Genetic Screening for the Prevalence of PRNP Mutations in Pakistan

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Table of Contents

List of Table

List of Figures

Abbreviations

TSE	Transmissible spongiform encephalopathies
PrP^{c}	Normal cellular prion protein
PrP^{Sc}	Pathogenic/scrapie prion protein
aa	Amino acid
GPI	Glycosylphosphatidylinositol
CJD	Creutzfeldt-Jakob disease
vCJD	Variant creutzfeldt-jakob disease
sCJD	Sporadic creutzfeldt-jakob disease
iCJD	Iatrogenic creutzfeldt-jakob disease
gCJD	Genetic creutzfeldt-jakob disease
Hgh	Human growth hormone
GSS	Grestmann-straussler-scheinker syndrome
FFI	Fatal familial insomnia
CSF	Cerebrospinal fluid
OPRI	Octapeptide repeats insertion
Rpd	Rapidly progressive dementia
ADKS	Alzheimer disease knowledge scale
M	Methionine
V	Valine
E	Glutamic acid
K	Lysine
EEG	Electroencephalography

Abstract

Prion diseases are highly contagious, rapidly progressive, and extremely life-threatening neuronal condition. CJD is the prime example of prion disease. Many cases of CJD have been reported up till now. It can be acquired, inherited or sporadic in nature. sCJD accounts for the highest prevalence globally i.e., 85% of the cases, gCJD encompasses 10-15% of the cases whereas iCJD cases include 1-2%. Its long incubation period (10-12 years) and short disease duration (3-12 months) makes it difficult to diagnose, which has contributed to higher death toll since its occurrence. World organizations are strictly monitoring CJD cases, in order to curtail the spread of this fatal disease but they are far behind in eradicating it completely. Hence it is imperative to identify CJD sources to reduce the number of cases. Therefore, this present research is the first initiative to assess the prevalence of CJD in Pakistani population. Results of this study indicated absence of mutation at codon 200 in the participants under study. Most prevalent genotype were M129-E200 (71%) and V129-E200 (29%) whereas M129-K200 and V129-K200 were absent in the participants. In parallel study a survey encompassing questions related to cognitive impaired just like CJD was also carried out to assess public knowledge regarding it. Results indicated that educated groups lacked knowledge in theoretical as well as non-theoretical aspects of CJD. This study is highly significant as it provides preliminary data on the susceptible cases of CJD and proof that the public knowledge also corresponds to the data gathered by genetic screening.

1. INTRODUCTION

1.1 Background

From the first case of "prion disease" discovered in 1960 by D. Carleton Gajdusek at Uwami, near Keiagana village to reporting of 1 to 1.5 cases per one million population shows the disease is progressive and highly transmissible (Liberski et al., 2019). Prion disease or Transmissible spongiform encephalopathies (TSE) are a group of rare progressive neurodegenerative disorders that affect both animals and humans. The causative agents of these novel diseases are pathogens known as "prions". These pathogens cause misfolding of protein, especially in the brain which results in multiple rapidly progressing neurodegenerative disorders (Sikorska et al., 2012).

1.1.1 Prion diseases and location

Prion diseases are highly transmissible and fatal sets of neurodegenerative disorders. Misfolding of host-encoded prion protein (PrP) results in these rare disorders. PrP protein comprises of 253 amino acids (aa). The transportation mechanism of PrP protein involves two steps. Firstly, when Prp is transported to the endoplasmic reticulum, 22 N-terminal aa is removed. Secondly, when the glycosylphosphatidylinositol (GPI) anchor gets attached to the C-terminal, it gets cleaved off and gets attached to out surface of the cell membrane. Prp exists in two forms- normal cellular form (PrP^{C}) and pathogenic form (PrP^{Sc}). Both conformers are encoded from the PRNP gene (16kb) present on the short arm of chromosome 20 with base pair ranging from 4,666,796-4,682,233. The human PRNP gene comprises of two exons, with an open reading frame (ORF) present on the second exon (Sikorska et al., 2012).

1.1.2 Normal PrP^{C} Vs Abnormal PrP^{Sc}

Normal prion protein (PrP^{C}) has richer concentration of alpha-helical content whereas pathogenic prion protein (PrP^{Sc}) contains a higher concentration of beta sheets (Vázquez-Fernández et al., 2017). PrP^{C} and PrP^{Sc} are different in their secondary and tertiary isoforms but not in their primary aa sequence Conformational changes in PrP^{Sc} render it from any proteolytic cleavage, chemical or physical degradation, making it insoluble. On the other hand, PrP^{C} is soluble, can undergo proteolytic cleavage, and gets degraded from

non-denaturing detergents (Ansoleaga et al., 2013). Oligomers from acquired PrP^{Sc} catalyze the conversion of PrP^{C} into pathogenic fibrils resulting in an increased concentration of PrP^{Sc} substrate for the conversion reaction. The propagation process results in the pathogenesis of prion disease (Aguzzi & Calella, 2009).

1.1.3 Animal vs human prion diseases

Prion diseases are found in animals as well as humans (Table 1). They are distinguished based on incubation periods, failure to induce inflammatory responses, and characteristic spongiform changes associated with neuronal loss. Although generally rare, bovine spongiform encephalopathy (causally linked to the zoonosis variant CJD) and kuru cogently illustrate that under exceptional circumstances the transmissibility of these diseases can lead to dramatic increases in disease incidence (Islam et al., 2018).

Table 1: Types of animal and human prion diseases. Prion diseases are divided into human and animal prion disease.

Prion diseases in Animals and their respective host		
1	Scrapie	Sheep and goat
2	Transmissible spongiform encephalopathy in non-human primates	Lemurs
3	Feline spongiform encephalopathy	Wild and domestic cats
4	Chronic wasting disease	Cervids
5	Bovine spongiform encephalopathy	Cattle
6	Exotic ungulate encephalopathy	Kudu and nyala

Prion diseases in humans and their respective host		
1	Acquired 1. Kuru 2. Variant CJD (vCJD) 3. Iatrogenic CJD	Human
2	Sporadic	

	1. Fatal insomnia 2. Sporadic CJD	Human
3	Genetic 1. Grestmann-straussler-scheinker syndrome (GSS) 2. Fata familial insomnia 3. Genetic CJD	Human

1.2. Research Objective:

The research was aimed at assessing the public knowledge regarding Creutzfeldt-Jakob disease and genetic screening of blood samples collected from the public for the prevalence of PRNP mutation. To achieve former a qualitative survey was designed and was made available to public using different platforms whereas in case of latter blood samples were collected at random and was examined for the mutations.

2. LITERATURE REVIEW

2.1 CJD- Creutzfeldt-Jakob Disease

The most common human prion disease is a rare form of progressive adult dementia known as Creutzfeldt-Jakob disease (CJD). This disease was not critical till late 1960s. Now research considers CJD as one of the chronic neurological disease prevalent in many parts of the world. The incidence of CJD is 0.5-1.5 cases per million per year. The prominence of this disease is not its incidence rate, rather it is the nature of the transmissible agent. The spread of bovine spongiform encephalopathy (BSE) in the UK and outside the UK (Europe, North America, and Asia) in human beings has raised serious concerns among people (Brandel, 2022).

2.1.1 Clinico-pathological Subtypes

CJD being the most common prion disease can be further classified into its subtypes depending upon its origin. There are three subtypes of CJD. Sporadic CJD (sCJD) accounts for 85% of the cases, genetic familial CJD accounts for 10-15% of the cases, and iatrogenic accounts for only 1% of the cases. Other forms of CJD involve vCJD that is more prevalent in certain regions i.e., France and UK. The first form of TSE that was ever studied in non-human primates was kuru. It has helped us in understanding the incubation period and transmission of this disease in humans (Gajdusek & Zigas, 1959).

2.1.1.1 Sporadic CJD

sCJD is the most predominant among all the CJD subtypes. sCJD affects the male and female populations and the onset of this disease is 60 years. It rarely occurs in people with age above 80 and below 40 (Tabaton et al., 2004). The initial diagnosis of this disease is symptomatic. 1/3rd of the patient initially complains about disorder in sleep, fatigue, and in appetite. Second, 1/3rd patients present cognitive and behavioral changes and the rest of the patients show aphasia, motor deficit, ataxia, and visual loss (Zanusso et al., 2003). Myoclonus and prominent cognitive decline in later stages of these diseases, indicating rapid progression of this disease. Patients of sCJD usually survival is between 5 months to 1 year after diagnosis (Favereaux et al., 2004). Diagnosis of sCJD involves

the MRI and the presence of 14-3-3 protein in CSF. Both diagnostic criteria are not completely specific and sensitive. MRI and electroencephalogram show synchronized triphasic or biphasic sharp-wave complexes but on repeating the examination process. Secondly, 14-3-3 can also be found in CSF in other comorbidities (strokes or encephalitis) involving neuronal damage. Pathogenesis of this disease is found in the spinal cord and brain. Vacuolization in dendrites and cell body of neurons and neuronal loss gives a spongiform appearance to deep nuclei and cortex. Diagnosis involving immunocytochemical staining and western blotting helps us identify pathogenic isoforms of PrP in the brain (Zerr & Parchi, 2018).

The mode of this disease is still unknown. Even interaction with disease patients or occupational exposure has not shown any increased risk among the population. Consumption of sheep with scrapie was thought to be its reason for spread in the US, UK, and France. But sCJD is common even in those scrapie-free areas i.e., New Zealand and Australia (Zerr et al., 2009). Exogenous i.e., ingestion traces of PrP^{Sc} can be found in gastrointestinal and tonsillar tissue, as in vCJD. Studies have shown even vegetarians have developed sCJD. Evidence shows that medical procedure among humans has also been the source of sCJD. According to some studies, sCJD can also occur due to endogenous PrP misfolding. Other reasons could be the genetic changes in the PRNP gene. But the progression of this disease with age is still under question (Hill et al., 2003).

2.1.1.2 Iatrogenic CJD

CJD disease transferred through contaminated medical procedures is considered iatrogenic CJD (iCJD) (Brown et al., 2012). The most common procedures for the transmission of iCJD is dura grafts, growth hormone replacement therapy, and corneal transplant. Evidence of iCJD caused using the surgical instruments are evident from the experiment conducted on a chimpanzee. In this experiment silver electrodes was used in a CJD patient to study abnormal movement in patients during surgery (Brown et al., 2006). Although before implanting these electrodes in chimpanzee they were sterilized with chemicals, but the chimpanzee eventually developed CJD. Later on, two young individuals after getting excision for epileptic foci developed spongiform encephalopathy

within 20 and 16 months of surgery. iCJD outbreaks started after the administering contaminated human growth hormone and dura matter graft started. The use of cadaveric grafts started in 1985 and since then 100 cases of CJD have been reported after 16 months to 18 years. 4 patients of human growth hormone (hGH) from cadaveric pituitary gland transplants under the age of 40 were reported in 1985. In the US, 8000 children received hGH after which it was replaced with licensed recombinant hGH in the market since the retrieval of the product from the market. Over 125 young individuals have developed iCJD within 30-5 years of getting the hGH. iCJD has a long incubation period which indicates the peripheral pathway of inoculation rather than the intra cerebral route as in dura mater graft. Whichever the condition is polymorphism at the codon 129 increases the susceptibility of iCJD (Brown et al., 2000).

2.1.1.3 Genetic CJD diseases

Mutation in the PrP gene (PRNP) corresponds to 10-15% of transmissible spongiform encephalopathies or prion diseases. These mutations are inherited in autosomal dominant nature but have variance in penetrance. Point mutations in the PRNP gene involve the addition of premature stop codon, substitution, or insertion of a few aa in the N-terminal region (Varges et al., 2017). Genetic linkage analysis and the high penetrance rate of PRNP mutations indicate the pathogenesis of this disease. Nevertheless, genetic screening is identifying new mutation in people with sCJD that is why it is preferred to use genetic prion disease than familial prion disease. Number of genetic CJD (gCJD) is either related to low rate of penetrance or rare polymorphism that increases susceptibility of prion disease or due to prior PrP presence (Lukic et al., 2015). In the past gCJD were classified on the bases of neuropathological and clinical phenotypes i.e., GSS, fatal familial insomnia (FFI) or rapidly progressive dementia caused by spongiform encephalopathy (Baiardi et al., 2021).

Genetic prion disease either occur due to aa insertion or point mutation which leads to the deposition of PrP protein in brain. Base pair insertion leads to atypical form of prion disease which is difficult to classify into CJD or GSS (Kovács et al., 2005a). However, mutation at stop codon shows cerebral amyloid angiopathy along with memory disorientation, disturbance an progressive dementia (Y145-129M), or aphasia, myoclonus

and cognitive impairment (Y226-129V/M) in some cases (Parchi et al., 2009). Some mutations are rare like Y163X known as Prp systemic amyloidosis, in which amyloids formation occurs in peripheral tissues. Lastly few mutations in PRNP gene shows no clinical phenotypes i.e., T193I-129M, R208C-129M-E219K etc., (M. O. Kim et al., 2018a). Researchers must investigate its pathogenesis and neuropathological phenotypes. All mutations correspond to specific phenotypes and pathogenicity, in general. But data has shown that with in the same family carriers of mutation can show different clinical symptoms. As an example of phenotypic variation codon 178 i.e., D178N shows two types of prion diseases depending upon its segregation with codon 129 i.e., valine (V) causing gCJD or with methionine causing FFI (Goldfarb et al., 1992). Hence, polymorphism at codon 129 defines the characteristics of genetic prion diseases.

gCJD occurs due to inheritance of autosomal dominant mutation in PRNP gene. More than 50 types of mutation have been found in the world. But the most common mutation that are found in 95% of the cases are at codon 210, 102, 178, 200 and insertion of six to five base pair in open reading frame of PRNP gene. Polymorphism of PRNP gene depends upon codon 129. It determines whether V or methionine (M) will be expressed in the protein. gCJD develops in earlier stages of life and has a longer clinical course than sCJD. Most prevalent gCJD is caused due to mutation in codon 200 and the clinical symptoms resemble that of sCJD. Other forms of gCJD differs from sCJD in their phenotypic expression. For example, GSS diseases develops at the age of 20-40 years with rapidly progressing ataxia along with spastic paraparesis in many GSS patients. In patients of GSS shows delayed dementia and myoclonus. Unlike sCJD the course may last from 5-11 years. Amyloid plaques are also an indication of pathological changes in this disease.

Mutation at codon 102 is also found in families. Phenotype of 102 resembles with other mutations is gCJD. FFI is a unique form of genetic prion disease. Clinical manifestation of FFI is dominated by insomnia, dementia, and autonomic dysfunction. Neural dysfunction is mostly found in a thalamic region of brain. Mutation in codon 178 causes FFI. Typical gCJD cases have witnessed mutation in this codon as well. Polymorphism at codon 129 determines the phenotypes. Homozygous or heterozygous for V indicates typical CJD whereas homozygous for methionine indicates FFI (Goldfarb et al., 1992).

2.2 Incidence of Genetic CJD

Incidence of gCJD varies from country to country as indicated by molecular and epidemiological studies (Ladogana et al., 2005a). Genetic variation among European and Asian populations also affects the distribution and frequency of PRNP mutations (Jeong et al., 2014). To facilitate the surveillance process, definite classification of gCJD cases is only done when they are neuropathological verified in association with either infectious PRNP mutation or has a family history of prion mutation. For sCJD presence of 14-3-3 protein in CSF and MRI scans validates the presence of this disease. Whereas neuropsychiatric symptoms that are progressive validates the diagnosis for presence of infectious PRNP protein. However, with advancement in diagnosis process ultrasensitive seeding assay have facilitated the diagnosis of infective PrP in olfactory mucosa or CSF. But in case of gCJD deposition of PrP^{Sc} depends upon the type of the mutation and pathway linked to it. In few gCJD cases conversion of PrP^{C} into PrP^{Sc} requires a mutant protein only, whereas in most cases a PrP^{Sc} can also emerge from a normal or a mutated PrP gene as well (Ladogana & Kovacs, 2018a). Evidence indicates that CJD from an infected individual can be transferred to healthy rodents. Similarly, CJD is infectious disease which if present in public can cause serious implications in masses as well. Hence the need of surveillance of gCJD is required.

2.3 Genotypes of gCJD

Different pathological and clinical phenotypes occur due to mutation at different codon sequence in PRNP gene.

2.3.1 P105T-129V

Sequence analysis of PRNP gene in a patient having 3 closely related relatives having CJD like symptoms showed a mutation at codon 105 i.e., proline (P) to threonine (T). Clinical phenotypes of these three individuals included progressive dementia and memory decline, myoclonic jerks, and pre-cerebral syndrome. Cerebrospinal fluid (CSF) showed no presence of 14-3-3 protein along with normal electroencephalography (EEG). Whereas lesions were scene in thalamus and cortex (Polymenidou et al., 2011).

Characteristic feature of gCJD cases also shows spongiform like appearance in cortex as well as neuronal loss, which were also evident in this mutation. Immunohistochemical assay revealed plaque like deposits in cortical synaptic pathway and in deeper cortical membranes. Unique PrP^{Sc} deposits were detectable in cellular area of cortex and cerebellar hemisphere. Synaptic patterns were seen in basal ganglia. Additionally, staining for alpha-synuclein, ubiquitin, tau, and beta amyloid showed no pathological symptoms (Polymenidou et al., 2011). Protein kinase resistant PrP protein was undetected. Whereas partially glycosylated Pk-resistant fragment as well as two un-glycosylated bands were seen through western blotting.

2.3.2 G114V-129M

Genetic analysis revealed the substitution of glycine instead of V in codon 114 was first found in a Uruguay family. China, India, Turkey, and USA also reported these mutations. Incomplete penetrance was observed in healthy population (Zarranz et al., 2005, Urwin & Molesworth, 2020). Onset of diseases varied from 45 to 32 years in Chinese family, from 28 to 18 years in Uruguayan family and 75 years in Indian family. Clinical characterization of this disease involved myoclonus, extrapyramidal and prominent pyramidal sign, and slowly progressive dementia with longer disease duration i.e., more than a year. 14-3-3 CSF test and varying patterns of EEG were not detected. Observation of neuropathological symptoms showed spongiform in cortex with pathogenic PrP^{Sc} like sCJD cases (Steinacker et al., 2016).

2.3.3 R148H-129M

Genetic and epidemiological studies of R148H-129M revealed change of arginine (R) to histidine (H) at codon 148. Cases of homozygous mutations were reported in China, Germany, and US (M. O. Kim et al., 2018b). One case of a heterozygous (M/V) individual was reported in US. Few cases have been reported involving this mutation which can be due to its low rate of penetrance and no family history (Pastore et al., 2005). Clinical features of this mutation resemble that of sCJD (psychiatric symptoms, gait disorientation, akinetic mutism, rapid cognitive deterioration and myoclonus). Brain MRI, EEG patterns and 14-3-3 protein were observed. Neuropathological symptoms of

this mutation mostly resemble sCJD symptoms with spongiform appearance in cerebellum, thalamus, cortex and basal ganglia. Pathogenic PrP was also detected through immune-staining (Krebs et al., 2005).

2.3.4 D178N-129V

Asparagine replaces aspartic acid at codon 178. This mutation was first recorded in 1979, Finland. Likely, Japan and China also reported cases with these mutation (Minikel et al., 2016). Phenotypic expression of this mutation depends upon the polymorphism at codon 129. Mutation of V at 129 causes gCJD whereas presence of M causes FFI. Clinical symptoms of D178 resembles that of sCJD homozygous for V at codon 129 but has minimum clinical duration of 15 months (Sun et al., 2015). Heterozygosity at codon 129 have longer survival and late onset of this disease. Symptoms involved memory loss, psychiatric and behavioral changes, and cognitive impairment (Marcon et al., 2014). Similarly, 86% cerebellar symptoms, 74% showed myoclonus, 21% visual impairment etc. EEG lacked typical periodicity but showed slow wave activity. Elevated tau levels were observed in few patients, some reported hyperintensity in cortical and basal ganglia region in brain MRI. Only two of the cases reported presence of PrP^{Sc} in CSF samples (Zerr et al., 1998a). Neuropathological and biochemical studies revealed that neuronal loss, spongiform degeneration and pathogenic astrogliosis were prominent in these cases. PrP^{Sc} staining showed diffused pattern in arears with higher spongiform changes. Less synaptic patterns were seen in areas like cerebellum, cortex where minimal deposition of PrP^{Sc} was observed. Kuru-plaques were undetected (Zerr et al., 1998b).

2.3.5 V180I-129M and V180I-129V

Changes at codon 180 from V to isoleucine (I) is more frequent in Japanese population than in European population. Epidemiological studies revealed that the penetrance rate of this rare mutation is very low, with most of the cases with negative family history (Minikel et al., 2016). Clinical presentation of V180 with 129M shows similarity with sCJD (Hayashi et al., 2020). Survival rate of this mutation is comparatively higher than other mutation i.e., 16.8 months whereas the onset of this disease happens in later stages of the age. Age of onset and duration of the disease is not affected by polymorphism at

codon 129. Clinically patients show slowly progressive dementia along with cognitive impairment. Comorbidities with this mutation involves akinetic mutism, psychiatric, extrapyramidal, and pyramidal signs. Neuropathological reports indicated degeneration in basal ganglia and in cerebral cortex. Longer disease duration with less involvement of brainstem and cerebellum. Cerebral and myoclonus signs were not evident in many cases. Immunohistochemistry of PrP^{Sc} protein showed deposition in cerebellar layers, para-hippocampal gyrus, and striatum in some cases whereas few reported no deposition of PrP^{Sc} in cerebral neo-cortex. Kuru like plaques were observed in patient heterozygous for codon 129 (Shi et al., 2014).

2.3.6 T183A-129M

Genetic studies conduct in past depicted a change of alanine instead of threonine in codon 183. First case of this mutation was reported in Brazilian family. Afterwards mutations were reported in Chinese, German, and North American family, previously diagnosed with dementia. Clinical presentation of this disease involves longer disease duration, earlier onset i.e., less than 50 years of age. Symptoms involving Alzheimer disease, dementia having early onset or frontotemporal dementia (Van der Kamp & Daggett, 2010). Diagnostic test did not indicate any period wave in MRI or periodic patterns in EEG, along with negative 14-3-3 protein CJD (Shi et al., 2015). Neuropathological examination of few patients showed high levels of spongiform with neuronal loss in putamen and deeper layers of cortical tissue. Other symptoms include plaques like depositions in putamen and cerebellum along with mini plaques in deeper layer of cerebellum.

2.3.7 T188K-R-A

This mutation has 3 different types of mutation (substitution) causing gCJD. Most mutations in this residue occur at codon 188 (T188K), when threonine (T) is changed to lysine (K). China has the second highest rate of incidence, 16 cases have been documented as of 2015 (Shi et al., 2015). Germany reported 3 cases with methionine at codon 129 on the mutant allele and an Austrian patient with dementia (Tartaglia et al., 2010). At ages 79 and 55, this mutation was also discovered in healthy carriers. A clinical feature of gCJD caused by T188K-129M haplotypes resembles sCJD type 1 with

homozygous mutation at 129 codons (Xiao et al., 2019). Age group affected by this mutation involves 59-50 with 4 months of disease duration. Clinical presentation of this disease type involved myoclonus, progressive dementia, pyramidal, visual and extrapyramidal signs (Xiao et al., 2019). EEG showed activity, hypersensitivity in basal ganglia and 14-3-3 protein showed 69% of sensitivity. Other two types involve T188R and T188A, reported in very few cases. In one example with the T188K mutation, histologic analysis showed extensive spongiform alterations across the cerebral cortex, occipital cortex, as well as putamen and caudate nuclei. The brainstem was completely devoid of spongiform change, but the cerebellar cortex displayed mild layer spongiform changes. Strong synaptic staining was seen after PrP^{Sc} immunostaining (Roeber et al., 2008).

2.3.8 E196A-129M

Alanine instead of glutamic acid substitution is seen in E196A genotypes was reported in only 3 Chinese patient (Wu et al., 2020). All three patients had akinetic mutism, myoclonus, progressive dementia, and cerebellar abnormalities. The CSF 14-3-3 test was positive, while the EEG in one patient exhibited periodic sharp wave complex and the brain MRI in the other two patients showed striatal high signal. The diagnosis of these cases as gCJD is based only on clinical and instrumental observations since no histologic or biochemical test (Zhang et al., 2014).

2.3.9 E196K-129M and E196K-129V

First person to have homozygosis for M at codon 129 and the glutamic acid (E) to lysine (K) alteration at codon 196 (E196K) for CJD was a French woman (Komatsu et al., 2014a). More than fifteen cases have been reported in different parts of the world i.e, China, France, Germany etc., Clinical characteristics of hereditary CJD associated with the E196K mutation include a later age at onset (73.2 years) and a median duration of 8 months. Cognitive impairment and psychiatric symptoms were frequently evident at the time of beginning, and as the disease progresses, patients also have myoclonus as well as cerebellar, pyramidal, and extrapyramidal symptoms. Most patients were reported to have akinetic mutism. Brain MRIs in roughly 57% of patients reveal hyperintensity in cortical regions or the basal ganglia, whereas EEGs typically do not exhibit the characteristic

periodic activity. All patients save one tested positive for the 14-3-3 protein, and three patients had increased levels of total tau (Shi et al., 2021). A study of the histology revealed the morphologic and immunohistochemical characteristics of CJD. Symptoms include PrP^{Sc} showed deposition in deeper layers of cerebellum. Glyco-form of this mutation was different from sCJD (Béjot et al., 2010).

2.3.10 E200K-129M and E200K-129V

The only PRNP (E200K) mutation that results in clustered cases is the change of E (glutamic acid) to K (lysine) at codon 200. While the first detected cluster was reported in Slovakia, this mutation was first discovered in a Polish CJD family (Spudich et al., 1995). Soon after, patients in Chile and Libyan Jews were also discovered to have the same mutation (Chatelain et al., 1998), Jewish population groups in Italy (Kovács et al., 2005b), Spain, Britain, Japan (Mancuso et al., 2009), and Argentina. Hungry also reported higher incidence of CJD cases (Ladogana & Kovacs, 2018a). Families of Tunisian and Greek descent, Italy, Spain, Britain. Hungary reported a significant frequency, especially in regions with historical and physical ties to the population of Slovakia (Goldfarb et al., 1990). The distribution of this mutation has now been documented more recently in China), Korea, and other European nations (Gao et al., 2019a, Choi et al., 2009). Incidence of CJD per year was reported higher (5-154 times) the global average.

Origin of E200K can be dated back to Spain, when some Sephardic population was expelled, during Middle Ages. According to research allelic mutations inherited from the ancestors. This research revealed that the families from Libya, Tunisia, Chile, Italy, and Spain share the same historic allele. Several families with Slovakian ancestry and one with Polish ancestry have yet another special allele linked to the condition (Colombo, 2000). Co-segregation of V at codon 129 is primary to the distinction between Eastern European and Mediterranean mutated alleles., although they are quite similar to one another in families from Austria, Germany, and Sicily.

Finally, the Japanese allele is unique from the others. These findings supported the hypothesis that the E200K mutation's distribution results from distinct mutational events.

Some mutation carriers never develop CJD because the mutation (E200K) exhibits disease inheritance is autosomal dominant, with varying penetrance. According to survival study, the E200K mutation's overall penetrance rate is 100% in Israeli Libyan Jews (Rosenmann et al., 1999), Italian cluster of Calabria is 67%, and 59.5% in the Slovakian community (Mitrová & Belay, 2002). A younger age at onset separates gCJD with the 129M -E200K from sCJD-MM129 type1 (Ladogana et al., 2005b), while this age is similar in Asian patients i.e., 61.1 years and Caucasian i.e., 60.4 years. The disease can manifest between 27 and 84 years of age, and codon 129 polymorphism is not likely to have an impact on this range. In Asian and Caucasian populations, duration of clinical course is 5 months, with survival from 1 to 74 months (Takada et al., 2017a).

Polymorphism at codon 129 influences the disease duration in affected patients' because homozygosity of methionine in patients had a shorter lifespan than heterozygous individuals. A few homozygous E200K mutation carriers were found as well. These patients did not differ significantly from heterozygous one's duration of or age at disease onset. Gait or balance issues, behavioral abnormalities, and a quickly progressing form of dementia are the typical presentations of E200K-M129 CJD (Cohen et al., 2016). Myoclonus and pyramidal or extrapyramidal symptoms are then typically developed by patients. Peripheral neuropathy, supranuclear gaze palsy, sleep difficulties, and cortico-basal syndrome are a few examples of unusual clinical manifestations (Friedman-Levi et al., 2011a).

In E200K mutant instances, the characteristic spongiform triad alteration, loss of neuronal function, and reactive Astro-cytosis is present. PrP^{Sc} immunostaining Diffuse/synaptic deposits, patchy aggregates in the neuropil or around vacuoles, plaques were revealed, which were devoid of amyloid features (Kovacs et al., 2011). The neuropathological distinguishing characteristics in mutated cases E200K are: (1) an increase in the prominence in the deeper cerebral layers that are independent of codon 129 polymorphism more typical of sCJD- VV2; (2) absence of MV at codon causes amyloid kuru-type plaques. as a distinguishing feature form sCJD MV2); and (3) the high prevalence of association with other proteins linked to Deposits of neurodegeneration deposits of neurodegeneration (Kovacs et al., 2012)

2.3.11 E200G-129V

Alteration of glutamate (E) to glycine (G) at codon 200 cause CJD. This mutation E200G was first identified in a US. At the age of 57 the asymptomatic patient, observed gait issues, memory disruptions and slowly progressive cognitive impairment. Extensive neuropsychologic testing revealed that the patient had mild cognitive impairment. No involuntary movements or myoclonus were observed. EEG and MRI scanned showed slowing of brain periodic activity and hypersensitivity in cortical region. The 14-3-3 test were debatable, but net tau protein (1351 pg/mL) was elevated. Immunohistochemistry revealed PrP^{Sc} granules like synaptic staining in the cerebral cortex with sponge appearance. Analysis of western blot revealed that type 2 PrP^{Sc} had a high proportion of diglycosylated species and a low proportion of unglycosylated species (M. O. Kim et al., 2014) .

2.3.12 V203I-129M and V203I-129V

CJD caused by a V to isoleucine mutation (I) at codon 203 and homozygosity of methionine at codon 129 was first reported in Italy. patient the mutation was then fixed. occurrences have been reported in Canada, France and Austria, and Canada (M. O. Kim et al., 2018c) . The V203I-129V haplotype has been identified for the first time in Europe, with one case of a 129 heterozygous patient in Italy and four cases in Asia. All had no family history of prion disease but in one (Mastrianni, 2010) .V203I-129M is almost identical to sCJD. Clinical duration of 4 months and mean age of onset is 69 years. Clinical symptoms include myoclonus, pyramidal, rapid progressive dementia, extrapyramidal signs, and mutism. PrP^{Sc} involves diffuse synapses in cerebral region. Western blot analysis resembled a typical PrP^{Sc} type 1 pattern in one case, (Kovacs et al., 2017).

2.3.13 R208H 129M and R208H-129V

China, Japan, European countries, and US reported mutated cases with arginine to histidine changes at codon 208.MM129 type 1 is like R208H-129M. Disease duration 7 months and age of onset 60+. 44 % patients showed positive result in MRI and 60 % showed promising results for CSF 14-3-3 protein (Shi et al., 2011) . The presentation of

gCJD with R208H-129V haplotype is very close to that of sCJD VV2. Typical periodic activity in EEG pattern. PrP^{Sc} deposition in this mutation has shown to have several features like that of CJD, including synaptic deposits. Tau protein restricted to the temporal lobe (Bagyinszky et al., 2018a) .

2.3.14 V210I-129 M and V210I-129V

V to isoleucine substitution at codon 210 (V210I) was first reported in and Italian family with previous CJD history. Japan, China, and Brazil also reported cases of V210I. In Italy, V210I represents 42% of all PRNP mutations (Xiao et al., 2020) .CJD with V210I-M129 shows similarity with sCJD-MM1. Mean age at onset of is 61 years and affects relatively young subjects. The clinical presentation is usually characterized by ataxic gait, cognitive impairment, and psychiatric dysfunctions (Appleby et al., 2022) . Typical activity witnessed in EEG (44% and 79% of cases). 85–100% of patients showed positive 14-3-3 while elevated levels of total tau in 100% of the cases (Franceschini et al., 2017) .Some patients with PrP^{Sc}, showed spongiform changes in the cerebellum and cerebral cortex.

2.3.15 E211Q-129M

E211Q mutation is very rare. France and Italy reported more than one case. Eleven cases have been recorded (Minikel et al., 2016) . E211Q-129M is almost same as sCJD. Clinical manifestations consist of EEG periodic synchronous discharges, positive 14-3-3 protein, cerebellar ataxia, myoclonus, and rapid progressive dementia. Spongiform modification and gliosis in striatum and iso-cortex, as well as synaptic deposits in cortex. Amyloid plaques were not found. A PrP^{Sc} pattern was discovered on a Western blot. (Bagyinszky et al., 2018b).

2.3.16 Octapeptide Repeat Insertions (OPRI)

Instead of single point mutation, a 24 base pair insertion in OPRI is also observed in few cases. For the first time, six OPR insertions were discovered in a British family. European countries, including the China, United States, and Japan. Dementia associated with OPRI includes late-onset and early- dementia GSS and CJD, (Areškevičiute et al., 2019) . The size of the OPRi is the most important determinant of its phenotypic

expression. Whereas other factors are PrP^{Sc} and SNR codon. Neuropathology of this mutation is different from CJD and GSS phenotypes. 6-OPRI cases showed eosinophilic globular PrP^{Sc} deposits and amyloid plaques. Western blots, which are rarely reported, are usually consistent with sCJD (Mead et al., 2007a). Some patients with 1 to 4 OPRI-129M mutations have also been reported. They have phenotype like typical CJD, including myoclonus, progressive dementia, and cerebellar symptoms. According to demographic studies the mean age of onset is 61 years with 6 months of disease duration (Imran et al., 2012). Another mutation with 5-7 OPRi linked disease exhibit a variety of phenotypes, including early-onset CJD with long disease duration, typical CJD, GSS-like disease or Huntington disease with chorea and dystonia. The most frequent insertion 6-OPRI and presents with progressive form of cortical dementia (Mead et al., 2007b) . 2-OPR deletion first identified in a woman, who developed rapidly progressive dementia (rpd) at age 86 and died 23 months later. The neuropathologic were inconsistent with those of sCJD-MM-1. Three additional unrelated patients have recently been reported in the Netherlands. Lastly, a case of OPRD was discovered as a novel polymorphism in some ethnic populations and is not thought to make one susceptible to CJD or any other type of human prion disease (Beck et al., 2001) .

2.3.17 Others

A case of Alzheimer's disease reported change at codon 215 i.e., isoleucine to V (Muñoz-Nieto et al., 2013) .Both were carriers and heterozygous for MV with no background of prion diseases. EEG and atrophy in brain shown by MRI scan, and both patients were positive CSF 14-3-3. Neuropathology revealed spongiform changes and neuronal loss, as well as active astrocytosis consistent with prion disease. There was no Western blot analysis. USA reported one case with Alanine to V mutation with absence of any relative or family showing CJD symptoms. Elevated tau levels with inconclusive 14-3-3 protein in CSF, and absence of typical periodic activity shown in EEG. PrP^{Sc} was characterized by severe degeneration, loss of neurons and cerebral and cortices showed granular deposition. (Takada et al., 2017b). Highest prevalence of M232R i.e., methionine to arginine substitution at codon 232, is reported in Japan. South Korea and China have also reported cases with this mutation (Bagyinszky et al., 2018c) .Characteristic similarity was observed in M232R and sCJD. Rapid progressive dementia is common among patients.

(Shiga et al., 2007) .Slow-type and rapid-type phenotypes of CJD show distinct neuropathologic lesions and immunostaining patterns PrP^{Sc}.

2.4 Prevalence of PRNP Mutation in Asia

CJD diagnosis in China was barely common, till the end of the 1980s. 11.1% of all instances of prion disease diagnosed between 2006 and 2021 were gPrD cases, 167 of which were later determined to be gCJD. In total, 19 distinct kinds of PRNP mutations were found in patients from China. With 65 cases (29.8%) of all gPRD cases, T188K is the mutation that occurs most frequently in China. Twenty provinces in China have reported T188k cases, with Shandong reporting the highest number of cases. The average patient survives 4 months on average after developing gCJD with a t188K mutation, which has a median onset age of 61 years. With 41 cases total, E200K is the second most frequent mutation to have been identified in China, accounting for 19.8% of all cases of gPRD.

Average onset age and survival time for gCJD with the E200K mutation are 57 and 6 years, respectively. With a total of 16 cases to date and 7.3% of all gPRDs, E196A is the third most frequent mutation in gCJD patients (Wang et al., 2021) .None of the e196A patients mentioned having a family history of CJD. The average age at which CJD manifests is 61 years old, and the average survival time is 6.5 months. With a total of 14 cases identified between 2006 and 2020, P102L mutation-related gCJD cases rank fourth most frequently in China. In comparison to the other three variants mentioned above, this mutation's median age at beginning of gCJD is 50 years, which is rather young. Other mutations with fewer than five cases have been reported in China, including E196K, V2031, R208H, V210I, G114I, R148H, P105L, V180I, T183A, and E200G (Komatsu et al., 2014b, Han et al., 1996). These mutations have been associated with 5, 3, 3, 2, 2, 1, 1, and 1 case, respectively.

India reported the first family CJD case with D178N from the South-East Asian region. Within 3 to 15 months of the disease's beginning, all the family members that had symptoms passed away. A change of aa was observed at codon 129 i.e., V/V polymorphism was discovered, but it had no symptoms. A neuropathological study

revealed significant neuronal loss and cortical atrophy. PrP immune-staining revealed tiny plaques. Two of his family members tested positive for the D178N mutation out of the 27 members of his family who underwent genetic testing. Three gCJD cases with the M232R mutation were discovered in Japan in 1996. Patients displayed myoclonus, aberrant EEG readings, and dementia that were progressing quickly. Immune PrP staining revealed diffuse grey matter. An examination of the histopathology revealed a loss of neurons. There was no evidence of plaque formation in the brain. Japan's national monitoring system was set up in 1999 to detect prion disorders in people. Of 2,394 cases identified, 365 were of gCJD. Overall, there were 1.2 incidents per million people annually. While the phenotype of CJD200 was uniform in Japan, it was shown to be heterogenous in Europe and America.

Genetic testing identified three mutations at codons D178N, E200K, and M232R in gCJD patients. Another instance included a 73-year-old lady who was diagnosed with gCJD and the V180I mutation. a monitoring study conducted in Korea between 2001 and 2019. Eight cases of gCJD were found in Taiwan between 1998 and 2017, according to surveillance research by CJDSU Taiwan. Four of the eight cases were P102L point mutations at codon 129, two were E196A-129M mutations, and one was R148H-129M mutation.

3. MATERIALS AND METHODOLOGY

3.1 Genetic Screening and Polymerase Chain Reaction

3.1.1 Collection of blood samples and Human DNA Isolation

Approximately 4ml of blood was collected at random from healthy individuals (n=100) in EDTA tubes. Age range of individuals was between 10 to 64 years. No biasness was made between male and female. Afterwards, DNA isolation was conducted using Mammalian Genomic Extraction kit (DI800, Solar-Bio, Beijing). Blood (200 µl) was withdrawn from each EDTA tube and added to an Eppendorf tube. Blood lysis buffer (600 µl) was also added into the Eppendorf (blood + lysis buffer) and incubated for 2-5 mins at room temperature. Centrifugation at 12000 rpm for 2 mins was performed. Supernatant was discarded. Afterwards, 200 µl of solution A was added in each tube containing pallet and was thoroughly mixed. RNase solution (20 µl) was added, mixed well and left at room temperature for 10 minutes to incubate. Furthermore, protein kinase K (20 µl) was added and incubated at 60 ºC for 45 minutes with frequent mixing after regular intervals, aiding the digestion process. Solution B (100 µl) was then added to the tubes and mixed well by inverting the tubes. Turbidity was seen, therefore placed in water bath at 60 ºC to remove it. Absolute ethanol (1140 µl) was added to the tubes and mixed thoroughly. Flocculant precipitation was seen in each sample. All of this was added to absorption column and centrifuged at 1200 rpm for two minutes. Flow through was discarded. Washing buffer (600 µl) was added to adsorption column and centrifuge at 1200 rpm at 2 mins. Flow through was discarded. Empty column was centrifuged again at 1200 rpm at 2 minutes. Any flow through left was discarded. These empty tubes were placed on hot plate to get rid of any liquid. Lastly, column was placed in a new clean centrifuge tube. Elution buffer (50 µl) was added to the membrane directly and centrifuged at 1200 rpm at 2 mins. Purified DNA was collected in the collection tube and stored at -20 ºC.

3.1.2 Quantification of DNA

Nanodrop was used in order to check the quality and quantity of DNA extracted from the samples. The quality (A260/A280) of DNA extracted was approximately 1.5, whereas the quantity of DNA was between 40-50 ng/µl.

3.1.3 Selection of Oligonucleotide Primers

Primers were based on extensive literature review and validated through online tools i.e., BLAST and sequencing. Forward primers for M129 (primer 1) (-GGCCTTGGCGGCTACA-, position 25819-25834, Tm 57.8 ºC, G + C content is 69 % with base pair length of 16) and V129 (primer 2) were used (-GCCTTGGCGGCTACG-, position - 25820-25834, T_m 55.8 ºC, G + C content 73 % with base pair length of 15). Whereas reverse primers included E200 (primer 3) (-CCATCATCTTAACGTCGGTCTC-, position 26047-26068, T_m 57.3, G + C content 50% with base pair length 22) and K200 (primer 4) (-CCATCATCTTAACGTCGGTC**T,** position 26047-26068, T_m 56.9ºC, G + C content 45 with base pair length 22). Above mentioned primers were used in combination i.e., M129-E200 (primer 1 and 3), M129-K200 (primer 1 and 4), V129-E200 (primer 2 and 3) and V129-K200 (primer 2 and 4) respectively (Calero et al., 2009).

3.1.4 DNA Amplification.

Amplification of genes was done by conventional PCR. Each reaction mixture contained reagents in a final volume of 25 µl: 12.5 µl of master mix (2X-SolarBio), 8.5 µl of nuclease free water, 0.3 µM of each primer and 50 ng of genomic DNA. The PCR amplification protocol for all the reactions was same, except for the annealing temperatures, depending on the primers being used. Initial denaturation at 96 C for 3 min, followed by 35 cycles of 96 ºC for 30 s, annealing at 55 ºC (VE), 56 ºC (VK), 57 ºC (MK) and 58 ºC (ME) respectively, depending upon the primer under study. Lastly final extension for 10 min at 72 ºC was done. PCR reaction was performed in thermocycler (TC9610, Multigene Opti-Max, China).

3.2 Gel Electrophoresis

Amplification of DNA was confirmed by performing gel electrophoresis of PCR product. Amplicon size of the desired DNA was validated by checking the location of our desired band with the DNA ladder. Size of the DNA ladder was 100-1500 base pair (bp).

3.2.1 Agarose Gel for DNA Quantification

After DNA extraction the quantity and quality of DNA was further validated by gel electrophoresis. To make 1% agarose gel following ingredients are required: distilled water, 10X TBE buffer, ethidium bromide and agarose powder. This mixture was poured into electrophoresis tank and left to set for few minutes. On a clear film PCR product was mixed with the dye and pipetted into the respective wells along with a DNA ladder. This protocol was performed at 100 volts for 45 minutes.

3.2.2 Agarose Gel for PCR Product Quantification

PCR product was validated on 2% agarose gel. To make 2% agarose gel following ingredients are required: distilled water, 10X TBE, ethidium bromide and agarose. This mixture was poured into electrophoresis tank and left to set for few minutes. On a clear film PCR product was mixed with the dye and pipetted into the respective wells along with a DNA ladder. This protocol was performed at 100 volts for 45 minutes.

3.3 Visualization of PCR Product

Gels were visualized on ChemicDoc^{TM} XRS + system (721BR19365, Bio-Rad,USA).

3.4 Protocol for DNA Sequencing

A reaction mixture of 30µl PCR product containing 50ng/ul of DNA was used in automated sanger sequencing. Purification was done using PCR clean up kit (740609, Thermo-Fisher, China). Reaction mixture (5µl) was used for PCR reaction. Condition for PCR reaction was as follows; initial denaturation at 98 ºC for 4 mins, followed by 35 cycles at 98 ºC, annealing at 60 ºC, extension at 72 ºC for 40 second and final extension at 72 ºC for 10 minutes. This mixture was loaded on to 96 well plate and observed in Microplate reader (5119500, Thermo-Fisher, China).

3.5 Cross-Sectional Survey Design

Cross-sectional studies are advantageous in estimating the prevalence of disease in certain population. Hence, for this research study the goal was finding the number of individuals who are aware about CJD also known as mad cow disease (Appendix B).

3.5.1 Selection of samples

An essential component of research design is the calculation of the precise sample size. Understanding that different study designs require various sample size calculation techniques and that a single formula cannot be applied to all designs is crucial. According to WHO (world health organization), the sample size of Pakistani population should be approximately 99,904, or at least 10,000 individuals as mentioned in this study (Zhu et al., 2019, Charan & Biswas, 2013). Pakistan being third world country does not have proper surveillance units which can monitor disease prevalence in larger population. The sample size selected was approximately $\geq$ 500 individuals. Sample size can be increased with effective collaboration with institutions and with proper resources.

3.5.2 Questionnaire Design

Knowledge assessment survey has been a part of many epidemiological studies. Carpenter et al developed Alzheimer's disease knowledge scale (AKDS) years ago to assess the public awareness on this disease and dementia. Yu Hong-Me also developed a modified version of Alzheimer knowledge assessment scale. Which has been proven reliable in assessing the knowledge of public regarding dementia and Alzheimer. Similarly using AKDS as standard, a questionnaire was devised containing "yes" or "no" questions belonging to different groups: life impact, symptoms, risk factors, treatment and management and protective factors. Score for each question was calculated by summing correct answers for every question and calculating the total score for each question.

Demographic question was also part of the questionnaire, to evaluate the background of individual. Question like these aided in segregation of population based on age, so that we can get a better understanding of groups that have better knowledge of CJD from the group that have less to no knowledge regarding the disease. Additionally, questions related to exposure to CJD patients or experience meeting them was also added.

3.5.3 Tool Validation

An online survey was conducted using Google forms. The questionnaire was validated by expert evaluation (Clinical Department of Neurology, University Medical Centre Göttingen, Germany) and tested on a small cohort study comprising of 86 individuals validated the language of the survey was sound and was easily understood by individuals with nonscientific background.

4. RESULTS AND DISCUSSION

4.1 Results-Polymerase Chain Reaction

4.1.1 Evaluation of Genotypes

Results for the identification of mutation at codon 200 along with polymorphism at codon 129 have been shown in Figure 1. The representative gel A shows the bands for M129-E200 was the most prevalent combination with prevalence in 71 samples. Gel B shows no bands as it represents mutated combinations M129-K200 and V129-K200. Gel C represents V129-E200 with bands in only 29 samples was the second most prevalent. All the genotypes found in 100 individuals is enlisted in Appendix A.

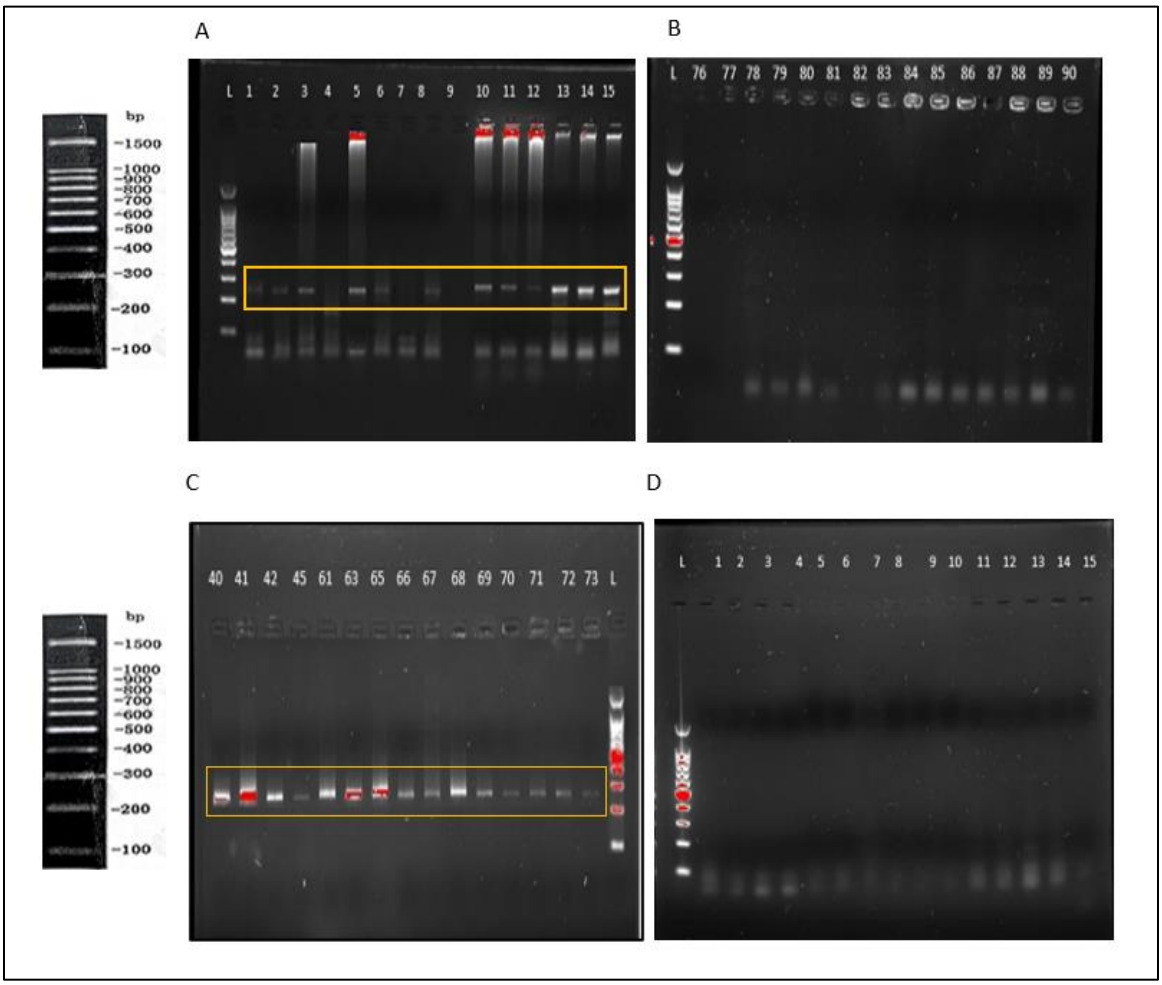

Figure 1: Representative gels for healthy and mutated sequence. Gel A and C shows the presence of M129-E200 and V129-E200 in the sample (n=100) whereas gel B and gel D shows the absence of mutations i.e., M129-K200 and V129-K200.

4.1.2 Genotype Frequency of PRNP Gene

As enlisted in Appendix A, the most prevalent genotype was M129-E200 . Therefore, Majority of the sample (n=71/100) had M129-E200 combination with highest penetrance. Whereas significant number of participants (n=29/51) showed V129-E200 with second highest prevalence depicting low penetrance with no mutation. Genotype frequency for M129-K200 and V129-K200 was absent from the samples under study, as shown in (Table 2) (Figure 2).

Table 2: Frequency of PRNP genotype. Prevalence of M129-E200 was estimated as 0.71 (71%), most prevalent genotype found in all of the samples, V129-E200 was the second most prevalent combination 0.29 (29%) and V129-K200 and M129-K200 were not present in any of the samples.

Frequency of PRNP Genotype				
No Sr.	**Genotype**	**Number of Individuals**	**Ratio of genotype to total**	**Genotype Frequency**
1	M/E	71	71/100	0.71
2	V/E	29	29/100	0.29
3	M/K	0	0	0
4	V/K	0	0	0
			Total	1.00

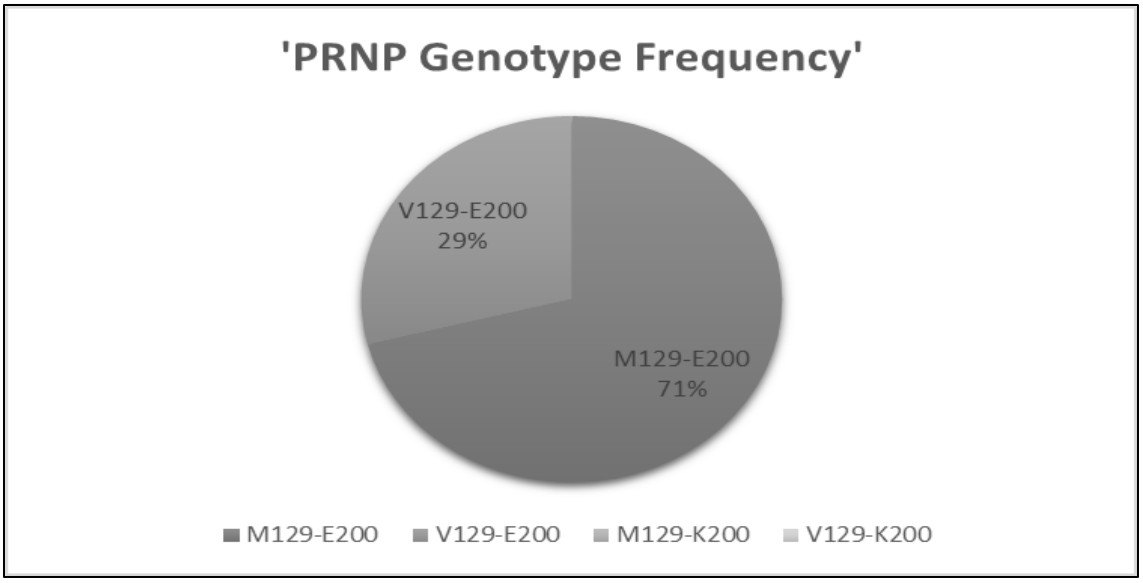

Figure 2: Pie chart showing total genotype frequency in the participants. M129-E200 was estimated as 0.71 (71%), most prevalent genotype found in all of the samples, V129-E200 was the second most prevalent combination 0.29 (29%) and V129-K200 and M129-K200 were not present in any of the samples.

4.1.3 Allelic Frequency

As indicated by Table 2 the most prevalent genotype was M129-E200 similarly Allelic frequency was the highest for M129-E200 with prevalence in 71 of the sample. Second most prevalent allelic combination was V129-E200 present in 29 of the individuals. Mutated sequence V129-K200 and M129-K200 were absent with allelic frequency 0, as shown in (Table 3).

Table 3: Frequency of PRNP alleles. M129-E200 was the highest i.e., 0.855 in 100 samples. Least prevalent allele was V129-E200 i.e., 0. 145. Mutated sequences were absent from the sample.

Frequency of PRNP alleles n= 100					
No Sr.	**Health / Mutant**	**Alleles**	**Number of present alleles**	**Ratio of no. of allele present in n to total no. of alleles (200)**	**Allele frequency**
1.	Healthy	M129-E200	71	171/200	0.855
2.		V129-E200	29	29/200	0.145
3.	Mutant	M129-K200	0	0/200	0
4.		V129-K200	0	0/200	0
				Total	1.00

4.1.4 DNA sequencing of PCR product

PCR products were sequenced. The sequencing results were validated through BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). As shown below the sequence obtained through sangers sequencing corresponds to Human PRNP protein at chromosome 20 (Figure 3).

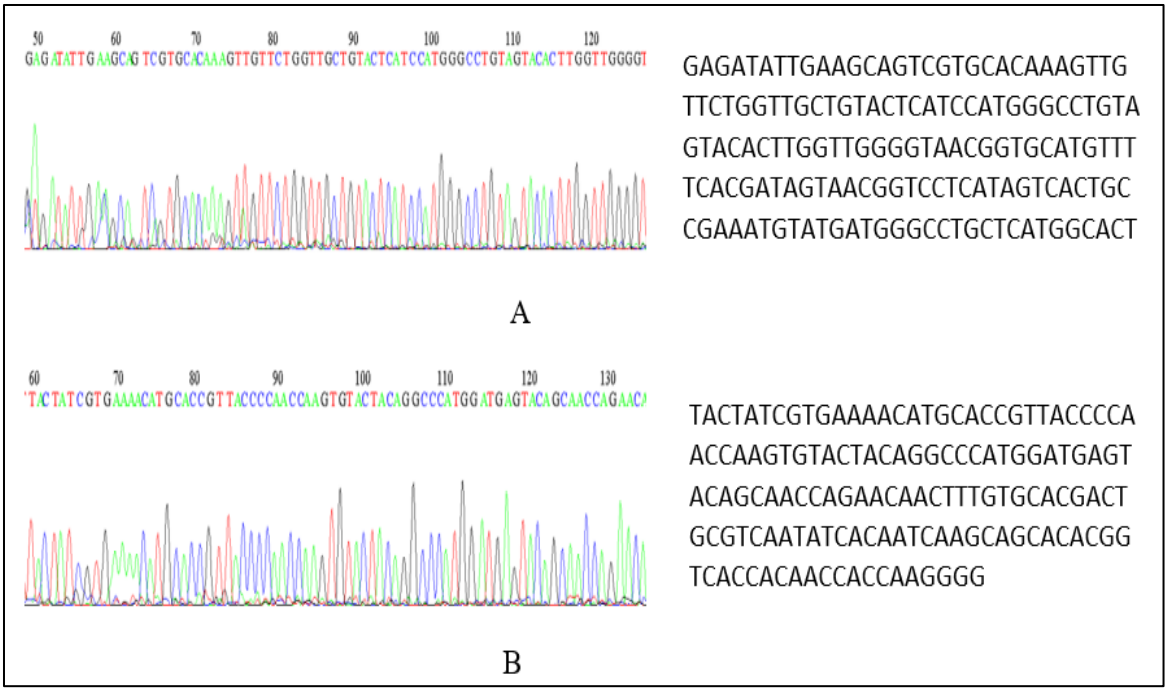

Figure 3: Chromatogram of amplified PRNP gene via Sanger Sequencing Method. A) shows the amplification of E200 (forward primer). B) shows the amplification of M129 (forward primer).

4.2 Results- Questionnaire

4.2.1 Demographic Characteristic of Respondents

Total number of participants in this study was 526. According to the demographic data 33.8% of the individuals belonged to 24-35 years of age group, 49.6% from 15-24 years of age, and 2.7% from 55-64 years of age group who are at risk the most. All these participants were from educated background ranging from intermediate to advance level. (Figure 4).

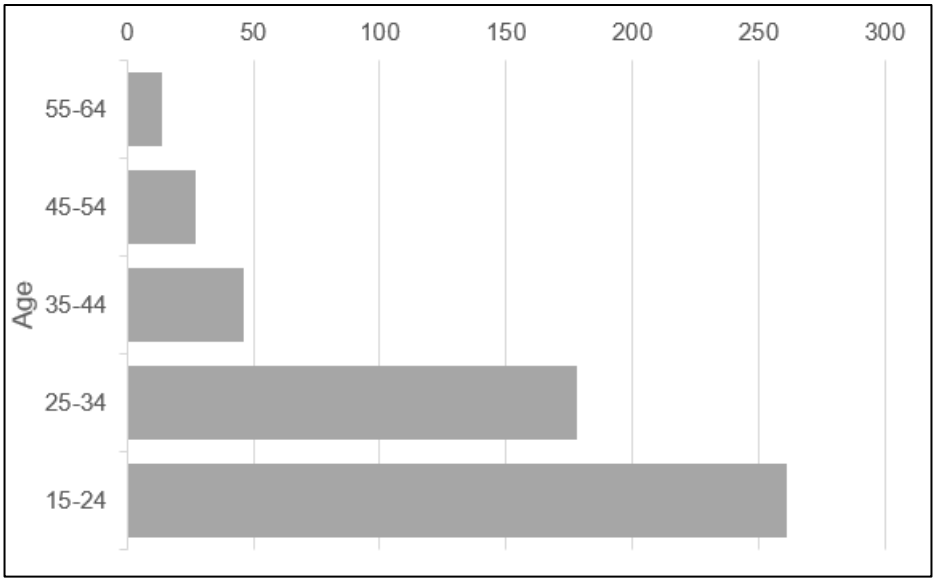

Figure 4: Age groups of the participants. Figure represents the age group of the respondents with 49.6 % of the individual belonged to 15-24 years. 33.8 % of the individuals were 25-34 years, 8.7% from 35-44 years, 5.1% from 45-54, and the lowest response was given by respondents from 55-64 (2.7%).

Question in the survey corresponded to following categories, symptoms (Figure 5), risk factors (Figure 6), life impact (Figure 7), treatment and measures (Figure 8), protective measure (Figure 9) and general knowledge (Figure 10).

4.2.2 Symptoms Based Knowledge Assessment

Respondents were asked questions whether they have difficulty in handling money (Figure 5A) and if they felt lost in familiar environment (Figure 5B). Participants were also asked question regarding better remembrance of recent events than past events (Figure 5C). If someone or anyone they know has suffered from depression and hallucinations simultaneously (Figure 5D). If anyone has suffered from loss of memory or forgetfulness (Figure 5F) or have trouble in remembering names (Figure 5E).

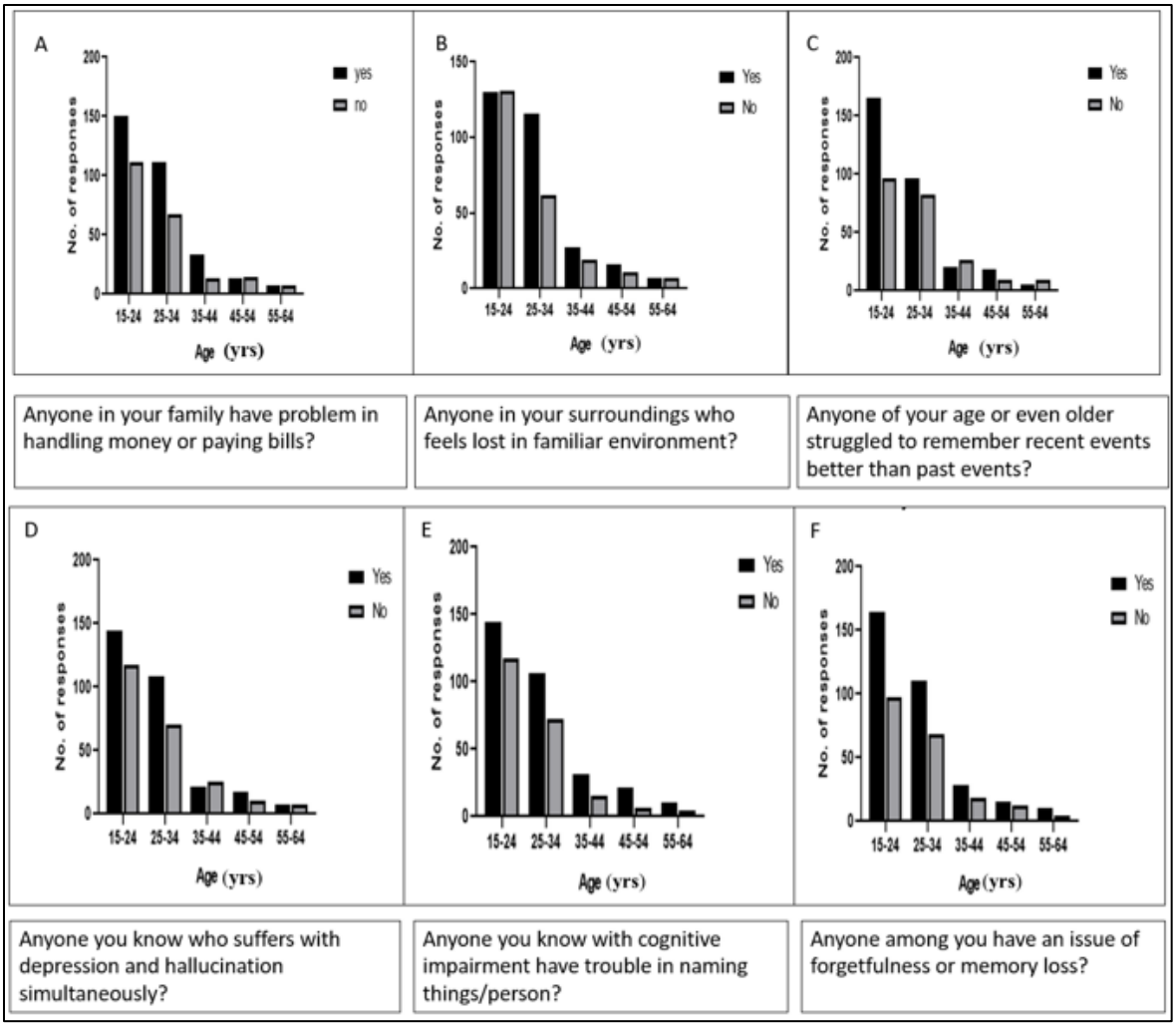

Figure 5: Histogram showing symptom-based analysis of the participants. Less number of the participants (36.1%) was able to identify the symptoms associated with cognitive impairment just like CJD.

4.2.3 Risk Factor Based Knowledge Assessment

Respondents were asked if they interacted with someone suffering from dementia (Figure 6A). If anyone they know has undergone surgery (cornea, brain, or spine) and faced decline afterwards (Figure 6B, 6C). Respondents were also asked if they think CJD increases with age (Figure 6D) or anyone they know died within a year of being diagnosed with dementia (Figure 6E). If they think that CJD can be cured (Figure 6F).

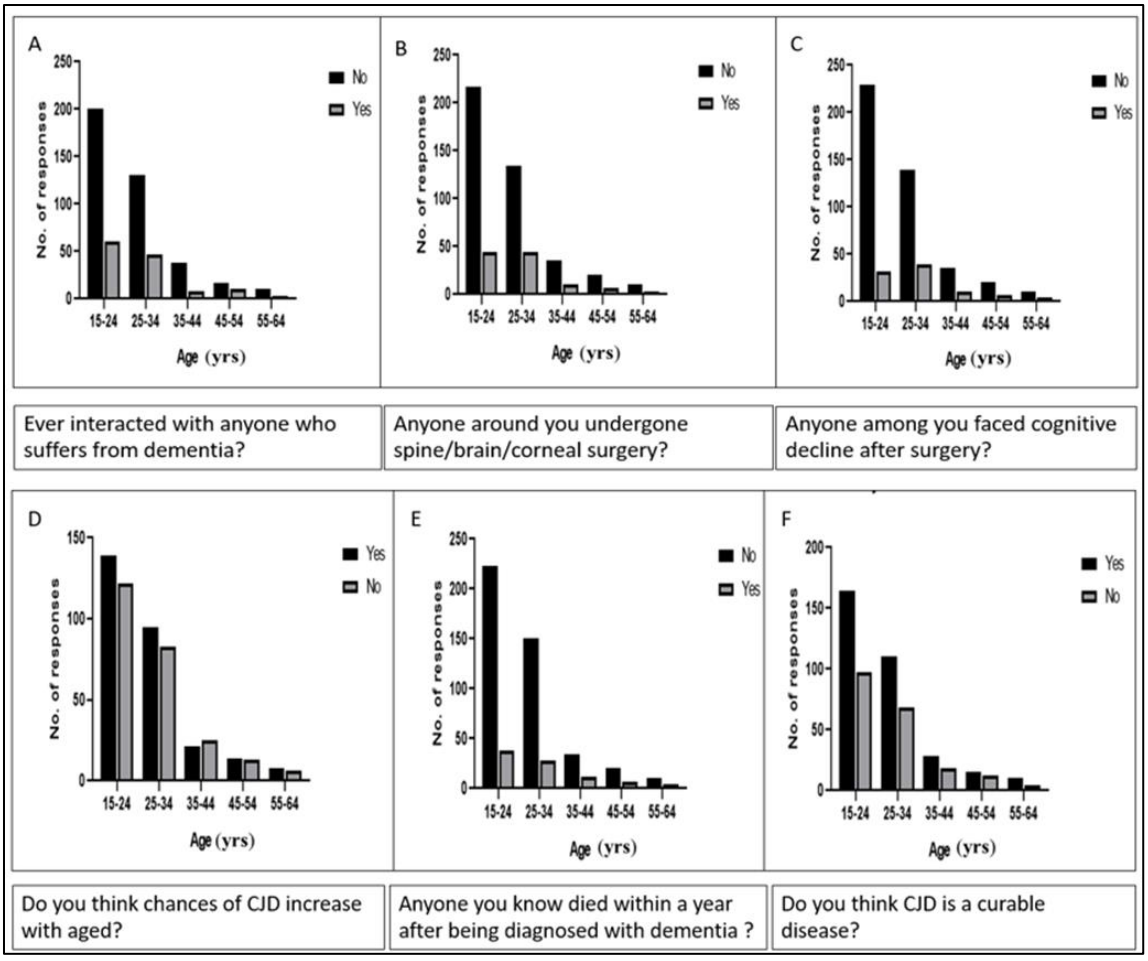

Figure 6: Histogram showing risk-based analysis of participants. Fewer number of participants responded yes (41.5%) that they are familiar with the risk factors associated with cognitive impairment like CJD.

4.2.4 Lifestyle Based Knowledge Assessment

Respondents were asked if they think CJD can be transferred from one place to another (Figure 7A). If they think CJD patient can be financially burdening for the family (Figure 7B) or if a CJD patient can cause psychological burden and illness in the family Figure 7C).

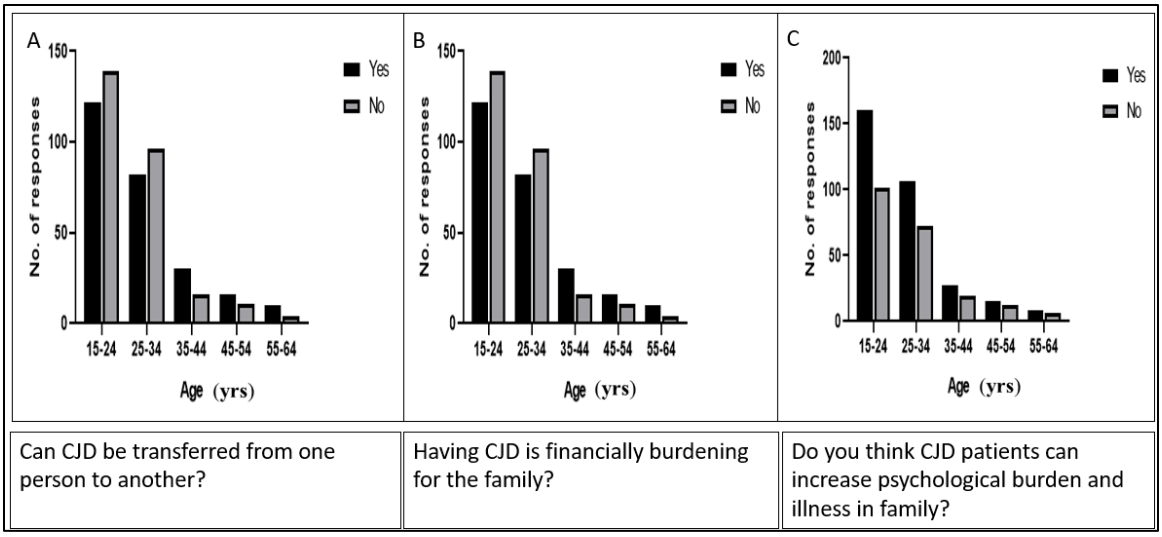

Figure 7: Histogram showing life impact-based analysis of participants. Majority responded yes (35.7%) that they are familiar with the psychological and financial constrains while handling a CJD patients.

4.2.5 Treatment and Measures Based Knowledge Assessment

Respondents were asked whether CJD is a curable disease or not (Figure 8A). Respondents were also asked if it is possible to prevent a person from getting CJD (Figure 8B).

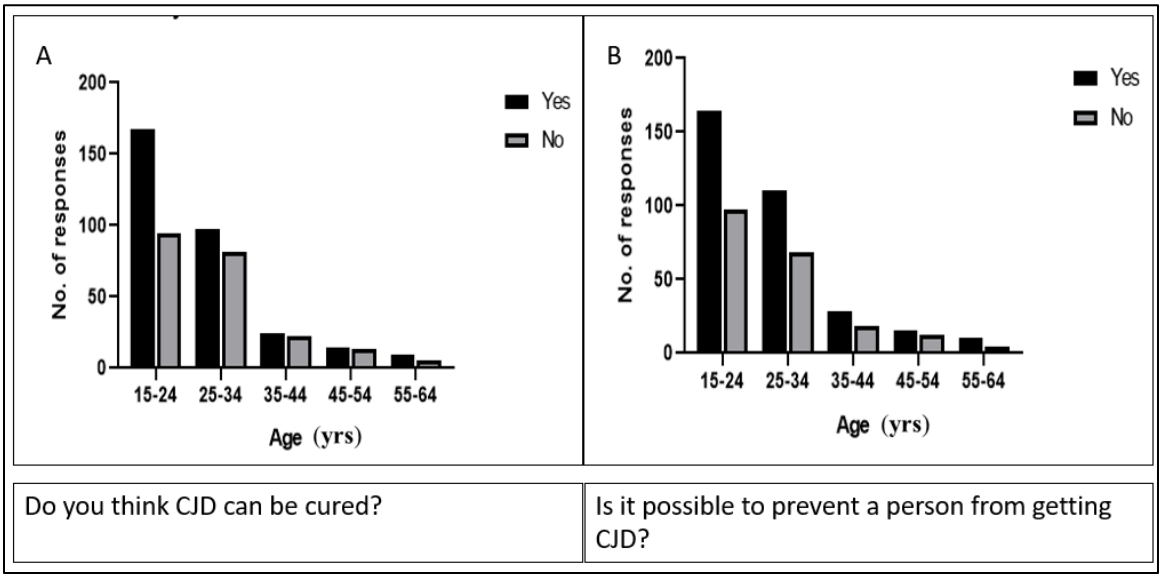

Figure 8: Histogram showing treatment and measures-based analysis of participants. Majority responded yes (84.5%) that they are familiar with the treatment and measures of CJD.

4.2.6 Protective Factor Based Knowledge Assessment

Respondents were asked that if they think playing chess/ reading book or newspaper can help in reducing cognitive decline (Figure 9A). They were also asked whether active participation of CJD patient in social activities can prove helpful for CJD patient or not (Figure 9B).

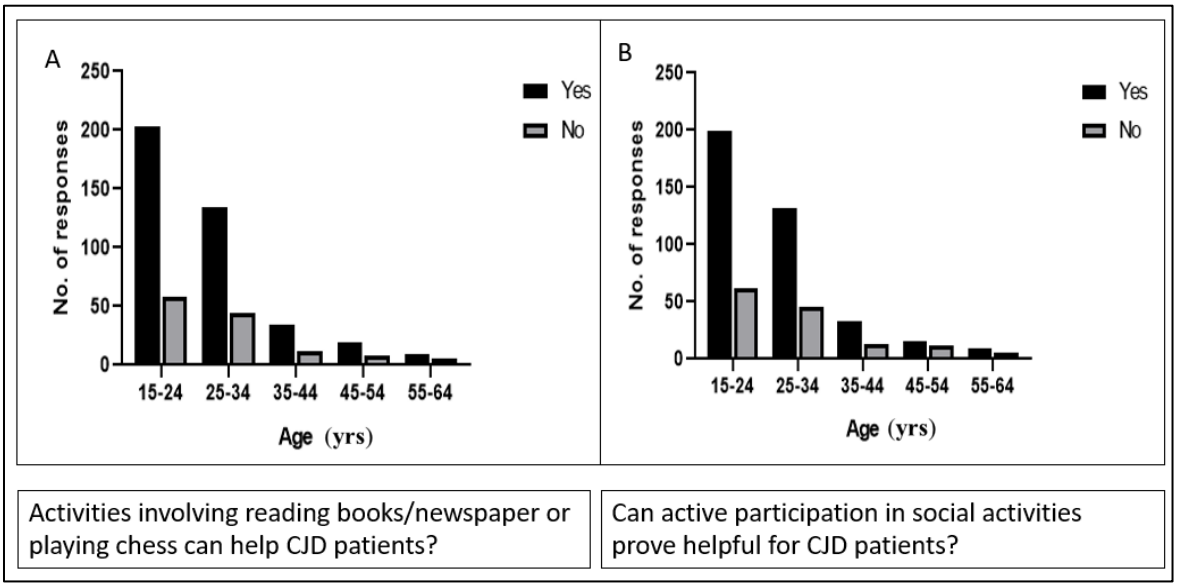

Figure 9: Histogram showing protective factor-based analysis of participants. Majority responded yes (90.1%) that cognitive involvement and social interactions can help a CJD patient.

4.2.7 General Knowledge Based Assessment

Respondents were asked questions whether they are familiar with mad cow disease in general as shown in Figure 10B and do they know what is dementia (Figure 10A).

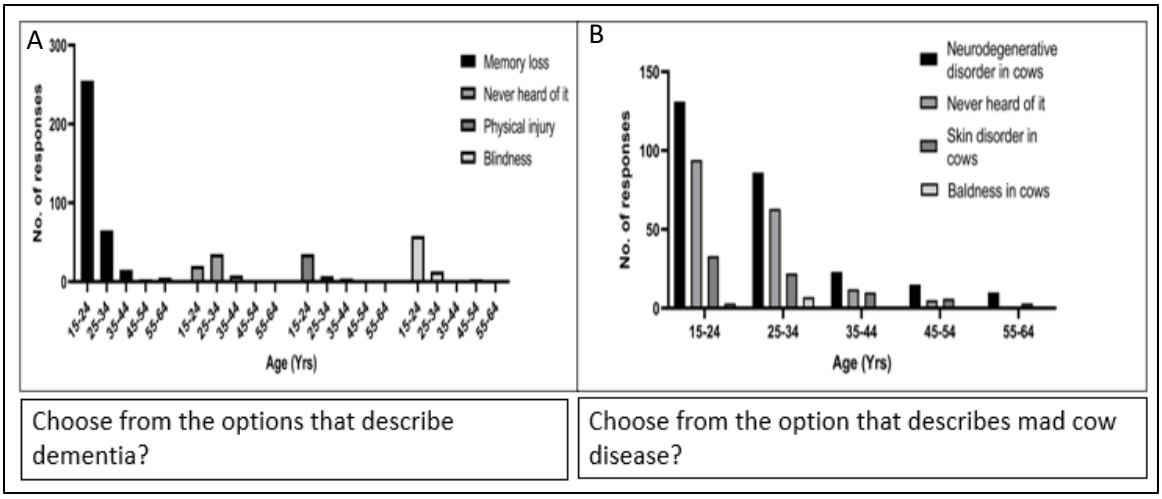

Figure 10: General knowledge-based assessment. In this figure 70% of the participants correctly identified what is dementia (A). Respondents (50%) were able to answer correctly regarding mad cow disease.

4.3 Discussion

CJD is a fatal and rare neurodegenerative disorder. Cases of CJD have been recorded worldwide by different organization i.e., NCJDRSU, CDC, and several CJD foundations in China and Japan. All these surveillance units have strictly monitored and recorded the definite and probable cases of CJD (Diack et al., 2014). According to these reports sCJD has the highest prevalence and death rate as compared to iatrogenic and gCJD. In 2019, 146 cases of CJD were recorded in UK whereas in US there are 32 confirmed cases of CJD who have previously undergone hormone replacement therapy (Sitammagari & Masood, 2022). sCJD dominates all other CJD cases i.e., 85% of the cases. iCJD comprises of 15-10 % of the cases. Whereas gCJD is the rarest form of CJD and comprises of only 1-2 % of the CJD cases (Ladogana & Kovacs, 2018b). 55 such mutations have been identified, each with their own clinical representation depending on age of onset and rapidity of disease progression (Belay, 2017) .

Epidemiological and Clinical studies have revealed that mutation causing CJD can be identified in younger population through blood screening and gene analysis. With these and other diagnostic method, the most prevalent mutation to be found in Caucasian and

East Asian population was E200K (Friedman-Levi et al., 2011b). For this mutation to cause neuropsychiatric disorder is to be inherited as a dominant gene. In 2018 according to Chinese monitoring unit 30 probable gCJD cases were identified (Gao et al., 2019b) .Likewise E200K cases were also reported in Japan, Korea etc. (D. Y. Kim et al., 2022) (Miyakawa et al., 1998) . Its clinic pathological characteristic depends upon the polymorphism at codon 129 (MM, MV or VV). Duration of disease in CJD patients is also influenced by polymorphism at codon 129 i.e., MM homozygosity shows shorter survival rate as compared to MV heterozygous patients (Pocchiari et al., 2004) . The prevalence of MM homozygosis at codon 129 was found to be 50% in Greek population, 40% in British population, 57% in Turkish population. Whereas the prevalence of MV and VV were 37.5% and 5.5% in Greeks, 48% and 11% in British and 34% and 9% in Turkish population. (Saetta et al., 2006) (Erginel-Unaltuna et al., 2001) . Apart from this the occurrence of M129- E200K is more frequent as compared to 129V-E200K. Cases of M129-E200K has been reported in Chile (39), Israel (377), Australia (172), Argentina (33), Japan (63), and 24. Only 3 cases of V129-E200K has been reported worldwide (Kosorinova et al., 2021) (Shi et al., 2008) (Appel et al., 2010). As we know gCJD due to single point mutation codon 200 (GAG to AAG) and should be inherited in an autosomal dominant manner.

As the research findings of our study does not show any mutation at codon 200, so none of the participant can have gCJD. On the contrary 71% (0.71%) of the individuals showed methionine (M) at codon 129, 29% (0.29%) were (V) at codon 129. Which shows that if any of the mutation at codon coding for gCJDs ((gCJD;171, 178, 180,188,196,200,203,208,210,211,232,102) and (GSS; 195 and 107)) comes with methionine, the propensity of this disease will increase, and it will also influence the disease onset and its duration as confirmed by studies mentioned above. Whereas the presence of valine in 29 samples indicates protectiveness against sCJD, iCJD and vCJD but can be a risk factor for mutation at codon 178, showing symptoms associated with FFI. Our genotypic study showed Pakistani population can acquire prion diseases. For that reason, we wanted to check whether our population can identify and report cases of CJD by evaluating their knowledge regarding cognitive impairment associated with CJD. Therefore, a survey was designed encompassing questions related to symptoms, risk

factors, life-impact, treatment and measure, protective measure, and general information regarding the disease (Liu et al.,2019).

Total number of 526 respondents participated in our survey “Creutzfeldt-Jakob diseases (CJD) health screening form”, which was an overall poor response as compared to surveys found in other countries. Predominantly the respondents were of middle age (between 24-35 years, comprising of 33%) and were educated $\geq$ 99.9%, indicating lower level of awareness in public. Knowledge was assessed on different parameters 1. Symptoms, 2. Risk factors, 3. Life impact, 4. Treatment and management and 5. Protective factors. From knowledge perspective the identification of risk factor by the respondents was low $\leq$ 50%, which indicates that half of the respondents were unaware about CJD associated risk factors. Individuals (20.7%) responded yes that they have observed declining brain function in individuals undergone brain/spin/corneal/ hormone replacement therapy. However, they might be confusing it with other comorbidities (cardiovascular disease, Alzheimer etc.) which can lead to low life expectancy.

Half of the individuals (52.7%) responded that CJD increases with age, which is true as CJD cases are more evident in older age group more than 60 years, specifically sCJD. Majority of the participants i.e., 77.9 % answered correctly that CJD can be transferred from one person to another person, as it is an infectious disease but only if it is inherited or acquired. The respondents (60.1%) believed that dealing with a CJD patients can be more psychologically and mentally burdening on the family members rather than financial burden 49.4%. more than half of the participants acknowledged the fact that anyone with mental disability faces social discrimination. This kind of discrimination can be stressful for the caregivers or the family of the patient. CJD treatment as well as management is very expensive. Medical, direct, indirect, and other non-medical procedure could be very financially suffocating for individuals, especially in developing countries like Pakistan where economic situation is already fragile.

We found that respondents have answered correctly about difficulty of CJD patients in handling money (40.3%), remembering recent events (43.7%) or environment (57.8%). Although, having declining brain functioning is one of the early symptoms in CJD

patients, but this may not allow easy detection hence leading to screening and differential diagnosis. Lack of knowledge about CJD can result in worsening of the situation whereas higher knowledge in other diseases have shown timely detection and diagnosis of that disease. Hence efforts are required to help identify between CJD patients and differentiate them from other cognitive disorders. Greater proportion (73-75%) of participants were able to identify that cognitive training, social involvement and healthy activities are helpful in early onset of CJD. There is no preventive measure for CJD but involvement but having a healthy lifestyle can prevent you from cognitive decline like in case of dementia.

Individuals (80%) responded that they have not heard about CJD but 50.4% of the individuals said that mad cow disease, also caused by PrP in animals is a neuro-degenerative disorders. Which shows the lack of knowledge among Pakistani population as they were unable to identify that CJD is also a transmissible spongy encephalopathy just like mad cow disease. Other answers were never heard of it (14.1%) and skin disorders (33.3%). Whereas 60% of the individuals were able to identify correct definition of dementia i.e., memory loss, from which we can imply that people may have knowledge about dementia but not about CJD as it is rare and less prevalent than other neuronal disorders.

On the knowledge assessment scale, awareness about CJD decreases with age. Knowledge score was higher among individuals aged between 15-24 as compared to 55-64. The result suggested that the young individuals have more awareness about CJD than older age group. Hence, we can infer that increased knowledge among younger generation have caused more awareness about prevalence of certain diseases than in older generation. Education and knowledge about CJD are closely associated. So, the people living in urban areas will have more knowledge about CJD than rural area. Other than this most of the neuro-physician and psychiatrics reside in urban areas, indicating many older patients cannot get required CJD treatments or the cases in rural areas are not recorded. Therefore, people who are less educated or lives in rural areas are more vulnerable to CJD. Imparting knowledge of CJD becomes more important to control and prevent the disease.

However limited number of samples demanded caution while using the result of this study. Furthermore, this study needs to be replicated on more diverse group of individuals to assess the actual knowledge and to quantify probable and definite cases of these diseases.

5. CONCLUSION AND FUTURE PROSPECTS

5.1 Conclusion

These research findings indicate that none of the individuals contained the mutated sequence for CJD but the presence of polymorphism in some cases may increase its susceptibility to acquire CJD in future. The most prevalent combination was M129-E200, showing absence of mutated sequence for gCJD but increased propensity for sCJD, iCJD, vCJD and other mutation associated for gCJDs due to methionine at codon 129. V129-E200 was also reported in 29 participants with no genetic mutation at codon 200 but polymorphism at codon 129, indicating protectiveness against sCJD, iCJD, vCJD and majority of the mutations associated with gCJDs. However, CJD is caused by having lysine instead of glutamic acid, as none of the individuals had the mutated nucleotide sequence due to which M129-K200 and V129-K200 was absent from the participants. Despite of having increased susceptibility, Pakistani population is still unaware about its technical aspect, as indicated by the survey study we conducted. Therefore, for its accurate assessment the stakeholders need to be involved.

5.2 Future prospects

Genotyping data gathered from this research provides preliminary step to explore other genotypes responsible for causing CJD. Moreover, it can help in identifying its source and prevent practitioners from its misdiagnosis. On the other hand, surveillance data can help law makers to include CJD in their law-making process to curtail this disease.

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Appendix A

Summary table for all genotypes; 51/100 were homozygous for M129-E200 combination, 29/100 were heterozygous for V129-E200. Mutated combinations M129-K200 and V129-K200 were absent in all samples.

Sample	**M129-K200**	**M129-E200**	**V129-E200**	**V129-K200**	**Genotype**	
1	No	Yes	No	No	M/E	Homozygous
2	No	Yes	No	No	M/E	Homozygous
3	No	Yes	No	No	M/E	Homozygous
4	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
5	No	Yes	No	No	M/E	Homozygous
6	No	Yes	No	No	M/E	Homozygous
7	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
8	No	Yes	No	No	M/E	Homozygous
9	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
10	No	Yes	No	No	M/E	Homozygous
11	No	Yes	No	No	M/E	Homozygous
12	No	Yes	No	No	M/E	Homozygous
13	No	Yes	No	No	M/E	Homozygous
14	No	Yes	No	No	M/E	Homozygous
15	No	Yes	No	No	M/E	Homozygous
16	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**

17	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
18	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
19	No	Yes	No	No	M/E	Homozygous
20	No	Yes	No	No	M/E	Homozygous
21	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
22	No	Yes	No	No	M/E	Homozygous
23	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
24	No	Yes	No	No	M/E	Homozygous
25	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
26	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
27	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
28	No	Yes	No	No	M/E	Homozygous
29	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
30	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous**

						for 200
31	No	Yes	No	No	M/E	Homozygous
32	No	Yes	No	No	M/E	Homozygous
33	No	Yes	No	No	M/E	Homozygous
34	No	Yes	No	No	M/E	Homozygous
35	No	Yes	No	No	M/E	Homozygous
36	No	Yes	No	No	M/E	Homozygous
37	No	Yes	No	No	M/E	Homozygous
38	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
39	No	Yes	No	No	M/E	Homozygous
40	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
41	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
42	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
43	No	Yes	No	No	M/E	Homozygous
44	No	Yes	No	No	M/E	Homozygous
45	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
46	No	Yes	No	No	M/E	Homozygous
47	No	Yes	No	No	M/E	Homozygous
48	No	Yes	No	No	M/E	Homozygous
49	No	Yes	No	No	M/E	Homozygous
50	No	Yes	No	No	M/E	Homozygous

51	No	Yes	No	No	M/E	Homozygous
52	No	Yes	No	No	M/E	Homozygous
53	No	Yes	No	No	M/E	Homozygous
54	No	Yes	No	No	M/E	Homozygous
55	No	Yes	No	No	M/E	Homozygous
56	No	Yes	No	No	M/E	Homozygous
57	No	Yes	No	No	M/E	Homozygous
58	No	Yes	No	No	M/E	Homozygous
59	No	Yes	No	No	M/E	Homozygous
60	No	Yes	No	No	M/E	Homozygous
61	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
62	No	Yes	No	No	M/E	Homozygous
63	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
64	No	Yes	No	No	M/E	Homozygous
65	No	**No**	**Yes**	No	**M/E**	**Heterozygous for 129 -Homozygous for 200**
66	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
67	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
68	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
69	No	**No**	**Yes**	No	**V/E**	**Heterozygous for**

						129 -Homozygous for 200
70	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
71	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
72	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
73	No	**No**	**Yes**	No	**V/E**	**Heterozygous for 129 -Homozygous for 200**
74	No	Yes	No	No	M/E	Homozygous
75	No	Yes	No	No	M/E	Homozygous
76	No	Yes	No	No	M/E	Homozygous
77	No	Yes	No	No	M/E	Homozygous
78	No	Yes	No	No	M/E	Homozygous
79	No	Yes	No	No	M/E	Homozygous
80	No	Yes	No	No	M/E	Homozygous
81	No	Yes	No	No	M/E	Homozygous
82	No	Yes	No	No	M/E	Homozygous
83	No	Yes	No	No	M/E	Homozygous
84	No	Yes	No	No	M/E	Homozygous
85	No	Yes	No	No	M/E	Homozygous
86	No	Yes	No	No	M/E	Homozygous
87	No	Yes	No	No	M/E	Homozygous
88	No	Yes	No	No	M/E	Homozygous
89	No	Yes	No	No	M/E	Homozygous
90	No	Yes	No	No	M/E	Homozygous

91	No	Yes	No	No	M/E	Homozygous
92	No	Yes	No	No	M/E	Homozygous
93	No	Yes	No	No	M/E	Homozygous
94	No	Yes	No	No	M/E	Homozygous
95	No	Yes	No	No	M/E	Homozygous
96	No	Yes	No	No	M/E	Homozygous
97	No	Yes	No	No	M/E	Homozygous
98	No	Yes	No	No	M/E	Homozygous
99	No	Yes	No	No	M/E	Homozygous
100	No	Yes	No	No	M/E	Homozygous

Appendix B

Questionnaire. Questions related to CJD were asked from the respondents to assess their knowledge.

1. Age
   - 15-24
   - 25-34
   - 35-44
   - 45-54
   - 55-64
2. Kindly add your field/education. *

3. Do you or anyone in your family have problem in handling money or paying bills? *
   - Yes
   - No
4. Is there anyone in your surroundings who feels lost in a familiar environment? *
   - Yes
   - No

5. Have you observed anyone of your age or even older struggling to remember recent events better than past events? *
   - Yes
   - No
6. Can you remember someone with depression suffering from hallucination as well? *
   - Yes
   - No
7. Choose yes if you/ blood relations have had trouble in naming person or a thing that is familiar to you? *
   - Yes
   - No
8. Choose from the option below that describes dementia? *

- Physical injury.
- Memory loss.
- Never heard of it.
- Blindness.

9. Have you ever interacted with anyone who suffers from dementia? *
   - Yes
   - No
10. Do you have a family history of memory issue/forgetfulness? *
    - Yes
    - No
11. Which option mentioned below describes "mad cow disease" accurately? *
    - Neurodegenerative disorder in cows
    - Skin disorder in cows.
    - Never heard of it.
    - Baldness in cows.
12. Have you heard about CJD before? *
    - Yes
    - No
13. Have you or anyone around you undergone spine/brain surgery/corneal transplant/ growth hormone replacement therapy? *
    - Yes
    - No
14. If yes to question 11, have you/anyone faced any decline in brain functioning after the surgery? *
    - Yes
    - No
15. Do you think chances of CJD increases with age? *
    - Yes
    - No
16. Has anyone you know died within a year after being diagnosed with Dementia? *
    - Yes
    - No

17. Do you think CJD can pass from one person to another? *
    - Yes
    - No
18. Do you think having a CJD patient is financially burdening for the family? *
    - Yes
    - No
19. Do you think psychological burdening and occurrence of psychological illness may increases in a family dealing with a CJD patient? *
    - Yes
    - No
20. Do you think CJD can be cured? *
    - Yes
    - No
21. Do you think it's possible to prevent a person from developing CJD? *
    - Yes
    - No
22. Do you think activities such as reading books or newspaper and playing chess or cards games are helpful for people with CJD? *
    - Yes
    - No
23. Do you think active participation in various social activities may protect the cognitive function of CJD patients? *
    - Yes
    - No