Analysis of Repeat Expansion in *HTT* Gene in Families with Huntington's Disease

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Certificate

This thesis submitted by Alina Murtaza is accepted in its present form by the Department of Biochemistry, Quaid-I-Azam University Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Biochemistry/Molecular Biology.

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Declaration

I hereby declare that the work presented in the following thesis is my own effort and the thesis is composed by me. No part of this thesis has been previously presented elsewhere for any other degree or certificate.

Alina Murtaza

I proudly dedicate this thesis to my family, who invested a remarkable amount of dedication and hard work to help me attain this milestone.

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All the praises and glories be to **Almighty Allah** the lord of the universe for His infinite mercy and blessings upon us, whomever He guides shall never go astray, and whomever He allows to astray shall never find the guidance, may the peace and blessing of Allah be upon to His noble **Prophet Muhammad S.A.W** and his companions.

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Alina Murtaza

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List of abbreviations

Abbreviations	Description
HD	Huntington's disease
ND	Neurodegeneration
NDD	Neurodegenerative Disorders
ALS	Amyotrophic Lateral Sclerosis
PD	Parkinson's Disease
AD	Alzheimer's Disease
LBD	Lewy Bodies Dementia
FTD	Frontotemporal Dementia
MD	Movement Disorders
SCA	Spinocerebellar Ataxia
FTLD	Frontal and Temporal Lobe Degeneration
DEM	Dementia
SP	Sporadic
TRDs	Triple Repeat Disorders
FMR1	Fragile X mental retardation 1
CAA	Cerebral Amyloid Angiopathy
CJD	Creutzfeldt-Jacob disease
VPSPr	Variably protease-sensitivity prionopathy
PiD	Pick disease
GGT	Globular glial tauopathy
CBD	Corticobasal Degeneration
AGD	Argyrophilic grain disease
MSA	Multiple System Atrophy
MND	Motor Neuron disease
BIBD	Basophilic Inclusion Body Disease
NIFID	Neurofilament intermediate filament inclusion disease
HDL2	Huntington's Disease like 2
FXTAS	Fragile X-associated tremor and Ataxia syndrome
SBMA	Spinal and Bulbar Muscular Atrophy
DRPLA	Dentatorubral-pallidolusian atrophy
GOF	Gain of function
LOF	Loss of function
RAN	Repeat-associated non-AUG
AOO	Age of onset
MSN	Medium spiny neurons
BEC	Bioethical Committee
EDTA	Ethylenediamine Tetra-acetic Acid
SDS	Sodium Dodecyl Sulphate
TE	Tris-EDTA
DMSO	Dimethyl sulfoxide
MgCl2	Magnesium Chloride
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction

ABSTRACT

Huntington's disease (HD) is a late-onset neurodegenerative disorder characterized by motor, cognitive and psychiatric abnormalities. HD is caused by repeat expansion in the huntingtin (*HTT*) gene located on chromosome 4p16.3. HD is frequently reported in Western populations, but there is a scarcity of studies from Pakistan. The present study aimed to analyze the clinical and genetic characteristics of HD in Pakistani families. For this study, three families with HD phenotype and healthy controls were recruited from different regions of Pakistan. The age of onset of HD in 8 patients of three families ranges from 20 to 49 years, except the single patient who presented juvenile-onset HD. Clinical features observed in these patients included chorea, tremors, bradykinesia, dystonia, facial dysmorphism, progressive dementia and low learning abilities. Brain magnetic resonance imaging (MRI) of 2 patients (H2-2, H3-1) presented severe striatum atrophy. Blood smear analysis was used to study acanthocytes in family H1 and H2 by light microscopy. The erythrocyte sedimentation rate (ESR) results of family H1 and H2 demonstrated a reduced sedimentation rate in the affected individuals as compared with healthy individuals. The average number of CAG repeats in the *HTT* gene of affected individuals was found to be between 40 to 70 CAGs and lies in the pathogenic range. The expanded and normal alleles of selected families were also confirmed by Sanger sequencing. One patient from family H2 with juvenile HD has 67 CAG repeats. Two members of family H1 have 44 CAG repeats in both alleles and their current ages were 13 and 24 years. Whereas the analysis of CAG repeats in normal controls by 2.5% agarose gel showed a range from 18 to 22. An inverse correlation between the age of onset of HD and CAG repeats was observed in our study. This study represents the first molecular characterization of HD in stani families and provides an initial estimation of repeat size in HD patients and healthy controls.

CHAPTER 01

INTRODUCTION

1. Introduction

1.1. Neurodegeneration

Neurodegeneration (ND) is etymologically composed of "neuro" meaning nerve cells (neurons) and "degeneration" means the process of losing structure and function (Przedborski et al., 2003; Sweeney et al., 2018). Neurodegeneration represents a heterogeneous group of neurological disorders characterized by the progressive loss of the structural and functional neurons in the brain (Wilson et al., 2023).

1.2. Neurodegenerative Disorders

Neurodegenerative disorders (NDD) are characterized by progressive dysfunction of neurons, glial cells, and synaptic networks (Kovacs et al., 2019). Neurodegeneration causes a collapse in both the structural and functional integrity of neurons. Neurons are unable to self-regenerate after neurodegeneration due to their terminally differentiated nature resulting in the disruptive core communicative circuitry that leads to impaired sensory, cognitive, psychiatric, and motoric abilities (Agrawal et al., 2020; Wilson et al., 2023). Despite the progressive loss of neurons, NDD shares overlapping clinical features i.e., motor function is affected not only in Huntington's Disease (HD) but also in Amyotrophic Lateral Sclerosis (ALS), Parkinson's Disease (PD) and Spinocerebellar Ataxia (SCA). Similarly, the cognitive system is affected by Alzheimer's Disease (AD), Lewy bodies Dementia (LBD), and Frontotemporal Dementia (FTD) (Gan et al., 2018). Another common feature is the localized aggregation of nuclear and cytosolic proteins (Kovacs & GG, 2016).

1.3. Classification of Neurodegenerative Disorders

NDDs are classified based on the following categories:

1.3.1. Clinical Symptoms Determined by Anatomical Region:

Basal ganglia, cerebral cortex, thalamus, lower motor neurons of the spinal cord, brain stem and nuclei, and motor cortical areas are involved in movement disorders (MD) i.e., cerebral (SCA), hyperkinetic (HD), hypokinetic (PD), and lower and upper motor dysfunctions (Gan et al., 2018). Frontal and temporal lobe degeneration (FTLD) is involved in frontotemporal dementia (FTD). Neocortical areas, hypothalamus,

entorhinal cortex, and limbic system are involved in dementia (DEM), high-order brain dysfunction and cognitive decline (Kovacs & GG, 2016).

1.3.2. Molecular and Neuropathological Profiling

It primarily involved the distinction between extracellular, intracellular, and synaptic protein aggregations. The subcellular locations of the intracellular deposits, i.e., cytoplasmic, nuclear and cell processes are also distinguished by using immunohistochemistry patterns (Jellinger, 2010).

1.3.3. Altered Proteins (Proteinpathies)

NDDs are majorly classified based on the aggregated proteins involved in most of the genetic adult-onset (GEN) and sporadic (SP) NDDs (Table 1.1). Proteinpathies are the major cause of DEM, FTD and MDs (Hardy et al., 1998; Jellinger, 2010; Kovacs & GG, 2014; Kovacs & GG, 2016).

1.4. Triple Repeat Disorders (TRDs)

TRDs represent a heterogenous group of NDDs caused by trinucleotide repeats (TRs) expansion beyond a pathological threshold (Kurokawa et al., 2023). In 1991, two different research groups discover CAG repeat expansion in the androgen receptor gene (OMIM:313700) in SBMA (Spada et al., 1991) and CGG repeat expansion in the 5'UTR of fragile X mental retardation 1 (FMR1) (OMIM: 309550) patients (Verkerk et al., 1999).

From 1991-2021 TR expansions in more than 50 genes have been discovered in the exonic, intronic, and UTRs of the genes (Figure 1.1), unraveling the basis of more than 25 genetic disorders that mainly affect CNS (Depienne et al., 2021).

Table 1.1. Classification of NDDs based on the aggregated proteins/ Proteinpathies (Depienne et al., 2021).

Disease Group	Aggregated Proteins	Disease Type	Mode of inheritance	Phenotype
AD	Aβ (Extracellular)	AD	GEN/SP	DEM
		Cerebral Amyloid Angiopathy (CAA)	GEN	DEM
Prion Disease	PrP (Extracellular)	Creutzfeldt-jacob disease (CJD), Variably protease-sensitivity prionopathy (VPSPr), Kuru, gCJD, PrP-CAA	SP	DEM/MD
Tauopathy	Tau (Intracellular)	Pick disease (PiD), Globular glial tauopathy (GGT), Corticobasal degeneration (CBD)	SP	FTD
		Argyrophilic grain disease (AGD)	SP	DEM
		FTDP linked to Chr17 caused by mutations in MAPT (FTDP-17T)	GEN	FTD/MD
	α-Synuclein (Intracellular)	PD	SP/GEN	MD
		Dementia with Lewy body (DLB), Multiple System Atrophy (MSA)	SP/GEN	DEM/MD
TDP-43 Proteinpathy	TDP-43 (Intracellular)	FTLD-TDP (Type A-D)	SP/GEN	FTD
		Motor neuron disease (MND)-TDP	SP/GEN	MD
	FUS/FET (Intracellular)	aFTLD- ubiquitinated inclusions (a-FTLD-U), Basophilic Inclusion body (BIBD), Neurofilament intermediate filament inclusion disease (NIFID)	SP	FTD/MD
		MND-FUS	GEN	MD
TRDs	Huntingtin	HD	GEN	MD
	Junctophilin 3	Huntington Disease like 2 (HDL2)	GEN	MD
	Ataxin1,2,3,7, CACNA1A, TBP	SCA 1, 2, 3, 6, 7, 17	GEN	MD
	FMRP	Fragile X-associated tremor and Ataxia syndrome (FXTAS)	GEN	MD
	ARP	Spinal and bulbar muscular Atrophy (SBMA)	GEN	MD
	Atrophin-1 (Intracellular)	Dentatorubral-pallidolusian atrophy (DRPLA)	GEN	MD

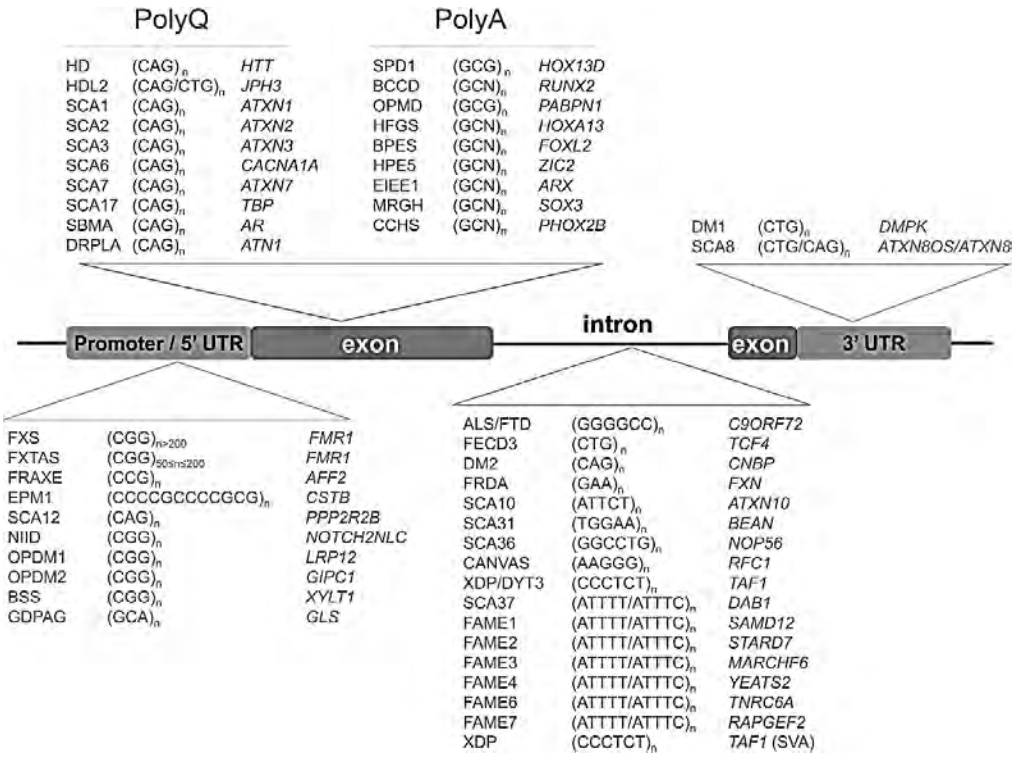

Figure 1.1. Localization of repeat expansions within different genes and their association with respective TRDs. TR expansions can affect coding regions (mainly encoding polyalanine and polyglutamine tract) or non-coding regions (mainly in introns, promoters, and UTRs) of genes (Depienne et al., 2021).

1.4.1. Pathomolecular mechanisms in TRDs

Repeat expansions cause architectural alterations in the DNA and promote a cascade of concurrent molecular processes by either gain of function (GOF) or loss of function (LOF) processes at the DNA, RNA, and protein level (Budworth et al., 2013). The pathogenesis of these processes is modulated by the somatic instability of repeats (Wells et al., 1996). Repeat expansions are mitotically unstable and every cell division is linked with a high risk of errors during DNA replication. Somatic instability in repeat expansions has been initially referred to as the DNA polymerase slippage error by forming stable secondary structures (Depienne et al., 2021). Recent observations in non-dividing cells, animal models and even from affected individuals revealed that instability of repeats majorly relies on the DNA mismatch repair processes that can affect the progression and onset of disease (Jones et al.,

2017; Benn et al., 2021). Majority of repeat expansion disorders are related to somatic mosaicism (Depienne et al., 2021). The pathomolecular mechanism in repeat expansion disorders has been broadly grouped into four categories.

1.4.1.1.Epigenetic gene silencing and R-loop formation

GC-rich expansions in 5'UTR and promoters are mostly linked with the CpG sites that are hypermethylated. Methylated expanded alleles with repeats beyond the pathological threshold are closed in the chromatin configuration, resulting in gene silencing or LOF (Figure 1.2A). CGG expansion in FMR1 triggers hypermethylation of neighboring FMR1 promoters and the CCG repeat tract that epigenetically silences the FMR1 locus (Malik et al., 2021). Active transcription across the expanded repeats promotes R-loop formation that triggers the DNA damage response cascade, contributing to the pathogenesis of the disease (Depienne et al., 2021.

1.4.1.2.RNA inclusions

Expanded repeats within RNA form stable secondary structures that have a high affinity to bind with RNA-binding splice factors (RBPs) resulting in the misplicing of transcripts and toxic RNA inclusions in the nucleus (Figure 1.2B) e.g., CAG/CTG expansion in HDL2 results in RNA toxicity and polyglutamine aggregations (Malik et al., 2021).

1.4.1.3.Protein aggregations

Abnormally encoded polyQ expansions alter the conformation of proteins that misfold into the insoluble polymer, forming intranuclear protein aggregates that elicit GOF toxicity (Figure 1.2C). CAG repeats expansion in HD forms intranuclear polyglutamine aggregates contributing to the disease pathogenicity (Jellinger et al., 2010).

1.4.1.4.Repeat-associated non-AUG (RAN) translation

It is a non-canonical protein synthesis mechanism at the repeat expansion site in which the synthesis of peptides is initiated without a start codon (AUG) in all three reading frames using both antisense and sense strand direction. RAN translation produces toxic polymeric proteins (Depienne et al., 2021).

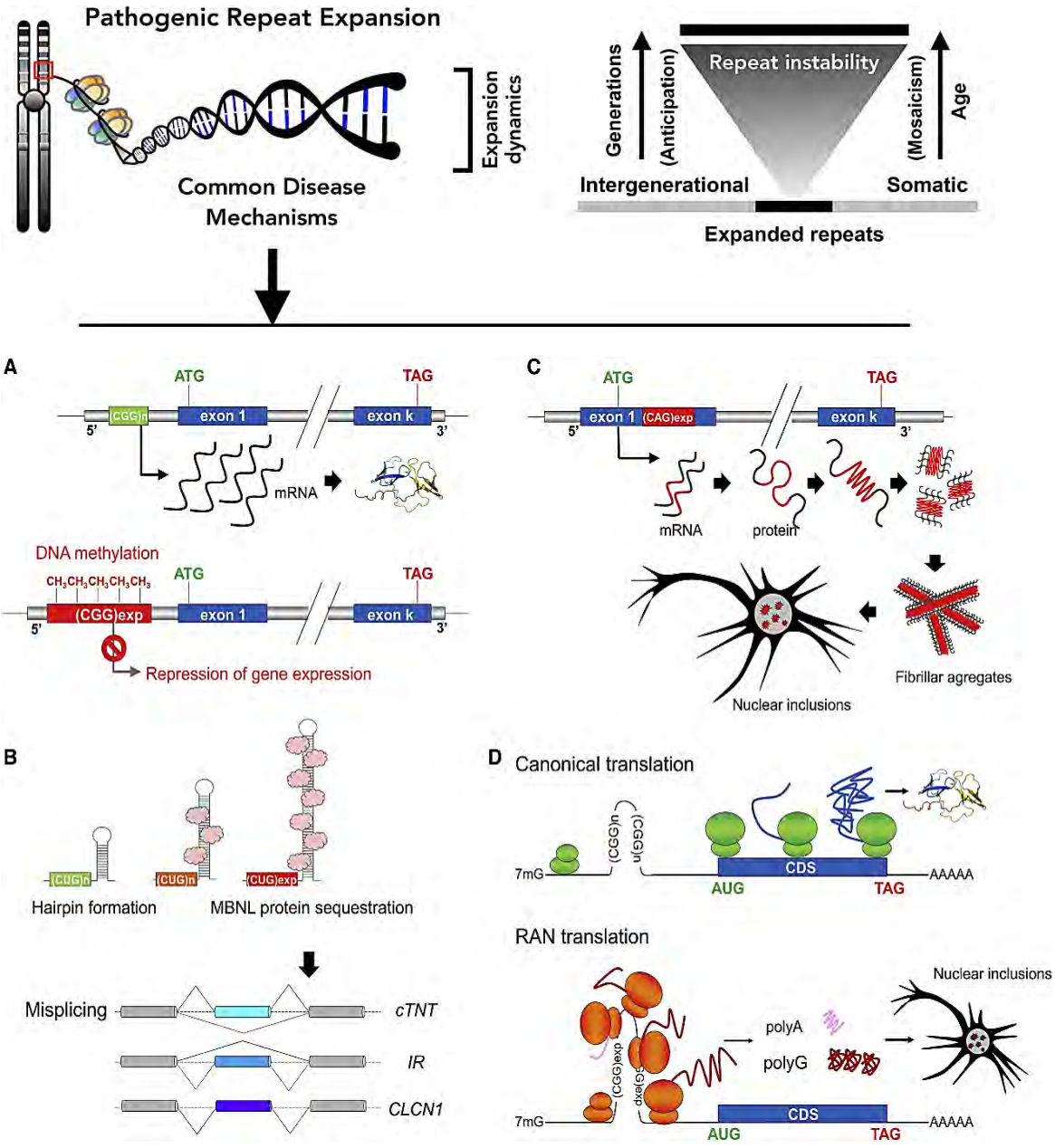

Figure 1.2. Molecular processes that drive the pathogenesis of nucleotide repeat disorders (Depienne et al., 2021; Malik et al., 2021). Repeat expansion disorders are associated with somatic mosaicism that correlates with the age of onset (AAO) and the size of repeat expansion. Repeat expansions can promote DNA hypermethylation that can lead to transcriptional silencing and LOF (A) or active transcription can form secondary structures (B). Alternatively, repeat expansions in protein-coding regions can cause misfolding (C) or RAN translation produces toxic RAN peptides (D).

1.5. Huntington's Disease (HD)

HD (OMIM: 143100) is an autosomal dominant progressive neurodegenerative disorder. HD is characterized physiologically by the atrophy of the striatum and cortex that leads to motor, cognitive and psychiatric abnormities. It is caused by the CAG repeats expansion in the *HTT* (IT15) gene (OMIM: 613004) that encodes mutant huntingtin protein (m*HTT*). Neuronal dysfunction in the striatum is responsible for the progression and appearance of the disease. Toxic GOF of the mutant huntingtin protein and LOF of the wild-type huntingtin leads to neuronal death.

1.5.1. Epidemiology

. Prevalence studies, using both clinical diagnostic and genetic standards depicted that in the Western population, 10.6-13.7/10,000 individuals are affected by HD. HD is endemic to all populations, but a higher frequency is reported in European ancestry (Bates et al., 2015; Kay et al., 2017). In Canada and British Columbia, 17.2/100,000 individuals are affected by HD. The prevalence of HD is very scarce in Asia and Africa. The low prevalence can be attributed to limited genetic testing and genetic bias towards CAG repeat size. The overall prevalence of HD in Asia is 0.4/100,000 individuals, which is very low in comparison to the European population (Pandey et al., 2018; Xu et al., 2018).

Different prevalence of HD across different ethnic groups is associated with genetic differences in the *HTT* gene. The average (normal) length of CAG repeats is longer in populations with a high prevalence of HD e.g., European ancestry has 18.4-18.7 average CAG repeats, African population has 16.9-17.4 average CAG repeats and East Asian has 17.5-17.7 average CAG repeats. In Pakistan, no epidemiological study related to HD has been conducted to date.

1.5.2. Clinical Manifestations

HD is characterized by a triad of motor, cognitive, and psychiatric abnormalities. There is clinical heterogeneity in HD even within members of the same family.

1.5.2.1. Motor Abnormalities

Motor abnormalities are defined as involuntary movements in the early and moderate stages of adult-onset disease and impaired voluntary movements in the advanced stages of HD. As the striatum's medium spiny neurons (MSN) are progressively lost, hypokinetic and hyperkinetic disturbances are observed (Tabrizi et al., 2013). The hyperkinetic phase involves chorea in the early stages of HD. Choreatic movements are involuntary, semi-purposeful, short-lived, and excessive movements. The progression of chorea involves the low amplitude occasional twitches of extremities and face to constant involuntary movements in the whole body. Abnormal eye movements, tongue protrusion, abnormal movements of facial and neck muscles, and head-turning followed by back extensions are the hallmarks of chorea. The hypokinetic phase involves bradykinesia, gait disturbance, dystonia, dyskinesia, and balance problems. As the disease progresses to the advanced phase, dystonia, rigidity, and bradykinesia dominate (Ghosh & Tabrizi, 2018).

1.5.2.2. Cognitive Abnormalities

Cognitive abnormalities may be seen 15 years prior to the onset of motor abnormalities (Paulsen et al., 2011). These abnormalities further progress to subcortical and frontal dementia in the advanced stages of the disease (Ghosh & Tabrizi, 2018). The TRACK-HD study described the early insufficiencies in psychomotor speed, spatial and visuomotor integrity, and visual attention (Tabrizi et al., 2013). It was observed that both explicit and implicit learning is compromised in HD patients. Attention deficits are more commonly observed in prodromal and diagnosed HD patients i.e., vigilance, response flexibility, and resource allocation (Paulsen et al., 2011). Executive dysfunction in concrete thinking is also one of the hallmarks of HD (Ghosh & Tabrizi, 2018).

1.5.2.3. Psychiatric abnormalities

A wide range of neuropsychiatric abnormalities affect HD patients. Depression is the leading symptom affecting 40% of HD patients (Tabrizi et al., 2013). Anxiety is the second major feature in HD patients. Depression and anxiety are highly observed in premanifest HD, and they are not related to the stage of the disease (Ghosh & Tabrizi, 2018). Apathy is

the most frequent and disabling neuropsychiatric symptom associated with disease staging and it progresses and worsens in the advanced stages of disease. It is characterized by passive behavior, difficulty with initiating normal activities, general loss of interest, and lack of emotional expression. Apathy, irritability, and anxiety are associated with functional decline (Paoli et al., 2017). Other neuropsychiatric symptoms can be treated with antidepressants and antipsychotics, but there is no medication or effective treatment for apathy (Ghosh & Tabrizi, 2018).

Other psychiatric symptoms observed in HD patients are aggression, compulsive thoughts, obsessive and repetitive behavior, and psychosis in the later stages of the disease. Suicidal thoughts are very common in HD patients (Tabrizi et al., 2013). Suicide is the second major reason for death in HD. Its risks become more evident when premanifest patients start developing HD and when independence is lost in the advanced stages of the disease (Naarding et al., 2001). A current study from the REGISTRY cohort included both the premanifest and manifest HD subjects demonstrated that 28% were suffering from apathy, 13% were facing depression, aggression, obsessive and compulsive behavior, and 1% were affected by psychosis (Duijn et al., 2014).

1.5.2.4.Other Neurological Symptoms

In addition to the motor, cognitive and psychiatric decline, HD-affected individuals may experience dysphagia (swallowing issues), insomnia, and apraxia (speech problems). Apraxia results due to dysphagia and difficulty in finding words. In the advanced stages of HD , anarthria (complete loss of speech) may occur in some patients (Tabrizi et al., 2013).

1.5.2.5.Acanthocytosis

A wide range of movement disorders are closely linked with acanthocytosis (Patel et al., 2021). Acanthocytosis is the transformation of the normal biconcave disk erythrocytes into irregularly shaped external projections. The associations of neuro-acanthocytosis with *HTT* expansion have been previously reported (Murphy et al., 2018). A recent study also observed acanthocytes in HD patients (Yu et al., 2022).

1.5.2.6.Peripheral symptoms

HD is primarily the CNS disease, but huntingtin is ubiquitously expressed throughout the cells of the body, resulting in some other systematic symptoms. HD patients experience severe weight loss, especially during the predormal stage which may lead to cachexia. Other symptoms include skeletal muscle atrophy and osteoporosis (Altıner et al., 2020). HD patients exhibit cardiac arrest. Heart failure occurs in about 30% of HD patients (Melkani et al., 2016). Endocrine dysfunction includes hypothyroidism, impaired glucose tolerance and testicular atrophy (Tabrizi et al., 2013).

1.5.3. Neuropathology in HD

In patients with HD, there is a slow loss of medium spiny GABAergic neurons in the striatum which results in a reduction in the striatum and thinning of the cortex. The atrophy spreads symmetrically throughout the brain as the disease progresses (Gutekunst et al., 2002). The subsequent loss of parenchyma affects different compartments of the brain like the cerebral cortex, white matter, thalamus, and cerebellum. The brain becomes considerably smaller than normal in the final stages, and the striatum is mostly affected by the degeneration of neurons. In addition, the hypothalamus and hippocampus are also affected (Estrada-Sánchez & Rebec 2013).

1.5.3.1.Grading of HD Based on Neuropathology

The caudate and putamen, as well as other CNS regions, exhibit substantial cell loss as part of HD's pathophysiology, which is brain specific. (Vonsattel & DiFiglia, 1998). Vonsattel developed the most widely used grading system for HD pathology, which includes five cellular degeneration severity classes (Veldman et al., 2018):

Grade 0-On a general level, the brain appears normal, but the head of the caudate nucleus histologically has lost 30–40% of its neurons.

Grade 1- The head of the caudate nucleus has 50% neuronal loss.

Grade 2-Caudate nucleus' ventricular profile causes striated atrophy less convex than normal.

Grade 3-Caudate nucleus' flat ventricular profile leads to severe striatal atrophy.

Grade 4-With the caudate nucleus having a concave ventricular profile, the striatum has atrophy and has lost up to 95% of its neurons.

1.5.4. Genetics

1.5.4.1.HD Gene

HD is a monogenic condition that is inherited via the autosomal dominant gene and is mainly caused by repeat expansion in the *HTT* gene located on chromosome 4p16.3. The 180 kb long *HTT* gene contains 67 exons. The common mutation is the expansion of trinucleotide repeats (CAG)n in exon 1 of the Huntingtin (*HTT*) gene. The N-terminus of the huntingtin protein has polyglutamine stretch which was encoded by the enlarged CAG repeat tract (Bean et al., 2021).

1.5.4.2. ACMG Guidelines for HD

The American College of Medical Genetics (ACMG) establishes the classifications of normal and mutant CAG repeats (Nance et al., 1998; Bean et al., 2021). The guidelines are the following:

1.5.4.2.1. Normal Allele

According to Bean, normal and non-pathogenic alleles are those that have 26 CAG repeats. The most prevalent lengths of normal alleles are 17–19 CAG repeats. Normal alleles segregate as stable polymorphic repeats in 99 percent of meiosis. There haven't been any cases of HD in this range documented (Nance et al., 2017).

1.5.4.2.2. Mutable normal allele

The mutable normal allele or intermediate allele is classified as having 27-35 CAG repeats. This region is commonly referred to as the meiotically unstable range (Bean et al., 2021). These alleles are meiotically unstable in sperm but are not related to the disease phenotype. In this region, a pathological increase of paternally derived alleles has been described. This risk may be greater than 21% for paternal alleles with 35 CAG repeats. There have been several reports of people with HD in this range from paternal inheritance. 35 CAG repeats

are considered an aberrant CAG repeat length by the Venezuela HD Research Consortium. However, no reports of maternally transmitted alleles in this range causing affected offspring have been reported (Nance et al., 2017).

1.5.4.2.3. HD alleles with incomplete penetrance

HD-associated alleles with 36-39 CAG repeats are classified as having incomplete penetrance (Bean et al., 2014). This range is classified as likely pathogenic. Alleles in this range are meiotically unstable and involved in the pathogenesis & phenotypes of HD. However, due to the gene's age-related penetrance individuals living up to or beyond normal life expectancy don't develop HD symptoms. Asymptomatic old people with alleles in this range are rare (Nance et al., 2017).

1.5.4.2.4. HD alleles with complete penetrance

HD alleles with 40 CAG repeats are considered to have complete penetrance. (Bean et al., 2021). This range is classified as pathogenic. Most HD animal models have used DNA fragments with more than 100 CAG repeats; however, the number of people documented with repeat numbers in this range is relatively small, perhaps less than 50 worldwide, and all are children with juvenile-onset HD (Nance et al., 2017).

1.5.4.3. Meiotic Instability and Anticipation

The intrinsic instability of CAG repeats during meiosis influences CAG repeat length Anticipation refers to the process by which the clinical manifestation develops at a younger age and becomes more severe as the condition is passed down from generation to generation. Because a larger CAG repeat length coincides with a younger age of onset, symptoms may appear at a younger age as the illness is handed down the family tree. Repeat mosaicism in all organs revealed CAG expansion in HD patients, with increased instability in the brain and sperm (Telenius et al., 1994). This is more likely to occur when transmission happens through the paternal bloodline due to differences in spermatogenesis and oogenesis (Jamali et al., 2018). Large increases in CAG repeat length exceeding 55 can occur in rare cases, resulting in Juvenile HD (JHD) when the age of onset is less than 20 years old. Paternal inheritance accounts for 90% of JHD instances (Depienne et al.,

2021). Multiple factors influence the instability of repeat expansions during meiosis and the transmission of bigger alleles to children, including the size of the repeat, its structure, and the age and gender of the transmitting parent (Wheeler et al., 2007).

1.5.4.4. Correlation of CAG repeat length with age of onset

The emergence of motor symptoms suggestive of HD is classified as disease onset. The CAG repeat length is inversely related to the age of onset of the disease and accounts for roughly 70% of the age of onset. There is an inverse relationship between CAG repeat length and age of onset. The larger the CAG repeat tract, the earlier the disease onset and vice versa. There are three types of HD based on the phenotype-genotype association (Sun et al., 2017).

1.5.4.4.1. Juvenile Huntington's Disease (JHD)

Juvenile HD (JHD) is a kind of HD that manifests itself at the age of 20 or younger, accounting for around 3-10% of all HD patients (Gonzalez-Alegre et al., 2006; Sequeiros et al., 2010; Schultz et al., 2020). Clinical symptoms of JHD patients are typically atypical. Rigidity, bradykinesia, dystonia, seizures, dysarthria, ataxia, behavioral instability, and cognitive decline are common symptoms, and chorea may emerge later (Lesinskienė et al., 2020). Paternal transmission is thought to account for 70-90% of JHD cases and is characterized by a high number of CAG repeats (>60). JHD progresses more quickly than adult HD. The average lifespan is 8 to 9.3 years (Sun et al., 2017).

1.5.4.4.2. Adult-onset Huntington's Disease

This is the most frequent type of HD, with disease onset between 30 and 50 years and 40-50 CAG repeats. Motor symptoms are the first sign of HD in people in their forties, followed by cognitive and mental decline. The usual age of an onset is roughly 35 years, with 45 CAG repeats on average.

1.5.4.4.3. Late-onset Huntington's Disease (LoHD)

Individuals with intermediate alleles have been shown to have HD clinical symptoms. Most of these patients had late-onset HD (Stoker et al., 2021; Petracca et al., 2022). The smallest

number of CAG repeats that induced the HD phenotype was 29. At the age of 60, the patient began to experience involuntary movements. (Sun et al., 2017) LoHD patients frequently have no family history of the disease, a shorter CAG length (averaging around 40 repeats), and motor manifestation at onset. (Volpi et al., 2021).

1.5.4.5. Genetic modifiers of HD

The main predictor of the course of HD is pure CAG repeat length, which accounts for roughly 70% of the variable in age at onset, but up to half of the remaining variability is also heritable (Tabrizi et al., 2020). The genome-wide association study (GWAS) conducted by the Genetic Modifiers of Huntington's Disease (GeM-HD) consortium discovered various HD modifiers on different chromosomes. (Lee et al., 2015; Moss et al., 2017; Tabrizi et al., 2020) (Table 1.2).

The findings of these genetic association analyses suggest that DNA repair activity is important in the etiology of HD, with repair protein variations influencing the pace of somatic expansion in tissues prone to neurodegeneration & repeat instability. (Iyer et al., 2015). Most HD-modifying mutations and pathways are linked to specific DNA repair systems, primarily mismatch repair, and influence somatic instability. These findings imply that downregulating MSH3, MutL, MutL and LIG1, inhibiting their interactions, or upregulating FAN1 and PMS1 could minimize somatic CAG expansion and improve the course of HD. (Schmidt et al., 2016).

Table 1.2. Effects of Genetic Modifiers on Huntington's Disease Onset (Tabrizi et al., 2020).

Gene	Function	HD modification
PMS1, PMS2, LIG1, TCERG1, CCDC82	Mismatch repair genes	*PMS2* was associated with a delay in onset of 0.8 years. *PMS1* with an onset 0.8 years earlier. Increased *CCDC82* acts to delay the onset of HD. *LIG1* is involved in somatic instability in the brain and delay in onset.
MLH1, MLH3, MSH1, MSH3, DHFR	Mismatch repair genes, Part of MutL endonuclease complexes	Associated with a delay in disease onset of HD.
RRM2B, UBR5, SYT1, GSG1L	*RRM2B*, which is involved in nucleotide synthesis, or UBR5, a ubiquitin ligase that might have a role in HTT aggregation	Associated with disease onset 1.6 years earlier than expected from CAG repeat length
FAN1 (Haplotypes)	*FAN1* encodes a nuclease with involvement in interstrand DNA crosslink (ICL) repair and replication fork recovery	One *FAN1* signal was associated with disease onset >6 years earlier than would be expected from CAG length alone, and the other was associated with disease onset 1.4 years later than expected.

1.5.5. Wild Type Structure of Huntingtin

Huntingtin is a large 350 kD protein containing 3,144 amino acids. It has the highest level of conservation among vertebrates. The polyglutamine tract in huntingtin lies at the N terminus and starts from the 18th amino acid. The average length of the poly Q tract in huntingtin protein produces a polar zipper that facilitates the binding of additional factors having polar residues. The poly Q tract appears to confer a significant neuronal function in

vertebrates, as suggested by the neurological damage in a poly Q-deleted knock-in mouse (Schulte and Littleton, 2011). Several HEAT repeats are present downstream of the polyglutamine tract. These HEAT repeats play an important role in interacting with other protein partners. Huntingtin protein is highly expressed in the neurons of the CNS, along with astrocytes, oligodendrocytes, and microglia (Guo et al., 2018).

1.5.5.1. Role of Huntingtin in Neurodegeneration

1.5.5.1.1. Aggregation and misfolding of Huntingtin

Extended CAG repeats present in exon 1 of the *HTT* gene are translated into the larger poly Q stretches. After proteolysis, these stretches are released which become more toxic compared to when they are bound to protein. Aggregates are formed in the brain because of the impairment of the ubiquitin-proteosome system (UPS) and autophagy (Cisbani and Cicchetti, 2012). Impairment of the UPS system was found in the mouse model and in the post-mortem HD brain tissue (Harding et al., 2018). A direct relationship between the aggregation of huntingtin and cellular toxicity was observed because the polyglutamine expansion is correlated with both the rate of aggregation and the beginning of the disease (Hackam et al., 1998).

1.5.5.1.2. Embryonic Development

HTT plays an important role in neuroblast differentiation in the striatum (basal ganglia and putamen) and cortex. In extra-embryonic tissues, *HTT* is also involved in iron transport. Extra embryonic tissues display iron transport abnormalities when there is low expression of *HTT*. Studies on mice demonstrate that *HTT* absence shows abnormalities of cortex and striatum. These mice also show defects in the development of the precursors of epiblast and die soon after birth (Ghosh & Tabrizi, 2018).

1.5.5.1.3. Abnormal trafficking of vesicles

Huntingtin is involved in the transportation of a range of organelles which include endosomes, lysosomes, synaptic precursor vesicles, amyloid precursor protein, brain-derived neurotrophic factor (BDNF), and GABA receptors (Zala et al., 2013; Saudou & Humbert, 2016). *HTT* is involved in the regulation of both anterograde and retrograde

transportation in dendrites and axons via dynein, kinesin, and huntingtin-associated protein (HAP1). In the case of HD, the mutant *HTT* binds more strongly to HAP1, dynein, and kinesin due to which vesicles prematurely detach from the microtubules and overall transport efficiency decreases (Colin et al., 2008).

BDNF is the most important cargo for corticostriatal circuits. When the BDNF levels are low, neurodegeneration occurs in these corticostriatal projecting neurons. In HD, post-translational modification such as phosphorylation is diminished, which is a key mechanism for controlling *HTT*-mediated transport. Kinesin 1 is recruited to facilitate the anterograde transport of vesicles by phosphorylating serine 421, which controls the direction of transport. Without phosphorylation, *HTT* travels in a backward direction by co-migrating with dynactin and dynein rather than kinesin (Drouet et al., 2014).

1.5.5.1.4. Dysregulation of Endocytosis

Two proteins that are involved in endocytosis are Huntingtin interacting protein 1 (HIP1) and HIP12. *HTT* interacts with these proteins and is involved in membrane invagination (Walter et al*.,* 2001). Mutant *HTT* protein participates in clathrin-mediated endocytosis. When mutant *HTT* protein interacts with proteins involved in clathrin-mediated endocytosis, endocytosis becomes deregulated (Wanker et al*.,* 1997). Some proteins like HIP1, HIP12, and SH3GL3 (*SH3 Domain Containing GRB2 like 3*) are also involved in clathrin-mediated endocytosis. Mutant *HTT* protein interacts with these proteins and leads to the deregulation of endocytosis (Sittler et al., 1998).

1.5.5.1.5. Activation of Cell death pathways

Wild-type *HTT* displays neuroprotective functions during various pro-apoptotic stimuli (Leavitt et al., 2001). The expression of wild-type *HTT* defends against excitotoxicity and ischemia. *HTT* inhibits apoptosis by blocking caspase 3,8 and 9 (Zhang et al*.,* 2006). *HTT* activates pro-survival pathways by acting as the substrate in the phosphoinositide 3-kinase (P13K)/Akt pathways. *HTT* increases the expression of the pro-survival genes and lowers the expression of death genes i.e., Bcl-2 and Bax (Jurcau, 2022). In HD brains, the inhibitory interaction of caspase-3 with *HTT* decreases due to the polyglutamine

expansions in mutant *HTT* (Rigamonti et al., 2000). p21-activated serine-threonine kinase (Pak2) is associated with apoptosis. The expression of Pak2 is seen throughout the brain (Jakobi et al*.,* 2003). Wild-type *HTT* interacts with Pak2 to prevent its cleavage by caspase 3 and caspase 9, thus protecting the neurons from Fas signal-induced apoptosis. The interaction between mutant *HTT* and Pak2 is weaker, thus the protective effect is reduced (Luo and Rubinsztein, 2009).

1.5.5.1.6. Transcriptional dysregulation

Wild-type HTT most frequently binds to the proteins that regulate transcription. These transcriptional regulators are cAMP Response Element Binding Protein (CBP) and Repressor Element-1 Transcription Factor (REST) (Laprairie et al., 2019). The production of neuronal survival signals is controlled by CBP, a transcriptional co-activator, which regulates histone acetylation and deacetylation (Li et al., 2004). Transcription factors can bind to DNA because of the CBP histone acetyltransferase activity (Steffan et al*.,* 2000).In HD, mutant *HTT* dysregulates transcription by inhibiting histone acetyltransferase activity resulting in the downregulation of the gene transcription (Xiang et al., 2018).

1.5.5.1.7. Mitochondrial Dysfunction

In HD, neurodegeneration is linked to altered mitochondrial activity that results in abnormalities in ATP synthesis and apoptosis (Panov et al., 2002). Mutant *HTT* may interact with the outer membrane of mitochondria and cause abnormalities in mitochondrial calcium (Choo et al., 2004). Mutant *HTT* disrupts the normal transport across the axons which can decrease the mitochondrial transport across synapses (Shirendeb et al., 2012).

Additionally, mutant *HTT* inhibits TIM23 (Translocase of the Inner Mitochondrial Membrane 23), which is part of a complex of proteins involved in inner mitochondrial transport. When mutant *HTT* interacts with TIM23, it leads to a defect in protein transport into mitochondria, which may contribute to respiratory failure and the death of neuronal cells (Yano *et al.,* 2014). As a result of mitochondrial dysfunction, there is the production of many reactive oxygen species (ROS) which can cause cell death (Sorolla et al., 2008).

1.5.6. Diagnosis of HD

The diagnosis of HD relies on familial history, clinical evaluation with definite motor symptoms, and most importantly the screening of CAG expansion in *HTT* through genetic testing. Neuroimaging findings can also support the diagnosis of HD.

1.5.6.1. Neuroimaging Findings

Neuroimaging tests are primarily performed to rule out other conditions overlapping with HD. These changes are even detectable before the motor onset of the disease (Figure 1.3). Magnetic Resonance Imaging (MRI) or computerized tomography (CT scan) presents symmetrical atrophy in the striatum (caudate and putamen loss) and to a lesser extent, the atrophy in the cerebral cortical grey matter and subcortical white matter is strongly suggestive of HD (Anderson et al., 2019; Arraj et al., 2021).

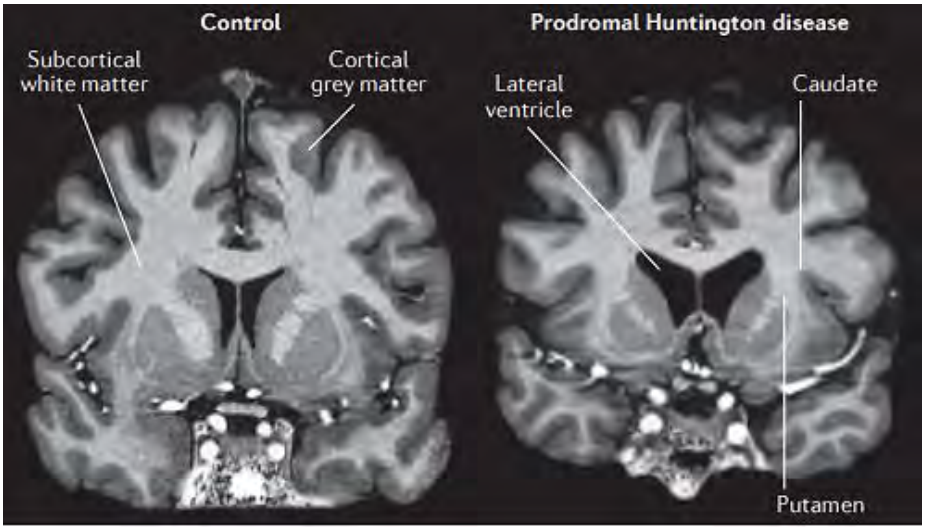

Figure 1.3. Qualitative MRI comparison of normal control case (Left) and HD patient (Right). Anatomical features (putamen in blue and basal ganglia in red) are shown in normal brain MRI. The grey and white matter atrophy with caudate and putamen loss (Striatum atrophy) was observed as compared to the normal healthy control (Anderson et al., 2019).

1.5.6.2.Clinical Evaluation

The clinical evaluation of HD relies on the triad of symptoms. Motor abnormalities include chorea, dystonia, bradykinesia, rigidity, impaired postural reflexes, gait abnormalities, and myoclonic jerks and tics. However, cognitive abnormalities include subcortical and frontal dementia, disorientation, and attention deficits. Psychiatric abnormalities in HD include anxiety, depression, irritability, aggression, apathy, obsession, and psychosis.

1.5.6.3. Erythrocytes Sedimentation Rate (ESR)

ESR is the most convenient diagnostic measure for neurodegenerative diseases. The presence of acanthocytes affects the ESR in normal and affected individuals (Darras et al., 2020).

1.5.6.4. Unified Huntington's Rating Scale (UHDRS)

UHDRS provides a diagnostic confidence score to assess the onset and stage of the disease (Youssov et al., 2013). UHDRS-Total motor score (UHDRS-TMS) can be used to assess motor abnormalities in HD patients. This score lies between 0-4 (0: no motor abnormality and 4: >99% to be HD). Score 4 defines the onset of motor symptoms and manifests HD. UHDRS-TMS assesses eye movements, parkinsonism, chorea, dystonia, gait, and speech. This score is sensitive over time (Kieburtz et al., 1996).

1.5.6.5. Genetic Testing for HD

Genetic testing can be predictive, and diagnostic based on circumstances. If the patient has symptoms typical of HD, the most useful confirmatory diagnostic test is CAG repeat testing. Predictive testing, conducted before the onset of HD phenotypes, is a crucial step for adults who are at risk of inheriting *HTT* gene mutation (Harper et al., 2000).

Reproductive considerations often drive the need for predictive testing. Individuals at risk have various reproductive options available to them, such as prenatal diagnosis and the option to terminate a pregnancy if the fetus has the expanded CAG repeat. Pre-implantation genetic diagnosis, which involves the selection and transfer of embryos without the CAG expansion, can also be pursued during vitro fertilization (Ghosh & Tabrizi, 2018).

1.5.6.6.Differential Diagnosis

Among 1% of the individuals presenting HD symptoms and tested negative in HD can have some other genetic mutations other than HD. Such conditions are known as HD phenocopies (Wild et al., 2008). The most common cause is the CTG/CAG expansions in JPH3, the major cause of Huntington's disease-like 2 (HDL2) and C9orf72 hexanucleotide repeat expansion mutation that are the major causes of FTLD and ALS (Estevez-Fraga et al., 2021).

1.5.7. HD Therapeutic Approaches

Different approaches are in research and in clinical trials to target the CAG repeat expansion in *HTT*. These approaches may have the potential to reduce the expression of huntingtin (Ross et al., 2014).

1.5.7.1.DNA and RNA targeting Therapies (Gene therapy)

Mutant *HTT* primarily involved in the pathogenesis of HD. RNA Interference (RNAi), anti-sense oligonucleotides (ASOs) and genome engineering techniques including the Zinc finger proteins, and CRISPR/Cas9 can target mutant repeats to lower the expression of *HTT* (Wild & Tabrizi, 2014).

1.5.7.2.Targeted Small Molecule Approaches

mHTT affects many cellular pathways and imparts its toxic effects by many cellular pathways i.e., inhibition of phosphodiesterase (PDE) 10A, histone deacetylases (HDACs), and potentiation of the neurotrophin BDNF. Different potential targets to modify the disease pathogenicity are in research and clinical trials stages (Gosh & Tabrizi, 201411.6:

1.6: Aims and Objectives

The present study aimed to recruit families of Huntington's disease with the following objectives:

- To analyze the clinical and genetic characteristics of HD patients
- Analysis of CAG repeat expansion in *HTT* gene.

CHAPTER 02

MATERIALS AND METHODS

2. Materials and Methods

2.1. Research Ethics Approval

Three families with Huntington's Disease (HD) agreed to participate in this research study. Informed consent was taken from the members of the affected family members to use their blood samples and medical records. Families were recruited from districts Jhang, Toba Tek Singh, and Chakwal of the Punjab province, Pakistan. This research study was approved by the Bioethical Committee (BEC) of the Quaid-I-Azam University, Islamabad. Additionally, blood samples were collected from 40 healthy individuals to get the idea of repeat size in the Pakistani population.

2.2. Family Recruitment

The recruited families with HD were labelled as Family H1, Family H2, and Family H3. Upon visiting the families, we interrogated the familial as well as the medical history including the age of onset of HD in the affected individuals. The initially presented clinical features of HD were also noted along with CT or MRI scan reports, current medications, and recommendations from the physician. The clinical features were observed including motor abnormalities (choreatic movements, bradykinesia, tremors, dystonia, dyskinesia, and facial dysmorphism), psychiatric abnormalities (aggression, depression, anxiety, restlessness, and apathy), and cognitive decline (memory issues, attention deficits, and learning disabilities).

The demographic features i.e., ages, height (inches), weight (Kg), and head circumference (cm) were also noted. Further, the birth history and growth conditions of affected individuals were also interrogated by the parents and normal members of three families.

2.3. Pedigree

The familial histories of the respective families helped in drawing the pedigrees by Haplopainter v1.043 (Thiele and Nurnberg, 2005). The pattern of inheritance was determined by examining clinical phenotypes and family history.

2.4. Sample Collection

Standard phlebotomy protocols were taken into consideration to collect the blood sample from the patient and normal family members (siblings and parents) (WHO, 2010). About 7ml of intravenous blood was taken from each member using the BD PrecisionGlide^{TM} Sterilized syringes (0.8mmX38mm, 21G,1.5T) (BD Singapore).

Standard potassium ethylene diamine tetra-acetate vacutainer tubes (K3 EDTA tubes) were used to store the drawn blood. Specific labels were given to each tube according to respective family IDs. The blood samples were stored at 4°C in the refrigerator in the Lab of Genomics, Department of Biochemistry, Quaid-I-Azam University, Islamabad.

2.5. Red Blood Cell (RBC) Morphology Analysis

To explore the effect of HD on RBC morphology, control, and HD-affected blood samples were visualized under an upright brightfield microscope (Olympus, Japan). For this purpose, blood smears were prepared and stained through Field's staining protocol for visualization of RBC. In Field's staining two stains were used, Field's stain A was methylene blue and Azure 1 dissolved in phosphate buffer solution and Field's stain B was Eosin Y in buffer solution.

2.5.1. Blood Smear Preparation

For each sample, a smear was prepared and stained usingthe Field's staining technique. Almost 3ul blood from control and HD-affected individuals were taken for preparation of thin blood smear on glass slides. For the fixation of cells, all blood smear slides were dipped in methanol (Daejung, South Korea) for 1 minute and then air-dried properly.

After fixation, the smears were first dipped in Field Stain B (H.F006, SDL) for 4 minutes which stained the cytoplasm and granules of different cells in the blood with pink color. Then the slides were washed with tap water and air-dried. Afterwards, the slides were dipped in Field Stain A (H.F002, SDL) for 45 seconds, which stained the nucleus of different blood cells with purple color. The slides were washed again and air-dried. Finally, slides were labeled and stored for visualization using microscopy.

2.5.2. Visualization of RBCs through Microscopy

For every sample, each slide was observed on a light microscope using a 100X immersion oil objective (Merck; UN3082). On each slide, RBC morphology was observed. Different images of RBC were taken through a 100X lens of a light microscope (Olympus CX41, Japan) attached to a camera (Tuksen; Model: ISH5000).

2.6. Erythrocytes Sedimentation Rate (ESR) measurement

The presence of acanthocytes in HD patients affects their sedimentation rate. HD-affected individuals have a lower sedimentation rate than normal controls due to the disrupted shape of erythrocytes (Darras et al., 2021). Fresh blood samples from the affected and normal controls were taken for ESR measurements, which were carried out in 200mm standard Westergren tubes by Aga Khan Laboratories, Pakistan. Color pictures of the tubes were taken every 10 minutes for 2 hours. For each tube, the blood sedimentation rate was calculated.

2.7. DNA Extraction

The phenol-chloroform method was used to extract the genomic DNA from the blood samples of all the individuals.

2.8. Phenol-chloroform Method

The organic phenol-chloroform method is a two-day DNA extraction protocol. The chemical composition of the solutions used in DNA extraction is mentioned in table 2.1. On the first day, 750µl of the stored whole blood was added with an equal volume of solution A (1:1) in the 1.5ml of the Eppendorf tube (Gene Era Biotech Co.®, California, USA). Both the blood and solution A were mixed by inverting the Eppendorf tube 4-6 times and then incubated at room temperature for about 20 minutes. The Eppendorf tubes were inverted occasionally during the incubation period to increase the lysis of blood cells. After the incubation time, the tubes were centrifuged for 1 minute at 12000rpm (Hettich Zentrifligen®, Mickro 120, Germany). Following centrifugation, the supernatant was removed using a 10% bleach solution. The nuclear pellet was dissolved in 400µl of solution

A by inverting the tubes several times. The tubes were centrifuged again for 1 minute at 12000rpm. The supernatant was decanted again. The pellets were resuspended in 400µl of solution B, 12µl of the 20% Sodium Dodecyl Sulphate (SDS) solution, and 5µl of the proteinase K (10mg/ml). For an overnight incubation, tubes were placed in the incubator at 37°C (HeraTherm^{TM} Thermo Scientific, USA).

On the second day, solutions C and D were freshly prepared. A total volume of 500µL of solution C (Phenol) and solution D (Chloroform and Isoamyl alcohol) was added to the Eppendorf tube stored in the incubator. The tubes were inverted and centrifuged for 10 minutes at 12000 rpm. The centrifugation resulted in two layers, the upper one was the aqueous phase containing DNA and the lower one contained the cellular debris. The aqueous phase was carefully transferred to a new microcentrifuge tube and the cellular debris was discarded. 500µL of Solution D was added in each tube containing the aqueous phase and centrifuged for 10 minutes at 12000 rpm. The aqueous phase was transferred into a new microcentrifuge tube and the supernatant was discarded. DNA precipitation was carried out by adding 500µL of the chilled isopropanol and 55µl of sodium acetate (3M, pH=6) to the tubes containing the aqueous phase. The tubes were inverted several times to precipitate the DNA.

The precipitated DNA was centrifuged for 10 minutes at 12000 rpm. The supernatant was discarded and 200µl of the 70% ethanol was added to the pellets for washing. The tubes were centrifuged at 8000 rpm for 7 minutes and the supernatant was disposed of. The DNA pellets were dried by keeping the tubes open at room temperature for half an hour. Depending on the size of the DNA pellet, 60-120µl of Tris-EDTA (TE)buffer was added to dissolve the pellet. The tubes were placed in the incubator for overnight incubation at 37°C. The next day tubes containing DNA were stored at 20°C.

2.9. 1% Agarose Gel Electrophoresis

Agarose gel separates the nucleic acid fragments based on their size. 1% agarose gel electrophoresis is used to analyze the quality of DNA. The chemical constituents of the agarose gel are given in table 2.2. For the formation of the gel, 1 gram of the agarose was measured on the weighing balance (Shimadzu weighing balance) and mixed with 100 ml

of 1X TBE buffer in the glass flask. This mixture was heated in the microwave oven for approximately 1.5 minutes to dissolve the agarose powder in the buffer completely. 5µl of the ethidium bromide (o.5 µg/mL) was added to the gel mixture and the gel was poured into the caster to solidify it.

After 30 minutes, the solidified gel was placed in the tank. 1X TBE running buffer was used to run the gel in the tank. 3µl of the loading dye (0.25%Bromo-phenol Blue+ 40% sucrose) and 3µl of the genomic DNA were loaded in the wells of the gel. The gel ran at 90 volts for 30 minutes. Gel Doc System (INGENIUS SYNGENE Bio Imaging®, UK). was used for the visualization of genomic DNA bands. The intensity of the agarose gel gives a rough estimation of the quality of DNA.

2.10. Nanodrop-based quantification of DNA

The quantification of the extracted DNA was done using Nanodrop (Colibri Titertek Berthold, Germany). Nanodrop measures the concentration of DNA in ng/µL based on the absorbance of light. Before measuring the concentration, the nanodrop was blanked using 2µl TE buffer.

The most optimized concentration of DNA for the polymerase chain reaction is 40ng/µl. For DNA samples with higher DNA concentrations, the DNA dilutions are made based on the dilution formula ($C_1V_1=C_2V_2$).

2.11. Selection of Candidate Genes

Genetic analysis of a particular disease involves the identification and selection of the candidate genes that are known to cause similar phenotypes. The recruited families have the phenotypes of autosomal dominant Huntington's Disease (HD). *HTT* is the main candidate gene in HD and the majority of HD patients CAG repeats expansion in Exon 1 of this gene.

2.12. Primer Designing

Primers are short oligonucleotides (18-24nts) sequences employed in PCR to amplify the specific regions within the genomic DNA. For the present study, primers were designed to

amplify Exon 1 of the *HTT* gene by using different bioinformatics tools. Sequence (NM_001388492.1) in the FASTA format was obtained from the Ensemble Genome Browser (https://asia.ensembl.org/index.html) (Martin *et al.,* 2023). To design the primer of the marked exon, the sequence was pasted in the Primer3 input browser (https://primer3.ut.ee/). The conditions of the primer3 were set to pick the right primers i.e., GC content (40-60%), Primer length (18-24bp), Tm (56-64°C), Tm difference (<1°C). The quality of the designed primers was assessed using different Insilico tools. The list of primers for Exon 1 of the *HTT* gene is given in Table 2.3.

The specificity of each primer was checked using the BLAT tool (https://genome.ucsc.edu/cgi bin/hgBlat) to select primers with only a single hit for the specific genomic region.

Before using the primers in the wet lab, the working conditions and the size of the primer were verified using in silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr). This tool checked whether the primer binds to the appropriate position in the genome, specifically targeting the regions that are free from the repeats.

The properties of the designed primers were inspected by using the oligo calc tool (http://biotools.nubic.northwestern.edu/OligoCalc.html). The hairpin formation in a primer, self-complementarity of primers, and mismatch binding of primers through 3' end complementarity was tested.

Multi Prime Analyzer (https://www.thermofisher.com) was used to analyze and compare multiple primer sequences simultaneously. It provided the Tm(°C), length of primer(bp), number of individual bases (G, C, T, and A), and primer dimer estimation.

2.13. Polymerase Chain Reaction (PCR)

PCR is used to amplify the small region of the DNA fragment to make billions of copies. The main steps in the PCR reaction involve denaturation, annealing, and extension. During denaturation, the DNA template is heated to separate the double-standard DNA into single strands. In the subsequent annealing process, specific primers bind to the complementary sequences in the single-stranded DNA. Finally, during extension DNA polymerase enzyme

extended these primers to make the new strand of DNA. Thermocycler (Biometra®, Germany) was used for the PCR reaction preparing 25µl of the reaction mixture in 200µl PCR tubes (Axygen USA).

For every PCR reaction, the concentration of each primer (forward and reverse) used was 20ng/µl and the DNA template concentration was 40ng/µl. The other reagents used for the open reaction PCR are mentioned in table 2.4.

In the case of Exon 1 of the *HTT* gene, the GC content is very high due to CAG and CCG repeat region. This region is highly prone to secondary structures. Enhancing agents i.e., Dimethyl Sulfoxide (DMSO) and betaine were added in the reaction mixture to enhance the amplification by reducing the formation of secondary structures.

After preparing the 25µl of the reaction mixture the PCR tubes were centrifuged. These PCR tubes were transferred into the thermocycler and the specific conditions were set according to the specificity of primers. The PCR profiles are given in table 2.5.

2.13.1. 2.5 % Agarose Gel Electrophoresis

2.5% agarose gel was used to examine the PCR products under of *HTT* gene. The bands were visualized in the Gel DOC system. The images of the PCR bands were saved in the Gel Doc system and later analyzed to eliminate the size of repeat expansion. The composition of the 2.5 % Agarose gel is given in table 2.5.

2.14. PCR Product Purification

The amplified PCR products contained the residual amounts of primer enzymes and dNTPs in the PCR tubes. These extra reagents were removed to obtain purified products before Sanger sequencing. Two different types of protocols were employed for the PCR product purification.

2.14.1. EXOSAP-IT^{TM} Product Purification

For the sequencing Exon 1 of the *HTT* gene of selected patients and healthy controls and normal siblings, PCR products were purified using EXOSAP-IT^{TM}. For the purification, 6µl of the PCR product was added in 2µl of the EXOSAP-IT^{TM} reagents (Thermo

Scientific). The reaction mixture was incubated at 37°C. During the incubation period, the surplus nucleotides and the primers were hydrolyzed by an enzymatic reaction. The EXOSAP-IT™ reagents were inactivated by incubating again at 80°C for 15 minutes. Clear bright bands confirmed the quality of the purified product for sequencing.

2.14.2. Gel Purification Protocol

Some affected individuals showed two bands on agarose gel. In such cases, each band was gel-purified for sequencing. These PCR products were purified employing the Gene Jet Purification Kit (Thermo Scientific, USA).

PCR products were run in the agarose gel for 1.5 hours to separate the bands. When both the bands were completely separated from each other, gel slices were excised by the sterilized scalp and stored in pre-weighted 1.5ml microcentrifuge tubes. The gel-containing tubes were weighed again to measure the weight of the gel slice. An equal volume of the binding buffer to the weight of the gel was added to the gel-containing tube. The gel mixture was incubated at 45°C for 30 minutes to completely dissolve the gel in the binding buffer. During the incubation time, the tubes were inverted to aid in the melting of the gel. The tubes were vortexed, and the gel mixture was loaded on the Gene Jet Purification column.

Columns were centrifuged at 8000 rpm for 1 minute. The flow-through column was discarded, and the columns were placed in the same collection tube. 100µl of the binding buffer was added to the column and centrifuged for 1 minute. Flow through the column was discarded and columns were placed again on the same collection tube. 700µl of the washing buffer was added to the column and centrifuged for 1 minute. The columns were placed again on the same collection tube. Centrifugation was repeated to remove all the impurities from the walls of the column. Purification columns were positioned on new Eppendorf tubes and elution buffer was added according to the intensity of bands and required volume. The tubes were centrifuged for 1 minute. The flow through the column was visualized using 2% agarose gel to confirm the quality of the purified PCR products. Purified products were stored at -20°C to use for Sanger Sequencing.

2.15. Sanger Sequencing

The purified products either by EXOSAP or gel purification were processed for the Sanger sequencing. For sequencing 4µl of the DNA template (30-90ng), 4µl of the Terminator Ready Reaction Mix, 1µl of the primer (forward and reverse), and 1µl of the ddH_2O were added in 200µl of the micro-map tubes. The tubes were centrifuged well and placed in the thermocycler for 25 cycles. The tubes were firstly incubated at 96°C for 10 seconds and then at 50°C for 5-10 minutes. The last incubation was done at 60°C for 4 minutes. The incubated samples were stored at 4°C.

2.15.1. Sequencing Product Purification

The sequencing products were purified by mixing 30µl of 100% isopropanol with 40µl of 75% isopropanol in a 1.5ml microcentrifuge tube. The sequencing products were incubated at room temperature for more than 15 minutes and then centrifuged for 20 minutes. The supernatant was discarded. The DNA pellet was washed with 75% isopropanol and centrifuged for 5 minutes. The supernatant was decanted, and the sample was placed in the vacuum centrifuge tube for 10-15 minutes. The samples were stored at 20°C. The DNA pellets were dissolved in 3µl of the loading buffer, centrifuged, and heated at 95°C for 2-3 minutes. Lastly, the tubes were chilled on ice and the products were sequenced on an ABI-310 sequencer.

2.16. Data analysis from Agarose gel electrophoresis

From the agarose gel, the size of the PCR bands in the affected and the normal individuals were compared with the size of the control sample and 100bp ladder. The size of the expanded band was estimated for each tested sample.

2.17. Mutation Analysis

The sequencing results were analyzed using BioEdit Software (Hall,1999). The reference sequence for exon 1 of the *HTT* gene was downloaded from the Ensembl Genome Browser. BioEdit aligned the sequence results with the reference sequence of the *HTT* gene using the ClustalW Multiple Alignment Option (Thompson et al., 1994). The changes in the

nucleotide bases and the chromatogram were analyzed. The expanded CAG repeats in the sequenced data were analyzed.

Mutation taster (https://www.mutationtaster.org/) was used to check the potential variants having the change in the amino acids that can be disease-causing. Polyphene-2 (http://genetics.bwh.harvard.edu/pph2/)predicts the possible effects of amino acid change on the structure and function of the protein.

Table 2.1: Genomic DNA extraction reagents

Stock Solutions	Chemical Composition	Concentration
Solution A	Tris HCL (pH=7.5)	10Mm
	Sucrose	0.32M
	Triton X-100 (v/v)	1%
	Magnesium Chloride ($MgCl_2$)	5mM
Solution B	Sodium Chloride (NaCl)	400mM
	Tris HCL (pH=7.5)	10mM
	EDTA (pH=8)	2mM
Solution C	**Saturated Phenol**	
Solution D	Isoamyl Alcohol	1 volume
	Chloroform	24 Volume
20% SDS	SDS	10g
	Water	50mL
Loading Dye	Bromophenol	0.25g
	Sucrose	40g
10X TBE	Boric acid	0.89M
	Tris	0.89M
	EDTA	0.89M

Table 2.2: Agarose gel electrophoresis reagents

Sr. No.	Reagents	Volume/Reaction
1	Agarose	1-2%
2	1X Tris-EDTA-Boric acid (TBE) Buffer	100ml
3	Ethidium Bromide	5-10µL

Table 2.3: List of HTT primers

Sr. No.	Primer Sequence	Tm°C	Product size (bp)
***HTT*-Ex1_01-F**	GCCTCACCCCATTACAGTCT	60	946bp
***HTT*-Ex1_01-R**	ACTCCCCTCGGTGAATTCAG	60	
***HTT*-Ex1_04-F**	CAGAGCCCCATTCATTGCC	62	246bp
***HTT*-Ex1_04-R**	GGCTGAGGAAGCTGAGGAG	62	

Table 2.4: PCR reagents

Sr. No.	PCR Reagents	Volume per Reaction
1	10X Taq Buffer $(NH_4)_2 SO_4$ (Thermo Scientific)	2.5µl
2	$MgCl_2$ (Thermo Scientific)	1.5µl
3	DMSO	1-2.5µl
4	Betain	1µl
5	Forward Primer(10µM)	1µl
6	Reverse Primer (10µM)	1µl
7	Template DNA (40-100ng)	1µl
8	dNTPs (10mM	0.5µl
9	Taq. Polymerase (Thermo Scientific)	0.2µl
10	Nuclease free water	Up to 25µl

Table 2.5: PCR profile

Steps	Cycles	Temperature (◦C)	Time Duration
Initial Denaturation		95	10 minutes
Denaturation	40Cycles	95	1 minute
Annealing		56-64	1-1.5 minutes
Extension		72	1-2 minutes
Final Extension		72	10 minutes
Pause		4	∞

CHAPTER 03

RESULTS

3. Results

3.1. Familial Characteristics

Three families (H1, H2 and H3) with inherited Huntington's disease (HD) were recruited from different regions of the Punjab province. 40 healthy controls from the age group (C1-C10: 20-30Years), (C11-C20: 30-40 years), (C21-C30: 40-50years), (C31-C40: 50-60Years) were enrolled in this study from different regions of Pakistan. These Controls were recruited to evaluate the number of repeats in normal Pakistani individuals.

3.1.1. Family H1

Patients of family H1 were suffering from HD. The analysis of 5 generations of pedigree depicted an autosomal dominant pattern of inheritance in the family H1. In the fourth generation, two individuals are affected while in the fifth generation, 3 individuals are affected with HD as shown in Figure 3.1. One of the parents of the affected individuals was affected by HD but he died before this study. Both the affected parent and asymptomatic offspring from one loop and two affected individuals and asymptomatic siblings from the other loop of family H1 were recruited in this study.

While doing physical observation, we examined that patient H1-1 has an early onset of HD with mild symptoms including i.e., mild tremors in the fingers, toes and shoulders, depression, anxiety, apathy, and low learning abilities. Patient H1-2 has an adult onset of HD with moderate manifestations like i.e., tremors in hands, legs and shoulder, dystonia, mild choreatic movements, depression, anxiety, severe aggression, apathy, insomnia, low learning abilities and attention deficits. Patient H1-3 has an early onset with severe symptoms of HD like severe full-body choreatic movements, tremors dystonia, bradykinesia, dysphagia, difficulty in walking, facial dysmorphism, depression, anxiety, suicidal thoughts, apathy, low learning abilities, attention deficits, and memory issues. The other siblings from this loop were still asymptomatic for HD.

From the second loop patient H1-6 has an adult onset of HD with severe symptoms i.e., severe choreatic movements, tremors, dystonia, dysphagia, facial dysmorphism, oromandibular dyskinesia, unable to walk, aggression depression, anxiety, memory issues

and low learning abilities. Patient H1-7 has an adult onset of HD with mild symptoms i.e., tremors in fingers and toes, anxiety, attention deficits and low learning abilities. The offspring of patients H1-6 and H1-7 are younger and are asymptomatic. The physical and clinical profiles of the members of family H1 are shown in Table 3.1.

The visualization of blood smears of patients H1-1, H1-2, and H1-3 showed acanthocytes in their blood as compared with individual H1-5 from family H1 and normal control as shown in Figure 3.2. The 2-hour ESR results of patients H1-1, H1-2, and H1-3 showed a reduced sedimentation rate as compared to individuals H1-4 and H1-5 as shown in Figure 3.3.

3.1.2. Family H2

Patients of family H2 were suffering from HD. By examining the 6 generations of the family H2 it was observed that there is an autosomal dominant inheritance of HD as shown in Figure 3.4. In the 6^{th} generation two individuals, i.e., H2-1 and H2-2, are affected by HD. The affected father of the patients died before the start of the study. Both the affected individuals, a normal mother and one asymptomatic sibling from family H2 were recruited in this study. During the physical assessment of family H2, we observed that patient H2-1 has a juvenile onset of HD with severe symptoms i.e., severe choreatic movements, tremors, bradykinesia, dystonia, dysphagia, apraxia, depression, anxiety, suicidal thoughts, insomnia, learning disabilities, memory issues. Patient H2-2 has an early onset of HD with mid symptoms i.e., mild tremors in hands and toes, depression, apathy, and irritability. The physical and clinical profile of family H2 is shown in table 3.2. The MRI findings of the patient HD1-1 demonstrated that there is severe atrophy in the striatum i.e., the basal caudate head, basal ganglia and putamen were enlarged as compared to the healthy control as shown in Figure 3.6.

The visualization of blood smears of patients H2-2 and H2-3 showed acanthocytes in their blood as compared with normal individual H2-1 and H2-4 from family H2 and control as shown in Figure 3.7. The 2-hour ESR results of patients H2-2 and H2-3 showed a reduced sedimentation rate as compared to individuals H2-1 and 2-4 as shown in Figure 3.8.

3.1.3. Family H3

Affected individuals of family H3 were also suffering from HD. There is one affected individual in the 3rd generation. The pedigree analysis represented that it is an autosomal dominant pattern of inheritance as shown in Figure 3.5. The affected father of patient H3-1 died before the start of the study. The late father of the patient H3-1 also has the same age of onset. During the physical observation of the patient, it was observed that patient H3-1 has an adult onset of HD with moderate symptoms i.e., tremors in hands and feet, mild chorea, bradykinesia, severe depression, anxiety, suicidal thoughts, insomnia, attention deficits, and low learning abilities.

The brain MRI findings of the patients depicted that the striatum is affected in patient H3-1 due to atrophy in the caudate nucleus basal ganglia and putamen as shown in Figure 3.6.

3.2. Genetic analysis of HD families

The main genetic cause for the pathogenicity of HD is the CAG repeat expansions in exon 1 of the *HTT* gene. For the genetic analysis of the individuals having the phenotypes of HD, exon 1 of the *HTT* gene was selected.

3.2.1. Family H1

The first loop of family H1 (H1-1, H1-2, H1-3, H1-4, H1-5) and second loop (H1-6, H1-7, H1-8, H1-9) was amplified for exon 1 of the *HTT* gene. The repeat size was analyzed using 2.5% agarose gel as shown in Figures 3.10 and 3.11. The size of the normal and expanded bands was compared by using the 100bp ladder. The CAG repeat size in the normal and expanded allele was estimated by agarose gel electrophoresis and confirmed by Sanger sequencing.

The sequencing results confirmed 12/44 CAGs in H1-1, 12/48 CAGs in H1-2, 12/49 CAGs in H1-3, 19 CAGs in H1-5, 19/49 in H1-6 and 44 CAGs in H1-8 (Homozygous HD) (Figures 3.12-3.18). The agarose gel estimation results showed ~19/31CAGs in H1-4 (intermediate penetrance of HD), ~38/40 in H1-7 (Homozygous HD), and ~44 CAGs in

H1-9 (Homozygous HD). Individual H1-8 and H1-9 are younger and asymptomatic for HD with homozygous expansion of HD (44 CAGs).

3.4.2. Family H2

Family H2 was amplified for exon 1 of the *HTT* gene. The repeat size was analyzed using 2.5% agarose gel as shown in Figure 3.19. From family H2, Sanger sequencing confirmed 19/67 CAG repeats in patient H2-2 presenting juvenile HD (JHD) as shown in Figures 3.20 and 3.21. Agarose gel estimation showed ~19/60 CAGs in patient H2-3, ~20 CAGs in H2-1, and ~20 CAGs in H2-4.

3.4.3. Family H3

From family H3, patient H3-1 has an adult onset of HD with heterozygous CAG repeat expansion i.e., 18/53CAGs. The agarose gel electrophoresis results are shown in Figure 3.19. The number of CAG repeats in the normal and mutant (expanded) alleles was confirmed by Sanger sequencing as shown in Figures 3.22 and 3.23.

3.4.4. Normal Controls

The PCR and agarose gel-based analysis was done in the case of controls to check the size of CAG repeats in normal individuals. One normal control was sequenced having 20 CAG repeats as shown in Figure 3.24. The size of the band of normal sequenced control (946bp:20CAGs) and mutant sequence control (1020bp: 45CAGs) was used to estimate the size of CAG repeats in normal individuals as shown in figures 3.25 and 3.26. In normal individuals, 18-22 CAG repeats are present.

3.4.5. Verification of Mutation

American College of Medical Genetics (ACMG) has provided standard guidelines about the variants of HD as discussed above in Chapter 1 (Bean et al., 2021). According to these guidelines, CAG repeat expansions in exon 1 of the *HTT* gene of H1-1, H1-2, H1-3, H1-5, H1-6, H1-7, H1-8, H1-9, H2-2, H2-3, and H3-1 lie in the pathogenic range. Individual H1-4 has intermediate penetrance of HD. Controls lie in the normal category. The summary of all the variants in the *HTT* gene of the affected individuals from family H1, H2 and H3 are given in Table 3.3.

3.4.6. Relationship between the Age of Onset and CAG Repeats

The relationship between the age of onset of HD and CAG repeats was observed among all the patients from families H1, H2, and H3. An inverse correlation between the age of onset and CAG repeats was observed and this correlation was obtained by using linear regression analysis through GraphPad Prism 9 as shown in Figure 3.27. The larger the expansion of CAG repeats the earlier the onset of HD and vice versa. The representation of CAG repeats in affected patients and normal controls is shown in Figure 3.28.

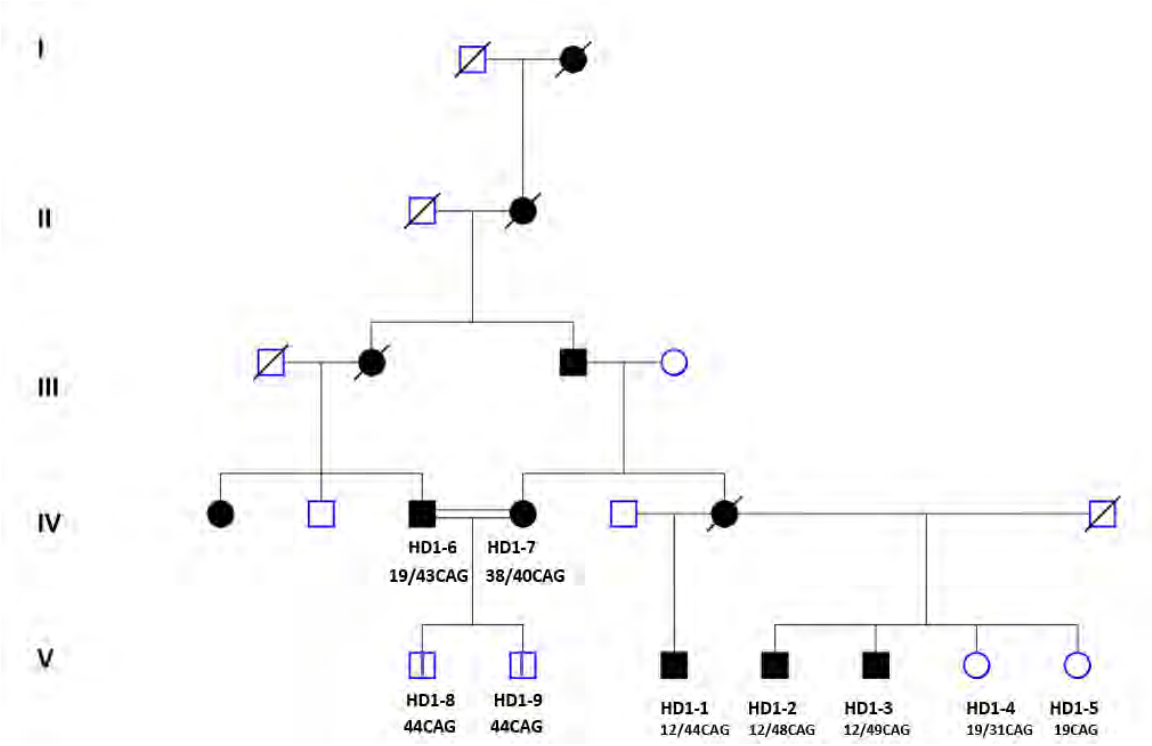

Figure 3.1. Pedigree of Family H-1. In the first loop H1-1, H1-2, and H1-3 are affected by HD (Filled black circle) and two normal siblings (H1-4, H1-5). From the second loop H1-6 and H1-7 are affected individuals while H1-8 and H1-9 carry a genetic mutation.

Table 3.1. Demographic and clinical profile of family H1. The motor, cognitive and psychiatric abnormalities are categorized from mild to severe.

Features	**Family H1**				
Patient ID's	**H1-1**	**H1-2**	**H1-3**	**H1-6**	**H1-7**
Age	25	40	36	50	53
Age of onset	24	37	24	35	48
Gender	Male	Male	Male	Female	Male
Height	5' 6"	5' 3"	5' 6"	5'	5' 7"
Weight	68kg	62kg	57kg	43kg	60kg
Choreatic movements	Asymptomatic	Mild	Severe	Severe	Asymptomatic
Tremors	Mild	Moderate	Severe	Severe	Mild
Bradykinesia	Asymptomatic	Mild	Severe	Severe	Asymptomatic
Dystonia	Asymptomatic	Mild	Moderate	Severe	Mild
Dysphagia	Asymptomatic	Asymptomatic	Moderate	Severe	Asymptomatic
Oromandibular dyskinesia	Asymptomatic	Asymptomatic	Moderate	Severe	Asymptomatic
Apraxia	Asymptomatic	Asymptomatic	Severe	Severe	Asymptomatic
Depression	Moderate	Severe	Severe	Severe	Moderate
Anxiety	Moderate	Severe	Severe	Moderate	Moderate
Apathy	Mild	Moderate	Severe	Severe	Mild
Aggression	Moderate	Severe	Severe	Severe	Moderate
Irritability	Mild	Mild	Moderate	Moderate	Mild
Suicidal thoughts	No	No	Yes	Yes	No
Insomnia	No	Yes	Yes	Yes	Yes
Attention deficits	Mild	Mild	Severe	Severe	Mild
Memory issues	Asymptomatic	Mild	Moderate	Severe	Asymptomatic
Learning disabilities	Moderate	Moderate	Severe	Severe	Moderate
Familial History	Mother	Mother	Mother	Father	Mother

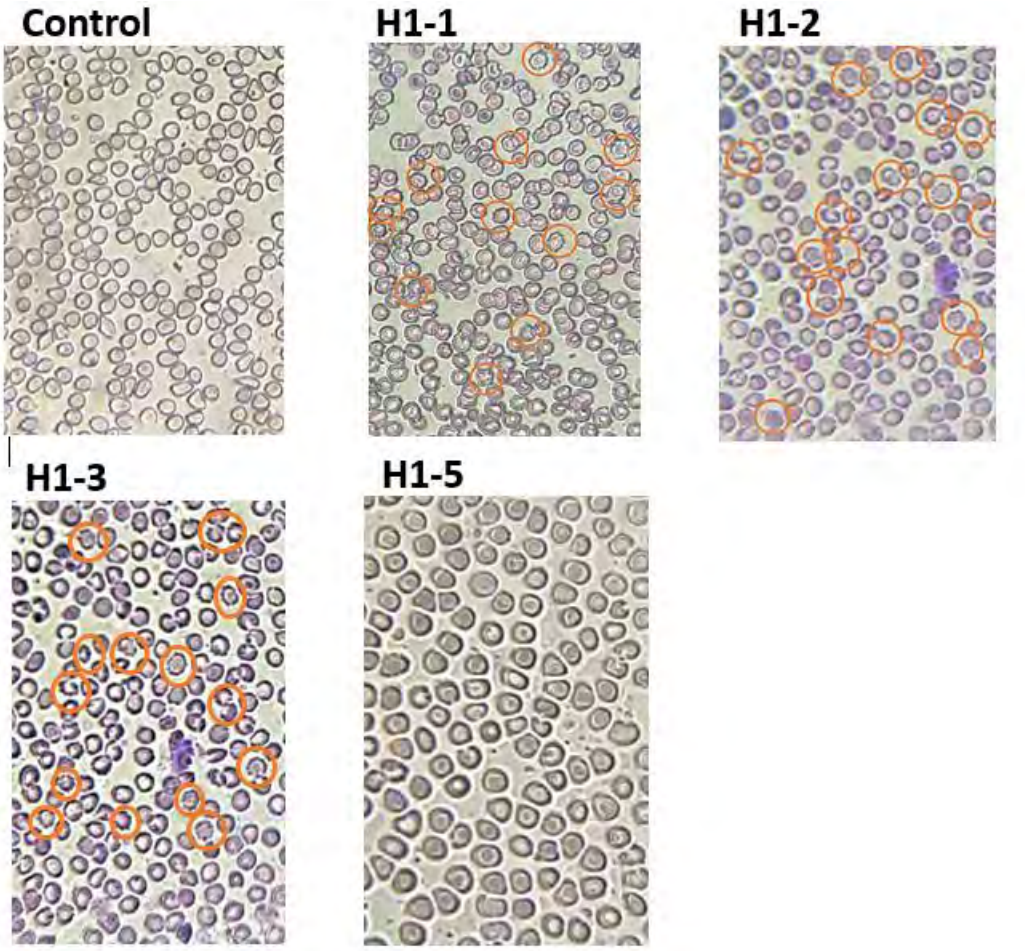

Figure 3.2. Acanthocytes were observed using 100X light microscopy in the affected individual of family H1 (H1-1, H1-2, H1-3) (represented by orange circles). No acanthocytes were observed in the normal control and normal individual of family (H1-5).

Figure 3.3. Comparison of ESR between HD patients and normal control. The difference of sedimentation of erythrocytes in affected individuals (H1-1, H1-2, and H1-3) is lower than in normal individuals (H1-4 and H1-5) after 2 hrs.

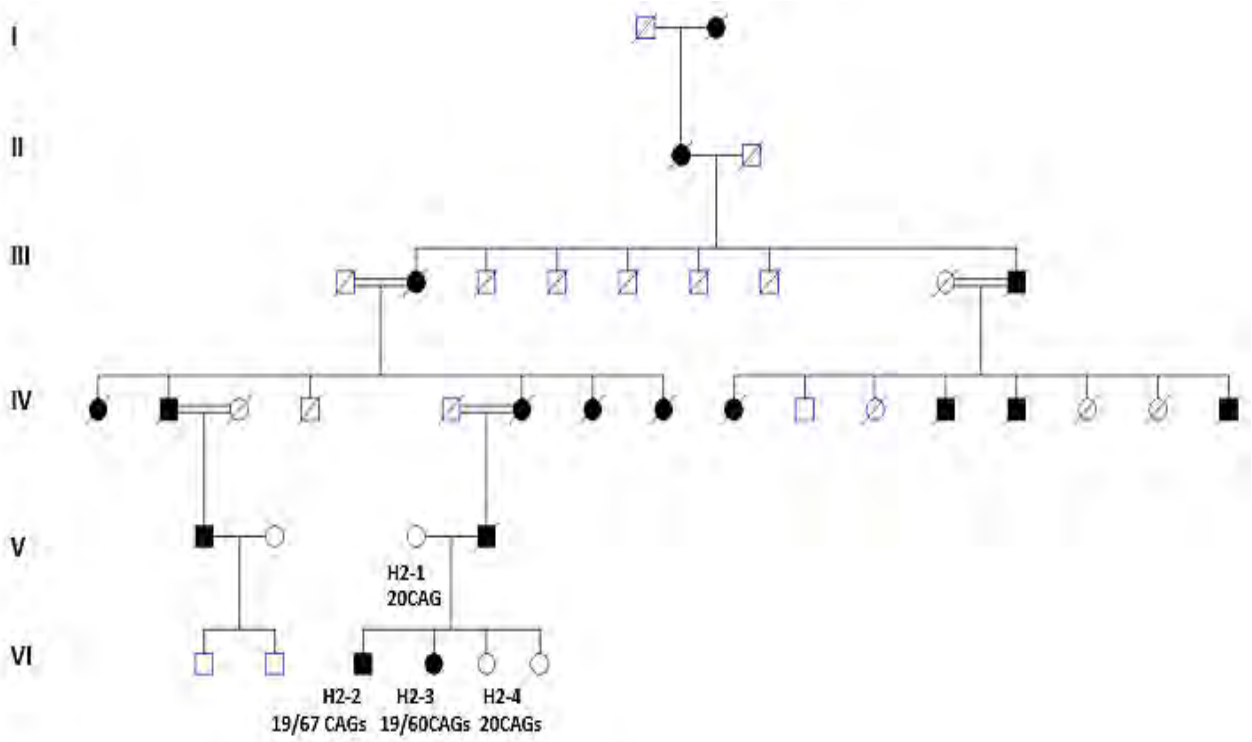

Figure 3.4. Pedigree of Family H-2. In the VI generation, H2-2 and H2-3 are affected by HD (Filled black circle) along with normal sibling HD2-4 and normal mother HD2-1.

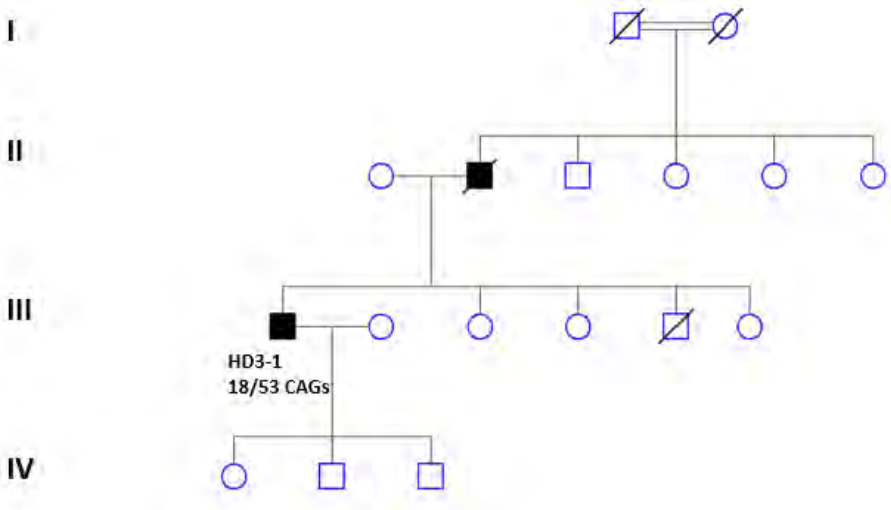

Figure 3.5. Pedigree of Family H-3. In the III generation, H3-1 is affected by HD.

Table 3.2. Demographic and clinical profile of family H2 and H3. The motor, cognitive and psychiatric abnormalities are categorized from mild to severe.

Features	**Family H2**		**Family H3**
Patient ID's	**H2-2**	**H2-3**	**H3-1**
Age	26	27	42
Age of onset	20	25	40
Gender	Male	Female	Male
Height	5' 9"	5' 3"	5' 6"
Weight	50kg	38kg	60kg
Choreatic movements	Severe	Asymptomatic	Mild
Tremors	Severe	Mild	Moderate
Bradykinesia	Severe	Asymptomatic	Mild
Dystonia	Moderate	Asymptomatic	Mild
Dysphagia	Severe	Asymptomatic	Asymptomatic
Oromandibular dyskinesia	Moderate	Asymptomatic	Asymptomatic
Apraxia	Severe	Asymptomatic	Asymptomatic
Depression	Severe	Severe	Severe
Anxiety	Severe	Severe	Severe
Apathy	Severe	Mild	Severe
Aggression	Severe	Severe	Severe
Irritability	Moderate	Mild	Mild
Suicidal thoughts	Yes	No	Yes
Insomnia	Yes	Yes	Yes
Attention deficits	Severe	Mild	Moderate
Memory issues	Moderate	Asymptomatic	Mild
Learning disabilities	Severe	Moderate	Moderate
Familial History	Father	Father	Father

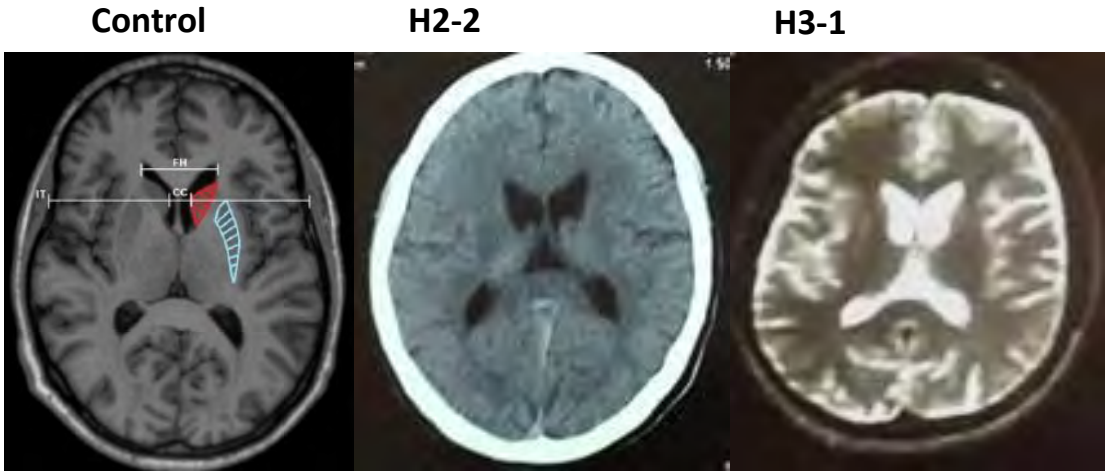

Figure 3.6. Brain MRI imaging of HD patients (H2-2 and H3-1) was compared with normal healthy control. Anatomical landmarks of the basal ganglia (putamen in blue and caudate in red) are shown on the normal T1 axial MRI, with measurement markers of atrophy (CC: inter-caudate distance; FH: frontal horn distance of lateral ventricles; IT: inner table of the skull). In contrast, severe caudate and frontal-predominant global atrophy are demonstrated in an MRI of HD patients. Normal brain MRI image adapted from Goh et al., (2018).

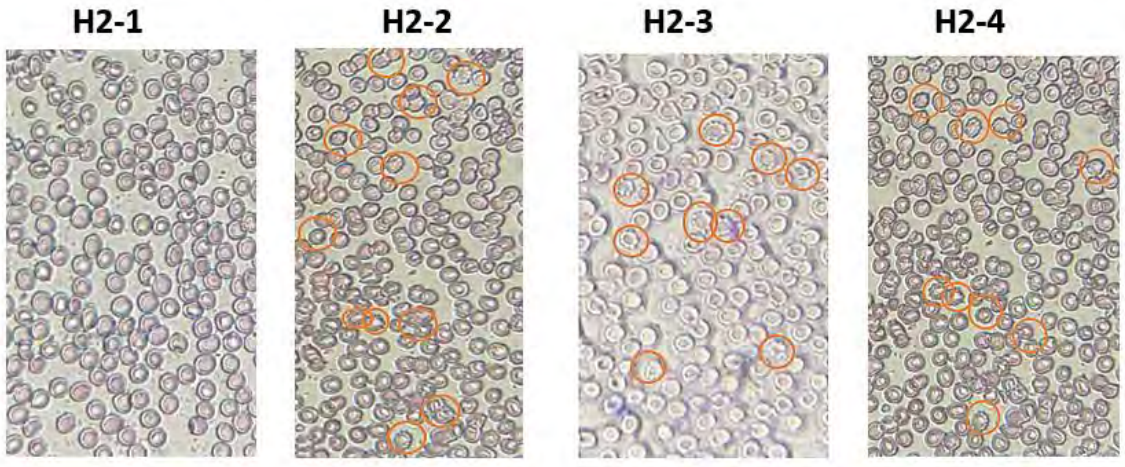

Figure 3.7. Acanthocytes were observed using 100X light microscopy in the affected individual of family H2(H2-2, H2-3, and asymptomatic H2-4) (represented by orange circles). No acanthocytes were observed in the normal mother (H2-1).

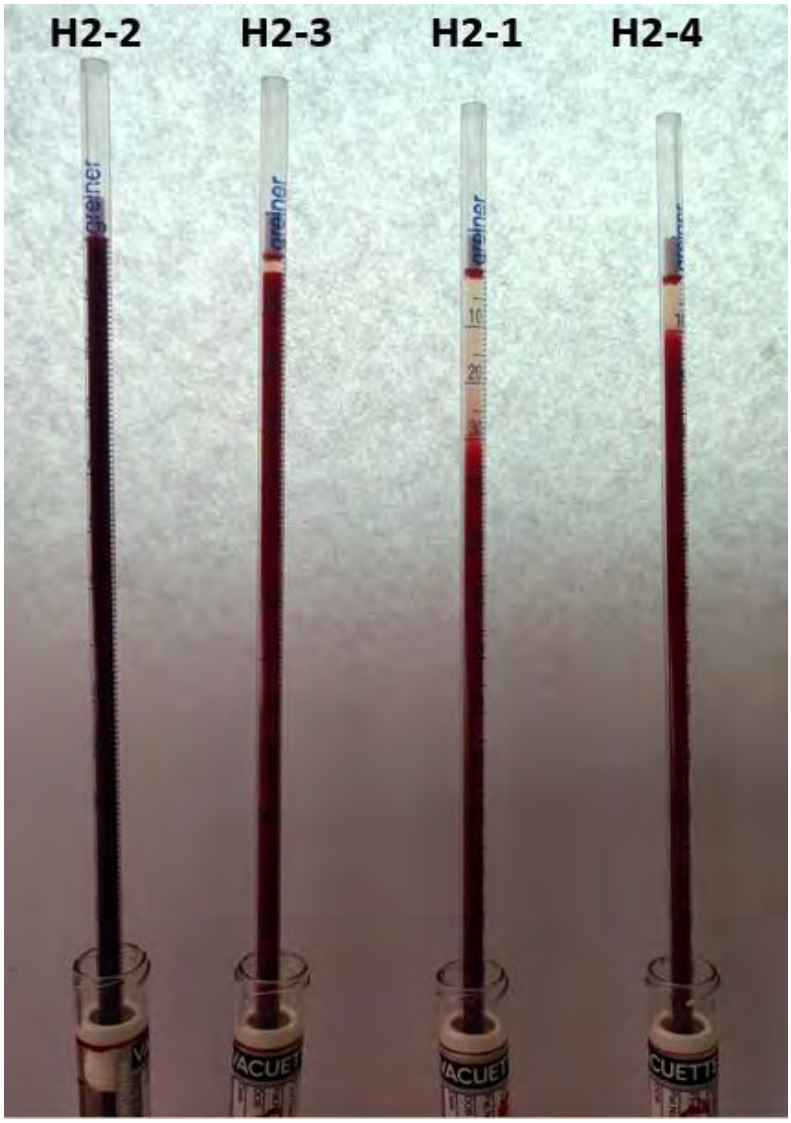

Figure 3.8. Comparison of ESR between HD patients and normal control from family H2. The difference in sedimentation of erythrocytes in the affected individuals (H2-2 and H2-3) is lower than in normal individuals (H2-1 and H2-4) after 2 hrs.

Figure 3.9. Agarose gel representation of Family H1 (first loop). Amplified exon 1 of the *HTT* gene represented an expansion of CAG repeats in affected individuals of the first loop of family H1 (H1-1, H1-2, H1-3: Heterozygous HD), H1-4 has an intermediate allele of HD and H1-5 is normal.

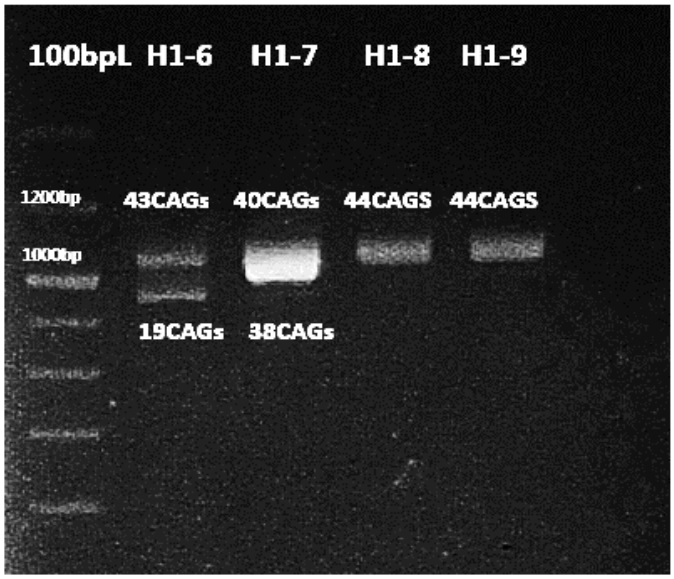

Figure 3.10. Agarose gel representation of Family H1 (second loop). Amplified exon 1 of the *HTT* gene represented an expansion of CAG repeats in affected individuals of the second loop of family H1 (H1-6: Heterozygous HD; H1-7, H1-8, H1-9: Homozygous HD).

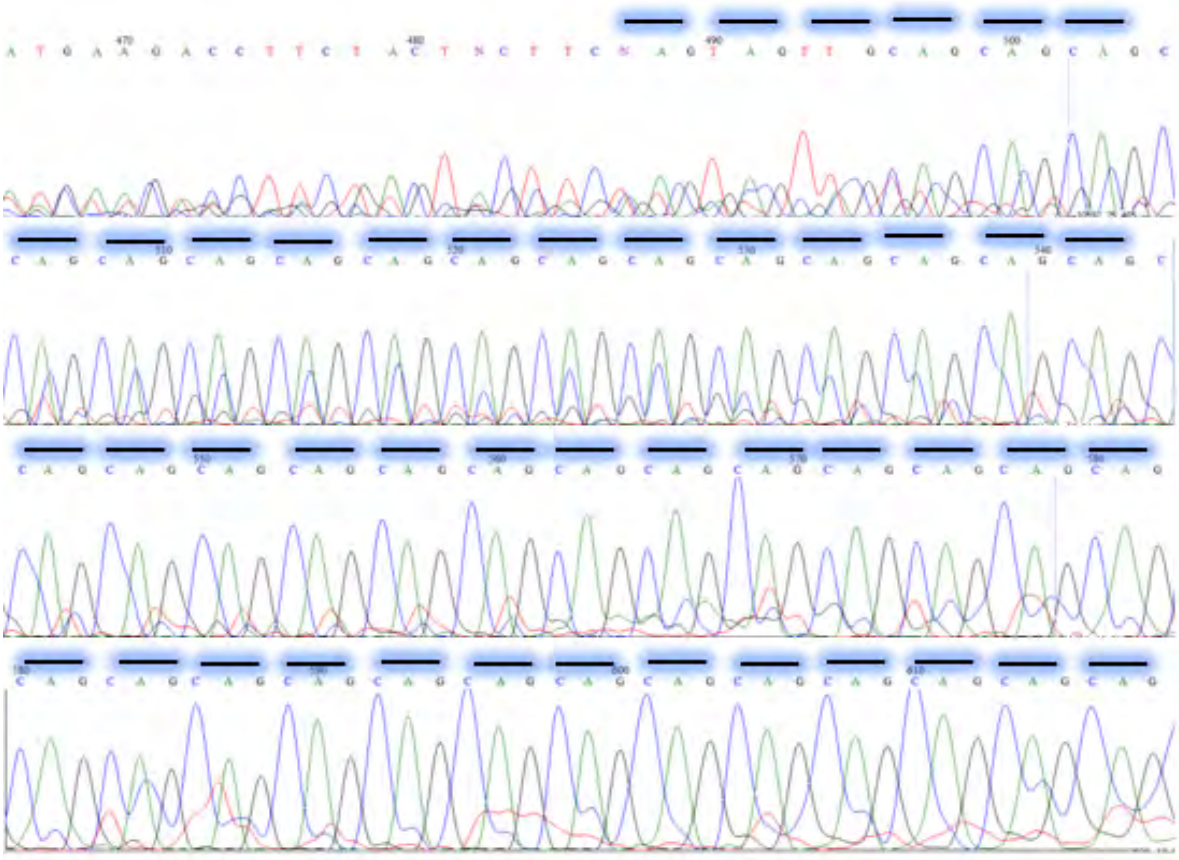

Figure 3.11. Sanger sequencing results of the individual HD1-1 (Family H1) having 44 CAG repeats in the mutant allele.

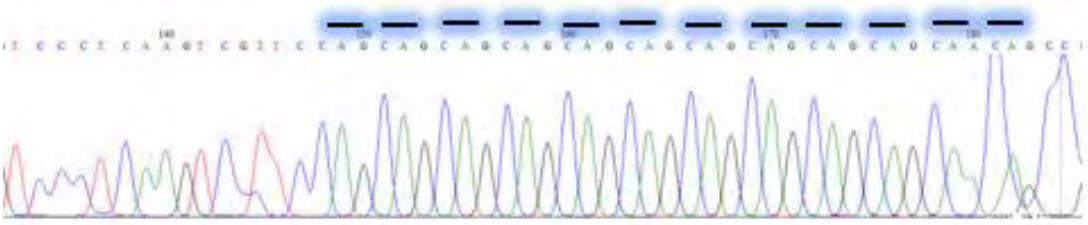

Figure 3.12. Sanger sequencing results of the individual HD1-1 (Family H1) having 12 CAG repeats in the normal allele.

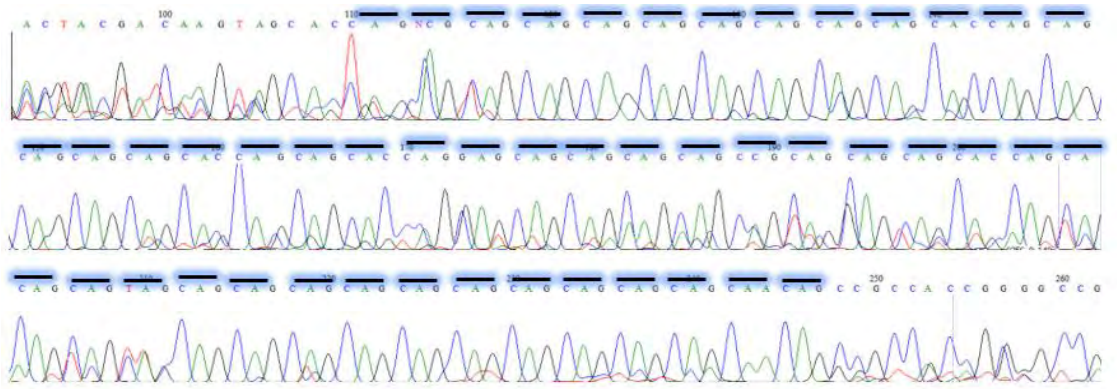

Figure 3.13. Sanger sequencing results of the individual HD1-2 (Family H1) having 48 CAG repeats in the mutant allele.

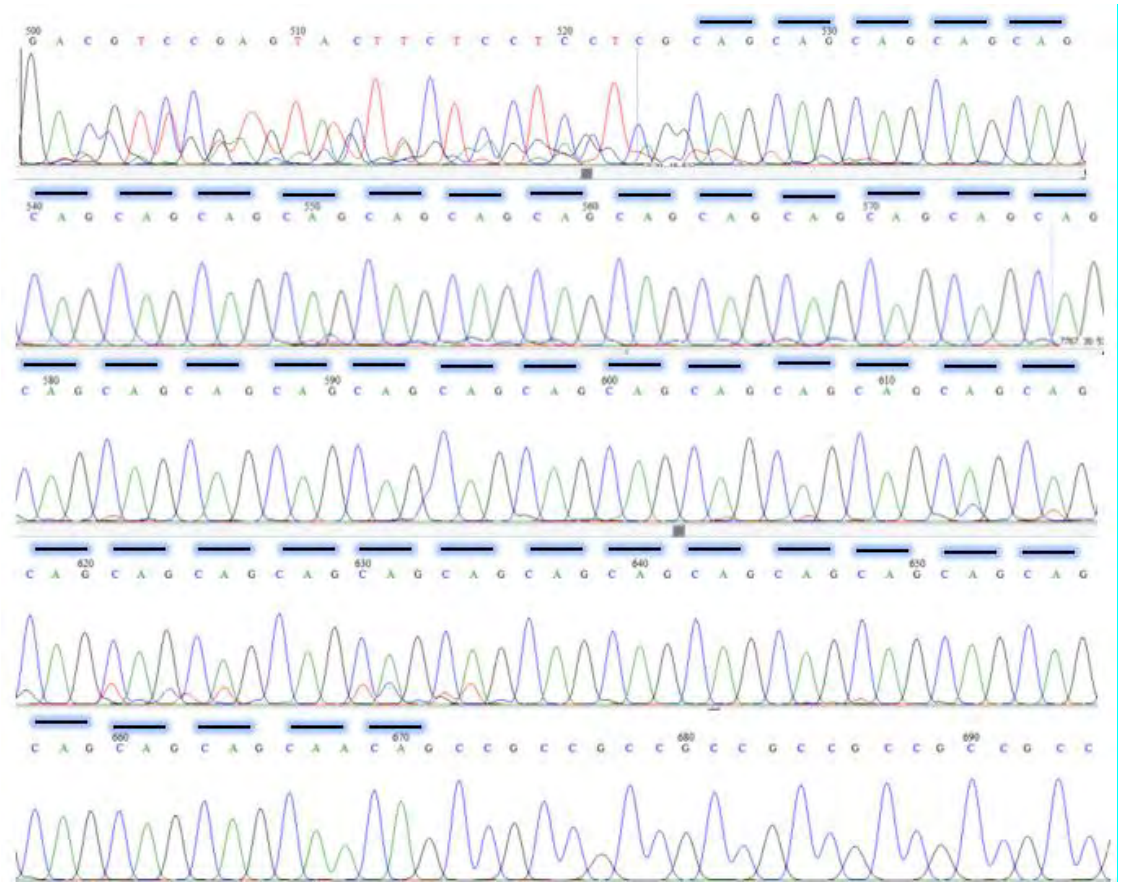

Figure 3.14. Sanger sequencing results of the individual HD1-3 (Family H1) having 49 CAG repeats in the mutant allele.

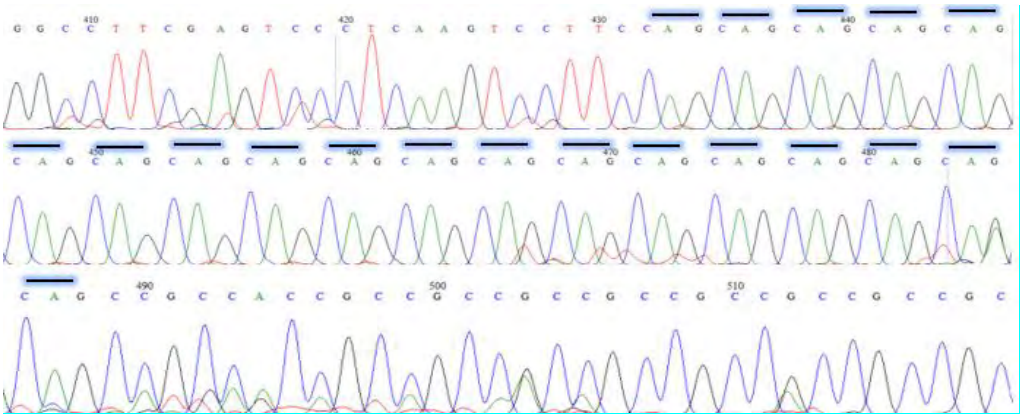

Figure 3.15. Sanger sequencing results of the individual HD1-5 (Family H1) having 19 CAG repeats.

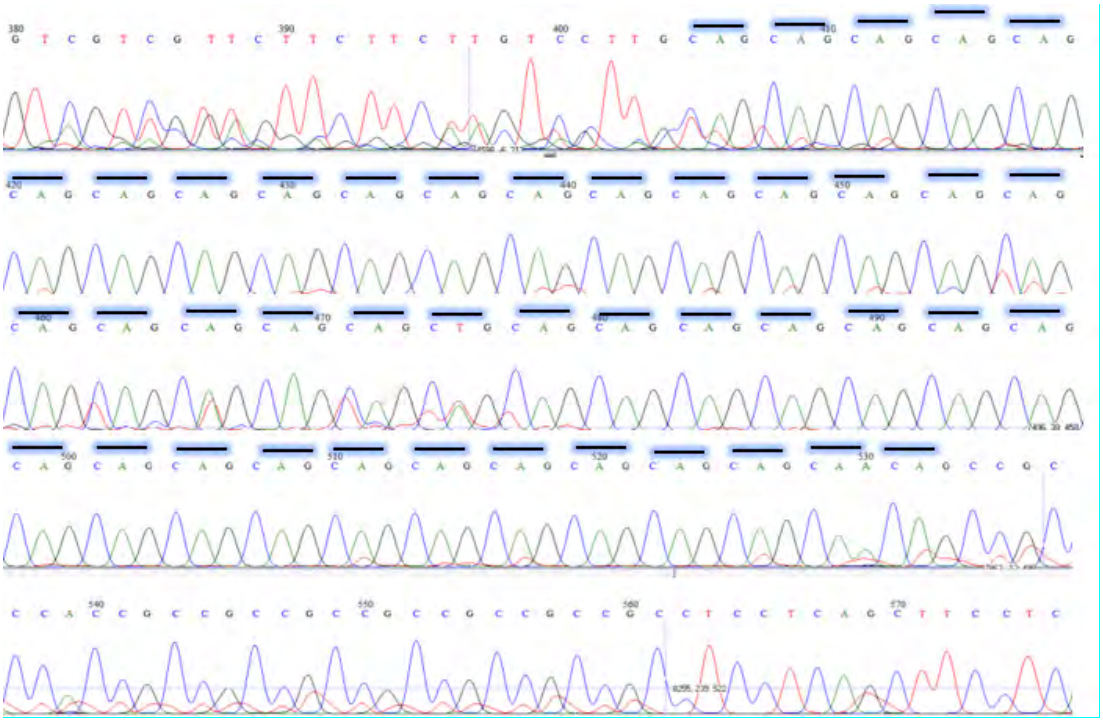

Figure 3.16. Sanger sequencing results of the individual HD1-6 (Family H1) having 43 CAG repeats in the mutant allele.

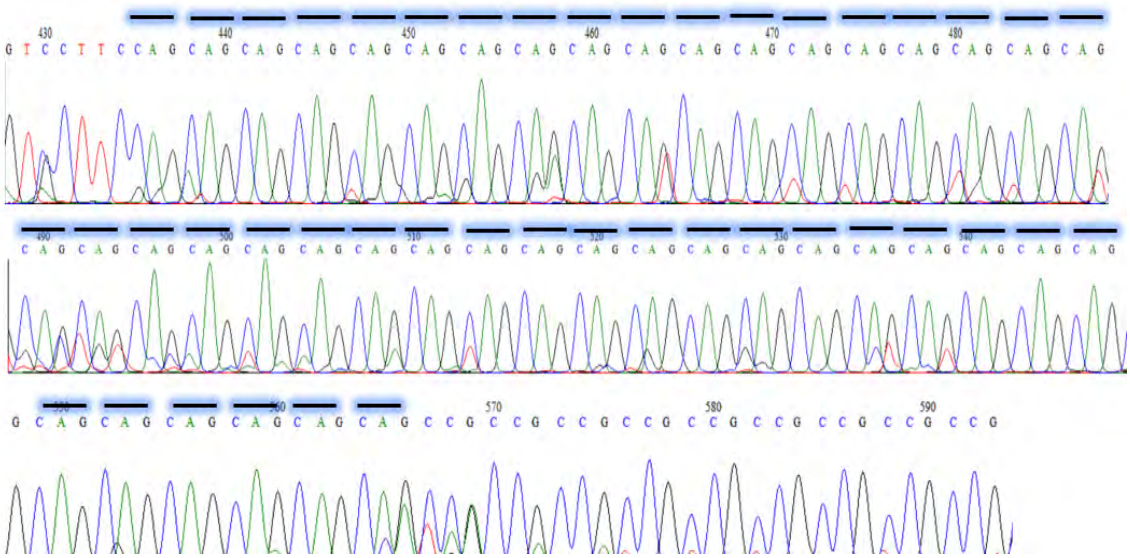

Figure3.17. Sanger sequencing results of the individual HD1-8 (Family H1) having 44 CAG repeats in both alleles (Homozygous)

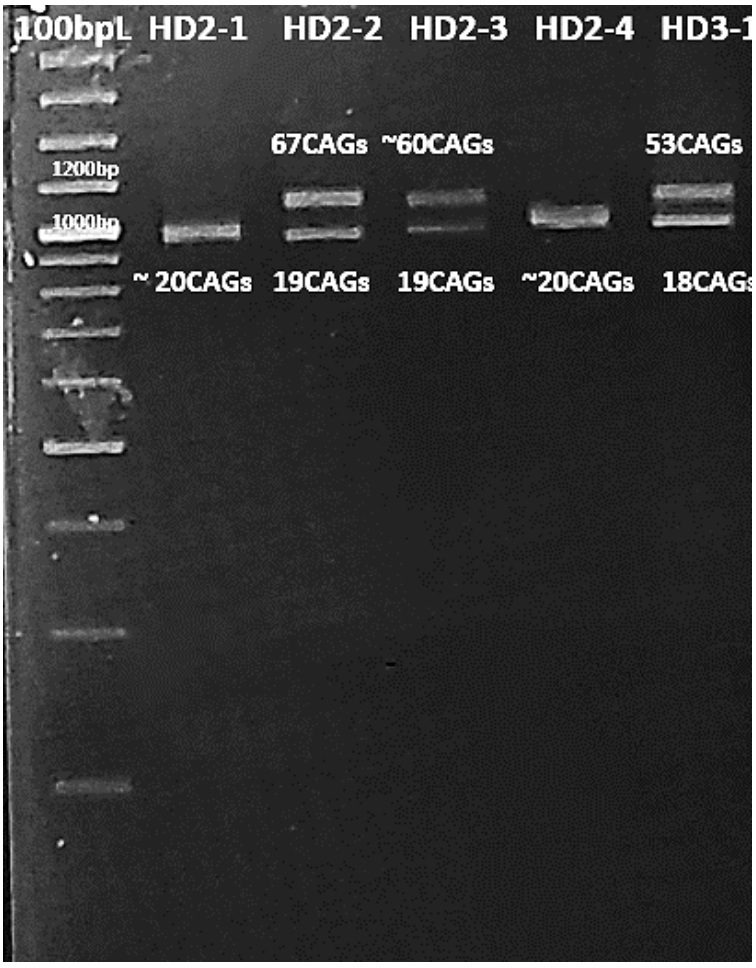

Figure 3.18. Agarose gel representation of Family H2 and H3. Amplified exon 1 of *HTT* gene represented an expansion of CAG repeats in affected individuals of the family H2 (H2-2, H2-3: Heterozygous HD, H2-4 has intermediate allele of HD and H2-1 is normal parent) and family H3 (H2-3: Heterozygous HD).

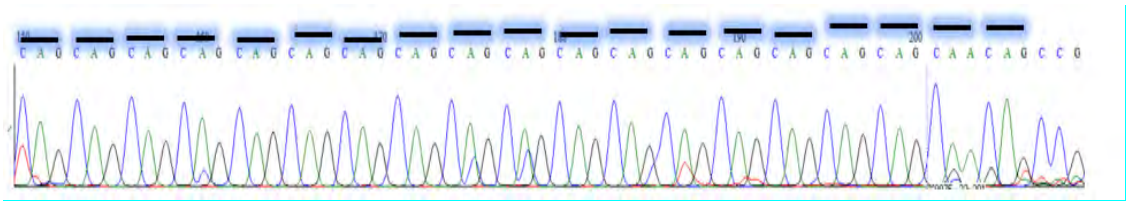

Figure 3.19. Sanger sequencing results of the individual HD2-2 (Family H2) having 19 CAG repeats in the normal allele.

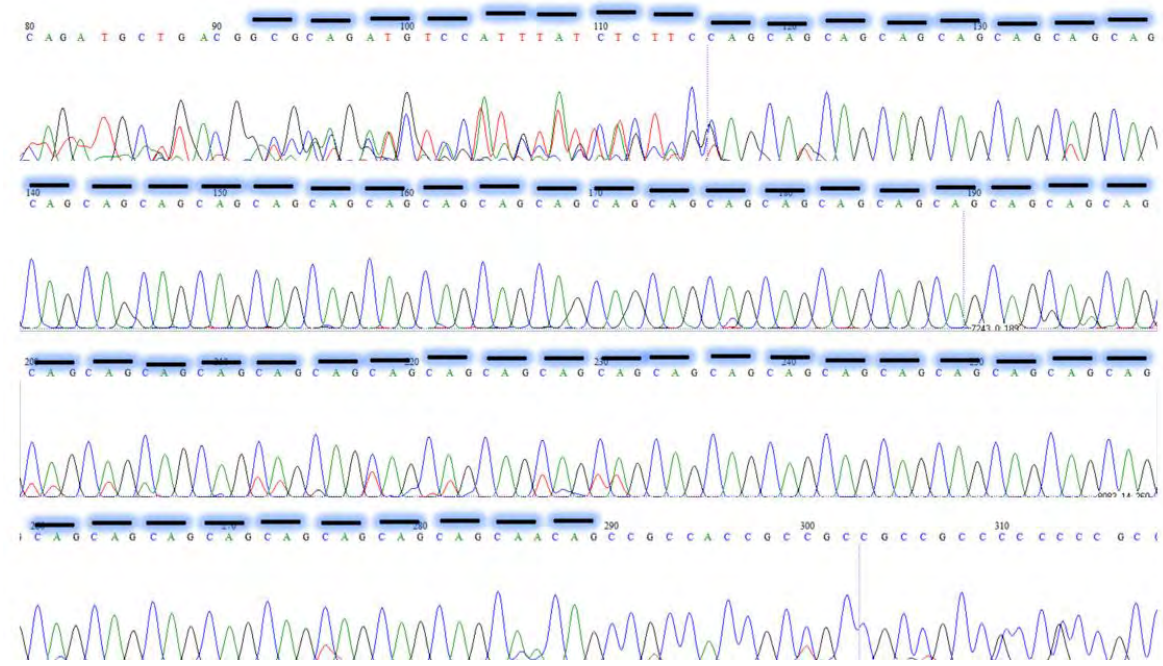

Figure 3.20. Sanger sequencing results of the individual HD2-2 (Family H2) having 67 CAG repeats in the normal allele.

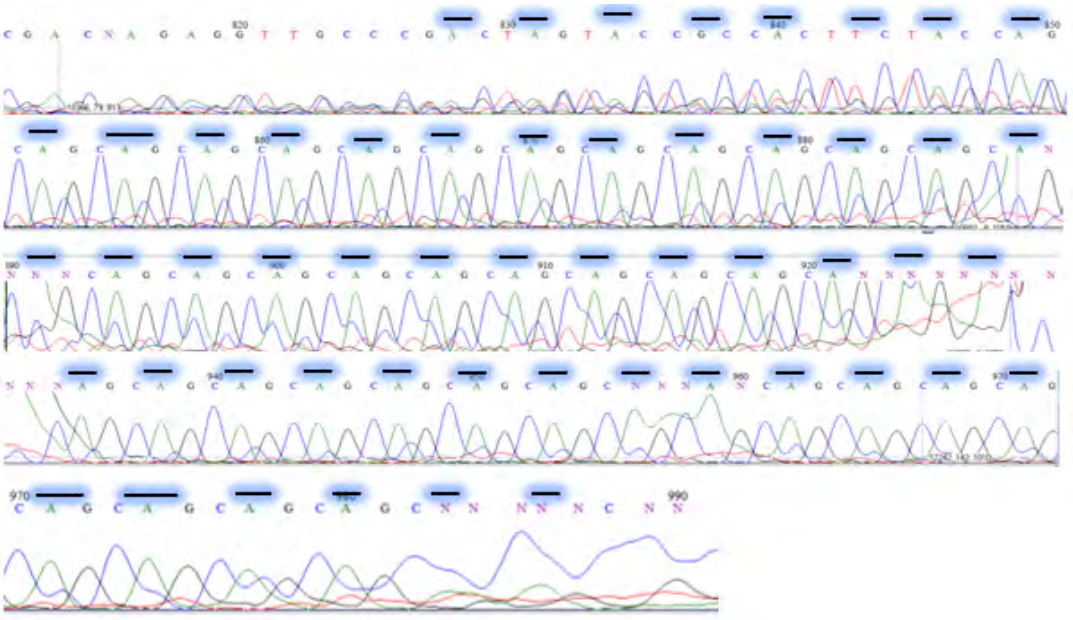

Figure 3.21. Sanger sequencing results of the individual HD3-1 (Family H3) having 53 CAG repeats in the normal allele.

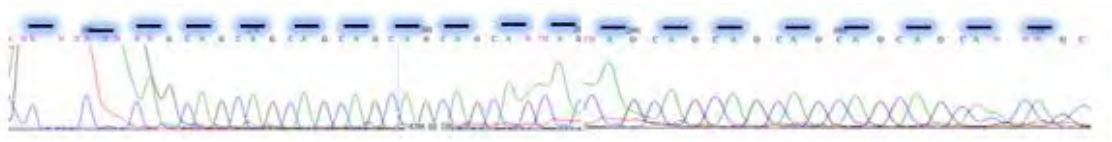

Figure 3.22. Sanger sequencing results of the individual HD3-1 (Family H3) having 18 CAG repeats in the normal allele.

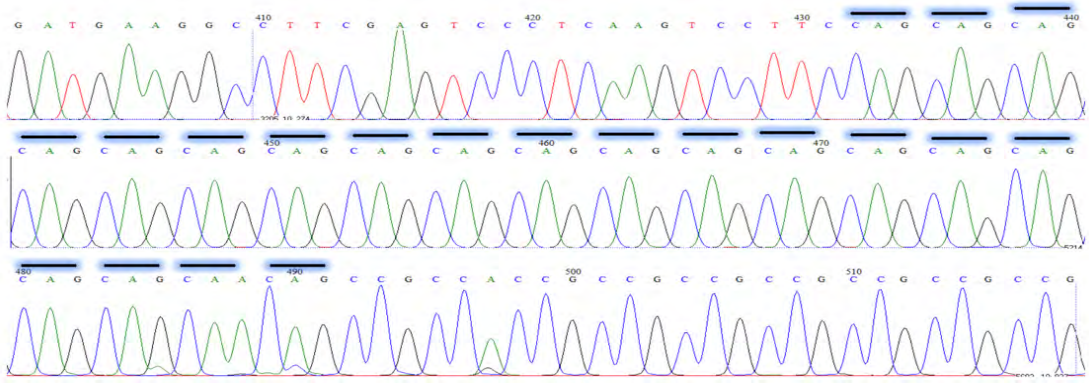

Figure 3.23. Sanger sequencing results of the normal healthy control having 20 CAG repeats.

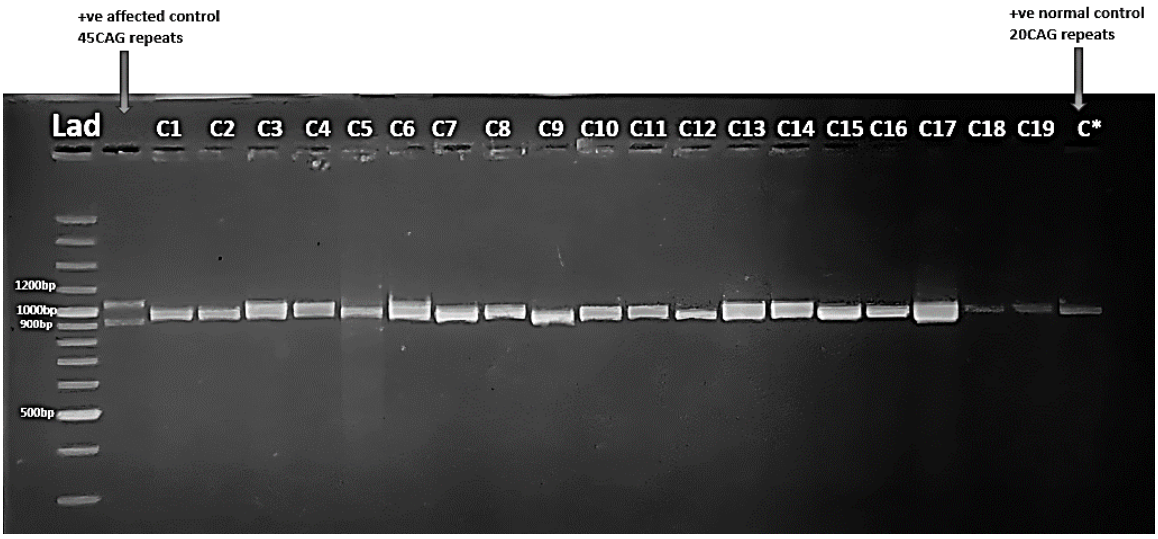

Figure 3.24. Agarose gel representation of healthy controls (C1-C19). Amplified exon 1 of the *HTT* gene represented a CAG repeat track in normal healthy controls. The results were compared with normal sequenced control (C*=20CAGs) and (positive control=45CAG).

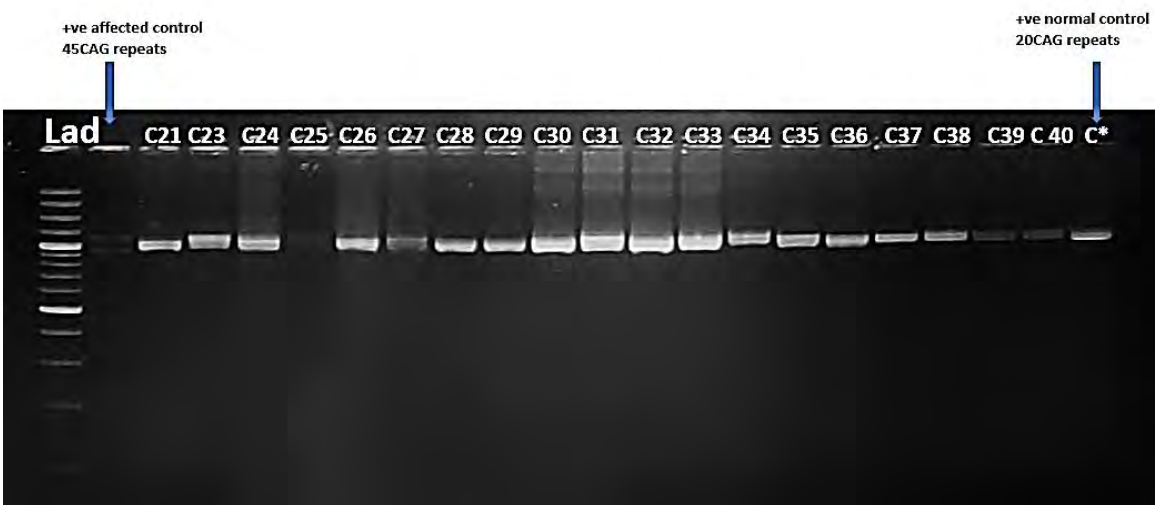

Figure 3.25. Agarose gel representation of healthy controls (C21-C40). Amplified exon 1 of the *HTT* gene represented a CAG repeat track in normal healthy controls. The results were compared with normal sequenced control (C*=20CAGs) and (positive control=45CAG).

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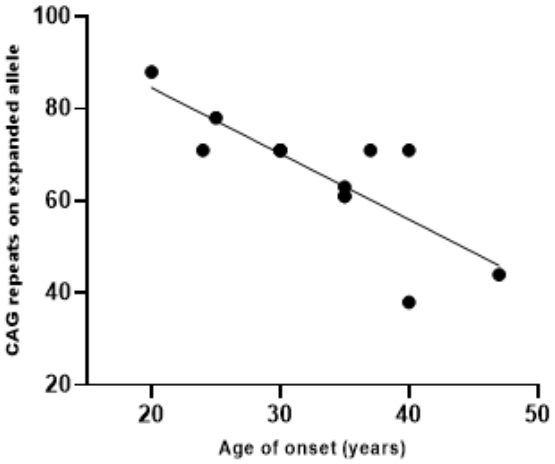

Figure 3.26. An inverse correlation between the age of onset and the CAG repeats was observed using GraphPad Prism 9 (n=10).

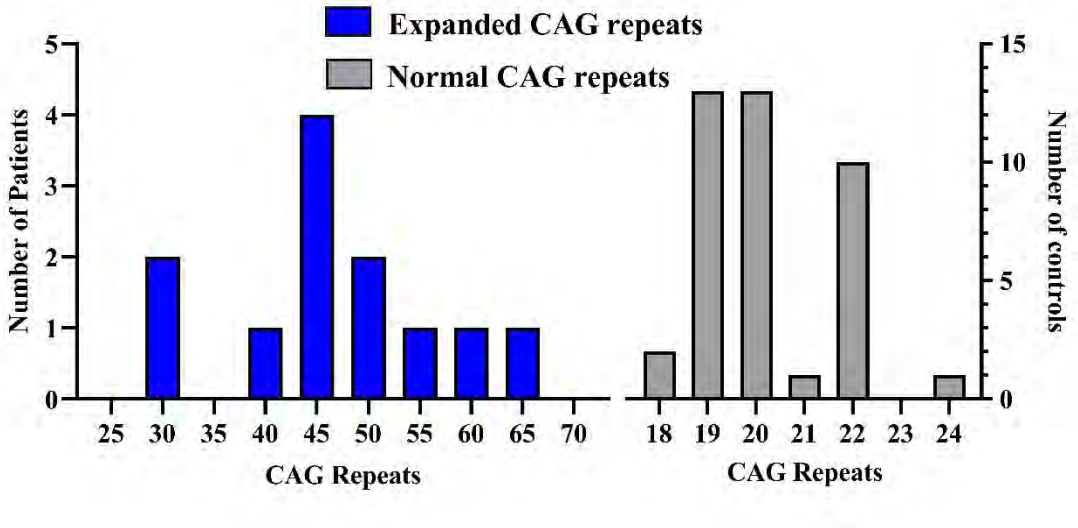

Figure 3.27. CAG repeat numbers in patients from family H1, H2, H3 and normal controls

Table 3.3. Summary of variants in *HTT* gene in affected individuals of Family H1, H2 and H3.

Family ID	Patient ID	Variant Coordinates	CAG repeats	Protein Change	Zygosity	Variant Classification (ACMG, 2021)
H1	**H1-1**	NM_001388492.1(*HTT*):c.54GCA [44]	44	p.Gln22[44]	Heterozygous	Pathogenic
	H1-2	NM_001388492.1(*HTT*):c.54GCA[48]	48	p.Gln22[48]	Heterozygous	Pathogenic
	H1-3	NM_001388492.1(*HTT*):c.54GCA[49]	49	p.Gln22[49]	Heterozygous	Pathogenic
	H1-6	NM_001388492.1(*HTT*):c.54GCA[43]	43	p.Gln22[43]	Heterozygous	Pathogenic
	H1-7	NM_001388492.1(*HTT*):c.54GCA[40]	40	p.Gln22[40]	Homozygous	Pathogenic
	H1-8	NM_001388492.1(*HTT*):c.54GCA[44]	44	p.Gln22[44]	Homozygous	Pathogenic
	H1-9	NM_001388492.1(*HTT*):c.54GCA[44]	44	p.Gln22[44]	Homozygous	Pathogenic
H2	**H2-2**	NM_001388492.1(*HTT*):c.54GCA[67]	67	p.Gln22[67]	Heterozygous	Pathogenic
	H2-3	NM_001388492.1(*HTT*):c.54GCA[60]	60	p.Gln22[60]	Heterozygous	Pathogenic
H3	**H3-1**	NM_001388492.1(*HTT*):c.54GCA[53]	53	p.Gln22[53]	Heterozygous	Pathogenic

CHAPTER 04

DISCUSSION

4. Discussion

HD is a progressive neurodegenerative disorder with an autosomal dominant mode of inheritance (Hong et al., 2021). HD is characterized by motor, cognitive and psychiatric decline due to the progressive loss of the medium spiny neurons of the striatum in the premanifest HD and pyramidal neurons of the cortex in the advanced stages of the disease (Barnat et al., 2020). The polyglutamine expansion causes the misfolding of huntingtin protein leading to intracellular aggregates that disrupt many neuronal pathways involved in HD (Bates et al., 2015). An unidentified common pathway responsible for RBC morphology and neurodegeneration resulted in spikey-shaped acanthocytes in HD patients (Jung et al., 2011; Yu et al., 2022).

HD is caused by CAG repeat expansions in exon 1 of the *HTT* gene located on chromosome 4. According to ACMG guidelines for HD, a normal-sized *HTT* allele has <26 CAG repeats with no disease phenotype. Individuals with CAG repeat expansion between 27-35 (intermediate range) are considered at risk of developing HD and the ones with 36-39 CAG repeats are at the reduced penetrance of developing HD. >40 repeats are pathogenic and involved in the full penetrance of HD (Bean et al., 2021). CAG repeat expansions are related to the age of onset of HD (Gusella et al., 2009). The mean age onset of HD is 35-40 years (Yu et al., 2014).

In the present study, we demonstrated the clinical and genetic features of HD in the Pakistani population. Consistent with the previous studies, parkinsonism, chorea, and depression are the initial motor and psychiatric manifestations in HD patients (Gatto et al., 2012; Shin et al., 2013; Yang et al., 2016; Orth et al., 2017; Chen et al., 2020). The phenotypes of HD are associated with atrophy in the caudate nucleus, basal ganglia and putamen observed through brain MRI imaging (Goh et al., 2018; Arraj et al., 2020). A large clinical trial in Australia and Europe depicted that brain atrophy may initiate 15 years before the onset of HD (Domínguez et al., 2013; Paulsen et al., 2014). Consistently, brain MRI imaging results of patients H2-2 and H3-1 demonstrated severe ventricular and striatum atrophy.

Acanthocytosis is the transformation of the normal biconcave RBC into irregularly shaped cells with external projections over the membrane (Lupo et al., 2016). Acanthocytes were frequently reported in neuroacanthocytosis syndromes

characterized by RBC acanthocytosis with neurological abnormalities and may serve as the diagnostic marker (Liu et al., 2014). Previous studies reported acanthocytes in chorea acanthocytosis and Huntington disease like-2 (HDL2) (Walker et al., 2003; Darras et al., 2022). Consistent with Yu et al., (2022) acanthocytes were observed in the blood smears of affected individuals of family H1 and H2 as compared with normal control under the light microscope. In an Irish family suffering from phenotypes of HD and acanthocytosis, coexisting compound heterozygous mutations in VPS13A with *HTT* CAG repeat expansion (37CAG repeats) were reported (Murphy et al., 2018). The abnormalities in VPS13A resulted in the loss of function of the chorein protein that is involved in the architecture of the cytoskeleton (Föller et al., 2012; Shiokawa et al., 2013).

Darras et al., (2022) observed a reduced ESR by acanthocytes in the patients of chorea acanthocytosis. We observed the ESR of the affected and normal members of family H1 and H2 and these results were consistent with the previous study. The acanthocytes affect the sedimentation rate of blood cells resulting in reduced differences in ESR of the affected individuals as compared with the normal controls.

The length of the CAG repeats differs among different ethnic groups (Bates et al., 2015). 7 patients from family H1, 2 patients from family H2 and one patient from family H3 were found to be carrying full penetrance CAG repeat expansion range from 40-67 CAGs with age of onset ranges from 20-50 years. >40 CAGs in *HTT* lie in the pathogenic range with full penetrance HD (ACMG,2021). The frequency of expanded CAGs is consistent with the Korean and Dutch cohorts (39-71CAGs) (Maat-Kievit et al., 2002; Shin et al., 2013). Studies from Chinese and Mexican cohorts reported (37-106CAGs) in the expanded allele of *HTT* in the affected individuals (Alonso et al., 2009; Li et al., 2019). The difference in repeat size may be caused by the genetic modifiers in specific ethnic groups.

The frequency of the intermediate alleles in our study is 7.6% (1/13) which is much lower than the one reported in previous studies (Ha & Jankovic, 2012; Chen et al., 2020). Studies reported that intermediate alleles have unstable *HTT* alleles which may expand due to the meiotic instability. The offspring of the individuals with intermediate

alleles are at risk of developing HD due to anticipation (Goldberg et al., 1995; Semaka et al., 2013).

Juvenile HD accounts for 5% of total HD cases all over the world (Cronin et al., 2019). From our study, one case of juvenile HD was observed with age onset at 20 years and 67 CAG repeats in the expanded allele. The initial symptoms in this patient were chorea, tremors, depression, and aggression. The patient presented severe atrophy of the striatum. HD homozygotes are rare compared with other neurodegenerative disorders (Squitieri et al., 2003). We identified two homozygotes with 44 CAG repeats in family H1. Both individuals are young (13 and 24 years) and still asymptomatic for HD. Both parents of these individuals have adult onset of HD. Alonso et al., (2009) depicted that in contrast with the description that homozygotes have a severe course of the disease as compared with heterozygotes, homozygotes had less severe and slow progression of HD (Squitieri et al., 2003).

In our study, the CAG repeats in normal alleles of the affected individuals range from 12-20CAGs. The average CAG repeat length in the general population ranges from 17-20 repeats (Falush et al., 2009). By comparing our findings with different populations around the world, the PCR-based results from normal controls of the Pakistani population depicted that the range of CAG repeats is 18-22 which is consistent with the European population having 17.8 CAGs in the normal cohort (Warby et al., 2011).

A negative correlation between CAG repeat size in the expanded allele and the age of onset is observed consistent with previous findings (Maat-Kievit et al., 2002; Alonso et al., 2009; Shin et al., 2013; Li et al., 2019).

In conclusion, our study presents the first clinical and molecular study of HD in the Pakistani population. The sample size from the normal controls and affected individuals should be increased in further studies to validate the number of CAG repeats in normal and affected individuals to determine the prevalence of HD in Pakistan. These results may have significant implications for a better understanding of Huntington's disease, its diagnosis and prognosis in the Pakistani population. It will set a paradigm to implement confirmatory genetic testing, pre-symptomatic testing, and therapeutic approaches.

CHAPTER 05

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