Assessment of APOE Genotype in Pakistani Population with Relevance to Rapidly Progressive Alzheimer's Disease

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Dedication

Dedicated to my parents

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Abbreviations

WHO	World health organization
AD	Alzheimer's Disease
Aβ	Amyloid beta
NFTs	Neurofibrillary tangles
PHF	Paired helical filament
MMSE	Mini mental state examination
CSF	Cerebrospinal fluid
MRI	Magnetic resonance imaging
CT	Computerized tomography
PET	Positron emission tomography
APP	Amyloid precursor protein
PSEN-1	Presenilin-1
PSEN-2	Presenilin-2
APOE	Apolipoprotein E
SAD	Sporadic AD
LOAD	Late onset AD
FAD	Familial AD
EOAD	Early onset AD
RpAD	Rapidly Progressive AD
CJD	Creutzfeldt Jakob Disease
LDLR	Low density lipoprotein receptors
GWAS	Genome wide association study
CAA	Cerebral amyloid angiopathy
BBB	Blood brain barrier
VLDL	Very low-density lipoprotein
LRP1	LDLR-related protein 1
IDE	Insulin degrading enzyme
PCR	Polymerase Chain Reaction

Abstract

According to the statistics provided by World Health Organization (WHO), approximately 55 million people are suffering from dementia globally making it a major public health concern. Alzheimer's Disease (AD) is the most common cause of dementia constituting 60-70% of cases worldwide. There are various environmental and genetic risk factors that contribute to the development of AD, among the genetic risk factors, APOE polymorphism is the major risk factor for AD. There are three isoforms of APOE gene E2, E3, and E4. The presence of different isoforms of APOE gene in different genotypical combinations determine how susceptible is the person to developing AD in the future. AD affects the patient's quality of life severely and makes even basic chores undoable, therefore it is imperative for people to be aware of the disease and stay clear of the myths surrounding it. The present study is the first one to assess the presence of APOE genotype in Pakistani population with relevance to AD, no study of this sort has been carried out in Pakistan before. The results showed complete absence of E2 allele in the tested population size. The most prevalent allele was E3 which was expressed in the form of E3E3 (95%) and E3E4 (5%) genotypes. E4/E4 genotype was also completely absent. In this study, a detailed survey was also conducted to assess the knowledge of AD in Pakistani people. In certain non-technical areas, the respondents exhibited good knowledge about the AD, but in the technical aspects where risk factors, diagnostic criteria and treatment options related to the disease were involved, the participants displayed poor knowledge, this shows that even the educated people in Pakistan are unaware about the basic knowledge of the disease. It is imperative to establish organizations that can create awareness among the masses regarding AD which is exponentially increasing in Pakistan.

Chapter 1

Introduction

1. Introduction:

1.1. Alzheimer's Disease (AD):

According to the fact sheet provided by the World Health Organization (WHO), nearly 55 million people have dementia worldwide making it a major public health hazard (Chowdhary et al., 2022). AD is a neurodegenerative disease which is a cause of approximately 60-70% of the dementia cases worldwide (Van Der Flier & Scheltens, 2005). It is one of the major causes of disability among the elderly population globally. AD has an adverse effect on the patients their families and economic conditions of the family and the countries with an estimated annual expenditure of 1 trillion US dollars globally (Breijyeh & Karaman, 2020a). The cure for AD has not been discovered yet, but there are treatments available that improve the symptoms and the overall quality of patient's life (Yiannopoulou & Papageorgiou, 2020).

1.2. Pathology

The accumulation of tau protein and amyloid beta (Aβ) in and around the brain cells is the potential cause of AD. It is characterized by senile plaques and neurofibrillary tangles (NFTs) formed by the deposition of Aβ and tau protein in different parts of the brain respectively (De-Paula et al., 2012). The consequences of these irreversible changes include cognitive decline in the form of loss of synapses and neurons in the region of the brain that controls memory which subsequently leads to memory issues and eventually dementia (Serrano-Pozo et al., 2011).

1.2.1. Senile plaques:

The extracellular senile plaques of Aβ exist in various morphological forms which includes compact, classic, diffused and neuritic plaques (Cras et al., 1991). Transmembranous amyloid precursor protein (APP) acts as a precursor for the synthesis of Aβ deposits in the presence of β-secretase and γ-secretase which are proteolytic enzymes (Perl, 2010). Cleavage of APP by these proteolytic enzymes generate numerous fragments of ammino acids including $A\beta_{40}$ and $A\beta_{42}$ (Armstrong, 2019). There are various kinds of Aβ monomers, one being large and insoluble in the form of amyloid fibrils that leads to the deposition and accumulation of amyloid plaques. The other type of monomer is soluble

with the potential of spreading throughout the brain (Tabaton & Piccini, 2005). Deposition of beta-amyloid causes neurotoxicity, neural dysfunction and stimulate the astrocytes and microglia which causes macroscopic atrophy, synaptic loss and increased cognitive decline (Chen et al., 2017).

1.2.2. NFTs:

NFTs are atypical aggregates of hyperphosphorylated tau protein that are often found in the form of helical structures known as paired helical filament (PHF) that accumulate in the neurons such as dendrites and axons destabilizing the cytoskeletal microtubules (Brion, 1998). Under normal condition, tau binds to the microtubules and stabilizes them, however in AD, tangles are formed inside the neurons because tau molecules separate themselves from the microtubules and attach with other tau molecules. The NFTs create hindrance in the neuronal transport system that eventually damages the synaptic communication (Metaxas & Kempf, 2016).

1.3. Clinical features of AD

1.3.2. Signs and symptoms of AD

Early symptoms of AD include cognitive decline which leads to memory impairment and frequent bouts of forgetfulness which eventually leads to dementia (Bäckman et al., 2004). This is followed by progression of anomia which is the inability of retaining and retrieving vocabulary (Huff et al., 1986). Anomia (Figure 1) is followed by aphasia (Figure 1) which is a language disorder that is caused by the cognitive dysfunction in the part of the brain that controls linguistic expressions and comprehension (Cummings et al., 1985). The AD patients also experience semantic impairment, difficulty in problem solving and concentrating on a particular task and often feel disoriented. Patients suffering from AD also experience neuropsychiatric symptoms which includes depression, apathy, auditory or visual hallucinations, delusions, irritability, and psychosis (Li et al., 2014). In the final stages of AD, the patient suffers from ataxia and eventually loses the mobility completely.

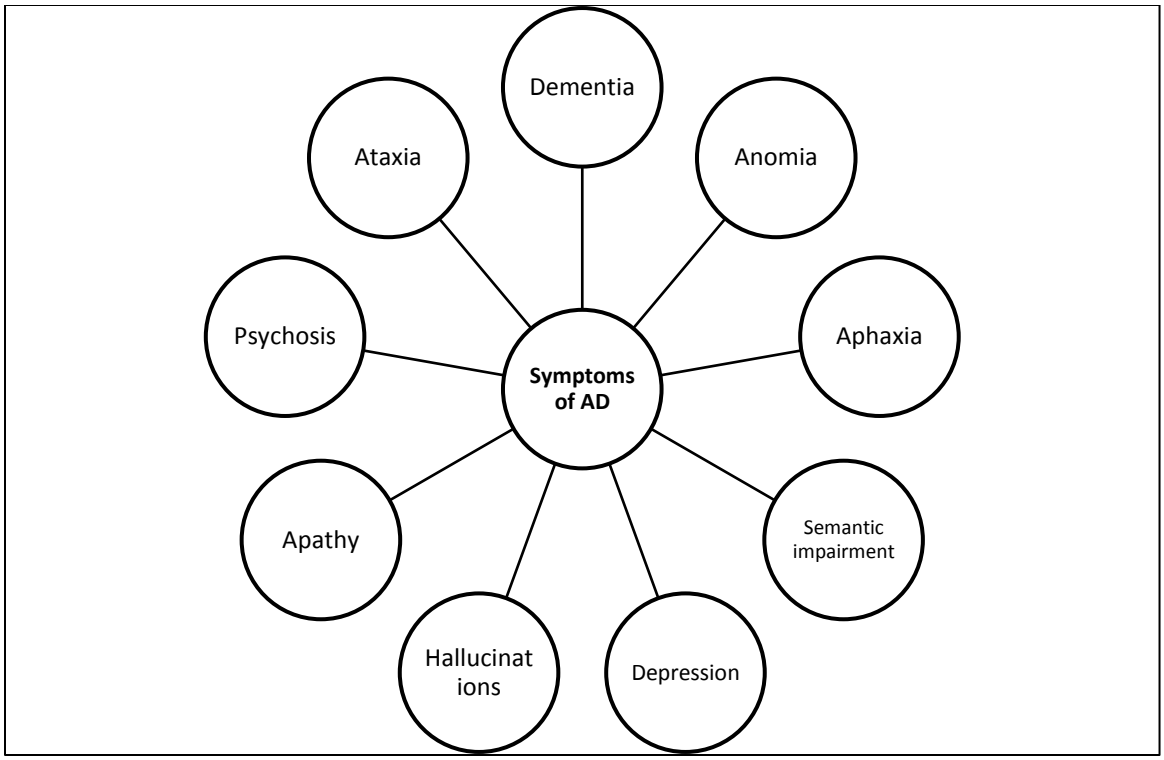

Figure 1: Symptoms of Classical AD. The figure illustrates the symptoms exhibited by patients suffering from classical AD.

1.3.3. Diagnostic criteria for AD

The general criteria used for the diagnosis of AD includes:

1. Mental status testing: It is checked by Mini Mental State Examination (MMSE), according to which decline of 3 MMSE points per year suggests AD (Schmidt et al., 2010).
2. Laboratory tests: Heightened levels of phosphorylated tau (p-tau) and decline of of $A\beta_{42}$ in cerebrospinal fluid (CSF) suggests AD (Andreasen & Blennow, 2005).
3. Brain imaging tests: Magnetic Resonance Imaging (MRI) (Vemuri & Jack, 2010), Computerized Tomography (CT), and Positron Emission Tomography (PET) are carried out to assess the degree of degeneration and to rule out other causes such as brain tumors, hemorrhages, and strokes (de Leon et al., 2016).
4. Even with all the diagnostic measures, definite diagnosis can only be made after the brain is autopsied and analyzed (Breijyeh & Karaman, 2020b).

1.4. Causes & risk factors of AD

- Alzheimer's is a multifactorial disease believed to have myriad of causes and riSK factors. Ageing: It is the most common risk factor of AD. It is irreversible and many problems come with it naturally which includes reduced brain volume, synaptic losses, deposition of senile plaques and NFTs in cerebral cortex which is enhanced with aging (Breijyeh & Karaman, 2020b). That is why majority of AD cases emerge in people about above 65 years of age (Guerreiro & Bras, 2015).
- Environmental factors: It includes poor diet, exposure to toxic metabolites, and infections (Wainaina et al., 2014).
- Genetic: Genetic factors play a significant role in the causation of AD. Mutations in genes such as APP, presenilin-1 (PSEN1), presenilin-2 (PSEN-2), and Apolipoprotein E (APOE) (González et al., 2018).
- Cardiovascular risk factors are also the risk factors for AD which includes hypertension, smoking, high cholesterol, and diabetes. These risk factors increase the risk of developing AD by 20-40% (Whitmer et al., 2005).

1.5. Subtypes of AD

Alzheimer's is a multifactorial disease that has been divide into several subtypes on the basis of age of onset, cognitive decline, genetic predisposition and pathological changes in the light of neuroimaging and neuropathology studies (Ferreira et al., 2020).

1.5.1. Sporadic AD

Sporadic AD (SAD) is also identified as late onset AD (LOAD). SAD is believed to be caused by a series of genetic and environmental risk factors that still need to be fully understood. It begins after the age of 65 and constitutes for about 95% of the cases. The APOE gene is responsible in the causation of SAD (Dorszewska et al., 2016). According to the recent discovery, Amyloid-β 42 filaments were found to be different in structure in SAD as compared to the Familial AD. Type I filaments were more common in brains of the patients with SAD (Yang et al., 2022).

1.5.2. Familial AD:

Familial AD (FAD) is a hereditary disease that is very rare and accounts for 5% of the cases only (Wolfe, 2019). The age of onset is usually less than 65 years; therefore, it is sometimes known as early onset AD (EOAD). In most cases of EOAD, the mutations are inherited in autosomal dominant pattern with 50% chance of inheriting the FAD causing gene if one of the biological parents is affected with the disease (Tanzi, 2012). Mutations in genes such as APP, PSEN1, PSEN-2, and APOE are responsible for the onset of FAD (Dorszewska et al., 2016). According to the current research findings, $A\beta_{42}$ filaments were found to have different structures in SAD and FAD with FAD being rich in Type II filaments (Yang et al., 2022).

1.5.3. Rapidly Progressive AD

Rapidly progressive AD (rpAD) has recently been categorized as an atypical subtype of AD (Abu-Rumeileh et al., 2018), however scientists have yet not reached at consensus regarding the clinical definition of the disease (Schmidt et al., 2010). It is characterized by rpAD cognitive dysfunction and dementia that develops within 1-2 years of the disease onset (Schmidt et al., 2011). Previously, due to the similarity in the progression of cognitive decline between rpAD and Creutzfeldt Jakob's Disease (CJD), rpAD was often misdiagnosed with CJD making diagnosis and treatment of rpAD even more difficult (Reinwald et al., 2004).

The genetic profile and biomarkers of rpAD and classical AD have numerous differences. Although the word rapid is used equivocally not clearly distinguishing whether it is used for survival time or rate of cognitive decline, however, rapid progression has roughly been defined as decline of 5 points per year according to the standardized psychometric test known as MMSE and disease duration of less than two years (Doody et al., 2001). There are believed to be many causes of rpAD including cardiovascular anomalies, autoimmune diseases, neurodegenerative pathology, genetic (APOE genotype) and environmental risk factors (Paterson et al., 2012).

1.6. Difference between AD and rpAD

Numerous clinical and molecular differences have been identified in the pathology of sAD and rpAD. Neurological symptoms such as cognitive dysfunction, language problem, ataxia and rapid memory loss are identified earlier during the disease progression in rpAD cases. RpAD cases exhibited heightened levels of tau and p-tau and low levels of $A\beta_{42}$ in cerebrospinal fluid (CSF) when compared to sAD (Llorens et al., 2017). Whereas 14-3-3 is only present in rpAD cases which can ultimately be utilized for differential diagnosis (Karch et al., 2016). Anatomically, brain atrophy and the volume of hippocampus showed no significant differences (De-Paula et al., 2012).

No major differences were noted in the structures of NFTs and senile plaques (Schmidt et al., 2012). Rapid progression and cognitive decline are also linked with heightened levels of PrP^c (Pillai et al., 2018). Even though, no significant difference was observed between sAD and rpAD with respect to PrP^c, but presence of various structures and interactions were observed in rpAD (Zafar et al., 2017). AD is characterized by the presence of high frequency of E4 allele while rpAD is associated with low frequency of E4 allele (Schmidt et al., 2010).

1.7. APOE Polymorphism

The APOE gene is a major cholesterol carrier and is involved in metabolizing fats in humans (Mahley, 1988) and it is mapped on chromosome 19q13.2 with 4 exons and 3 introns. It is expressed in various organs including liver and brain. APOE is vital in maintaining lipid homeostasis in brain and its periphery (Mahley & Rall, 2000). Astrocytes are primarily responsible for the synthesis of APOE in the brain. APOE carry cholesterol and other essential lipids to the neurons via low density lipoprotein receptors (LDLR) (Pitas et al., 1987). APOE gene is found to be polymorphic at 2 single nucleotides which includes rs429358 and rs7412. The polymorphism generates three alleles E2, E3, and E4 and six APOE genotypes. The difference between these isomers arise from amino acid residues 112 and 158 and the presence of the bases cysteine and arginine (Phillips, 2014).

Although the differences among the different isoforms only arise by one or two amino acids (Table 1), but the functionality and structural differences are significant enough to cause disease risk which includes AD (C. C. Liu et al., 2013a).

Table 1: SNP mutations in APOE. The table shows the sequence of two SNP mutations in APOE gene forming three isomers E2, E3 and E4.

	SNP: rs429358	SNP: rs7412	Protein 112	Protein 158
E2	TGC	TGC	Cys	Cys
E3	TGC	CGC	Cys	Arg
E4	CGC	CGC	Arg	Arg

1.8. APOE genotype's association with AD and rpAD

According to the genome wide association studies (GWAS) E4 allele is categorized as the risk factor for SAD (William Rebeck et al., 1993). As mentioned in table 2, the probability of the development of AD in people with one E4 allele increases by 2-3-fold while individuals who are homozygous for E4 allele have 10-15-fold increased risk of developing and almost 60-80% of people suffering from AD carry E4 allele (Farrer et al., 1997).

Carriers of E4 alleles show increased pathological changes in the brain which includes Aβ, and tau pathologies as compared to individuals who did not carry the E4 allele. E2 allele has protective role against AD so individuals carrying E2 allele have decreased risk of developing AD (Zannis et al., 1982) and those carrying E3 allele have neutral risk (Troutwine et al., 2022).

Table 2: Mean age of onset. This table shows the AD frequency and mean age of onset of AD in people who are non-carriers, homozygous, and heterozygous for E4 allele.

	Non carrier	Homozygous	Heterozygous
AD frequency	20%	47%	91%
Mean age of onset	84 years	76 years	68 years

On the other hand, rpAD is associated with low frequency of E4 allele (Cohen et al., 2015). In a study carried out to assess the rate of recurrence of E4 allele in people with rpAD, the results showed that only 38% of the patients had APOE allele in contrast to AD patients and none of them was found to be homozygous for E4 allele (Schmidt et al., 2010).

1.9. Worldwide Prevalence of APOE genotype and AD

The Table 3 indicates the frequency of E2, E3 and E4 alleles in the population worldwide (Liu et al., 2013) . The presence of E4 was found to be exponentially more in people with AD. The prevalence of APOE genotype varies among people with different ethnicities. The association between E4 allele and AD was observed to be weaker in Hispanics and African Americans and stronger in Japanese and Caucasians (González et al., 2018). The most prevalent allele in APOE genotype is E3 allele with 85% cases in Asia, 82% in North America, 79% in Europe, 77% in South America 69% in Africa (Singh et al., 2009). Prevalence of E4 allele also varies among different continents with 40% frequency in Central Africa, 37% in Oceania and 26% in Australia (Huebbe & Rimbach, 2017), 25% in Asia, 25% in northern Europe, 10% in South China and Mediterranean region (Egert et al., 2012). The occurance of E2 allele in Oceania and Africa is 11.1% and 9.9% respectively which is higher than average (Singh et al., 2009).

Table 3: Allele Frequency worldwide. The table lists the Allele frequencies of APOE genotype in the general population worldwide and AD population.

APOE Isoform	Allele Frequency (general)	Allele Frequency AD
E2	8.4%	3.9%
E3	77.9%	59.4%
E4	13.7%	36.7%

1.10. Role of E4 gene in Aβ deposition

APOE significantly enhances the breakdown of Aβ both within and between cells (Jiang et al., 2008). It interacts with Aβ to produce senile plaques and cause cerebral amyloid angiopathy (CAA) in cerebral cortex of the brain (Ellis et al., 1996). The individuals carrying E4 allele are more susceptible to developing Aβ depositions in the shape of senile

plaques as compared to the non-carriers (Schmechel et al., 1993). Clearance of Aβ takes place primarily from three possible routes interstitial fluid, blood brain barrier (BBB) and enzymatic degradation (Huynh et al., 2017). APOE is involved in Aβ clearance via BBB (Figure 2), but E4 allele prefers to bind with very low-density lipoprotein (VLDL) which clears the APOE/ Aβ complexes at slower rate as compared to other receptors that are bound to other isoforms of the APOE gene. This leads to excessive buildup of Aβ in the brain (Deane et al., 2008).

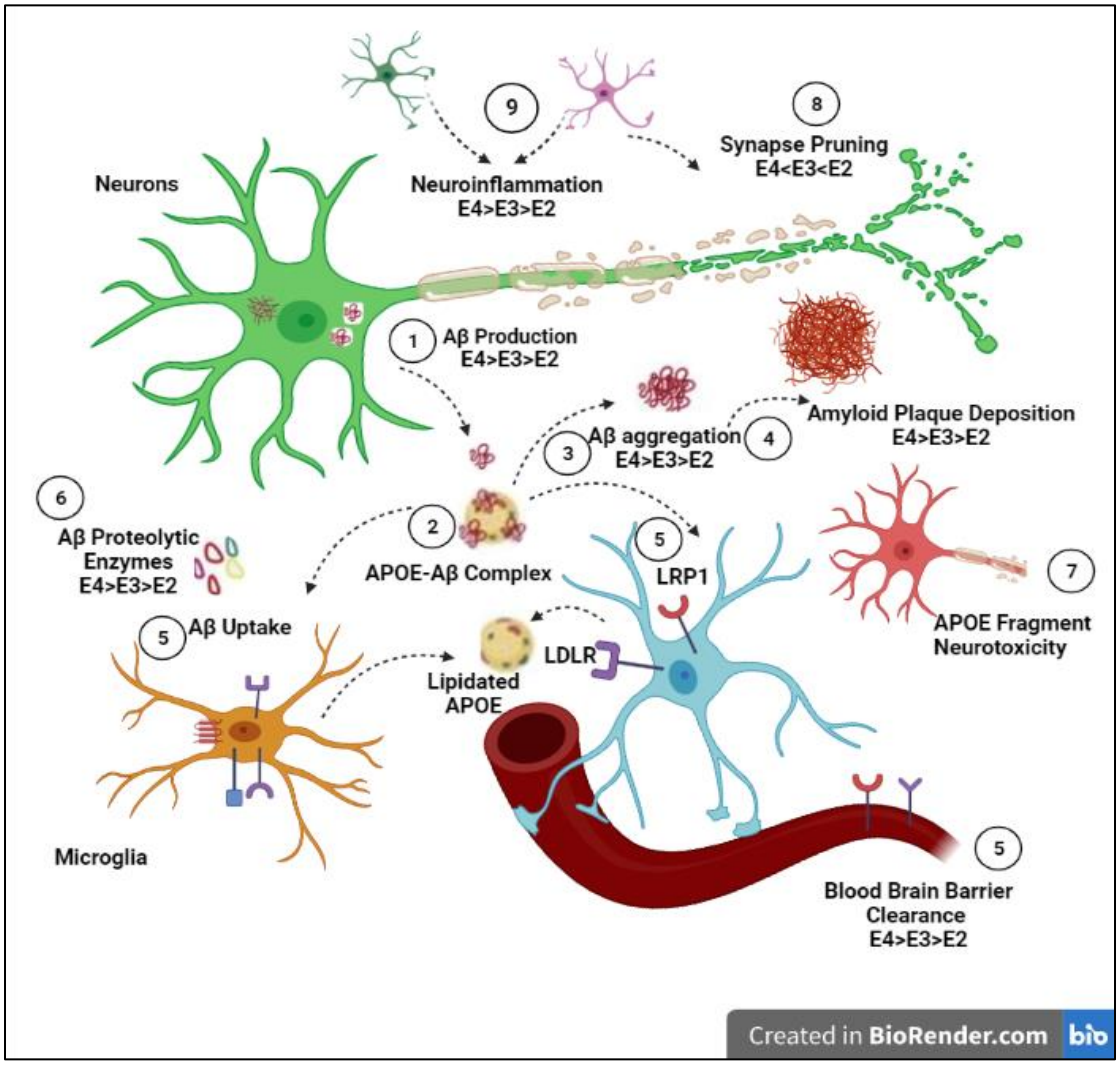

Figure 2: Role of APOE gene in Aβ deposition 1) APOE is secreted by the microglia and astrocytes 2) which interacts with Aβ. 3) in a manner that is isoform specific. APOE enhances the rate of Aβ accumulation 4) which subsequently deposits in the form of

senile plaques. 5) By the help of microglia and astrocytes, cell surface LDLR, heparan sulphate and LDLR-related protein 1 (LRP1) facilitate the endocytosis of Aβ. E4 promotes the aggregation of Aβ and slows down the clearance of Aβ by hindering the transport across BBB. 6) Over expression of E2 promotes the enzymatic degradation of Aβ by Insulin-degrading enzyme (IDE) and neprilysin. 7) Proteolysis of Aβ produces fragments that are neurotoxic.8) synaptic pruning capacity is reduced due to accumulation of C1q protein which subsequently induces neurodegeneration. 9) E4 causes enhanced production of cytokines which leads to neuroinflammation and neurodegeneration (Raman et al., 2020).

1.11. Aim of this study

AD cases are rising exponentially in Pakistan, however not much is being done to document the cases and to categorize them as AD or rpAD. Even though E4 gene is has a strong correlation with the development of AD, no study has been carried out to check the prevalence of APOE gene frequency in Pakistani population yet. General public seem to be unaware about the disease and lack appropriate scientific knowledge that is required to spot a potential AD patient in their family, which they mostly conflate with myths and superstitions making matters worse. Therefore, the objective of this study is to assess the frequency of APOE genotype in the population of Pakistan via peripheral blood samples and to estimate the prevalence, knowledge and myths related to AD through a well-designed questionnaire.

Chapter 2
Methods and Materials

2. Materials and Methodology

This chapter discusses the materials and methodology used in the entire course of the research study.

2.1. Blood Sample Collection

In total, 100 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. DNA extraction:

Commercially available DNA Extraction kit (Solar Bio Cat#D1800, China) was used in the extraction of genomic DNA. Each kit is designed to extract 50 DNA samples; hence two kits were used for the extraction of 100 DNA samples.

2.2.1. Protocol

In the first step, 200 µl blood was taken and pipetted into the centrifuge tube followed by 600 µl of lysis buffer for the lysis of red blood cells which was then centrifuged at 12000 rpm for 2 min. After centrifugation, a pellet was formed while the lysed supernatant was discarded. After discarding the supernatant, solution A (200µl) was added to the pellet and mixed carefully. After that, 20 µl of RNase A was added to the Eppendorf tube, it was vortexed and then placed in the incubator for approximately 10 minutes at rtp. Then, 20 µl of proteinase K was added, vortexed and incubated at 60 degrees for 45 minutes. The tubes were inverted several times during the process of digestion. Then, solution B (100µl) was added and mixed fully. Then, 1140µl of absolute ethanol was added in the tube, the mixture was then mixed thoroughly and was added to the adsorption column followed by incubation at rtp for 2 min. Then the mixture was centrifuged at 12000 rpm for 2 min, flow through was thrown away and the collection tube was used again. Adsorption column was washed with 600 µl of washing buffer and then placed in the centrifuge 2 min at 12000 rpm. Flow through was discarded again and the collection tube was reused. The preceding step was repeated. The sample was centrifuged at 2000 rpm for 2 minutes. The column was put in the heating block at 50 degrees for 10 minutes for it to completely dry. This step is critical

for the removal of ethanol from the column otherwise it will interfere with enzyme digestion and PCR. Then, 50µl of elution buffer was added and the sample was placed in the incubator at rtp for 5 minutes. The mixture was then centrifuged at 12000 rpm for 2 minutes. The solution obtained in the centrifuge tube after the centrifugation was the DNA. The presence of DNA was confirmed by gel electrophoresis. All these steps were repeated for 100 samples.

2.3. Determining DNA Quality and Quantity

The quantity and quality of the DNA was determined by Colibri NanoDrop (Titertek-Berthold, Germany).

2.4. Primers

Primers were selected from the published literature (Table 4).

Table 4: Primer Sequence. List of all the primers used in this study.

Name	Primer Sequence	Length (bp)	Temperature (°C)
E2 Forward	GCGGACATGGAGGACGTGT	19	63.2
E2 Reverse	CCTGGTACACTGCCAGGCA	19	62.7
E3 Forward	CGGACATGGAGGACGTGT	18	63.2
E3 Reverse	CTGGTACACTGCCAGGCG	18	64.0
E4 Forward	CGGACATGGAGGACGTGC	18	64.7
E4 Reverse	CTGGTACACTGCCAGGCG	18	64.0

2.5. Polymerase Chain Reaction (PCR)

2.5.1. Reaction Mixture

Reaction mixture for PCR product was made using commercially available master mix (Wizbio Solutions, cat#W1401-2, South Korea). A total of 25µl of reaction mixture was made (Table 5).

Table 5: List of PCR ingredients. List of all the ingredients along with their quantities used to make 25µl PCR mix.

	Ingredients	**Quantity (µl)**
1)	PCR Master Mix	12.5 µl
2)	Nuclease Free water	8.50 µl
3)	Forward Primer	1.00 µl
4)	Reverse Primer	1.00 µl
5)	DNA Template	2.00 µl
		=25 µl

2.5.2. Reaction Conditions

A total of 12.5 µl of PCR master mix (Wizbio Solutions, cat#W1401-2, South Korea), 8.5µl of Nuclease free water, 1µl of forward primer, 1µl of reverse primer and 2µl of DNA template 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 annealing temperatures of 55°C, 56°C, 57°C, 58°C and 59°C at 35 seconds (the temperature of the primers was calculated by adding the temperatures of forward and reverse primers and then dividing 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 of the respective alleles. These steps were repeated with all the 100 samples for E2, E3, and E4 respectively, therefore PCR was run 300 samples.

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 location of the bands against the DNA ladder. DNA ladder of 100-1500 base pairs was used.

2.6.1. For DNA (1% agarose)

For DNA, 1% of agarose (Invitrogen, cat#16500500, USA) gel was made. 50X TBE buffer (Solarbio, cat# T1060, China) was used.

2.6.2. For PCR product (2% agarose)

For PCR product, 2% of agarose (Sigma Aldrich, cat#39346, USA) gel was made. 10X TAE buffer (Solarbio, cat# T1051, China) was used.

2.6.3. Gel analysis

The gels were analyzed using Bio Rad ChemiDoc^{TM} XRS, serial number:721BR19365.

2.7. DNA Sequencing

To validate the results APOE genotyping, one sample from E3E3 genotype and one from E3E4 genotype were DNA sequenced via automated Sanger sequencing method.

2.7.1. Automated Sanger Sequencing protocol

A total of 30 µl PCR product containing 50 ng genomic DNA was taken and purified to remove contaminants and other impurities using Qiagen PCR cleanup kit, chain termination PCR was performed on 5µl DNA. The PCR cycling conditions included initial denaturation at 98ºC for 4 min, followed by 35 cycles at 98ºC for 10 sec, after that annealing was done at 60ºC for 30 sec, extension at 72ºC for 40 sec and final extension at 72ºC for 10 min. Each band in the capillary gel was read by the computer, fluorescent tags in each band are excited by laser resulting in the emission of light which is detected by the computer. The output can be seen on the chromatogram, on which different colored waves represent different bases.

2.8. Designing Questionnaire

The questionnaire was designed in accordance with cross-sectional study design to investigate public awareness, knowledge, and myths related to AD in general, and rpAD in particular. The questionnaire was designed in the light of several existing research papers and Alzheimer's Knowledge Scale developed by Carpenter (Carpenter et al., 2009) with only slight modifications. A total of 954 responses were collected from the google forms, the link of the form was posted and shared online in different groups with people who could read. Questions related to family history of AD were asked which can help in rough estimation of prevalence of AD in Pakistan. The questionnaire contained 28 items, including questions related to symptoms, general knowledge about AD and myths and assumptions surrounding AD patients. Sociodemographic data such as age, gender, living location (urban/rural) and educational background was also collected. In addition to that, questions related to the personal experiences of the respondents with AD patients (if any) were also asked. Questions related to family history of AD were asked which can help in rough estimation of prevalence of AD in Pakistan. Despite being comprehensive in nature, the language of the questions asked was kept simple and unequivocal to facilitate the patients in answering the questions without an ounce of difficulty.

2.8.1. Tool Validation

The questionnaire was designed and sent to the Clinical Department of Neurology, University of Medical Center Göttingen for verification. To make sure that the questionnaire designed was comprehensible for the masses, a small pilot survey was conducted, to assess whether the respondents understand the language and terminologies used in the questionnaire. The feedback of the respondents was duly acknowledged, and the required modifications were made.

Chapter 3

Results

3. Results

3.1.DNA Extraction (Nanodrop)

The quality and quantity of DNA of all 100 samples was measured using Colibri Nanodrop. The DNA extracted from the blood samples had an A_{260}/A_{280} ratio between 1.5 to 1.7 and the quantity was approximately ± 50 ng/µL.

3.2. PCR results (gels)

3.2.1. E2, E3 and E4 Genotype:

The representative gels in Figure 3 show that E3 was the most prevalent allele present in homozygous pattern in 95 samples and in heterozygous pattern in 5 samples. E4 allele was the 2nd most prevalent allele, but it was present in only heterozygous pattern in 5 samples as E3E4. E2 allele was completely absent in the given population (Appendix table 1).

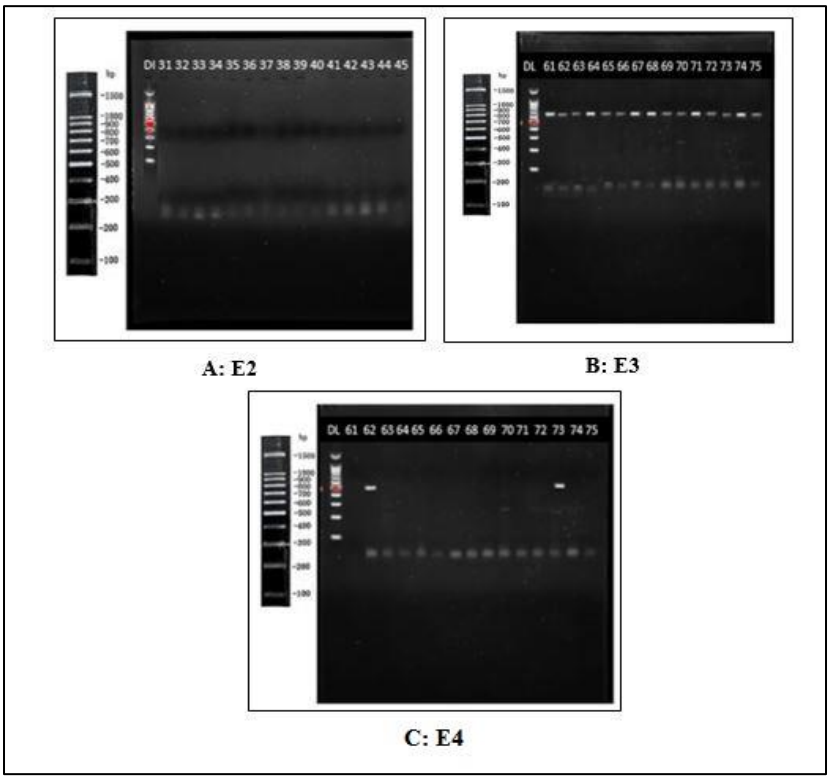

Figure 3: Gel electrophoresis results. The figure shows representative gels for E2, E3, and E4. Gel A shows that no band could be seen for E2 allele, therefore E2 allele was absent in all 100 samples. Gel B shows that E3 allele was present in all the 100 samples in homozygous pattern, except for 5 samples in which it is present in heterozygous pattern with E4 allele. Gel C shows that E4 was present in only 5 samples in heterozygous pattern E3E4.

3.3. Allele Frequency

As mentioned in Table 7, the most prevalent allele in 100 samples was E3, it was present in all the samples with and allele frequency of 0.975 (97.5%). The least prevalent allele was E2, which was completely absent in the entire sample size with allele frequency of 0. E4 allele was present in 5 samples with an allele frequency of 0.025 (2.5%).

Table 6: Allele Frequency. The table shows the frequency of APOE alleles in the given dataset (n=100). E3 allele was found to be the most prevalent followed by E4. E2 allele was completely absent from the dataset.

Frequency of APOE alleles n=100			
Alleles	**Number alleles present in n**	**Ratio of no. of allele present in n to total no. of alleles (200).**	**Allele frequency**
E2	0	0/200	0
E3	195	195/200	0.975
E4	5	5/200	0.025
			=1.00

3.4. Frequency of APOE Genotypes

The most prevalent genotype was E3E3 with genotype frequency of 0.95 (95%). E2/E2, E2E3, E2E4, and E4E4 genotypes were completely absent. E3E4 genotype was the second most prevalent with an allele frequency of 0.05 (5%). The summary of APOE genotypes is depicted in Table 8 below.

Table 7: Genotype Frequency. The table shows the genotype frequencies of APOE isoforms in the given population size (n=100). E3E3 was found to be the most prevalent genotype followed by E3E4. E2E2, E2E3, E2E4 and E4E4 genotypes were completely absent.

Frequency of APOE Genotypes			
Genotype	**Number of individuals**	**Ratio of genotype to total**	**Genotype Frequency**
E2E2	0	0/100	0
E2E3	0	0/100	0
E2E4	0	0/100	0
E3E3	95	95/100	0.95
E3E4	5	5/100	0.05
E4E4	0	0/100	0
Total	n=100		1.00

3.5. DNA Sequencing

The results obtained from the DNA sequencing was verified in BLAST (blastn, NCBI), it showed similarity with the APOE 3 and 4 alleles (Figure 4). The chromatogram for the respective sequence was also obtained in the DNA sequencing results (Figure 5).

E3

CGGACATGGAGGACGTGTGCGGCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTC
GGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCG
GCTCCTCCGCGATGCCGATGACCTGCAGAAGCGCCTGGCAGTGTACCAGGCCGGGGCCCGCG
AGGGCGCCGAGCGCGGCCTCAGCGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGGGCCCA
GGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCCGGACCCGCGACCGC
CTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTGGAGGAGCAGGCCCAGC
AGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAAGAGCTGGTTCGAGCCCCTGGTGG
AAGACATGCAGCGCCAGTGGGCCGGGCTGGTGGAGAAGGTGCAGGCTGCCGTGGGCACCAGC
GCCGCCCCTGTGCCCAGCGACAATCACTGAACGCCGAAGCCTGCAGCCATGCGACCCCACGC
CACCCCGTGCCTCCTGCCTCCGCGCAGCCTGCAGCGGGAGACCCTGTCCCCGCCCCAGCCGTC
CTCCTGGGGTGGACCCTAGTTTAATAAAGATTCACCAAGTTTCACGCACTGGTACACTGCCA
GGCG

E4

CGGACATGGAGGACGTGCGCCTACAAATCGGAACTGGAGGAACAACTGACCCCGGTGGCGG
AGGAGACGCGGGCACGGCTGTCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGA
CATGGAGGACGTTGCGGCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCA
GAGCACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCT
CCGCGATGCCGATGACCTGCAGAAGCICCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGGCG
CCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGGGCCGCGTG
CGGGCCGCCACTTGGGCTCCCTGGCCGGCCAGCCCTACAGGAGCGGGCCCAGGCCTGGGGCG
AGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCTGGTACACTGCCAGGCG

Figure 4: DNA sequencing results. The above figure shows the sequence of E3 and E4 isomers of APOE gene amplified using E3's F-CGGACATGGAGGACGTGT, R-CTGGTACACTGCCAGGCG, and E4's F-CGGACATGGAGGACGTGC, R-CTGGTACACTGCCAGGCG primers.

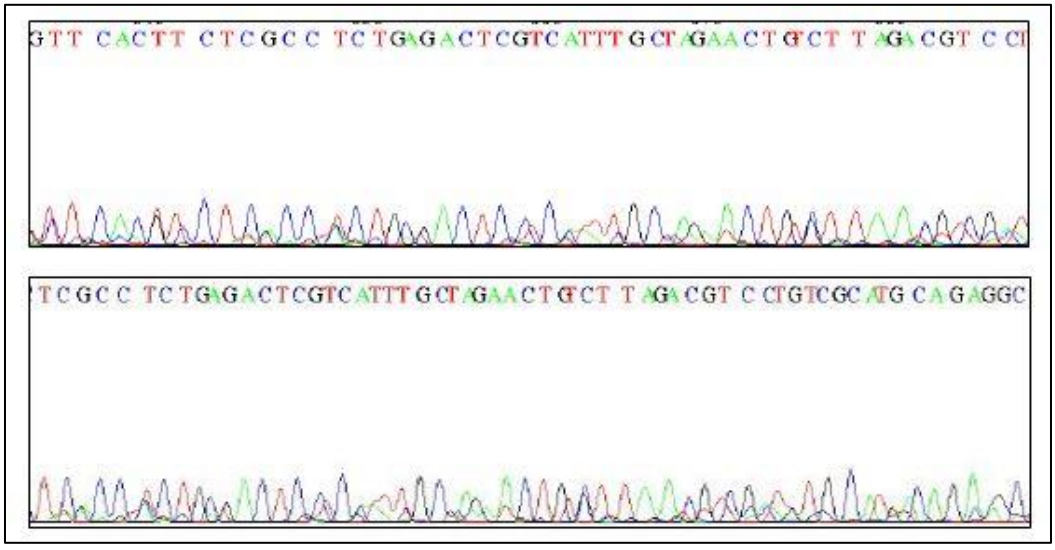

Figure 5: Chromatogram. Representative chromatogram for E3 and E4 alleles.

3.6. Questionnaire Results
3.6.1. Demographic details

A total of 954 individuals answered the questionnaire with age range from 25–64-year-old (Figure 6A). Majority of the respondents were females with the percentage of 72.6% (Figure 6B). Most of the participants (90%) were either studying in the university or graduated (Figure 6C). An overwhelming majority of the population belonged to the city when asked about the type of residence (Figure 6D).

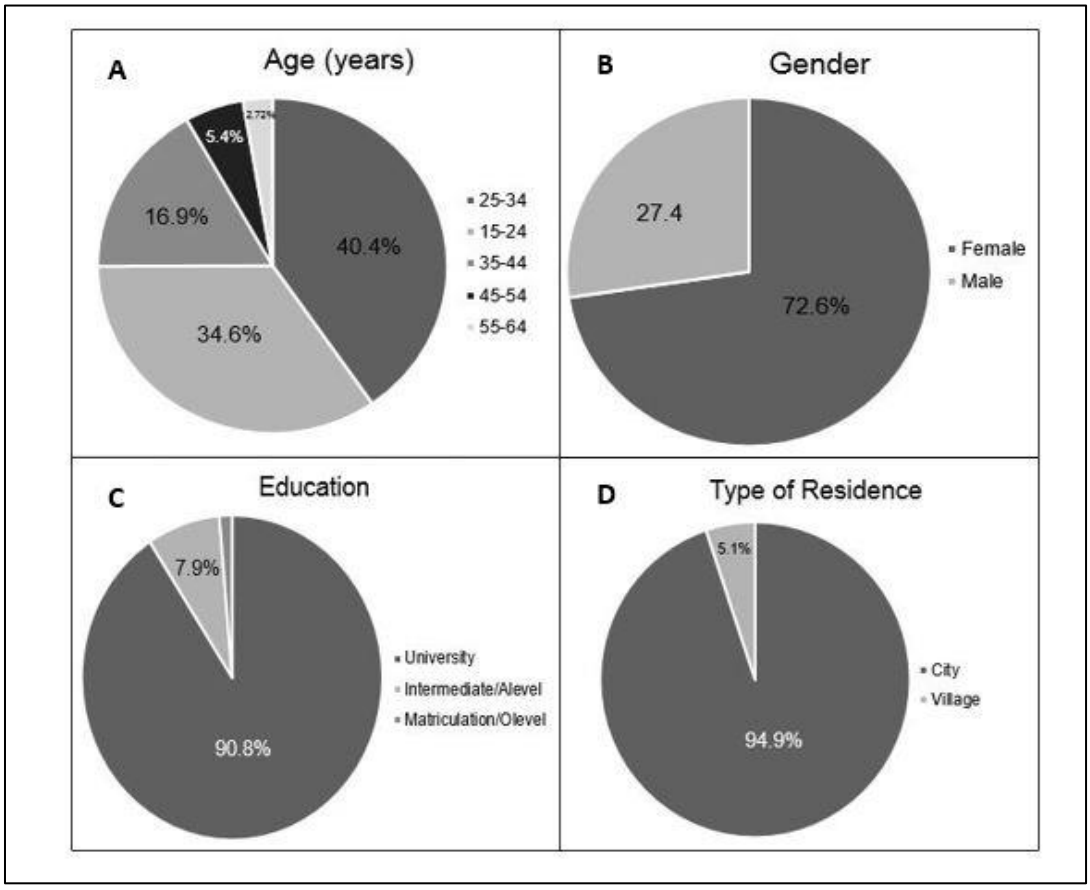

Figure 6: Demographic details of the respondents. A represents that vast majority of the participants were from the age group of 25-34 years. The least number of responses were from the age group 55-64 years. **B** shows majority of the participants were females with 72.6% response rate as compared to males with only 27.4%. **C** shows the education of the respondents, 90.8% respondents were either enrolled in a university currently or were graduated. **D** shows the type of residence of the respondents, 94.9% of the respondents lived in the urban areas while only 5.1% were from rural areas.

3.6.2. General information regarding AD and rpAD

Respondents were asked whether they were aware of the terms AD (Figure 7A) and rpAD (Figure 7B) and if they had family history of memory loss (Figure 7E). Participants were also asked if they ever had an interaction with someone suffering from dementia (Figure 7C) or if they knew about someone who died after being diagnosed with dementia (Figure 7D).

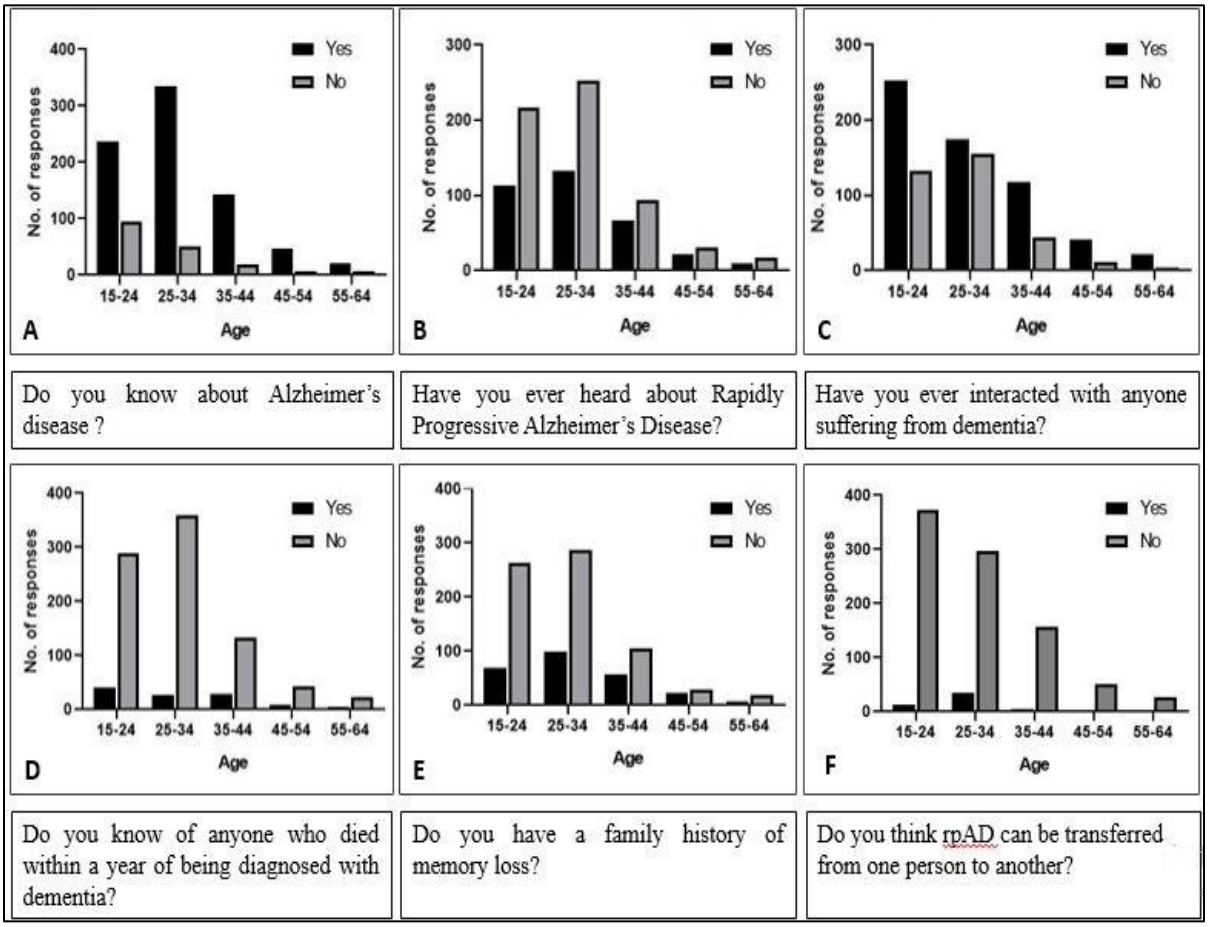

Figure 7: General information regarding AD and rpAD. Almost 81.7% **(A)** respondents were aware of AD while only 36.1% **(B)** knew about rpAD. **C** illustrates that 63.7% respondents have interacted with people with dementia. **D**, **E** and **F** show that majority of the respondents answered no to the respective questions.

3.6.3. Knowledge about AD risk factors

Respondents were asked about risk factors associated with AD. An overwhelming majority of respondents were not aware of the risk factors, Figure 8 below illustrates the answers of the participants.

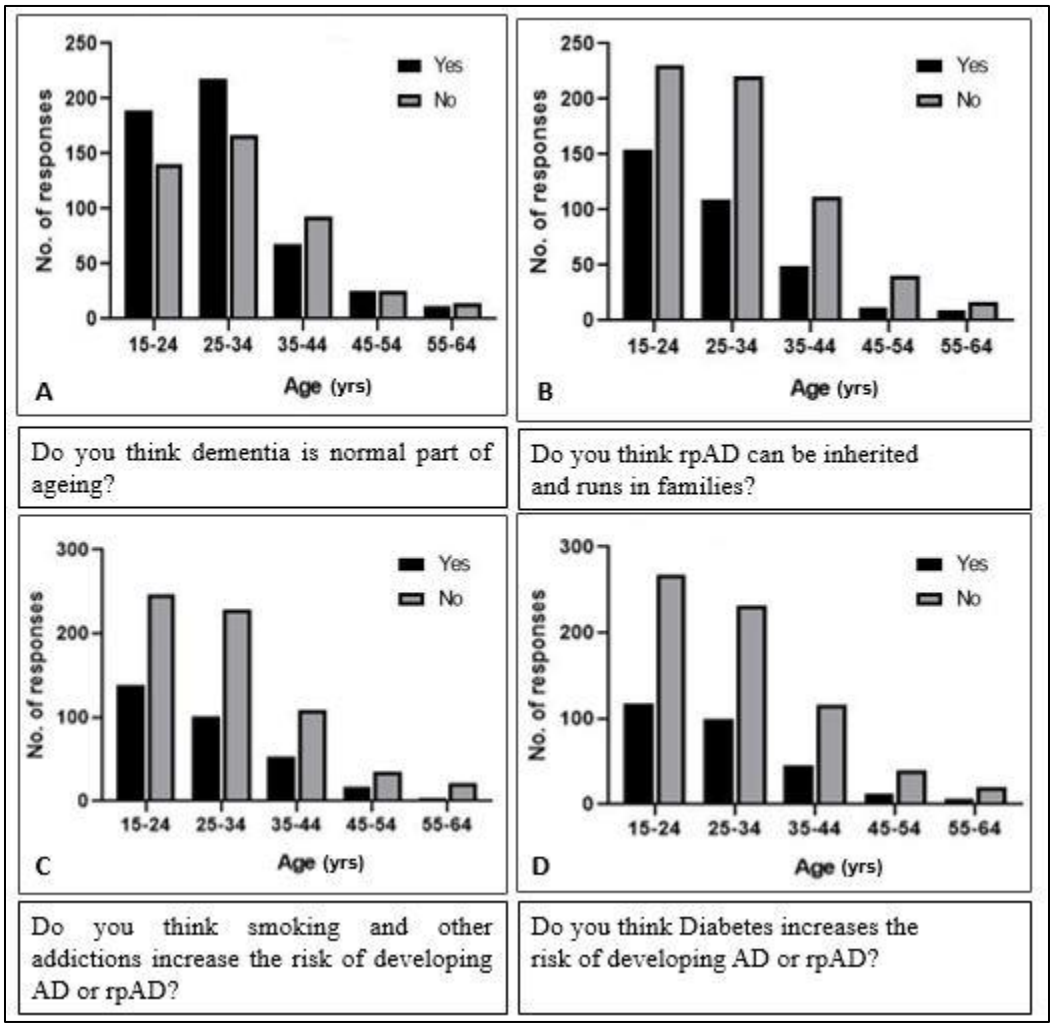

Figure 8: Knowledge about Risk Factors. A show that a total of 53.8% respondents believe that dementia is a natural part of aging while only 46.2% believed otherwise. **B** shows that majority of the respondents (65.1%) believe that AD cannot be inherited and does not run in families, while only 34.9% believed the opposite. **C** shows that 67.1% respondents believe that smoking is a risk factor for AD, while only 32.9% believed otherwise. **D** shows that an overwhelming majority of 70.6% respondents believed that diabetes is a risk factor for AD.

3.6.4. Knowledge about AD symptoms

A total of 954 respondents answered questions related to symptoms of AD. Majority of the respondents were aware of the symptoms exhibited by AD patients (Figure 9).

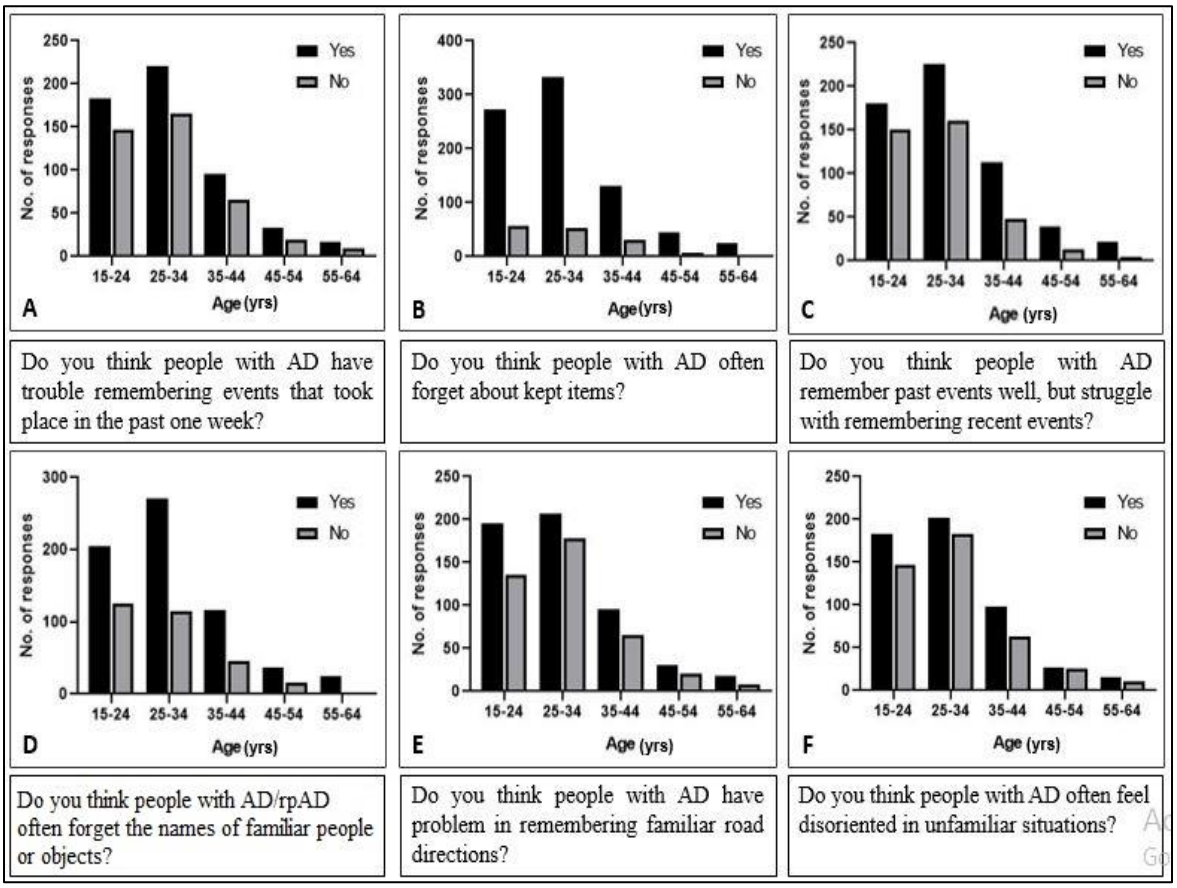

Figure 9: Knowledge about Symptoms. A shows that for that question a total of 57.5% respondents answered yes while 42.5% answered no. **B** shows that an overwhelming majority of 84.3% answered yes and only 15.7% respondents answered no. **C** shows that a total of 60.7% of the respondents answered in affirmation and only 39.3% answered no. **D** shows that majority (68.3%) of the respondents answered yes while only 31.7% answered no. Graph E shows that a total of 57.3% respondents answered yes while 42.7% answered no. Graph F shows about 55.1% respondents answered yes while 44.9% answered no.

3.6.5. Knowledge about Treatment and Diagnosis:

Questions regarding the treatment options and diagnosis were also asked from the participants. An overwhelming majority of people were not aware about any particular treatment option or diagnostic criteria available to treat AD (Figure 10).

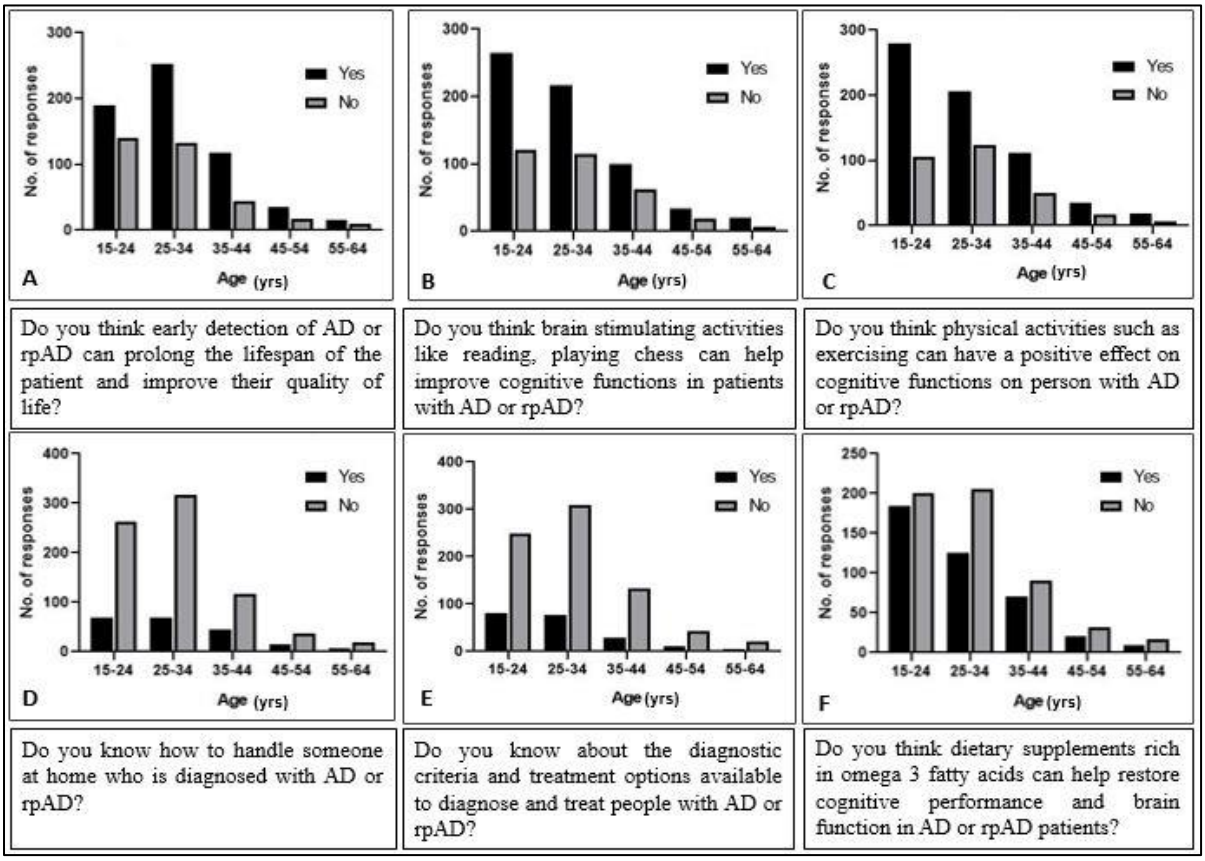

Figure 10: Knowledge about Treatment and Diagnosis. A show that majority of the respondents (64%) believed that early detection of rpAD can prolong the lifespan of AD patients. **B** and C show that 66.45% and 68.2% respondents believe that brain stimulating, and physical activities have a positive effect on the cognitive functions of the AD patient respectively. **D** shows that majority of the respondents (78.7%) did not know how to handle someone with rpAD at home, only 21.3% knew how to do so. **E** shows that Only 21.1% respondents claimed to be aware about the diagnostic criteria and treatment options available, 78.9% respondents did not know. **F** shows that majority of the respondents with an overall percentage 57.2% believed that dietary supplements cannot help in restoring cognitive functions in people with AD while 42.8% believed otherwise.

3.6.6. Myths related to AD

Questions related to myths related to AD were also asked. An overwhelming majority of the respondents did not believe in these results.myths, pertaining to the fact that they were educated segment of the society. Figure 11 depicts these results.

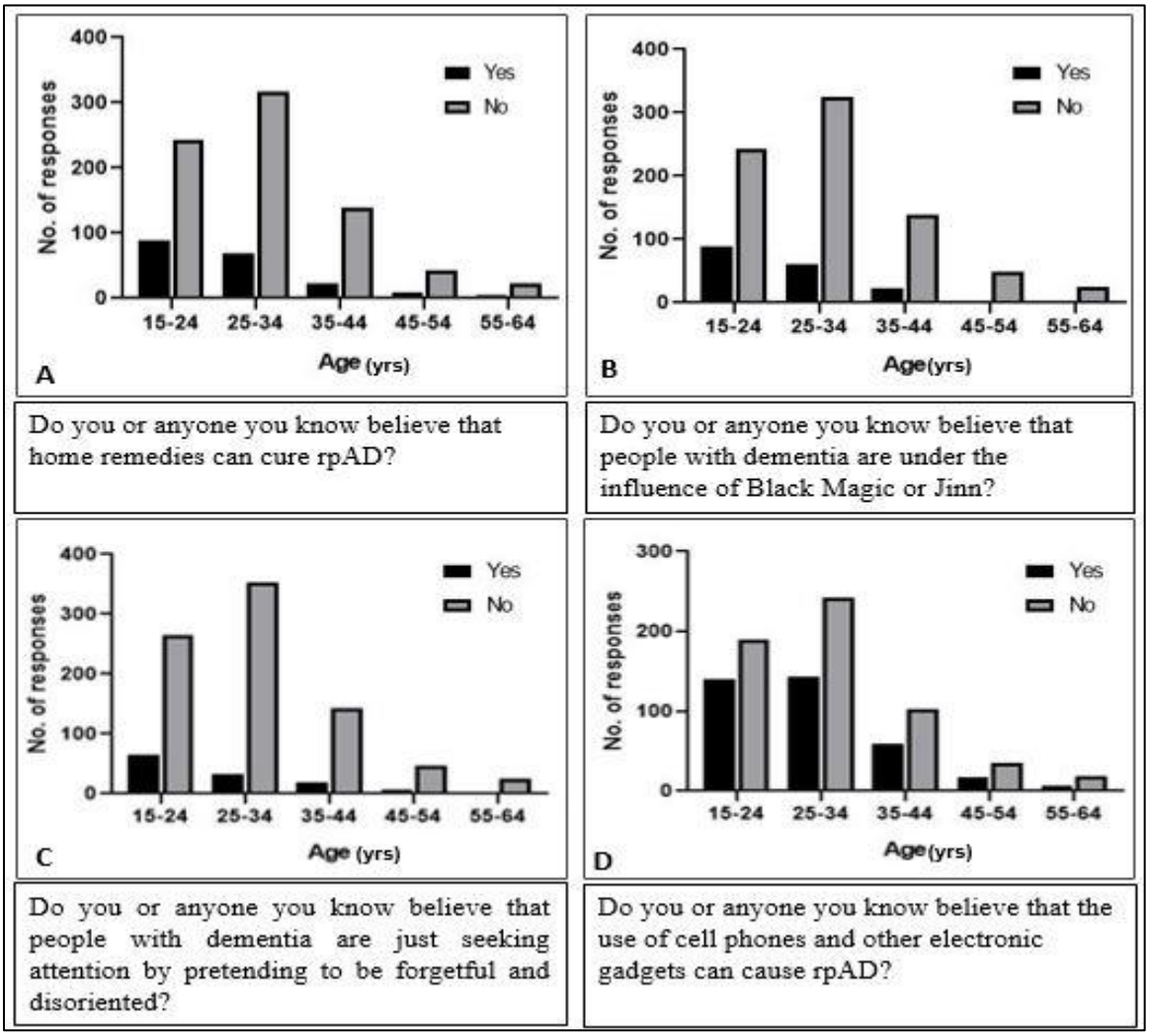

Figure 11: Myths related to AD. A show that an overwhelming majority of 80% do not believe that home remedies can cure rpAD. **B** shows that vast majority of 81.7% respondents do not believe in the superstition that AD is caused by black magic. **C** shows that majority of the people do not believe that AD patients are seeking attention by pretending to be forgetful. **D** shows that 61.6% respondents believe that the use use of electronic gadgets do not cause rpAD.

Chapter 4

Discussion

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, APOE polymorphism is one of the major risk factors for AD (Huynh et al., 2017). There are three alleles of APOE gene E2, E3, and E4. The presence of different isoforms of APOE gene in different genotypical combinations will decide how susceptible is the person to developing AD in the future. AD affects the patient's quality of life severely and makes even basic chores undoable, therefore it is imperative for people to be aware of the disease and stay clear of the myths surrounding it (Jiang et al., 2008). The present study is the first one to assess the presence of APOE genotype in Pakistani population with relevance to AD, no study of this sort has been carried out in Pakistan before. In this study, a detailed survey was also conducted to assess the knowledge of AD in Pakistani people.

E2 gene has a protective role against AD, and it was first discovered in 1994 in a study that documented that AD patients had low frequency Of E2 allele (Maciej Serda et al., 1994). The frequency of E2 allele in healthy population worldwide is only 8.4 % and in AD patients it is 3.9% (C. C. Liu et al., 2013a). E2 is known to reduce Aβ pathology in humans, the autopsy of AD patients carrying E2 allele showed lower density of Aβ containing senile plaques when compared to E3E3 (Nagy et al., 1995). PET imaging also confirmed that Aβ accumulation happens at much lower rate in non-demented individuals carrying E2 allele as compared to E3E3 homozygotes (Jansen et al., 2015). Previous studies conducted on humans show that E2 is involved in low Aβ pathology which indicates that E2 reduces the risk of developing AD through Aβ-dependent pathways (Z. Li et al., 2020). In this study, the genotyping for E2 allele was done using PCR, but E2 allele was not found in any combination in any of the 100 subjects. The frequency of E2 was found to be 0 and the genotype frequency was also 0. Therefore, the absence of E2 allele in non-demented individuals indicate that they are deprived of the protective role that may have been provided by E2 if present. This increases the risk of developing AD in Pakistani population.

E3 is the most common isoform of APOE gene with 77.9% prevalence in general population worldwide and 59.45 in AD patients (C. C. Liu et al., 2013a). It is believed to play a neutral role with respect to AD (Frieden & Garai, 2012). E3E3 is the most common genotype of E3 genotype worldwide (de-Almada et al., 2012). In this study, the findings indicate that all 100 samples contain E3 allele. The allele frequency of E3 allele in 100 samples was 100%. The most common genotype in the subjects was E3E3 with 95% prevalence, the second most common genotype was E3E4 with 5% prevalence. Although, E3 does not play any role in the development of AD, the absence of protective E2 and the presence of E3 with E4 indicates that there is a relatively high risk of AD among the subjects as compared to if they had E2 allele.

E4 is the major risk factor for AD (M. Di Battista et al., 2016) and the second most prevalent isoform of APOE genotype after E3. Allele frequency of E4 allele among general population worldwide is 13.7% and among AD patients, the allele frequency is 36.7% (C. C. Liu et al., 2013b). The probability of AD in individuals with one E4 allele increases by 2-3-fold while individuals who have two E4 alleles in homozygous pattern. have 10-15-fold increased risk of developing AD. E4 allele is involved in exacerbation of Aβ deposition (Tachibana et al., 2019). In this study, 5% of subjects were positive for E4 allele in heterozygous form E3E4. E2/E4 and E4/E4 genotype was completely absent. The genotype frequency of E4 allele among 100 subjects was 5% which suggest that 5% of the subject population is at risk of developing AD.

While increase frequency of E4 allele increases the risk of AD, decreased frequency is believed to increase the risk of rpAD (Cohen et al., 2015), however full consensus on this theory has not been reached yet. Considering the aforementioned theory, when the allele frequency of E4 allele is lower, the chances of developing rpAD increases, in our study, E4 allele is only present as E3E4 in 5 people and the rest of the people were positive for E3E3 and none of them was positive for E4/E4 which indicates low frequency of E4 allele. With the complete absence of protective E2 allele and presence of E4 allele in heterozygous condition may increase the chance of developing rpAD. Since E3 is a neutral allele neither involved in causing AD nor protecting against it, with the absence of E2 allele from the population, the entire 95% population is at the risk of developing AD, but

the risk is low. 5% population is at the greater risk of developing AD since it contains E4 allele.

This is the first study that has been conducted in Pakistan to assess the knowledge of Pakistani people regarding AD to date. A total of 954 responses were collected within the age range of 15-64 years old with majority of the respondents between 25-34 years of age (40.4%). 72.6% of the total respondents were females and the remaining 27.4% were males. When asked whether dementia is normal part of ageing or not, 53.8% of the respondents answered in yes which is consistent with previous studies carried out globally showing people have this misconception that dementia is normal part of ageing which is not true (Pacifico et al., 2022). 63.7% of the respondents claimed that they have interacted with someone suffering from dementia. 26.2% of the respondents had a history of memory loss. Some questions related to symptoms involving memory were also asked. 60.7 respondents answered that they remember past events really well as compared to the recent events. 84.3% of the respondents answered that they often forget about kept items and 68.3% confirmed that they often forget the names of familiar people and objects while 57.3% admitted that they have a problem in remembering familiar road directions. This shows that dementia is perceived as a vague concept of memory decline and is being confused with everyday short term memory loss (Nielsen & Waldemar, 2016).

When asked about AD in particular, 81.7% respondents claimed that they've heard about the disease, which is consistent with the study carried out in China (D. Liu et al., 2019), while only 36.1% knew about rpAD. Only 5% of the respondents believed that AD could be transmitted from one person to another. Currently there is no example of AD being transmitted from one person to another via surgical procedures, however, transmissible AD theory is gaining traction but consensus among the scientific community has not been reached yet (Abbott, 2018).

Questions related to risk factors were also asked. Although family history is not necessary in the development of AD, but AD can run in families, if one of the parents suffers from Alzheimer's, the chances of their kids having AD also increases (Davies, 1986), when asked 34.9% respondents believed that AD can be inherited while 48.7% were unsure and answered 'maybe'. When asked whether smoking and other addictions and diabetes

increase the risk of developing AD, 32.9% responded 'yes', 22.2% said no while the remaining were unsure. According to various research studies, smoking considerably increases the risk of developing dementia (30%) and AD (40%) (Zhong et al., 2015), while diabetes is also categorized as the major risk factor for AD (Hölscher, 2011). The answers of the respondents reflected poor knowledge regarding the risk factors that are so important in causing AD, the population of Pakistan needs awareness on this matter.

When asked about the diagnostic criteria and treatment options available for AD, an overwhelming majority of respondents (78.9%) were unaware about it. Majority of the respondents were university graduates and still they did not know about diagnostic criteria and treatment options, this shows a lot of work needs to be done to educate the masses regarding the disease that is so prevalent. Most respondents believed that physical exercise (68.2%), and brain stimulating activities (66.4%) can help improve the cognitive functions of AD patients, however only 42.8% of the respondents believed that taking omega 3 fatty acids can help with cognitive performance of AD patients even though research shows that omega 3 fatty acids can help improve brain function (Ajith, 2018).

In Pakistan, many neuropsychological conditions are attributed to black magic, so a question related to this was also added, only 18.3 percent believed that people suffering from AD are under the influence of black magic. Since majority of the respondents were well educated, the responses related to this particular question are not representative of the entire population, people who live in villages believe otherwise. Majority (61.6%) of the respondents believe that mobile phones and other electronic gadgets do not have any role to play in the development of AD, which is consistent with other studies, no evidence has yet been found that confirm electronic gadgets' role in causing AD (Mortazavi et al., 2013). Regarding the behavior related to AD patients, 58.5% believed that people with AD are more unpredictable, violent, and aggressive as compared to people without AD, which is true, the cognitive decline increases aggressive and violent behavior in AD patients (Yu et al., 2019).

In certain non-technical areas, the respondents exhibited good knowledge about the AD, but in the technical aspects where risk factors, diagnostic criteria and treatment options related to the disease were involved, the participants displayed poor knowledge, this

shows that even the educated people in Pakistan are unaware about the basic knowledge of the disease. It is imperative to establish organizations that can create awareness among the masses regarding AD which is exponentially increasing in Pakistan.

Chapter 5
Conclusion

5. Conclusion

The findings of this study indicate that the population of Pakistan is at the risk of developing AD and rpAD since the protective E2 allele is completely absent from the population and the most prevalent genotype is E3E3 which is neutral i.e., it neither plays any part in protecting against the disease or causation of the disease. E4 allele which is a key risk factor for AD is present in 5 individuals as E3E4 genotype which puts them at the risk of developing AD. RpAD is believed to be caused by low frequency of E4 allele, since none of the subjects tested positive for E4/E4 genotype this shows that E4 allele is present, but in low frequency which subsequently increases the risk of rpAD in population. This study identifies the risk at a microscopic level, with a greater number of samples, the study can be carried out at a massive scale, which will give an accurate idea regarding the gravity of the situation. Even though, Pakistani population is at the risk of developing AD, very few people are aware of the technical aspects of the disease which includes diagnosis and treatment, as suggested by the questionnaire that we conducted. It is imperative and the need of the hour for the government to develop institutes and centers to promote awareness regarding AD among the people of Pakistan.

6. APPENDICES

6.1. Appendix A

PCR results

PCR was performed on 100 samples, as depicted in Table 6, the results showed that E2 allele was completely absent from the given population in any of the genotype form. E3 was the most prevalent allele and was present in E3E3 and E3E4 pattern, while E4 was the second most prevalent allele present in E3E4 pattern.

Appendix Table 1: PCR results. The table shows the APOE genotype present in each of the 100 samples, E2 allele was absent in all the subjects, 96 samples were homozygous for E3 allele and only 5 samples were positive for E4 allele (E3E4).

Sample	E2	E3	E4	Genotype
1	-	+	-	Homozygous E3E3
2	-	+	-	Homozygous E3E3
3	-	+	-	Homozygous E3E3
4	-	+	-	Homozygous E3E3
5	-	+	-	Homozygous E3E3
6	-	+	-	Homozygous E3E3
7	-	+	-	Homozygous E3E3
8	-	+	-	Homozygous E3E3
9	-	+	-	Homozygous E3E3
10	-	**+**	**+**	**Heterozygous E3E4**
11	-	+	-	Homozygous E3E3
12	-	+	-	Homozygous E3E3
13	-	+	-	Homozygous E3E3
14	-	+	-	Homozygous E3E3
15	-	+	-	Homozygous E3E3
16	-	+	-	Homozygous E3E3
17	-	+	-	Homozygous E3E3
18	-	+	-	Homozygous E3E3
19	-	+	-	Homozygous E3E3
20	-	+	-	Homozygous E3E3
21	-	+	-	Homozygous E3E3
22	-	+	-	Homozygous E3E3
23	-	+	-	Homozygous E3E3
24	-	+	-	Homozygous E3E3
25	-	+	-	Homozygous E3E3
26	-	+	-	Homozygous E3E3
27	-	+	-	Homozygous E3E3
28	-	+	-	Homozygous E3E3
29	-	+	-	Homozygous E3E3

30	-	+	-	Homozygous E3E3
31	-	+	-	Homozygous E3E3
32	-	+	-	Homozygous E3E3
33	**-**	**+**	**+**	**Heterozygous E3E4**
34	-	+	-	Homozygous E3E3
35	-	+	-	Homozygous E3E3
36	-	+	-	Homozygous E3E3
37	-	+	-	Homozygous E3E3
38	-	+	-	Homozygous E3E3
39	-	+	-	Homozygous E3E3
40	-	+	-	Homozygous E3E3
41	-	+	-	Homozygous E3E3
42	-	+	-	Homozygous E3E3
43	-	+	-	Homozygous E3E3
44	-	+	-	Homozygous E3E3
45	-	+	-	Homozygous E3E3
46	-	+	-	Homozygous E3E3
47	-	+	-	Homozygous E3E3
48	-	+	-	Homozygous E3E3
49	-	+	-	Homozygous E3E3
50	-	+	-	Homozygous E3E3
51	-	+	-	Homozygous E3E3
52	-	+	-	Homozygous E3E3
53	-	+	-	Homozygous E3E3
54	-	+	-	Homozygous E3E3
55	-	+	-	Homozygous E3E3
56	-	+	-	Homozygous E3E3
57	-	+	-	Homozygous E3E3
58	-	+	-	Homozygous E3E3
59	-	+	-	Homozygous E3E3
60	-	+	-	Homozygous E3E3
61	-	+	-	Homozygous E3E3
62	**-**	**+**	**+**	**Heterozygous E3E4**
63	-	+	-	Homozygous for E3E3
64	-	+	-	Homozygous for E3E3
65	-	+	-	Homozygous for E3E3
66	-	+	-	Homozygous for E3E3
67	-	+	-	Homozygous E3E3
68	-	+	-	Homozygous E3E3
69	-	+	-	Homozygous E3E3
70	-	+	-	Homozygous E3E3
71	-	+	-	Homozygous E3E3
72	-	+	-	Homozygous E3E3
73	**-**	**+**	**+**	**Heterozygous E3E4**
74	-	+	-	Homozygous E3E3

75	-	+	-	Homozygous E3E3
76	-	+	-	Homozygous E3E3
77	-	+	-	Homozygous E3E3
78	-	+	-	Homozygous E3E3
79	-	+	-	Homozygous E3E3
80	-	+	-	Homozygous E3E3
81	-	+	-	Homozygous E3E3
82	-	+	-	Homozygous E3E3
83	-	+	-	Homozygous E3E3
84	-	+	-	Homozygous E3E3
85	-	+	-	Homozygous E3E3
86	-	+	-	Homozygous E3E3
87	-	+	-	Homozygous E3E3
88	-	+	-	Homozygous E3E3
89	-	+	-	Homozygous E3E3
90	-	+	-	Homozygous E3E3
91	-	+	-	Homozygous E3E3
92	-	+	-	Homozygous E3E3
93	-	+	-	Homozygous E3E3
94	-	+	-	Homozygous E3E3
95	-	+	-	Homozygous E3E3
96	-	+	-	Homozygous E3E3
97	**-**	**+**	**+**	**Heterozygous E3E4**
98	-	+	-	Homozygous E3E3
99	-	+	-	Homozygous E3E3
100	-	+	-	Homozygous E3E3

6.2. Appendix B

Questionnaire

Public awareness, knowledge and, myths about Rapidly Progressive Alzheimer's Disease (rpAD) in Pakistan

Age

- 15-24
- 25-34
- 35-44
- 45-54
- 55-64

Gender

- Male
- Female

Education

- Matriculation/Olevel
- Intermediate/Alevel
- University

Type of residence

- City
- Village

1. Do you think dementia is normal part of ageing?

- Yes
- No

2. Have you ever interacted with anyone suffering from dementia?

- Yes
- No

3. Do you have a family history of memory loss?

- Yes

- No

4. Do you think people with AD/rpAD have trouble remembering events that took place in the past one week?

- Yes
- No

5. Do you think people with AD/rpAD often forget about kept items?

- Yes
- No

6. Do you think people with AD/rpAD remember past events well, but struggle with remembering recent events?

- Yes
- No

7. Do you think people with AD/rpAD often forget the names of familiar people or objects?

- Yes
- No

8. Do you think people with AD/rpAD have problem in remembering familiar road directions?

- Yes
- No

9. Do you think people with AD/rpAD often feel disoriented in unfamiliar situations?

- Yes
- No

10. Do you know about Alzheimer's disease (AD)?

- Yes
- No

11. Have you ever heard about Rapidly Progressive Alzheimer's Disease? (rpAD)?

- Yes
- No

12. Do you know of anyone who died within a year after being diagnosed with dementia?

- Yes
- No

13. Do you think rpAD can be inherited and runs in families?

- Yes
- No

14. Do you think rpAD can be transferred from one person to another?

- Yes
- No

15. Do you know early detection of rpAD can prolong the lifespan of the patient and improve their quality of life?

- Yes
- No

16. Do you think smoking and other addictions increase the risk of developing rpAD?

- No
- Yes

17. Do you think brain stimulating activities like reading, playing chess can help improve cognitive functions in patients with rpAD?

- Yes
- No

18. Do you think physical activities such as exercising can have a positive effect on cognitive functions on person with rpAD?

- Yes
- No

19. Do you think dietary supplements rich in omega 3 fatty acids can help restore cognitive performance and brain function in rpAD patients?

- Yes
- No

20. Do you think Diabetes increases the risk of developing rpAD?

- Yes
- No

21. Do you or anyone you know believe that home remedies can cure rpAD?

- Yes
- No

22. Do you know how to handle someone at home who is diagnosed with rpAD?

- Yes
- No

23. Do you know about the diagnostic criteria and treatment options available to diagnose and treat people with rpAD?

- Yes
- No

24. Do you or anyone you know believe that people with dementia are under the influence of Black Magic or Jinn?

- Yes
- No

25. Do you or anyone you know believe that people with dementia are just seeking attention by pretending to be forgetful and disoriented?

- Yes
- No

26. Do you think people suffering from dementia are unpredictable and are more prone to being violent and aggressive?

- Yes
- No

27. Do you think people suffering from dementia are stigmatized in our society?

- Yes
- No

28. Do you or anyone you know believe that the use of mobile phones and other electronic gadgets can cause rpAD?

- Yes
- No

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