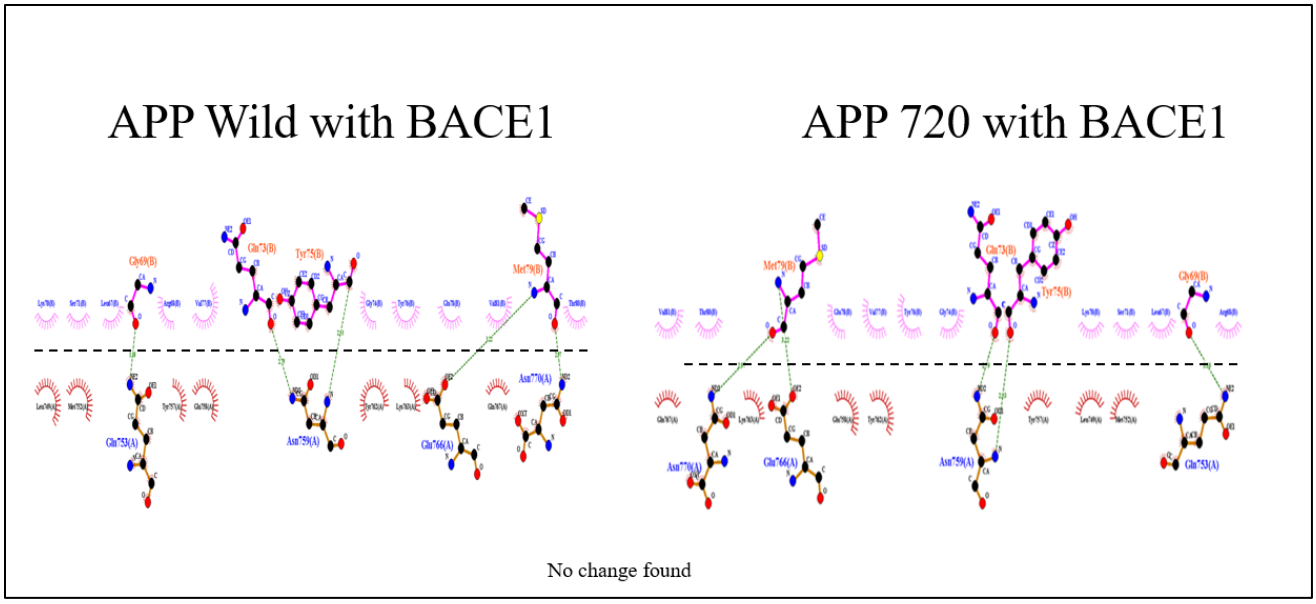


Figure 3.4 Wild-type APP and mutated 720 with BACE1. In this Figure wild-type APP and mutated L720G APP interaction with BACE1 is shown.

The comparative analysis was also done of wild APP with mutated APP at 720 codon shown in Figure 3.5. No changes were seen between Wild-type APP and mutated 720 APP with ADAM10 in Ligplot+ images as shown in Figure 3.5. Similarly docking score also did not change and remained same for both. The docking score of Wild-type APP and 720 mutated APP with ADAM10 was same i.e., -215.28.

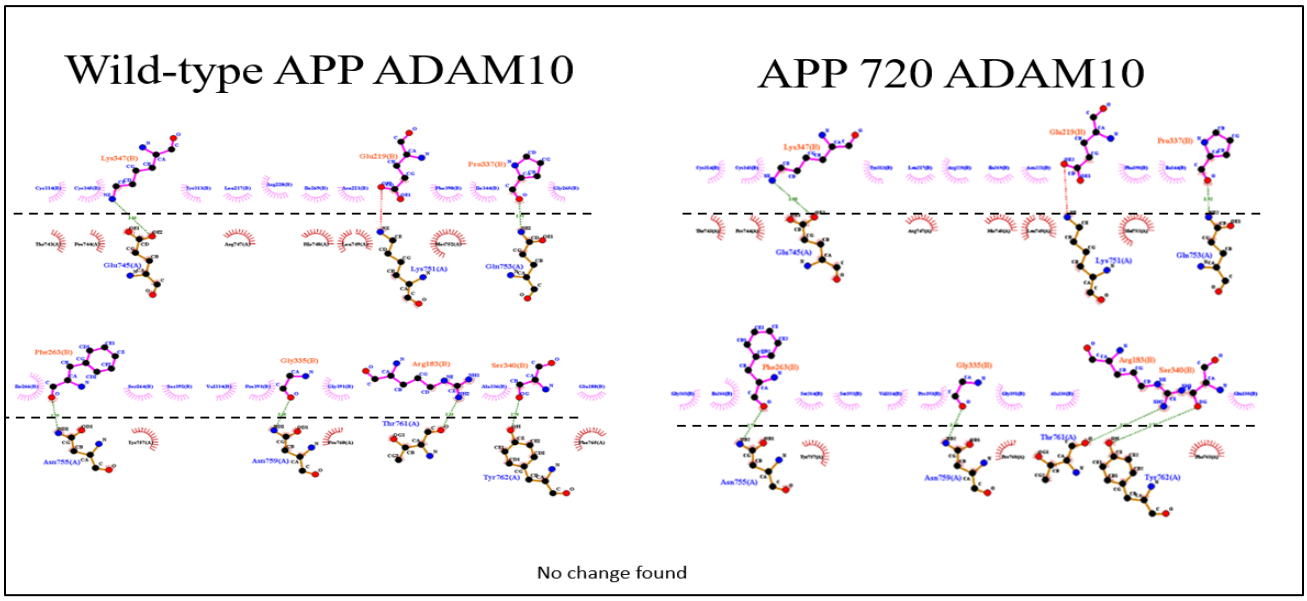


Figure 3.5 Wild-type APP and mutated 720 APP with ADAM10. In this Figure wild-type APP and mutated L720G APP interaction with ADAM10 is shown.

A comparison of Wild-type APP and 720 mutated APP with Nicastrin was made after 2D visualization on ligplot+ software. After the comparison was made, it was observed in Figure 3.7 that mutated 720 APP has no interaction with tyrosine while wild type APP has. Therefore the docking score also changed. The associations between the ligand and the protein is linked to the process of docking. In the case of non-covalent binders, the diverse assortment of interactions between the protein and the ligand include hydrogen bonds, ionic contacts, and van der Waals interactions (which encompass dispersion, polar, and induced interactions). Utilizing docking as a method, both compound optimization and virtual screening are employed. Extensive and extensive research endeavors have revealed that docking does not invariably generate precise estimations of binding affinity. A comparison of Wild-type APP and 710 mutated APP with ADAM10 was made after 2D visualization on ligplot+ software. After the comparison was made, there were observed additional residues between wild type and mutated APP, thus docking score of both proteins also varied shown in Figure 3.6.

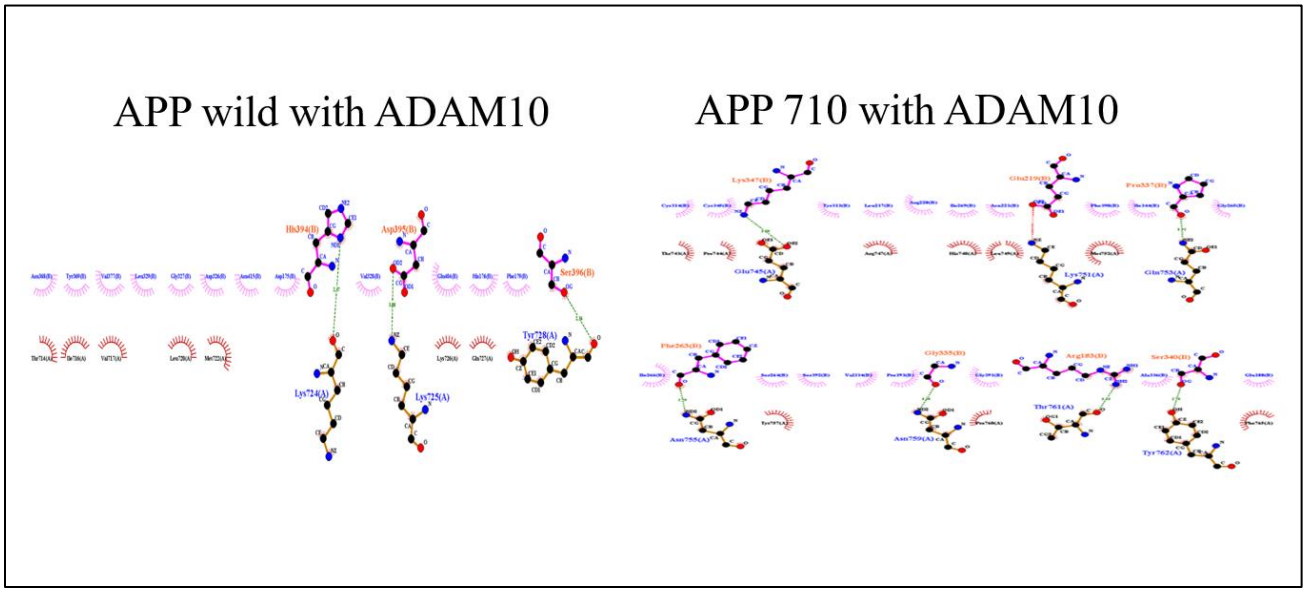


Figure 3.6 Wild-type APP and mutated 710 APP with ADAM10. In this Figure wild-type APP and mutated 710 APP interaction with ADAM10 is shown.

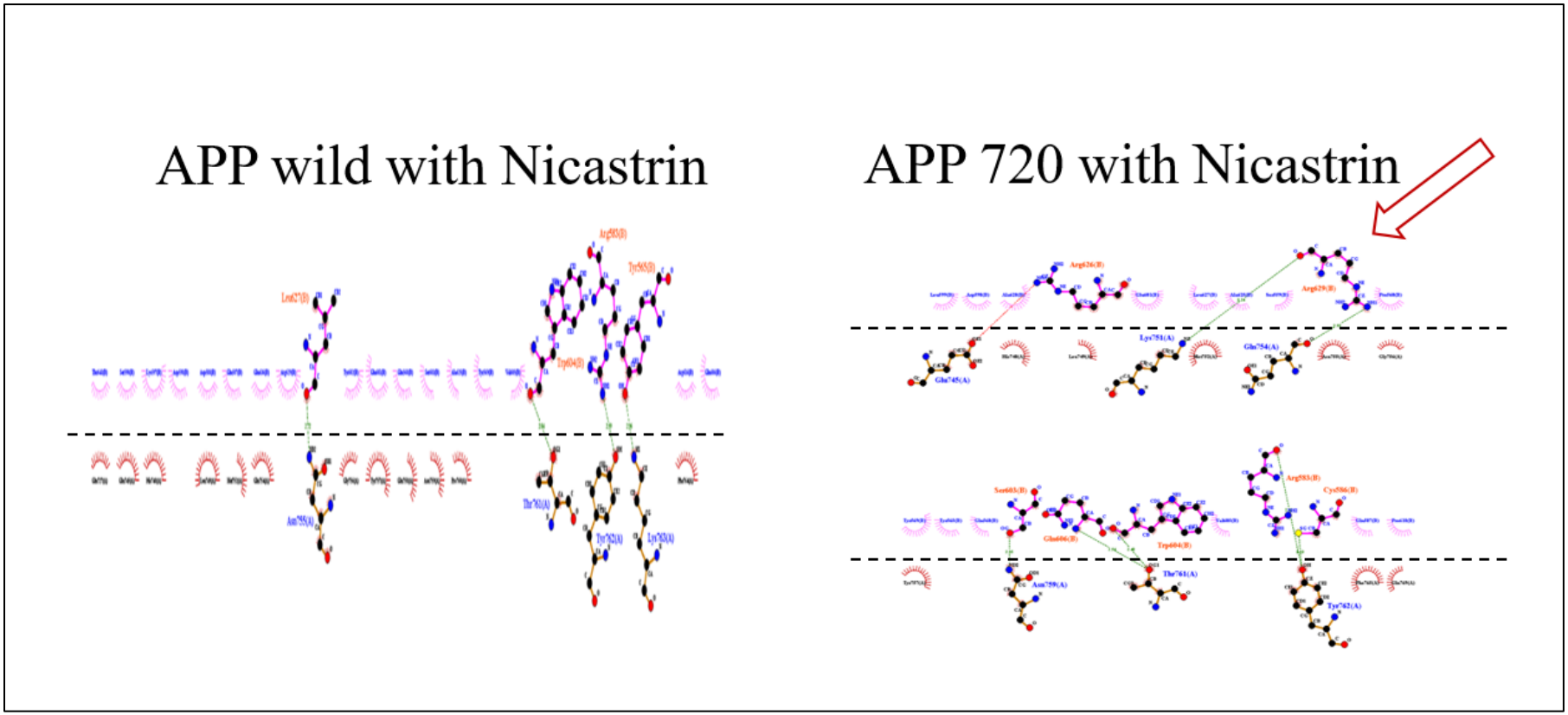


Figure 3.7 Wild-type APP and mutated 720 APP with Nicastrin. In this Figure wild-type APP and mutated 720 APP interaction with Nicastrin is shown. APP wild with Nicastrin gave docking score of -227.28. After creating the mutation at 720 codon of APP the docking score change to -247.91. It shows that 720 mutated APP has enhanced binding with Nicastrin as compared to wild type APP.

A comparison of Wild-type APP and 710 mutated APP with BACE1 was made after 2D visualization on ligplot+ software. After the comparison was made, a drastic change was observed between wild type APP and 710 mutated APP with BACE1 shown in Figure 3.8. APP 710 has not shown any interaction with BACE1.

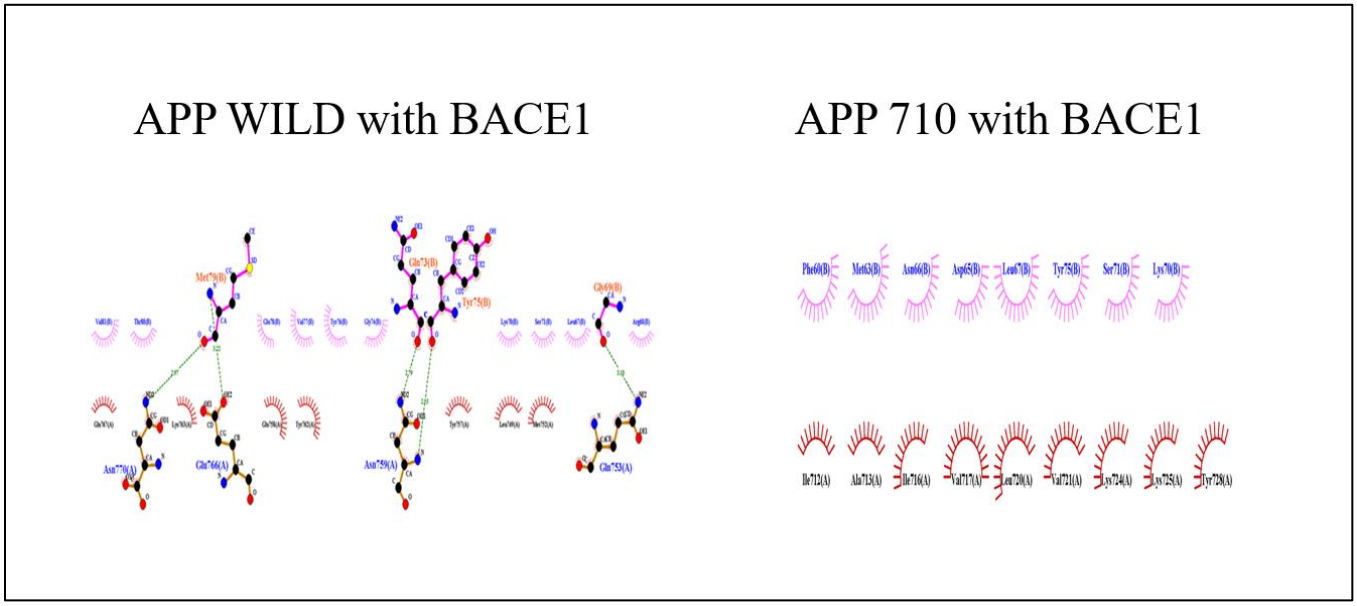


Figure 3.8 Wild-type APP and mutated 718 APP with BACE1. In this Figure wild-type APP and mutated 710 APP interaction with BACE1 is shown.

Wild type APP and mutated APP at 710 position interaction with Nicastrin was also compared after visualizing the graphs on Ligplot as shown in Figure 3.9. Mutated APP at 710 position interacted with Nicastrin at so many residues while wild type did not. Therefore the docking score of both interaction also changed.

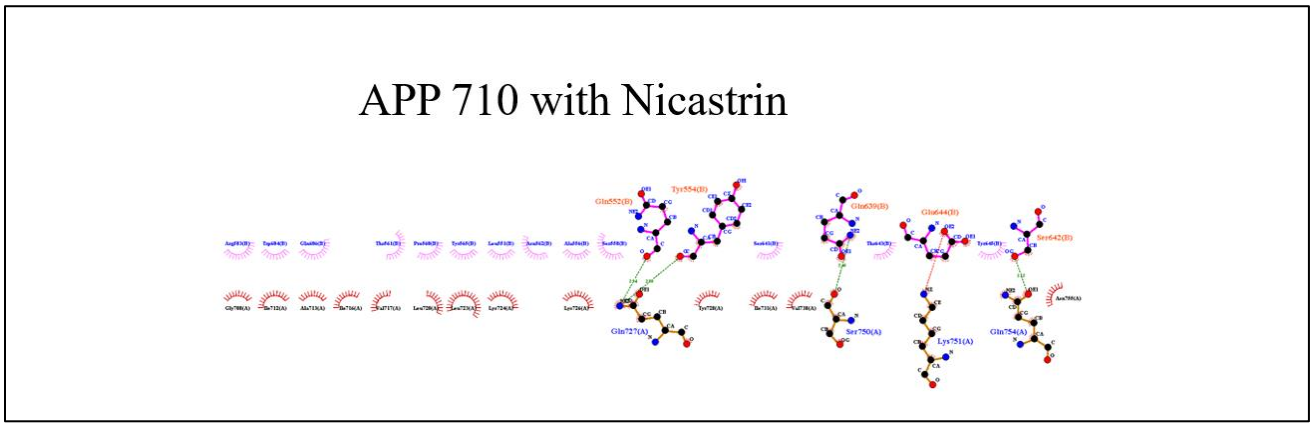


Figure 3.9 Wild-type APP and mutated 710 APP with Nicastrin. In this Figure wild-type

APP and mutated 710 APP interaction with Nicastrin is shown.

3.1.2 Docking score

These Figures show the graphs indicating docking score of ligands i.e., Nicastrin, ADAM10, and BACE1 with wild APP and mutated APP at three codons (718, 710, and 720). Figure 3.10 shows the docking score of BACE1 with wild-type APP and mutated APP. It can be observed that there is a slight change in docking score between wild-type APP and mutated APP. Wild-type APP with BACE1 gives -175.45 docking score while mutated APP with BACE1 gave -176.26. This slight change of docking score is due to the change in the residues and in result generation of amyloids due to differential binding of mutated protein in AD.

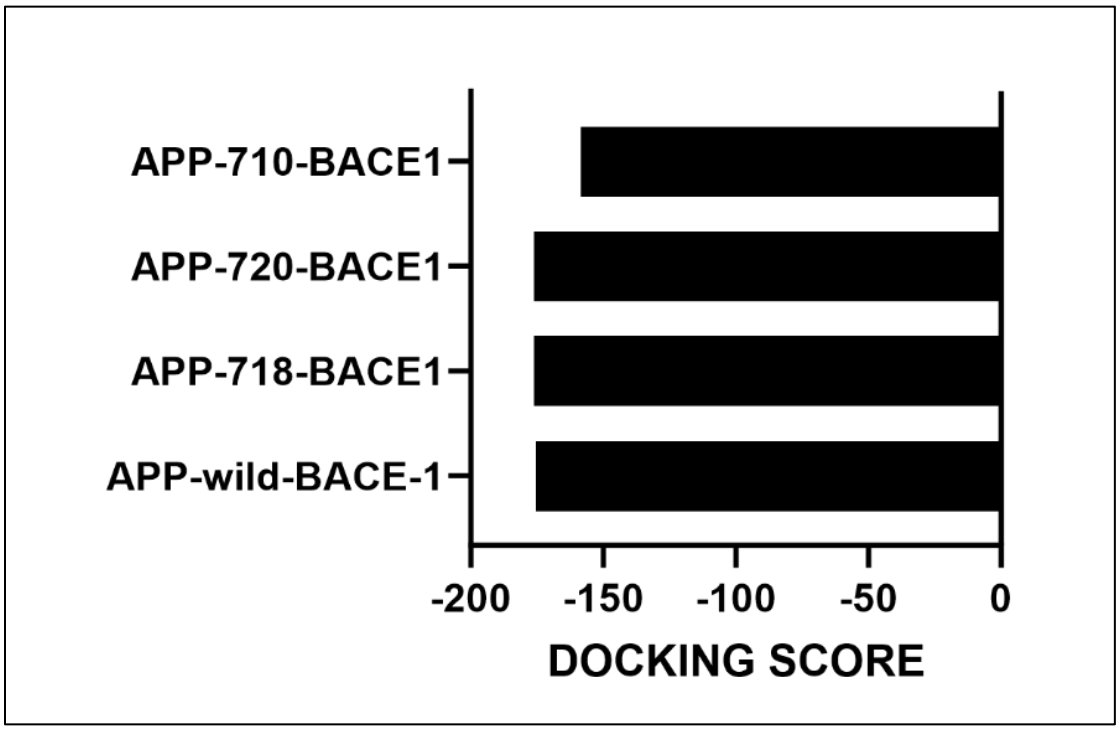


Figure 3.10 Docking Score of mutated and wild type APP with BACE1. This graph shows docking score of I718L, L720G, and V710G mutated APP and wild type APP with BACE1.

Figure 3.11 shows the graph plotted for the docking score of wild and mutated APP with Nicastrin. It can be seen that docking score has been changed from -227.91 to -238.09 that is due to presence of extra hydrogen bonding. Hydrogen bonding interaction causes the formation of protein abnormal folding and denaturing. Hence the protein binds with different targets with different intensity that eventually ends up causing the fibrillation of protein.

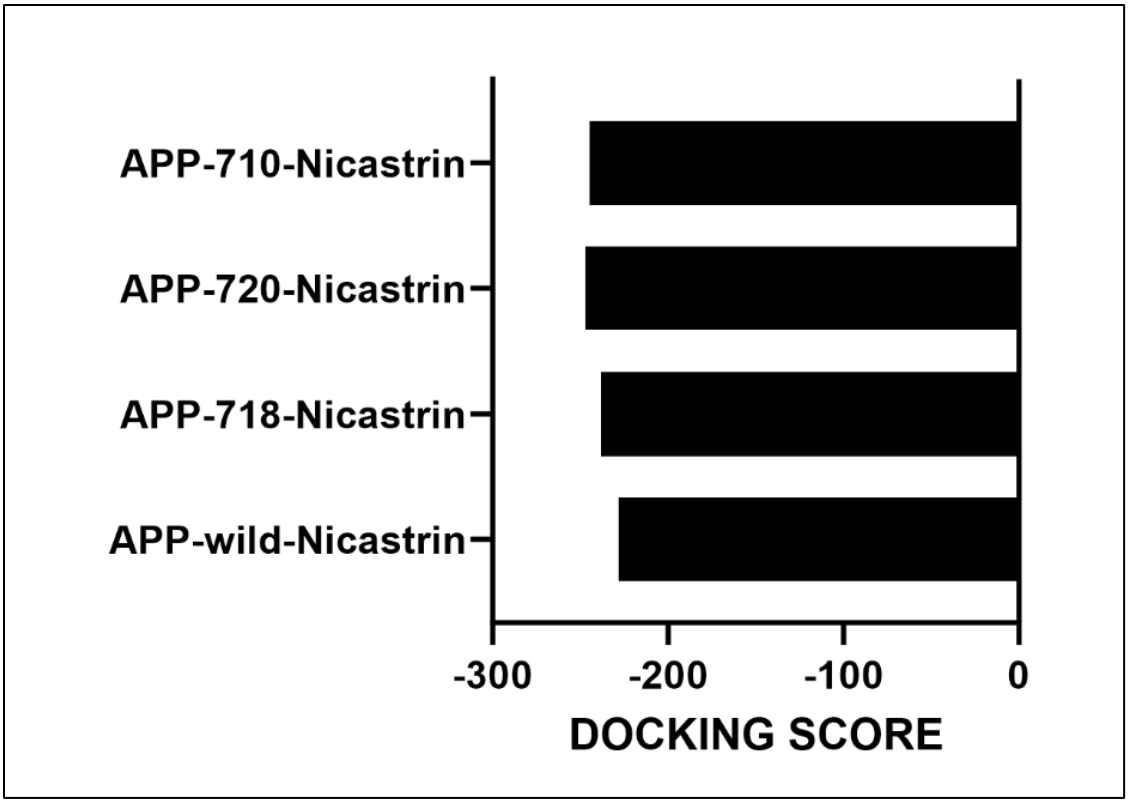


Figure 3.11: Docking Score of mutated and wild type APP with Nicastrin. This graph shows docking score of I718L, L720G, and V710G mutated APP and wild type APP with Nicastrin.

It can also be seen in Figure 3.12 when we compare the Wild-type APP and 710, 718, and 720 mutated APP with ligand ADAM10. The docking score has changed from, -215.28 to -206.36. This is due to the extra interaction of mutated APP with ligands that cause irregular folding and denaturing of protein. . Hydrogen bonding interaction causes the formation of protein abnormal folding and denaturing. Hence the protein binds with different targets with different intensity that eventually ends up causing the fibrillation of protein.

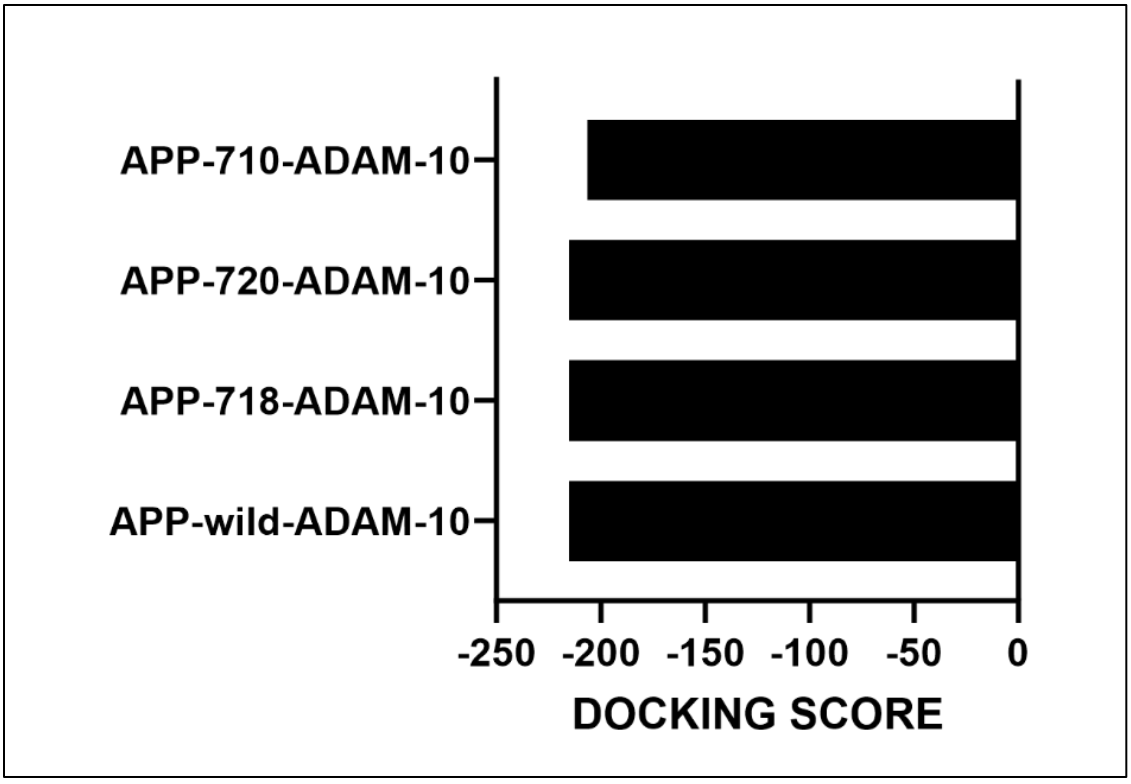


Figure 3.12: Docking Score of mutated and wild type APP with ADAM10. This graph shows docking score of I718L, L720G, and V710G mutated APP and wild type APP with ADAM10.

3.2 Evaluation of SNPs

3.2.1 DNA Extraction (Nano drop)

The quality and quantity of DNA of all 50 samples was measured using Colibri Nanodrop (Germany). The DNA extracted from the blood samples had an A260/A280 ratio between 1.5 to 1.7 and the quantity was approximately ± 50 ng/µL.

3.2.2 PCR results (gels)

3.2.3 APP genotype

The representative gel in Figure 3.13 shows that Wild-type APP type band at 305 bp is present in 48 samples. The bands in this gels are at 305bp and show the normal APP sequence amplification. This means that these samples do not contain mutation of APP in their genes.

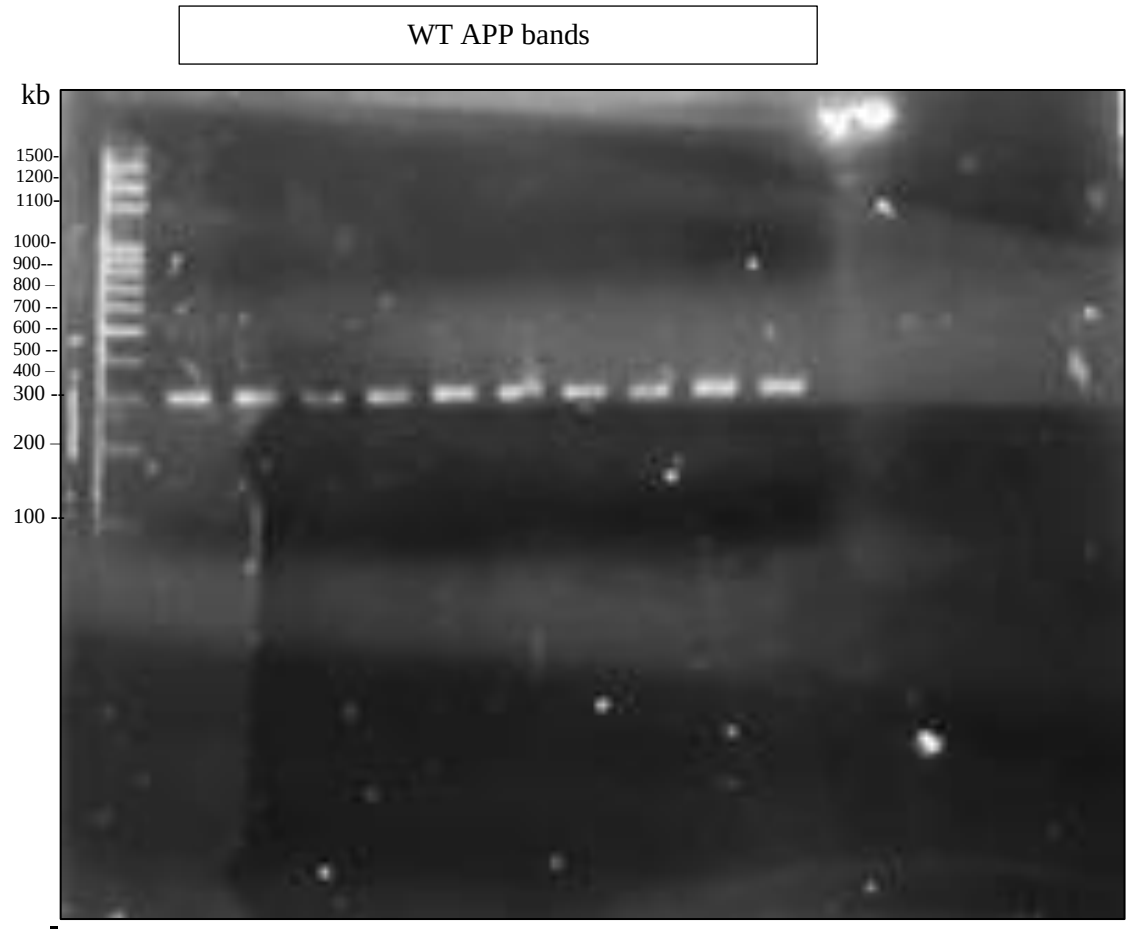


Figure 3.13: Gel electrophoresis results of presence wild type APP. Wild-type APP bands at 305 bp are shown at agarose gel in studied samples.

The representative gel in Figure 3.14 shows that APP 718 (I>L) band at 510 bp is present in following samples. The bands in this gels are at 510 bp and show the mutated APP sequence amplification. This means that these samples contain mutation of APP in their genes

Figure 3.14: Gel Electrophoresis results of presence of mutated APP. APP I718L bands at 510 bp are shown at agarose gel in studied samples.

3.2.4 Allele Frequency

The most prevalent allele was APP healthy allele which was observed in 48 out of 50 samples with the allele frequency of 0.96 (96%). APP mutated allele was absent in 48 samples and present in two samples only with allele frequency of 0.4 (4%). The Table 4.2 shows the frequency of APP alleles in the given dataset (n=50). APP healthy allele was found to be the most prevalent in the dataset, APP mutated present in only two samples of dataset.

Table 3.2 Frequency of APP alleles: Frequency of targeted alleles to total alleles are shown in the Table 4.2.

Allele	No. of alleles	Ratio of targeted alleles to total allele.	Allele frequency
Wild-type APP	100	96/100	0.96
APP I718L mutated	4	4/100	0.04
			= 1.00

3.2.5 Frequency of APP Genotypes

The most prevalent genotype was APP healthy with genotype frequency of 0.96 (96%). The summary of APOE genotypes is depicted in Table 4.3 below.

Table 3.3 Genotype Frequencies. The table shows the genotype frequencies of APP in the given population size (n=50).

Genotype	Number of individuals	Targeted genotype	Genotype Frequency
APP_healthy	48	48/50	0.96
APP_mutated	2	2/50	0.04
Total	n=50		1.00

CHAPTER 4: DISCUSSION

Dementia cases in the world are increasing exponentially with 10 million new cases every year with AD being the primary cause of dementia globally (De-Paula et al., 2012). There are numerous environmental and genetic risk factors that contribute to the development of AD. Among the genetic risk factors, APP polymorphism is also a risk factors for AD (Huynh et al., 2017). Amyloid precursor protein APP is a trans-membrane protein that plays important functions in the brain and nervous system such as it plays its role in synaptic plasticity and synaptogenesis. The roles of APP are much more clearly understood, and many of the functions played by contact receptors and released substances on the tissue level instead of the cellular level. One trans membrane region, a tiny cytoplasmic tail, and a sizable extracellular domain make up the entirety of full-length APP, which makes up 90% of the protein mass present in the brain region. Because of this kind of structure, scientists have hypothesized that APP might serve as a receptor that shows interaction with ligands (Kang et al., 1987).

The majority of APP missense variations are linked to Alzheimer Disease AD in autosomal-dominant manner that mostly start appearing at the age of 60 or above in humans. Recently, it was shown that a mutation in the APP codon 673 was linked to AD. More than 30 mutations are linked to APP that cause the onset of AD. The APP gene consists of total 18 exons. Alternative splicing of these exons of APP leads to the formation of several proteins that range from 695 to 770 amino acids. Aβ peptide is encoded when the APP exon 16 and 17 is spliced (Giaccone et al., 2010). So far up till now, scientists have reported 26 missense polymorphisms or mutations that are present in the gene of APP. Aβ sequence of the APP gene contains most of these missense mutations, while some occur at the side region of it (Goate et al., 1991). The toxic peptide that is Amyloid-beta Aβ peptide is cleaved by two enzymes known as β-secretase and γ-secretase from the APP protein. Beside this, in our study three mutations were generated in APP protein sequence using Pymol at codon 710, 718 and 720.

We have studied these three mutations and performed their docking with our ligands ie.e BACE1, ADAM10 and Nicastrin. We considered I718L APP mutation to be pathogenic. Our docking results of APP wild with BACE1, ADAM10 and Nicastrin showed docking scores of -175.45, -215.45, and -227.91 respectively. When mutated APP at 718 codon from isoleucine to leucine docked with same ligands (BACE1, ADAM10 and Nicastrin) showed docking score -176.26, -

215.28, and 238.09 respectively. To our knowledge, there is no evidence of change in interaction of APP wild and mutated APP at 718 and 720 position with ADAM10. As our results also showed that mutated APP at codon 720 showed -176.26, -215.28, and -247.25 docking score when docking was performed on Hdock with ligands (BACE1, ADAM10 and Nicastrin). Another mutation that was generated codon 710 of the APP protein sequence showed docking score of -158.40, -206.36, and -244.60 when it was docked with same ligands that were (BACE1, ADAM10 and Nicastrin). When docking score of APP wild and mutated APP is compared with Nicastrin, it was be observed that docking score is decreased. Based on these results, we observed that mutations in APP gene cause differential binding of APP with its ligands. The intensity of binding is varied upon generating mutations in APP gene sequence. In our study, this interaction of APP wild and 718 mutated APP with Nicastrin was seen on ligplot+ software. After the comparison was made, it was observed that 718 APP has interacted with Tyrosine 565 residue of Nicastrin which was not present in Wild APP with Nicastrin. It was also observed that 718 APP has additional interaction with serine residue with BACE1. This also caused the difference of docking scores of both APP with Nicastrin that was -227.91 and with 718 APP with Nicastrin it was -244.60 and with BACE1 as well.

In silico methods are used in this study to understand how APP and mutation in APP codons interact with different ligands. In our study, APP protein sequence is downloaded from Alpha fold and its interaction with BACE1, Niscastrin component, and ADAM10 (α-secretase) was checked using HDock and their 2D visualization was checked on Ligplot+. In our study we predicted that three mutations I718L, L720G, and V710G we generated in APP are pathogenic and the binding affinity of these mutated APP will get affected when they interact with γ-secretase, and β-secretase. After performing docking on Hdock when docking score of Wild-type APP and mutated APP is compared with Nicastrin, it was observed that docking score is decreased. Interaction of Wild-type APP and 718 mutated APP with Nicastrin was seen on Ligplot+ software. After the comparison was made, it was observed that 718 APP has interacted with Tyrosine 565 residue of Nicastrin which was not present in Wild APP with Nicastrin. It was also observed that 718 APP has additional interaction with serine residue with BACE1. This also caused the difference of docking scores of both APP with Nicastrin that was -227.91 and with 718 APP with Nicastrin it was -244.60 and with BACE1 as well.

These changes such as Tyrosine and serine residue change, and extra hydrogen bonding cause the

polymerization of protein which aggregates in the brain and cause neuro-toxicity. Based on previous studies, it was demonstrated that changes in residues of protein causes its misfiolding and denaturation. In our study we have analyzed through computational tools that docking score of BACE1 with wild-type APP and mutated APP are slightly different. Wild-type APP with BACE1 gives -175.45 docking score while mutated APP with BACE1 gave -176.26. This slight change of docking score is due to the change in the residues and in result generation of amyloids due to differential binding of mutated protein in AD. Similarly in our study we have observed the wild type APP and mutated APP with Nicastrin. Our study revealed that docking score has been changed from -227.91 to -238.09 that is due to presence of extra hydrogen bonding. Hydrogen bonding interaction causes the formation of protein abnormal folding and denaturing. Hence the protein binds with different targets with different intensity that eventually ends up causing the fibrillation of protein. We also used third ligand i.e. ADAM10 and it was seen when we compare the Wild-type APP and 710, 718, and 720 mutated APP with ligand ADAM10. The docking score has changed from, -215.28 to -206.36. This is due to the extra interaction of mutated APP with ligands that cause irregular folding and denaturing of protein.

A previous study revealed that Aβ mutations are present at 670 and 671 codon of exon 16 of APP. As the result of these mutations, at codon 670 the amino acid of APP protein replaces lysine with asparagine, and at 671 position the amino acid transformed to leucine from methionine. Due to these changes in the amino acid sequences, levels of $A\beta_{40}$ and $A\beta_{42}$ are amplified which causes the large production of Aβ and causes neurotoxicity (Levy et al., 1990). There are many other mutations that are reported at 710, 717, 718, and 720 codons of APP gene sequence. Based on our *in-silco* results, we have selected 718 mutation for our PCR analysis as it was interacting with BACE1, and Nicastrin. It is present in Asian population and it has most prominent symptoms that were cortical atrophy and dementia.

Previously, mutations have been observed in individuals with familial Alzheimer disease (AD) at codon 717 of the APP amyloid precursor protein gene. These mutations include a substitution to isoleucine from valine APP V717I and a transition of valine to glycine APP V717G. In an Iranian family affected with familial Alzheimer disease (FAD), genetic mutations were observed at codon 714 of the amyloid precursor protein (APP) gene. These mutations had the substitution of amino acid threonine (Thr) with alanine (Ala) at codon 714 of APP gene sequence. In this study the genotyping of polymorphism in the amyloid precursor protein (APP) gene, at codon 718 inside the

transmembrane domain was done using PCR in 50 subjects. This polymorphism at 718 codon of APP gene sequence includes the substitution of isoleucine with leucine. In this study, it was found out that APP mutated at 718 codon was present in 4% samples in the given investigating subjects. All other samples had the normal APP protein sequence which was confirmed by PCR using primers in the investigating subjects.

Based on this and previous studies, it can be said that substitutions at the codons of APP sequence may also play a crucial role in the processing of APP cleavage and the subsequent aggregation of Amyloid beta in the brain which lead to the progression of Alzheimer Disease. Many individuals with underlying polymorphisms in the amyloid precursor protein (APP) gene may have familial Alzheimer disease (FAD) that may appear later in life. Another potential reason might be the incomplete penetrance of APP mutations or the lack of detection of dementia by family members. In Pakistani culture, mild cognitive decline is often seen as a natural part of the ageing process. The extent of memory impairment may have been significant, leading to the provision of inaccurate information about the timing of symptom start. It is also plausible that there may be an underestimation of the family incidence of moderate cognitive impairment among Pakistani individuals with Alzheimer disease.

CHAPTER 5: SUMMARY

Alzheimer's disease (AD) arises as a formidable global health challenge, specifically impacting the elderly population. In 2010, an estimated 35.6 million cases were reported, a number projected to reach a staggering 66 million by 2030. This emphasizes the urgent need for comprehensive research in order to comprehend the complexities of this neurodegenerative disorder. Considering this urgent issue, the current research stands out as a groundbreaking endeavor, being the initial of its sort to examine the Amyloid Precursor Protein (APP) genotype and its association with AD in the Pakistani population.

The research takes a multifaceted approach that combines an exploration of the structural dynamics of both the wild-type APP and its mutated forms. The mutated variants under examination include 710 (V>G), 718 (I>L), and 720 (L>S). This structural analysis is extended to evaluate the interaction of these APP variants with key ligands that have been implicated in the pathological progression of AD. The ligands under investigation include BACE1, ADAM10, and Nicastrin.

In the realm of *in silico* results, a noteworthy point is made regarding the interaction between the mutated APP at the 718 codon and specific residues of BACE1 and Nicastrin. This interaction is identified as a catalyst for abnormal denaturation of the protein, a phenomenon that has significant implications for the generation of amyloid plaques, a hallmark feature of AD. The detailed molecular insights provided by this study contribute significantly to our understanding of the genetic and molecular foundations of AD, particularly in the unique context of the Pakistani population.

Moreover, a comprehensive Single Nucleotide Polymorphism (SNP) analysis is integrated into the study, revealing a 4% prevalence of the 718 (I>L) mutation within the examined population. This prevalence underscores the significance of this specific mutation within the genetic landscape of AD in the Pakistani demographic. Symptomatically, the identified mutation is associated with distinct clinical manifestations, including Hippocampus atrophy, dementia characterized by noticeable oral tendencies, and amnesia. These findings not only advance our understanding of the genetic markers associated with AD but also provide crucial insights into the potential clinical outcomes linked to specific mutations.

Beyond the scientific exploration, the study carries significant implications for public health in Pakistan. The exponential increase in AD cases within the country necessitates proactive measures.

The thesis advocates for the establishment of organizations dedicated to raising awareness about AD, its risk factors, and preventive strategies. Fostering a robust informational infrastructure becomes imperative for early detection, intervention, and the overall well-being of individuals affected by AD in Pakistan. In conclusion, this thesis represents a pivotal contribution to the field of Alzheimer's research, particularly in the Pakistani context. By unraveling the molecular intricacies of the APP genotype and its role in AD pathology, the study not only expands our scientific understanding but also advocates for a proactive public health approach to tackle the rising tide of Alzheimer's in Pakistan.

CHAPTER 6: FUTURE PROSPECTS AND CONCLUSION

The profound insights provided by this thesis establish a strong basis for future investigations and clinical endeavors in the realm of Alzheimer's disease (AD) within the Pakistani population. The discovery of the prevalence of the 718 (I>L) mutation and its correlation with specific clinical symptoms creates opportunities for more focused inquiries. Subsequent studies can delve further into the intricate molecular mechanisms underlying the interaction between mutated APP and crucial ligands, thus paving the way for the development of targeted therapeutic interventions.

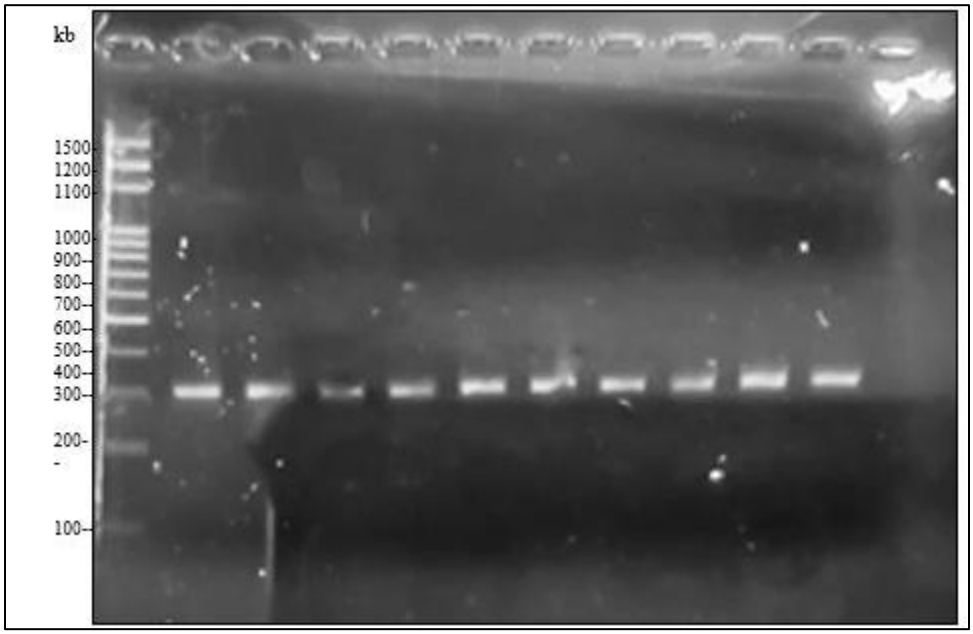
Furthermore, as our understanding of the genetic landscape of AD expands, there emerges the potential for genetic screening and personalized medicine. Comprehensive comprehension of the specific mutations that contribute to the onset and progression of AD allows for more accurate diagnostics and tailored treatment approaches. The integration of genomic information into clinical practice has the potential to revolutionize the approach to AD by enabling earlier detection and intervention, which would ultimately enhance patient outcomes. Collaboration among genetic researchers, clinicians, and public health experts is imperative in translating these scientific breakthroughs into tangible advantages for the affected population. The establishment of a framework for ongoing genetic studies and clinical trials is crucial in advancing our knowledge of AD and developing effective interventions.

In conclusion, this thesis signifies a significant milestone in Alzheimer's research as it provides a comprehensive examination of the role of the APP genotype in AD within the distinctive context of the Pakistani population. The thorough structural analysis, along with the identification of the 718 (I>L) mutation and its clinical implications, contributes significantly to our comprehension of the molecular foundations of AD. The findings emphasize the need for heightened awareness and proactive measures in Pakistan, where the prevalence of AD is increasing. The thesis advocates for the establishment of organizations focused on disseminating information about AD, highlighting the importance of early detection, intervention, and public health initiatives.

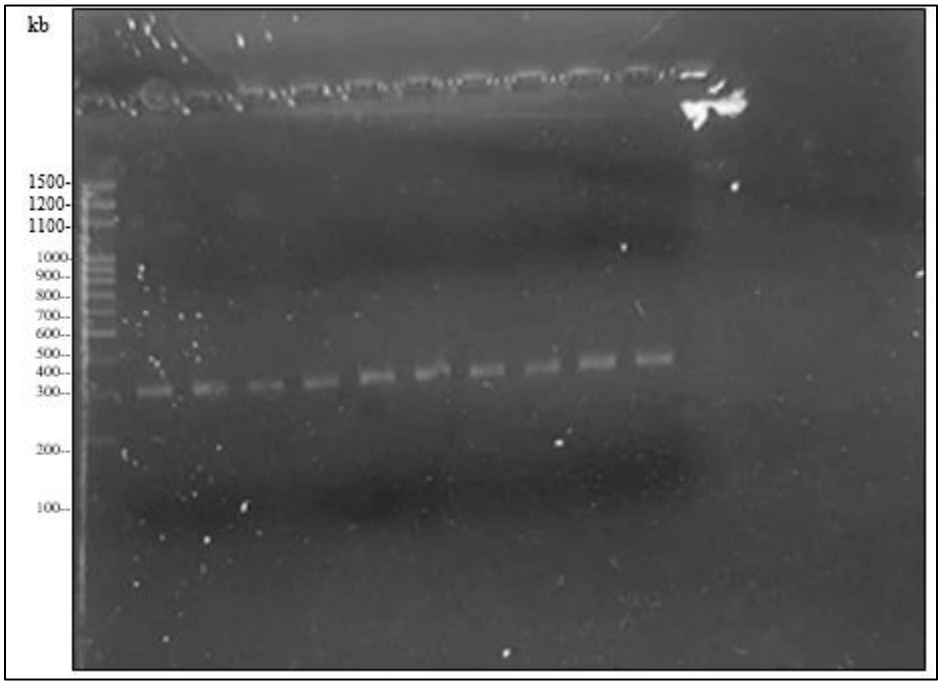
As we stand on the verge of a new era in genetic research and personalized medicine, the implications of this study extend far beyond its immediate results. The identification of particular genetic markers linked with AD extends possibilities for investigation, clinical practice, and public health strategies. This work not only deepens our understanding of AD within the Pakistani context but also provides valuable insights to the global endeavor to combat this formidable

neurodegenerative disease. Moving forward, sustained collaborative efforts and a dedication to further research are crucial in transforming the knowledge gained from this thesis into practical solutions that benefit individuals affected by AD in Pakistan and beyond. The journey towards unraveling the complexities of Alzheimer's disease continues, guided by the hope that these insights will ultimately lead to more effective treatments and, ultimately, a brighter future for those impacted by this challenging condition.

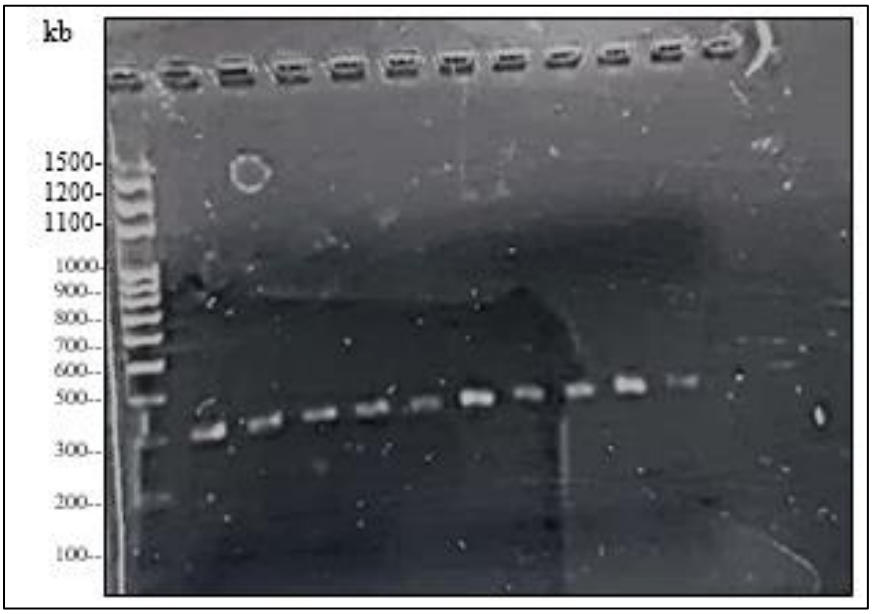
APPENDICES



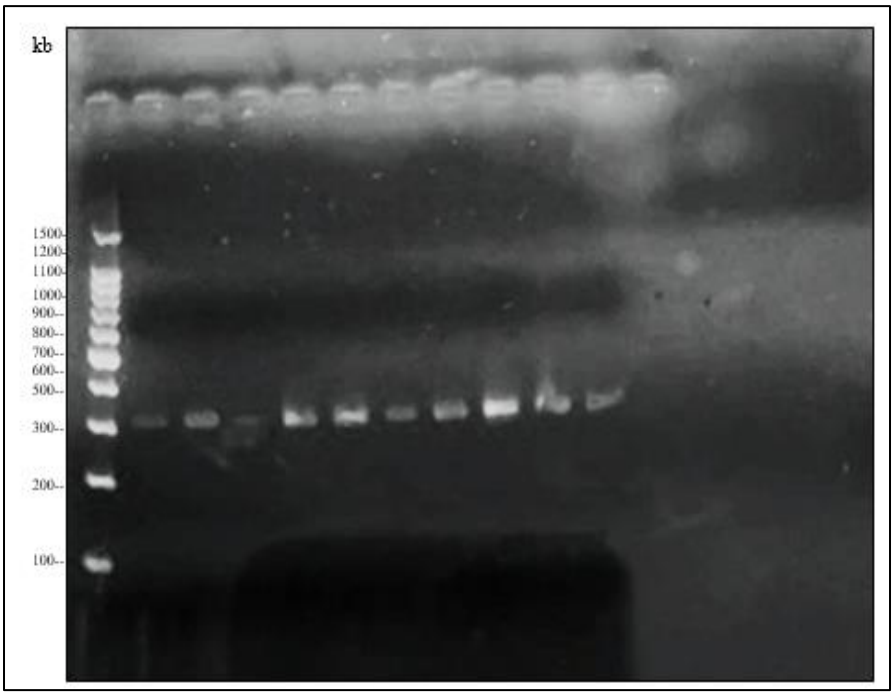
Supplementary Figure 7.1: This figure represents the wild type APP present in tested samples.



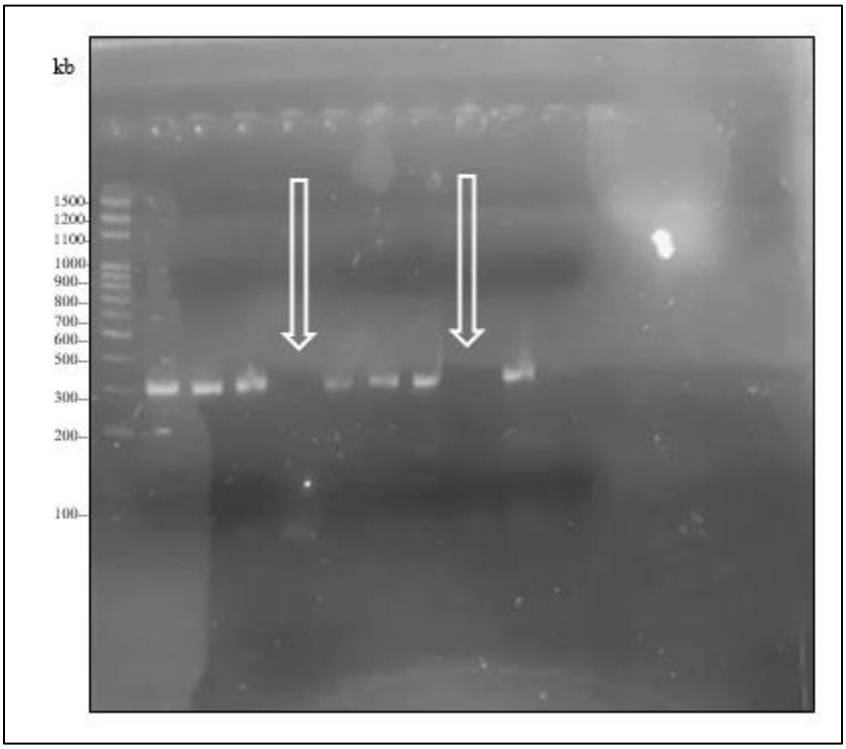
Supplementary Figure 7.2: This figure represents the wild type APP present in tested samples.



Supplementary Figure 7.3: This figure represents the wild type APP present in tested samples.



Supplementary Figure 7.4: This figure represents the wild type APP present in tested samples.



Supplementary Figure 7.5: In this Figure No healthy band were seen in 2 samples. Wild type APP bands were seen in other samples.

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