SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

THIRUVANANTHAPURAM, KERALA

UNRAVELLING THE GENETIC PATTERN IN PATIENTS WITH NEURONAL BRAIN IRON ACCUMULATION

Thesis submitted in partial fulfilment of the rules and regulations for PDF Movement Disorders course of

Sree Chitra Tirunal Institute for Medical Sciences and Technology

By

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DECLARATION

I, Dr. Mitesh Vinodray Chandarana, hereby declare that this project was undertaken by me under the supervision of the faculty, Department of Movement disorders, Sree Chitra Tirunal Institute for Medical Sciences and Technology.

Thiruvananthapuram
Date: 30/12/2020

Dr. Mitesh Vinodray Chandarana

Forwarded:

The candidate, Dr. Mitesh Vinodray Chandarana, has completed the project under my guidance.

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INDEX

INTRODUCTION

Introduction:

Neurodegeneration with brain iron accumulation (NBIA) encompasses a clinically and genetically heterogeneous group of inherited neurodegenerative disorders, characterized by excessive iron accumulation in the deep nuclei of basal ganglia, and to a lesser extent in substantia nigra and other adjacent areas. The common clinical features of NBIA include infantile to adult onset progressive extrapyramidal symptoms (dystonia, chorea, parkinsonism), pyramidal signs, seizures, cognitive decline, neuropsychiatric, and ocular symptoms in varying combinations. The prevalence data is rather incomplete, but all forms of NBIA are considered to be quite rare with less than 1/1000000 affected(1).

Though this group of disorders has been hitherto identified as early as 1922, with the advances in imaging modalities and genetic testing the spectrum of disorders in this group are still expanding. Recently, improvements in gene sequencing technologies have largely facilitated rapid gene discovery, and many single-gene disorders are now included in this group(2). Till date, total 10 NBIA genes have been identified; eight of them are autosomal recessive, one is autosomal dominant, and one is X-linked dominant(1) (Table 1). Identification of these various genes have advanced our understanding of the disease pathogenesis. However, the role of iron deposition in the disease pathophysiology remains uncertain, particularly the question of whether iron deposition has a causal role or it is simply an epiphenomenon of underlying neurodegeneration still needs to be answered with precision. The final common pathway by which the iron accumulates is also not clear(2). Till date, only two of

these 10 genes (*FTL* and *CP*) have been found to encode proteins that have a direct role in iron metabolism, while rest of the eight genes (*PANK2*, *PLA2G6*, *C19orf12*, *WDR45*, *FA2H*, *ATP13A2*, *DCAF17* and *COASY*) encode proteins that are involved in coenzyme A (CoA) synthesis and metabolism, lipid metabolism, mitochondrial function, or autophagy(3).

Despite genetic and clinical heterogeneity, these syndromes of NBIAs manifest with degeneration of the globus pallidus and substantia nigra along with iron accumulation. In normal ageing brains of humans as well as animals, iron accumulates in globus pallidus, substantia nigra, red nucleus and dentate nucleus. The exact reason behind this selective regional iron accumulation remains unclear, although it is postulated that some specialized neuronal population in the globus pallidus and substantia nigra are selectively programmed to transcribe larger amounts of ferritin and thereby create a ferritin-rich iron depository in the brain(3). Ongoing elucidation in the pathogenesis of each NBIA syndrome will have implications for the design of novel therapies to treat these patients(4).

A unified view of the NBIA disorders has been driven by genetics. Beyond providing a diagnostic path and clinical benefits to patients, broader scientific gains have been followed from the disease gene discovery. New cellular processes and pathways have been identified; selective neuronal and brain region vulnerabilities have been revealed, and our understanding of the pathophysiology of common related neurodegenerative disorders has enhanced.

Table 1: Genes associated with various types of NBIA

NBIA subtype	*Gene Associated*	Mode of Inheritance
Pantothenate kinase-associated neurodegeneration (PKAN)	*PANK2*	Autosomal recessive
Phospholipase A2-associated neurodegeneration (PLAN)	*PLA2G6*	Autosomal recessive
Mitochondrial membrane protein-associated neurodegeneration (MPAN)	*C19orf12*	Autosomal recessive
Beta-propeller protein-associated neurodegeneration (BPAN)	*WDR45*	X-linked dominant
Fatty acid hydroxylase-associated neurodegeneration (FAHN)	*FA2H*	Autosomal recessive
Coenzyme A synthase protein-associated neurodegeneration (CoPAN)	*COASY*	Autosomal recessive
Neuroferritinopathy	*FTL1*	Autosomal dominant
Aceruloplasminemia	*CP*	Autosomal recessive
Kufor-Rakeb syndrome (KRS)	*ATP13A2*	Autosomal recessive
Woodhouse-Sakati syndrome (WSS)	*DCAF17*	Autosomal recessive

REVIEW OF LITERATURE

Historical Background and Nomenclature:

The research related to brain iron deposition began in late 19th century (1886) by Zaleski, who performed a quantitative analysis of single human brain and correlated brain iron levels with observations on stained slices and microscopic sections(5). In 1917, Hunt reported a case of juvenile parkinsonism associated with progressive pallidal atrophy. Julius Hallervorden reported a family of five affected sisters in 1922 and confirmed the presence of high levels of brain iron in globus pallidus and substantia nigra pars reticulata histopathologically(6). Around the same time, a German neuropathologist, Hugo Spatz (1888-1969), classified brain regions into 4 groups according to staining intensity of iron and observed more intense staining in globus pallidus and substantia nigra(7). This research lead to the origin of the eponym Hallervorden–Spatz syndrome (HSS), which was used for decades to describe all patients with a pathologic or radiological evidence of high iron in basal ganglia, irrespective of the clinical phenotype(8). This category possibly comprised of several distinct disorders, but recognition of clinical phenotypes and discovery of accompanying genes lead to reclassification of this syndrome. Because of their involvement in examining brains from institutionalized disabled children and adults during the World War II (Third Reich Nazi euthanasia program), both Hallervorden and Spatz were discredited, and use of the disease label has been discontinued.

Over the next few years, new cases were reported, and variants were recognised. In 1952, Franz Seitelberger described the early-onset form, which was later labelled as ‘‘infantile neuroaxonal dystrophy’’ (INAD) by Cowen and Olmstead, differentiating them from classical Hallervorden–Spatz syndrome(9). In 2001, Taylor et al mapped the gene for a specific subtype of HSS, which comprised of extrapyramidal symptoms, pigmentary retinopathy with autosomal recessive inheritance in a large consanguineous family(10). Mutations in this gene encoded pantothenate kinase 2 (*PANK2*) on chromosome 20p13, which was later identified in additional families with a similar phenotype. However, still in some of the families diagnosed with this HSS subtype, *PANK2* mutations were not found. Following the discovery of *PANK2* gene, this HSS subtype was later designated as pantothenate kinase-associated neurodegeneration (PKAN)(11). Majority of cases reported in the literature as “Hallervorden-Spatz syndrome” were probably PKAN, but it also included other forms of NBIA. In 2002, a new disease nomenclature was established and a common term “Neurodegeneration with Brain Iron Accumulation (NBIA)” was proposed. The defect in the causative gene or protein is referenced as part of the name (e.g., pantothenate kinase) and then linked to the common term “-associated neurodegeneration,” (e.g., pantothenate kinase-associated neurodegeneration) to link these disorders to their etiology(12).

NBIA describes a clinically and genetically heterogeneous group of progressive extrapyramidal disorders associated with radiological evidence of abnormal brain iron deposition, particularly in basal ganglia. Patients who were previously diagnosed with Hallervorden-Spatz syndrome fall into this category, including those now recognised to have PKAN. In addition to PKAN, it also includes nine other genetic forms (eight

variants are autosomal recessive, one autosomal dominant and one X-linked dominant).

Pathogenesis:

With the discovery of various genes associated with NBIA syndromes, our knowledge and understanding about the pathogenesis of the disease has improved. The role of iron in the pathogenesis of disease is not clear, as is the mechanism/s by which iron accumulates. Moreover, it is also uncertain whether iron deposition has the primary role in the pathogenesis of NBIA or is just an epiphenomenon of widespread neurodegeneration(2). According to the mutant genes and encoding proteins, pathophysiology of NBIAs have been divided into those diseases caused by abnormal iron metabolism, reduced CoA synthesis, impaired lipid metabolism, mitochondrial dysfunction, and defective autophagy(2,13).

a) Abnormal Iron Metabolism and NBIA (Aceruloplasminemia and Neuroferritinopathy)

Iron is integral for the metabolism of mammalian cells in the brain. It is an essential factor for many processes such as synthesis of myelin, DNA synthesis and repair, energy production, oxidative phosphorylation, and phospholipid metabolism(14). The exact mechanism by which iron uptake occurs in the brain is uncertain, but it is possibly mediated by divalent metal transporter 1 (DMT1), transferrin receptor (TFRC), and ferroportin (FPN1). Majority of brain iron is transported in the interstitial fluid, which is bound to transferrin. For this process, iron must be oxidised to its ferric form, mediated by ceruloplasmin (CP) which is linked to glycophosphatidylinositol (GPI) in the astrocytic membrane(14). The cytoplasmic

iron (taken up from endosomes by DMT1) is either stored by ferritin heavy and light chain complex, or transported to mitochondria by mitoferrin. Among all NBIA subtypes, only two genes encoding for proteins involved in iron homeostasis have been identified to cause NBIA: ceruloplasmin *(CP)* gene for aceruloplasminemia and ferritin light chain *(FTL1)* gene for neuroferritinopathy(15,16).

Ceruloplasmin (CP) is a copper ferroxidase containing majority (95%) of plasma copper, and facilitates iron export from cells mediated by ferroportin. It also catalyses the oxidation of ferrous iron to ferric form to be able to bind to transferrin(13). Mutations in *CP* gene lead to internalisation and degradation of ferroportin and defective iron export, which result in increased astrocytic iron levels and resultant oxidative stress(17). *FTL1* mutations responsible for neuroferritinopathy, result in a reduction in the iron storage capacity of ferritin, and formation of abnormal intra-cytoplasmic and intra-nuclear ferritin inclusion bodies in the glia and neurons(2). Pathogenic mutations also lead to loss of intact proteinaceous shell of spherical ferritin, which protects the sequestered iron. As a result, iron gets leaked out from ferritin and results in oxidative stress(18).

b) Abnormal brain lipid metabolism and NBIA (PLAN, FAHN and MPAN)

The *PLA2G6* gene, the mutations in which cause PLAN, encodes calcium-independent phospholipase A2 enzyme. This enzyme catalyses the conversion of glycero and sphingophospholipids into free fatty acids, and plays a crucial role in cell membrane phospholipid homeostasis. Loss of function mutations lead to increased levels of acyl CoA and phospholipids, and reduction of free fatty acids(19). This

disrupted phospholipid homeostasis alters the lipid content of plasma membrane or endosomes, leading to alterations in membrane permeability and fluidity(20).

Fatty acid hydroxylase-associated neurodegeneration (FAHN) is associated with mutations in the *FA2H* gene, which encodes for enzyme fatty acid 2-hydroxylase. It is responsible for hydroxylation of fatty acids in sphingolipids, which are essential components of myelin sheaths(13). These mutations lead to an altered profile of hydroxylated sphingolipids, which can have a secondary impact on structural properties of lipid membranes. Mutations in *C19orf12* are associated with mitochondrial membrane protein-associated neurodegeneration (MPAN). It encodes for a small transmembrane protein that is localised to mitochondria and endoplasmic reticulum (ER), the function of which is not known(2). Studies have shown that *C19orf12* is co-regulated with other genes associated with fatty acid biogenesis, and branched-chain amino acid degradation, indicating a link with lipid and CoA metabolism, and mitochondria(21).

c) Abnormal CoA synthesis, metabolism and NBIA (PKAN and CoPAN)

CoA is an essential cofactor for multiple metabolic pathways in humans as well as other living organisms such as microsomal fatty acid synthesis and oxidation, cholesterol and sphingolipids synthesis, citric acid cycle, post-translational modifications, and membrane trafficking(22). CoA biosynthesis occurs largely in cytosol and it consists of five enzymatic steps, which utilises pantothenate, cysteine, and ATP(2). Pantothenate kinase 2 (PANK2) catalyses the first and the rate-limiting step in CoA synthesis, which phosphorylates pantothenate into 4'-phosphopantothenate. Amongst all pantothenate kinase (PANK1 to 4), PANK2 is the

only one which localises to mitochondria and nucleus(23). So, *PANK2* mutations, associated with PKAN, lead to decreased CoA synthesis, and resultant multiple metabolic defects and subsequent mitochondrial dysfunction(2). Multiple mechanisms appear to be involved in the pathogenesis of PKAN such as mitochondrial dysfunction, altered lipid metabolism, and oxidative stress.

CoA synthase (COASY) is a bifunctional enzyme, which catalyses the last two steps in CoA synthesis by 4'-PP adenyltransferase (PPAT) and dephospho-CoA kinase (DPCK) respectively(13). CoA synthase protein-associated neurodegeneration (CoPAN), which is caused by loss of function mutations in the *COASY* gene, leads to decreased total CoA and acetyl CoA levels(24). Acetyl-CoA plays a role in autophagy regulation, so reduced levels of acetyl-CoA secondary to these mutations lead to induction of autophagy(2).

d) Defective autophagy process and NBIA (BPAN and Kufor-Rakeb syndrome)

Two major degradation systems responsible for cellular recycling in eukaryotes are proteasome and lysosome. The lysosomal system contains three different types of autophagy mechanisms: macroautophagy, microautophagy, and chaperone induced autophagy(25). Autophagy induction can be secondary to various factors such as cellular stress due to amino acid or growth factor limitation, protein aggregates, damaged DNA, hypoxia, or reactive oxygen species (ROS)(26). The autophagic dysregulation has been observed in some of the common neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Huntington's disease, along with the accumulation of misfolded protein suggesting a link between the two(2).

In beta-propeller protein-associated neurodegeneration (BPAN), mutations in *WDR45* gene which encode a β-propeller scaffolding protein, is involved in the pathway of autophagy. This protein is a member of the WD40 repeat protein family, a key component in various essential biologic processes such as signal transduction, cell cycle regulation, and apoptosis(2). Mutations result in a decreased expression of WDR45 protein, which is associated with accumulation of aberrant autophagic structures, and resultant disruption of maturation of autophagosome.

Kufor-Rakeb syndrome (KRS) is caused by mutations in *ATP13A2* that encode for a 5 P-type ATPase, located in lysosome(26). This ATPase transports cations and other substrates. The mutant protein ATP13A2 is retained in the ER and leads to ER stress. Mutations lead to severe disturbances of lysosomal function, with reduced autophagosome clearance and impaired substrate degradation(27). Furthermore, increased number of lysosomal vesicles with fragmented mitochondria and undigested material accumulate, leading to increased generation of mitochondrially derived ROS(2).

1) Pantothenate Kinase-Associated Neurodegeneration (PKAN)

It is the most common form of NBIA and it accounts for approximately 40-50% of all NBIA cases(12). It is an autosomal recessive disease caused by mutations in the *PANK2* gene at chromosome 20p13-p12.3. It encodes for mitochondrial enzyme pantothenate kinase 2, which is the most important step in CoA synthesis(1). Most mutations are missense (detected in all seven exons), but deletions, duplications, as well as splice site mutations have also been described. The two most frequent

mutations are 1231-G>A and 1253-C>T(12). It usually presents from childhood to mid-adulthood. PKAN has heterogeneous clinical presentations and is divided into two types based on age at onset, rate of disease progression, and presenting signs and symptoms: 1) Classic PKAN and, 2) Atypical PKAN.

Classic PKAN

It is characterised by an early age of onset at around 3-4 years of age and rapid progression. Approximately 90% of classic PKAN cases present before the age of 6(8). It is caused by total loss of function of the mutant protein pantothenate kinase 2. Patients usually present with gait and postural difficulty due to dystonia of lower extremities. As the disease progresses, dystonia becomes multifocal or generalised involving axial musculature with characteristic dystonic opisthotonus or arching of back. Predominant oromandibular dystonia is the hallmark feature, particularly jaw opening dystonia(28). It can be associated with a peculiar geste "antagonsite" with the patient touching his/her chin with both hands clenched into a fist with elbows flexed, called as "mantis sign" because it resembles a praying mantis(29). It can be associated with other extrapyramidal manifestations like parkinsonism or chorea as well as behavioural changes, mild intellectual disability, cognitive decline, and pyramidal tract signs like spasticity, hyperreflexia and bilateral extensor plantar response. Pigmentary retinopathy with abnormal electroretinogram is a common feature of classic PKAN, seen in approximately 40-60% of cases(30). Oculomotor abnormalities such as impaired saccadic pursuits, hypometric or slow vertical saccades, square wave jerks, poor convergence or vertical supranuclear gaze palsy can also occur(30). Seizures are rare in PKAN. Acanthocytes can be seen on peripheral blood smears. Majority of affected children usually lose ambulation and become wheelchair-bound

10-15 years after the disease onset(31). Patients experience episodic rapid functional decline with longer intermittent periods of relative quiescence. Speech and swallowing are affected as the disease progresses. Death is usually secondary to aspiration pneumonia, cardiovascular complications, nutritional compromise, and rarely status dystonicus(1).

Atypical PKAN

It usually presents in the second or third decade of life (rarely in 6^{th}-7^{th} decade). The atypical form has less severe extrapyramidal features with a slower rate of progression compared with the classical form. It results from partial loss of function of mutant protein pantothenate kinase 2. Patients usually present with neuropsychiatric manifestations, dystonia or parkinsonism. Dystonia is usually focal limb or cranial dystonia at onset, followed by subsequent generalisation. Parkinsonism can be levodopa responsive(12,32). Although uncommon, late-onset PKAN cases presenting with isolated cranial dystonia such (e.g. blepharospasm) as the only manifestation have been described(33). Neuropsychiatric symptoms such as emotional lability, impulsivity, depression, and obsessive-compulsive disorder are more common in atypical PKAN than in the classical form(8,34). Many of such cases are difficult to diagnose initially due to atypical presentations. Speech disturbances can also be present early in the disease course, which include dysarthria, pallilalia, hypophonia or frank stuttering. The extrapyramidal symptoms in atypical PKAN are age dependent, with earlier the age of onset, more severe the dystonia. In contrast, patients having a later age at onset (in their 20s or 30s) have more prominent parkinsonian features like bradykinesia, rigidity, freezing of gait and postural instability(35). Action induced dystonia with dystonic tremors are frequent in atypical PKAN. Other extrapyramidal

manifestations include chorea, motor and/or vocal tics, and isolated freezing of gait(28). Cognitive decline and oculomotor abnormalities are common, but retinitis pigmentosa is rare(30). Eventually, patients develop gait difficulty secondary to dystonia, spasticity and rigidity. Majority of individuals with atypical PKAN have normal lifespan due to slower rate of disease progression.

MRI brain typically shows T2-weighted anteromedial hyperintensity surrounded by hypointensity in bilateral globus pallidus interna, termed as classical "eye of the tiger" sign(12,36). This finding is not absolutely sensitive or specific for PKAN. Cases with proven genetic mutations have been described without this sign. In contrast, "eye of the tiger" sign can be seen in other disorders like MPAN, CoPAN, neuroferritinopathy, multiple system atrophy, corticobasal degeneration, and carbon monoxide poisoning survivors(37,38). The central hyperintense area is due to primary tissue insult leading to edema or necrosis, while surrounding hypointensity is indicative of iron deposition, probably as a secondary effect. In pre or early symptomatic patients, these hyperintense lesions predominate(8). Hypointense lesions gradually appear and dominate with the disease progression, and central hyperintensity gradually decrease and eventually disappear(39). Substantia nigra iron deposition, if present, is milder as compared to globus pallidus, and it appears later in the disease course(35). Optic nerves and cerebellum are spared.

2) Phospholipase A2-Associated Neurodegeneration (PLAN)

Phospholipase A2-Associated Neurodegeneration (PLAN) is the second core NBIA syndrome, caused by recessive mutations in *PLA2G6* gene on chromosome 22q. It encodes for group VIA calcium independent phospholipase A2, which hydrolyses the phospholipids and plays a crucial role in maintaining the homeostasis of cell

membrane phospholipids(40). PLAN has three distinct but partially overlapping clinical phenotypes depending upon the age at onset: Infantile neuroaxonal dystrophy (INAD), atypical neuroaxonal dystrophy (aNAD) of childhood and adult-onset dystonia-parkinsonism(41).

Classic infantile neuroaxonal dystrophy (INAD)

It is the most common form of PLAN, accounting for 85% of cases. It presents between 6 months to 3 years of age, with delayed developmental milestones, marked axial hypotonia and gait disturbances (due to ataxia, spasticity, dystonia or rigidity)(42). Early hypotonia and areflexia secondary to peripheral nerve denervation are later replaced by progressive pyramidal dysfunction with spastic tetraparesis and bulbar symptoms as the disease advances(1). Other prominent features are optic atrophy, nystagmus, strabismus, and generalised seizures. Electroencephalography (EEG) shows abnormal fast rhythms and epileptiform discharges. In the pre-genetic era, the diagnosis of INAD was confirmed by characteristic finding of dystrophic axons in peripheral nerve or conjunctival histopathology(42). This phenotype is relentlessly progressive, and most of the children die in the first decade.

Atypical neuroaxonal dystrophy (aNAD)

This childhood-onset PLAN presents between 1 to 6 years of age, initially with cerebellar syndrome (gait ataxia and dysarthria), mild intellectual disability, truncal hypotonia, and optic atrophy. As the disease progresses, spastic tetraparesis, dystonia, cognitive decline, nystagmus, seizures, and neurobehavioral manifestations predominate(28). This PLAN subtype has a more variable clinical presentation and a slower rate of progression.

Adult-onset PLAN (dystonia-parkinsonism)

This subtype was recognised after the discovery of causative *PLA2G6* gene and recent advances in molecular genetic technology. This led to expansion of the phenotypic spectrum of PLAN. Patients usually present in late adolescence or early adulthood with subacute onset of focal or segmental dystonia (blepharospasm, foot dystonia), akinetic-rigid parkinsonism along with pyramidal signs, cognitive decline, psychiatric features, and oculomotor abnormalities(43). Common eye movement abnormalities include slow velocity of saccades, supranuclear gaze palsy, nystagmus and eyelid opening apraxia(44). Autonomic dysfunction (bowel and/or bladder dysfunction) can also be seen. Seizures and cerebellar symptoms are generally not seen(28). The parkinsonism of adult-onset PLAN is levodopa responsive, consistent with Lewy body pathology. But, patients usually develop early motor fluctuations and dyskinesia(43).

Regardless of phenotype, iron accumulation may be absent or very mild in the early stage of disease and it may never appear in INAD and aNAD(45). Iron deposition typically occurs in globus pallidus and substantia nigra. The most common neuroimaging finding in infantile and childhood-onset PLAN is cerebellar hemispheric and vermian atrophy, which precedes the iron deposition(38). Adult-onset PLAN shows iron deposition in globus pallidus and/or substantia nigra but MRI may be completely normal also. Therefore, PLAN should be considered in differential diagnosis of patients presenting with dystonia-parkinsonism even with normal brain MRI(5). Optic atrophy and non-specific white matter changes can also been seen on brain MRI. Pathological studies have shown diffuse alpha-synuclein positive Lewy

body pathology, affecting the basal ganglia and neocortex particularly in late-onset cases and advanced stage of disease(46). Tau pathology is also seen with the accumulation of hyperphosphorylated tau as threads and neurofibrillary tangles. Early-onset cases demonstrate variable decline in cerebellar cortical neurons (granule cells particularly) with significant astrocytosis(46).

3) Mitochondrial Membrane Protein-Associated Neurodegeneration (MPAN)

Mitochondrial Membrane Protein-Associated Neurodegeneration was first described by Hartig *et al* in a Polish cohort of 24 patients(21). It is an autosomal recessive NBIA syndrome caused by mutations in *c19orf12* gene on chromosome 19q12. The most common mutation is an 11-bp deletion which leads to a premature stop codon and truncation of mutant protein.

MPAN typically manifests between 1st to 3rd decade of life. The most common presenting symptoms in early-onset disease are early spastic paraparesis, dystonia (extremities or rarely oromandibular), levodopa unresponsive parkinsonism, optic atrophy, and psychiatric symptoms such as depression, anxiety, emotional lability, inattention and impulsive-compulsive behaviour(47). Additional findings include cognitive decline, dysarthria, dysphagia, and bowel/bladder incontinence. With the disease progression, pyramidal signs are gradually replaced by lower motor neuron signs (areflexia, muscle weakness, and amyotrophy). Electrophysiological testing shows pure motor axonopathy in approximately 40% of cases(47). The disease progresses slowly, and most children survive into adulthood. The adult-onset cases have a more variable presentation with prominent neuropsychiatric symptoms,

dementia, parkinsonism, and gait difficulty. Many late-onset MPAN cases follow an aggressive disease course with death within 5-10 years of onset(48).

MRI brain shows evidence of iron deposition in globus pallidus and substantia nigra in T2 and susceptibility-weighted images (SWI), even in the early stage of the disease. T2-weighted sequences show characteristic hyperintense linear streaking of medial medullary lamina of the globus pallidus(47). This finding helps to differentiate MPAN from other NBIA syndromes. In advanced disease, cortical and cerebellar atrophy can be seen. The common pathological findings include axonal spheroids (both centrally and peripherally), neuronal loss, gliosis, iron deposition, and occasional tau inclusions. The post-mortem studies have shown extensive alpha-synuclein positive Lewy bodies and Lewy neurites in the basal ganglia and neocortex(21,49).

4) Beta-Propeller Protein-Associated Neurodegeneration (BPAN)

Beta-propeller protein-associated neurodegeneration is the only X-linked dominant NBIA amongst all subtypes. It was previously termed as "static encephalopathy with neurodegeneration in adulthood" (SENDA) before the discovery of the causative gene in 2012(50). It is caused by mutations in *WDR45* gene, which encode for a β-propeller protein having a role in autophagy regulation(51). Majority of cases are due to de novo mutations, and some males are also affected with BPAN secondary to somatic mosaicism resulting from post-zygotic mutations(35). The phenotype of males with BPAN is similar to that of females.

BPAN is a biphasic disease with childhood-onset of delayed motor and language milestones, seizures, sleep disorders, wide based ataxic gait, hand stereotypies and pyramidal dysfunction mimicking Rett-like syndrome followed by rapidly progressive onset of dystonia, parkinsonism, and cognitive decline in adulthood (3^{rd} decade)(28). Parkinsonism is responsive to levodopa, with the emergence of early motor fluctuations and dyskinesia complicating the management. Other manifestations include spastic paraparesis, oculomotor abnormalities, frontal release signs, dysautonomia, and dysphagia, which appear as the disease progresses(5).

The characteristic neuroimaging findings consist of iron accumulation in globus pallidus and substantia nigra, seen in T2-weighted and SWI sequences(38). MRI brain can be normal in childhood in BPAN. The hypointensity is more marked in substantia nigra where it appears as a "linear streak". This hypointense streak is surrounded by a characteristic hyperintense "halo" on T1-weighted sequences extending into cerebral peduncles, virtually pathognomonic of BPAN(52). It represents the release of neuromelanin from degenerating nigral neurons. Other imaging findings include cerebellar atrophy, and corpus callosum thinning in the advanced stage(51). Neuropathological findings of BPAN include giant axonal spheroids in substantia nigra, globus pallidus, brainstem, and thalamus, neuronal loss, reactive astrocytes, and tau positive neurofibrillary tangles in the basal ganglia, cortex, and hippocampus(53).

5) Fatty acid hydroxylase-associated neurodegeneration (FAHN)

It is an autosomal recessive form of NBIA, caused by mutations in the *FA2H* gene encoding for the enzyme fatty acid 2-hydroxylase. Previously, *FA2H* mutations were originally known to cause hereditary spastic paraplegia-35 (HSP35) and leukodystrophy, leading to overlapping syndromes(54). Subsequently, the link

between *FA2H* mutations and FAHN was first identified in 2010(55). Fatty acid 2-hydroxylase enzyme leads to production of 2-hydroxylated fatty acids, which incorporate into 2-hydroxyceramide and 2-hydroxydihydroceramide. These ceramide components serve as precursors for the synthesis of lipid molecules essential for myelin synthesis and also plays a role in cell cycle regulation(55,56).

The clinical presentation is identical to INAD variant of PLAN, with childhood-onset of gait impairment, severe ataxia, other cerebellar symptoms, dystonia, spastic quadriparesis, intellectual dysfunction, optic atrophy, and strabismus(5). Seizures appear relatively late in the course of disease. Dysarthria and dysphagia also appear as the disease progresses. Axonal neuropathy is a less consistent feature(57). Majority of patients lose ambulation before the adulthood(55).

MRI brain in FAHN shows T2-weighted hypointense signals in bilateral globus pallidus consistent with iron deposition, cortical and pontocerebellar atrophy, corpus callosum thinning, and bilateral subcortical as well as periventricular confluent white matter hyperintensities(38,58). Iron deposition can also be present to a lesser extent in substantia nigra. FAHN demonstrates clinical as well as radiological overlap with leukodystrophies and HSP35. Pathological studies of human brains in FAHN are not available, but mouse models have shown significant central demyelination and axonal loss, abnormally enlarged axons, and abnormal cerebellar histology. In contrast, unlike in PLAN, pathological changes in peripheral nerves were not observed(59).

6) Coenzyme A synthase protein-associated neurodegeneration (CoPAN)

It is an autosomal recessive NBIA, characterised by homozygous mutations in *COASY* gene. The mutant protein, Co-A synthase, is essential for final two steps in CoA synthesis(24). Till date, it has been only described in two Italian families. It shares phenotypic similarity with classic PKAN(60). It usually presents in the first decade of life with dystonia of extremities with gait impairment, spasticity, and cognitive decline. Later in the disease course, parkinsonism, oromandibular dystonia, axonal neuropathy, obsessive-compulsive behaviour also appear(24). The disease progresses relatively slowly.

The pattern of MRI findings in CoPAN shows hypointensity in bilateral globus pallidus as well as substantia nigra with focal hyperintensity in medial portion of GP (secondary to pallidal calcification), mimicking classical "eye of the tiger" sign(24,61). Isolated involvement of striatum with T2-weighted hyperintensity in bilateral caudate and putamen, and minimally in thalami has also been reported(61). Neuropathological features are similar to PKAN.

7) Neuroferritinopathy (NFT)

Neuroferritinopathy is the only autosomal dominant subtype amongst all NBIA syndromes. It occurs as a result of mutations in the *FTL1* gene on chromosome 19q13.3, which encode for ferritin light chain(16). The mutant protein leads to structural changes in ferritin and disruption the iron carrying capacity of ferritin, which results in brain iron accumulation(1). Majority of mutations are single nucleotide insertions, found in exon 4. Most of the cases have been reported from the Cumbrian region of England due to founder effect, but isolated cases have also been reported from other parts of the World(62).

Unlike other NBIAs, it presents relatively later in mid-adulthood (4^{th} or 5^{th} decade), with chorea (orofacial or generalised), dystonia (orofacial and/or limb; action-induced), parkinsonism, tremor, and tics, consistent with Huntington's disease phenotype(62). Chorea and dystonia have a predilection for involvement of the oromandibular region. Parkinsonism, cognitive decline particularly frontal executive dysfunction, and behavioural disturbances (anxiety, depression and psychosis) appear later in the course of disease. Other clinical features include oculomotor abnormalities (slow saccades, vertical supranuclear gaze palsy, and eyelid opening apraxia), sleep disturbances, and palatal tremor(63). Pyramidal signs and cerebellar ataxia are usually rare or absent.

The diagnosis of neuroferritinopathy can be made by a combination of low serum ferritin levels usually < 20 ug/dL) and characteristic neuroimaging findings. Unlike other NBIAs, MRI brain shows a more diffuse pattern of iron accumulation in caudate, putamen, globus pallidus, thalamus, substantia nigra, red nucleus, and dentate nucleus(38,64). Iron deposition is also evident in the cortical region described as "pencil lining cortex"(65). This sign is not specific for NFT, as it can also be seen in aceruloplasminemia. Also, cystic necrotic changes in bilateral striatum and globus pallidus in T2-weighted images are observed in the late stage of the disease, which differentiate neuroferritinopathy from other NBIAs(38,62). Mild cortical as well as cerebellar atrophy may also be seen. Pathological studies demonstrate ferritin positive inclusions in posterior putamen and cerebellum, and tau and ubiquitin reactive neuroaxonal spheroids(66). Extracerebral pathology in the form of hepatic iron deposition may also been seen(67).

8) Aceruloplasminemia (ACP)

Aceruloplasminemia is a recessively inherited form of NBIA, caused by mutations in *CP* gene on chromosome 3q encoding ceruloplasmin(68). It is a copper-bound ferroxidase, which is required for efflux of cellular iron (mediated by ferroportin) and oxidation of ferrous to ferric iron. Therefore, *CP* gene mutations lead to cellular iron overload, ultimately resulting in iron deposition in brain, pancreas and retina, and subsequent neurodegeneration(69).

Like in neuroferritinopathy, the onset of symptoms usually occurs in 4^{th} or 5^{th} decade of life. Patients present with a classical triad of diabetes mellitus, retinal macular degeneration, and microcytic anaemia, followed by neurological symptoms relatively later in the disease. Most common presenting neurological symptoms are cerebellar ataxia, and craniofacial dyskinesia (blepharospasm, oromandibular and/or cervical dystonia, facial grimacing, orofacial chorea)(70). Other common neurological symptoms are parkinsonism, tremor, cognitive decline, and psychiatric symptoms(28). Patients with single heterozygous variants present with an incomplete clinical phenotype, which poses diagnostic difficulty(71).

Laboratory investigations in aceruloplasminemia show microcytic anaemia, low or absent serum ceruloplasmin, low serum iron and copper levels, and higher serum ferritin (2-40 fold)(70). Urinary copper levels are normal. MRI brain shows more widespread iron deposition in striatum, pallidum, thalami, substantia nigra, red nuclei, dentate nuclei, and superior and inferior colliculi. Like in NFT, iron deposition can also occur in cerebral cortex, appearing as characteristic “cortical pencil lining”(38,72). Patients with heterozygous mutations can have pallidal hypointensity,

cerebellar atrophy, and white matter hyperintensities(73). FDG-PET shows hypometabolism in basal ganglia and thalamus. Hepatic iron accumulation can also been seen in MRI(35). Common neuropathologic findings include severe neuronal loss in putamen, globus pallidus, substantia nigra and dentate nucleus, and gliosis(28).

9) Kufor-Rakeb Syndrome (KRS)

It is a rare autosomal recessive form of NBIA, which was described first in a Jordanian family in 1994(74). It is also referred to as PARK9-associated parkinsonism. The causative gene mutation was identified later in *ATP13A2* gene on chromosome 1p in Chile in 2006(75). Pathogenic mutations encode lysosomal 5 P-type ATPase, leading to lysosomal dysfunction. Majority of mutations are homozygous, but compound heterozygous mutations have also been reported. Moreover, heterozygous mutations have been found in patients with Parkinson's disease suggesting that heterozygous carriers are at increased risk of developing PD(76).

The disease usually manifests in adolescents before age of 20 years, with a variable combination of parkinsonism, dystonia, spasticity, oculomotor abnormalities, cognitive impairment (dementia and hallucinations), and neuropsychiatric symptoms. Parkinsonism is usually levodopa responsive, but patients are at risk of development of early motor fluctuations and dyskinesias(74). Dystonia can manifest late in some of the cases. The common eye movement abnormalities include slowing of vertical and horizontal saccades, vertical supranuclear gaze palsy, and saccadic pursuits(77). Other features such as characteristic facial-faucial-finger polyminimyoclonus, oculogyric crises, olfactory dysfunction and dysautonomia may be present(76).

MRI brain findings of Kufor-Rakeb syndrome include diffuse cortical, subcortical, cerebellar, and brainstem atrophy. Basal ganglia iron deposition (striatum and globus pallidus) is not evident early in the course of disease, and it may not be present in all patients(38,78). Dopamine transporter imaging shows bilateral symmetrical reduction of striatal tracer uptake, consistent with presynaptic dysfunction(79). The post-mortem human brain studies of patients with Kufor-Rakeb syndrome are lacking, but sural nerve biopsy has shown acute axonal degeneration with mild chronic inflammation, and cytoplasmic inclusion bodies in Schwann cells, epineural and perineural cells(80).

10) Woodhouse-Sakati Syndrome (WSS)

Originally described by Woodhouse and Sakati in six Saudi Arabian patients in 1983, Woodhouse-Sakati syndrome is a rare autosomal recessive neuroendocrine disorder characterized by facial dysmorphism (long triangular face, hypertelorism, prominent nasal bridge), alopecia, hypogonadism, diabetes mellitus, intellectual disability, sensorineural deafness, extrapyramidal features, and ECG abnormalities(81). Later on, cases have also been reported from North-East Europe, India, Turkey, and Israel. The pathogenic mutation is in the *DCAF17* (*C2orf37*) gene on chromosome 2q31.1, which encodes DDB1- and CUL4-associated factor 17, a nucleolar protein that may function as a substrate receptor for CUL4-DDB1 E ubiquitin-protein ligase complex(82,83). This protein is highly expressed in brain, liver, skin, and seminiferous tubules but not in pancreatic islet cells(82). Till now, eleven different

mutation variants have been reported. The most common mutation is c.436delC:p.L146fs in exon 4 of the *DCAF17* gene.

Childhood-onset alopecia, hypogonadism, and multiple endocrinopathies are hallmark features of WSS, which are present in all reported cases. Hypogonadism has a mixed origin, with women having a hypergonadotropic hypogonadism and men tend to have hypogonadotropic hypogonadism(84). The disease follows a progressive course with the appearance of neurologic and endocrine manifestations during adolescence and early adulthood. The common neurological features include extrapyramidal signs, mild intellectual disability, dysarthria, dysphagia, spastic paraparesis, sensorineural deafness, seizures, and polyneuropathy. The extrapyramidal features consist of dystonia (focal, segmental, or generalized), chorea, and less commonly tremor and ataxia which progress to cause gait difficulties and immobility later(85). The other endocrine manifestations include diabetes mellitus and hypothyroidism which affect 40-50% of individuals and low serum insulin-like growth factor 1(IGF-1) level, which is seen in virtually all patients(86). Based on clinical manifestations and disability, two phenotypes of the disease were proposed. Type-I was defined when neurological manifestations were moderate to severe, and caused severe disability and significant impairment in quality of life. While, absent or mild neurological features that don't affect the activities of daily living defined type-II WSS(84).

Characteristic MRI findings include a partially empty sella, a small pituitary gland, iron deposition in globus pallidus and later in substantia nigra as well as red nucleus and non-enhancing periventricular and frontoparietal dominant subcortical white

matter hyperintensities on T2-weighted and FLAIR images(87). The presence of these classical imaging findings distinguishes WSS from other subtypes of NBIA.

Clinical Evaluation and Diagnosis:

The diagnosis of NBIA relies on a combination of 1) characteristic clinical phenotypes associated with various NBIA subtypes, 2) the neuroimaging evidence of iron accumulation together with other characteristic imaging findings, and 3) the genetic confirmation of a specific subtype of NBIA by targeted gene panel or whole-exome sequencing. In the pre-genetic and pre-imaging era, the diagnosis of NBIA could only be made during autopsy(38). A detailed clinical history and thorough neurological as well as systemic examination are key to the diagnostic evaluation. The clinical characteristics such as age at onset, birth and developmental history, family history, extrapyramidal as well as other neurological and non-neurological manifestations are extremely helpful in the evaluation of NBIA syndromes. Although clinical features are often non-specific, the presence of certain clinical findings such as oromandibular dystonia, cerebellar ataxia, facial dysmorphism, seizures, oculomotor abnormalities (particularly strabismus and nystagmus), endocrine abnormalities, optic atrophy, pigmentary retinal degeneration, and peripheral neuropathy helps in suspecting a specific subtype of NBIA (Table 2). For example, optic atrophy is seen in some of the specific NBIA subtypes like PLAN, MPAN, FAHN, and PKAN, while retinitis pigmentosa is characteristically seen in PKAN and CoPAN. These clinical characteristics are supplemented by specific laboratory findings, MRI brain, and electrophysiological testing in diagnosing the specific NBIA subtype.

Common laboratory investigations that should be done in all clinically suspected cases of NBIA include hemogram for anaemia, serum and urinary copper, and serum ceruloplasmin. Other investigations such as serum iron and ferritin levels, blood sugars as well as glycosylated haemoglobin, peripheral smear for acanthocytes and hormonal profile for hypogonadism should be performed in appropriate settings. For example, serum ferritin is abnormally low in neuroferritinopathy, while it is higher in aceruloplasminemia. Serum hormonal levels are altered in patients with Woodhouse-Sakati syndrome, and blood sugar levels are raised in aceruloplasminemia as well as Woodhouse-Sakati syndrome.

Electroencephalogram can be helpful in patients presenting with active seizures or a history of seizures in the past as in PLAN, BPAN, FAHN, and Woodhouse-Sakati syndrome. The nerve conduction study (NCS) and electromyography (EMG) should be performed in patients with suspected peripheral neuropathy.

Table 2: Clinical clues for the diagnosis of specific NBIA subtype/s	
Oromandibular dystonia	- PKAN (Classic and atypical) (Jaw opening dystonia in particular) - Neuroferritinopathy - Aceruloplasminemia - CoPAN - MPAN (rare)
Cerebellar ataxia	- PLAN (INAD and aNAD variants) - Aceruloplasminemia - FAHN - BPAN
Seizures	- PLAN (INAD and aNAD variants) - BPAN - FAHN - Woodhouse-Sakati syndrome
Levodopa responsive parkinsonism	- Adult onset PLAN - Kufor-Rakeb syndrome

	- Atypical PKAN - BPAN
Peripheral neuropathy	- PLAN (INAD and aNAD variants) - MPAN (pure motor axonopathy) - CoPAN - FAHN (less common)
Facial dysmorphism, alopecia, hypogonadism, sensorineural deafness, ECG abnormalities	- Woodhouse-Sakati syndrome
Optic atrophy, strabismus, and nystagmus	- PLAN (INAD and aNAD variants) - MPAN (strabismus, nystagmus less common) - FAHN - PKAN (less common)
Retinal degeneration	- PKAN (Classic > atypical), CoPAN, PLAN (Pigmentary degeneration) - Aceruloplasminemia (Macular retinal degeneration)
Diabetes mellitus	- Aceruloplasminemia - Woodhouse-Sakati syndrome

MRI brain is an essential component of diagnostic evaluation of patients with NBIA. The common neuroimaging finding in all NBIA cases is the iron accumulation symmetrically in the bilateral basal ganglia (particularly globus pallidus and substantia nigra) with or without in other basal ganglia nuclei, and nuclei of brainstem and cerebellum (red nuclei and dentate nuclei). It particularly helps in differentiating the subtypes of NBIA to a certain extent and guiding the clinician towards the appropriate genetic testing. These iron-rich areas appear hypointense in T2-weighted images, and isointense in T1-weighted sequences(38,72). Calcium also appears hypointense on T2-weighted and isointense in T1-weighted sequences, but both can be differentiated by computed tomography (CT) scan of the brain, in which calcium appears hyperdense as compared to surrounding brain parenchyma and iron appears

isodense. Moreover, iron deposition appears hypointense on both diffusion-weighted (DWI) and apparent diffusion coefficient (ADC) sequences. The addition of iron sensitive sequences such as gradient echo T2*-weighted sequences (GRE), and susceptibility-weighted images (SWI) accentuate this hypointensity (visible as "blooming")(64). This along with using MRI scans with higher magnetic field strengths (3T and 7T MRI) increase the sensitivity of detecting iron on these sequences. Iron can also be quantitated by T2 mapping as well as quantitative susceptibility mapping (QSM) sequences(88). The MRI finding of basal ganglia iron deposition is usually non-specific, but the presence of other characteristic imaging findings helps to differentiate amongst various subtypes of NBIA (Table 3). Besides, iron deposition in basal ganglia can also be observed in some of the neurodegenerative diseases such as multiple system atrophy, corticobasal degeneration, Friedrich's ataxia, Alzheimer's disease, Parkinson's disease, progressive supranuclear palsy, multiple sclerosis, Huntington's disease, and dentatorubropallidolyusian atrophy (DRPLA)(89–91). Newer imaging techniques, such as magnetic resonance spectroscopy can also be helpful, but their utility has not been established yet(31).

NBIA subtype	Iron deposition	Other characteristic finding/s
PKAN	Globus pallidus mainly ("Eye-of-the-tiger" sign), substantia nigra (mild)	Occasional calcification
PLAN	Globus pallidus, substantia nigra (Can be absent in adult onset variant)	Cerebellar atrophy, optic nerve atrophy, mild white matter changes
MPAN	Globus pallidus, substantia nigra	Hyperintense "streaking" of medial medullary lamina Cortical and cerebellar atrophy,
BPAN	Substantia nigra > globus pallidus	T1 hyperintense halo surrounding a hypointense

		streak in substantia nigra Corpus callosum thinning Cerebellar atrophy (rare)
FAHN	Globus pallidus > substantia nigra	Corpus callosum thinning Confluent white matter hyperintensities Pontocerebellar atrophy
CoPAN	Globus pallidus ("Eye of the tiger" sign), substantia nigra	Bilateral T2-weighted striatal hyperintensity (occasionally)
Neuroferritinopathy	Globus pallidus, striatum, thalamus, red nucleus, substantia nigra, dentate nucleus, cortex	Cystic necrotic pallidal changes on T2-weighted images "Pencil lining cortex" "Eye-of-the-tiger" sign (occasionally)
Aceruloplasminemia	Globus pallidus, striatum, thalamus, red nucleus, substantia nigra, dentate nucleus, colliculi, cortex	"Pencil lining cortex" Cerebellar atrophy and white matter changes (heterozygous cases)
Kufor-Rakeb syndrome	Globus pallidus, striatum and substantia nigra (Can be absent)	Global cerebral, cerebellar and brainstem atrophy
Woodhouse-Sakati syndrome	Globus pallidus, substantia nigra	Partially empty sella Confluent white matter hypeintensities

Table 3: Neuroimaging findings of different NBIA subtypes

Because of non-specificity of clinical features and findings of brain iron accumulation on MRI, it is difficult at times to lead to the diagnosis of a specific NBIA subtype and order a single gene testing. However, with advancements in genetic technology, testing using a panel including known NBIA genes or whole-exome sequencing helps in confirming the diagnosis of a specific subtype. Though genetic testing confirms the diagnosis, not all NBIA cases have above described mutations and some unknown NBIAs also exist(28). Moreover, the interpretation of a mutation in one of the known NBIA genes in asymptomatic cases or cases with minimal symptoms becomes

difficult, and not all NBIA related gene mutations are pathogenic. Therefore, caution is required while interpreting these genetic results.

Treatment:

Despite multiple studies undergoing for disease specific treatments, the treatment of NBIA has largely been symptomatic till date and often unsatisfactory. The ideal recommended approach to manage a patient with NBIA is a combination of medical, surgical, and nursing as well as supportive care/physiotherapy(28).

Medical Management

Symptomatic treatment is the mainstay of management. Dystonia can be managed with standard medications such as trihexyphenidyl, levodopa, baclofen, and benzodiazepines and spasticity can be treated with oral or intrathecal baclofen and benzodiazepines. But, the response to these medications is variable and not long-lasting(92). Botulinum toxin injection therapy is useful for cases with drug-resistant dystonia, particularly oromandibular and focal hand dystonia which impair one's ability to speak, eat or write. Parkinsonism in patients with NBIA should be treated with levodopa. In typical PKAN, parkinsonism is levodopa non-responsive; but patients with adult-onset PLAN, Kufor-Rakeb syndrome, MPAN, BPAN, and sometimes atypical PKAN respond to levodopa, but they usually develop early motor fluctuations and dyskinesia as well as hallucinations(28). Dopamine agonists should be avoided in NBIA patients with parkinsonism, due to increased risk of worsening of

neuropsychiatric and cognitive symptoms. Seizures can be treated with standard antiepileptic drugs.

For patients with severe spasticity or dystonia who are refractory to oral medications or those who are unable to swallow medications because of dysphagia, intrathecal baclofen pump can be used. However, the side effects associated with it such as catheter dislodgement, catheter infection, programming errors, and need to periodically refill the pump make this treatment option a last resort after all treatment modalities have failed(31).

As the role of iron deposition in the pathogenesis of majority of NBIA subtypes is uncertain, iron chelation holds its place as an investigational therapy till date. Deferiprone, an oral iron chelator, was tested in a preliminary study in nine patients with PKAN. It was given at a dose of 25 mg/kg/day over 6 months. It demonstrated a significant reduction in the iron content of globus pallidus (15-60%), but there was no clinical improvement noted(93). It was well tolerated without any serious adverse events, with most common adverse effects being nausea and gastritis. On the other hand, neuroferritinopathy and aceruloplasminemia are only NBIAs, gene defects of which are directly related to iron metabolism. Considering this fact, several iron-chelating agents have been tried in aceruloplaminemia with variable results. Deferasirox in patients with aceruloplasminemia has shown a reduction in the hepatic iron content, but there was no reduction of brain iron overload or clinical improvement(94). While both deferiprone and deferasirox have been demonstrated to delay the onset of neurological symptoms in isolated case reports of aceruloplasminemia(95,96). Other agents that have been found useful in

aceruloplasminemia include zinc sulphate and antioxidants(97). The data on the efficacy of iron chelators in other NBIA subtypes are sparse and not encouraging.

The most challenging aspect in the medical management of patients with NBIA is the development of status dystonicus or dystonic storm. It is particularly common in children with classic PKAN(1). This exacerbation can occur with or without any precipitants, and can be life-threatening and is generally refractory to medical treatment.

Surgical management

The data in the literature regarding the effectiveness of deep brain stimulation (DBS) in NBIA is insufficient. The studies are mostly limited to isolated case reports, small case series, and confined to patients with PKAN. Bilateral globus pallidus interna (GPi) DBS in patients with PKAN has shown improvement in dystonia rating scale(98), although long term follow-up studies are required in future to establish its long term effectiveness. It is life-saving particularly for patients with status dystonicus.

Supportive care

Good supportive care utilising a multidisciplinary approach including physiotherapy, speech and language therapy, occupational therapy, psychology support, and nursing care remains the mainstay of supportive management of patients with NBIA and can delay the complications of the disease(28). In the advanced stage of disease, gastrostomy tube placement can improve the nutritional status of patients with dysphagia.

AIMS AND OBJECTIVES

Aims and Objectives

1. To study the genetic pattern in patients with a clinico-radiological diagnosis of NBIA

2. To correlate the clinical profile and imaging characteristics with the genetic results obtained by targeted gene sequencing and establish patterns of genotype-phenotype correlations.

MATERIALS AND METHODS

Materials and Methods

Background:

The current study is a case series of patients with a clinico-radiological diagnosis of NBIA evaluated at SCTIMST. Patients who had already been evaluated in SCTIMST previously and had a clinico-radiological diagnosis of NBIA and were under follow up were also enrolled in the study. In addition, patients who were evaluated and suspected with NBIA during the study period were also enrolled in the study. The study procedures were explained and written informed consent were taken. Their clinical and imaging characteristics were noted and their blood samples were sent for genetic testing to the genetic laboratory.

Study Design: Case series

Sample size: Total 18 patients with a clinico-radiological diagnosis of NBIA were included

Inclusion Criteria:

Patients with clinical symptomatology of an extra pyramidal syndrome, spasticity, seizures and neuropsychiatric abnormalities in varying combinations with a MRI evidence of iron deposition in the brain

Exclusion Criteria:

1. Patients without a progressive illness and a static course
2. History of definite perinatal insult
3. History of traumatic brain injury
4. History of intracranial bleed or step wise progression of symptoms

Study Methodology :

Patients who fulfilled inclusion and exclusion criteria were included in the study. The patients underwent a standard clinical interview and neurological examination to confirm the clinical diagnosis and eligibility to participate in the study. The demographic details, detailed clinical history including parental consanguinity, family history, and treatment response were documented. General systemic and neurological examination were carried out and documented. Video recordings of patients who were previously assessed and clinically suspected as NBIA were evaluated for identifying the clinical phenomenology. The appropriate blood investigations (Serum Ceruloplasmin, ferritin, creatine kinase, 24 urine copper etc.) to rule out differential diagnoses of NBIA were done. MRI Brain (1.5 Tesla) were done with the following sequences : T1 axial, T2 axial high resolution, 3D Fluid Attenuation and Inversion Recovery (FLAIR), Susceptibility Weighted Imaging (SWI) and Diffusion Weighted Imaging (DWI), and were analyzed by an experienced radiologist. The regions and patterns of iron accumulation in the brain were noted. Other ancillary investigations such as fundus examination, slit lamp examination for Kayser-Fleischer (KF) ring, neuropsychological assessment, electroencephalogram (EEG), nerve conduction study (NCS) were done on an individual basis according to the clinical presentation of the patient. Genetic testing were done for the ten known genes of NBIA (namely, *PANK2, PLA2G6, C19orf12, WDR45, FA2H, ATP13A2, DCAF17, COASY, FTL and CP*) and results by the target gene sequencing were recorded.

Test Methodology: Genetic testing with targeted gene sequencing was done with the following test methodology: Ten ml of blood was collected from the patient and transferred to the commercial laboratory on the same day. Targeted gene sequencing

with selective capture and sequencing of selected set of genes that have suspected association with disease was done. DNA extracted from blood was used to perform targeted gene capture using a customized capture kit. The libraries were sequenced to mean >80-100X coverage on Illumina sequencing platform. The sequences obtained were aligned to human reference genome (GRCh37/hg19) using BWA program and analyzed using Picard and GATK version 3.6 to identify variants relevant to the clinical indication. The GATK best practices framework were followed for identification of variants in the sample. Gene annotation of the variants were performed using VEP program against the Ensembl release 87 human gene model. Clinically relevant mutations were annotated using published variants in literature and a set of diseases databases - ClinVar, OMIM, GWAS, HGMD and SwissVar. Common variants were filtered based on allele frequency in 1000Genome Phase 3, ExAC, EVS, dbSNP147, 1000 Japanese Genome and our internal Indian population database. Non-synonymous variants effect was calculated using multiple algorithms such as PolyPhen-2, SIFT, Mutation Taster2, Mutation Assessor, and LRT. Only non-synonymous and splice site variants found in the NBIA panel genes were used for clinical interpretation.

Statistical methods:

The descriptive data is presented in graphs and tables. The statistical analyses of all clinical datasets were performed by using the Medical Statistics Software SPSS, Version 20.0 (IBM, Armonk, New York). Parametric data is presented as mean and standard deviation.

Ethical considerations

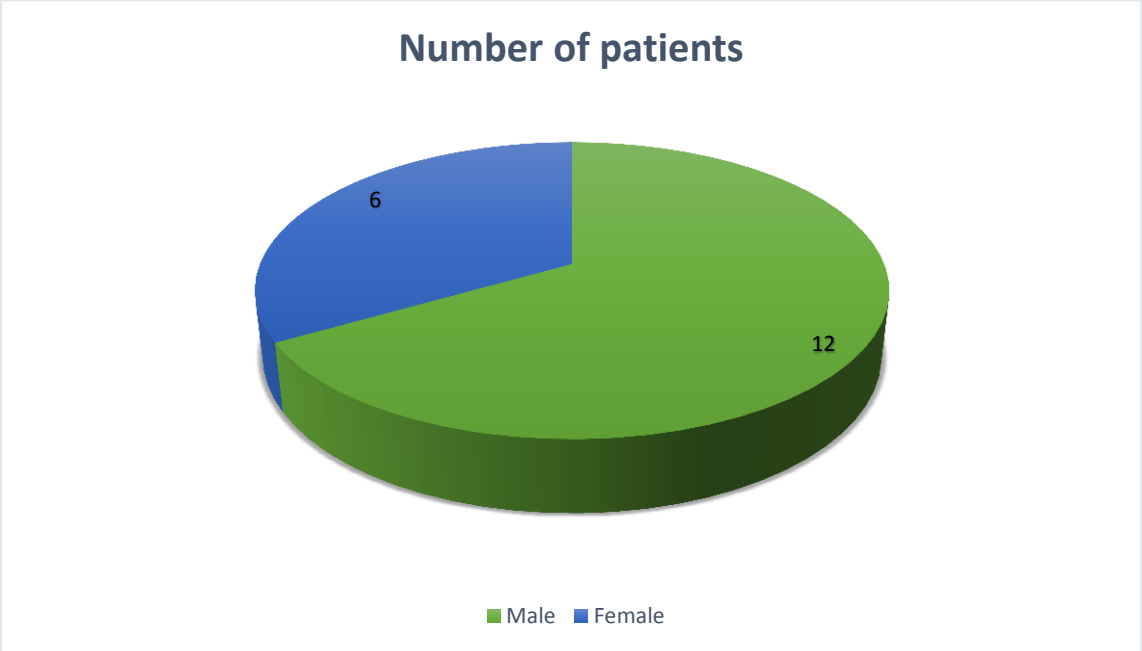
This registry has the approval of the Institutional Ethics Committee and informed consent was obtained from the patient or caregiver.

RESULTS

RESULTS

Twenty patients fulfilled the inclusion and exclusion criteria. Two patients declined to give consent for blood sample for genetic testing. Finally, 18 patients were included in the study, evaluated, and data were analysed. Thirteen patients were sporadic, while four patients were siblings (2 families). One patient had a history of similar illness in the elder sibling, who died of the illness. History of consanguinity was present in 7 patients (5 families). All patients had normal ceruloplasmin levels, and none had Kayser-Fleischer rings. Peripheral smear for acanthocytes, serum ferritin, blood sugar levels, hormonal profile, and electrophysiological testing were done according to the clinical presentation of patients.

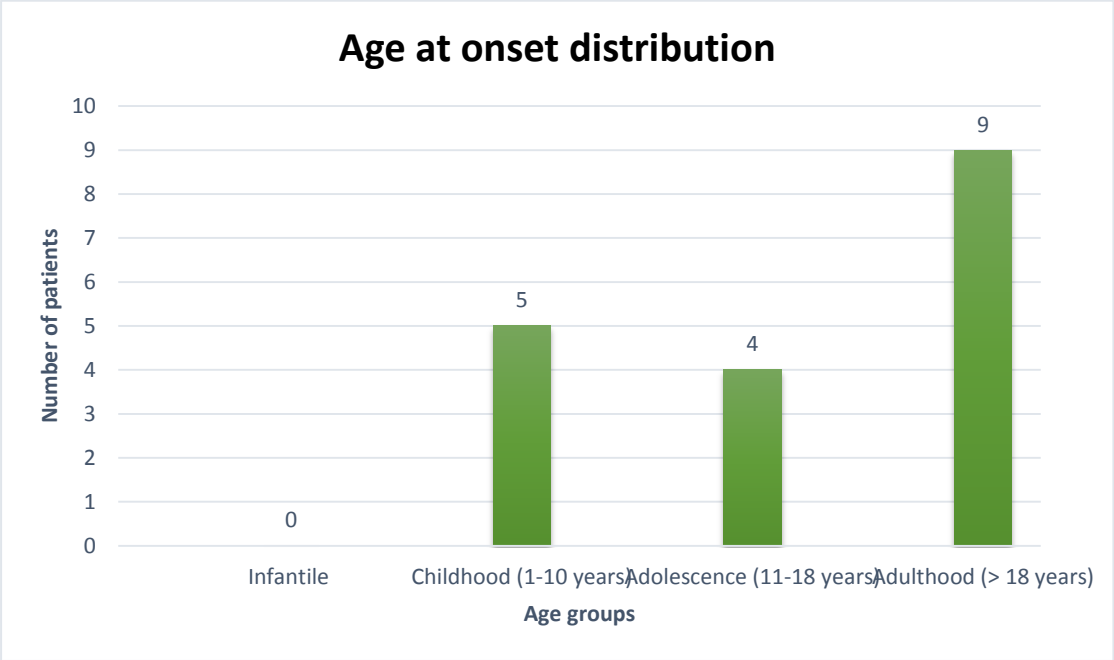
Figure 1: Gender distribution of patients



Out of 18 patients, 12 (66.7%) were males, and 6 were females.

Mean age at onset was 24.72 years (SD= 17.69) (Range: 2-63 years)

Figure 2: Age at onset distribution

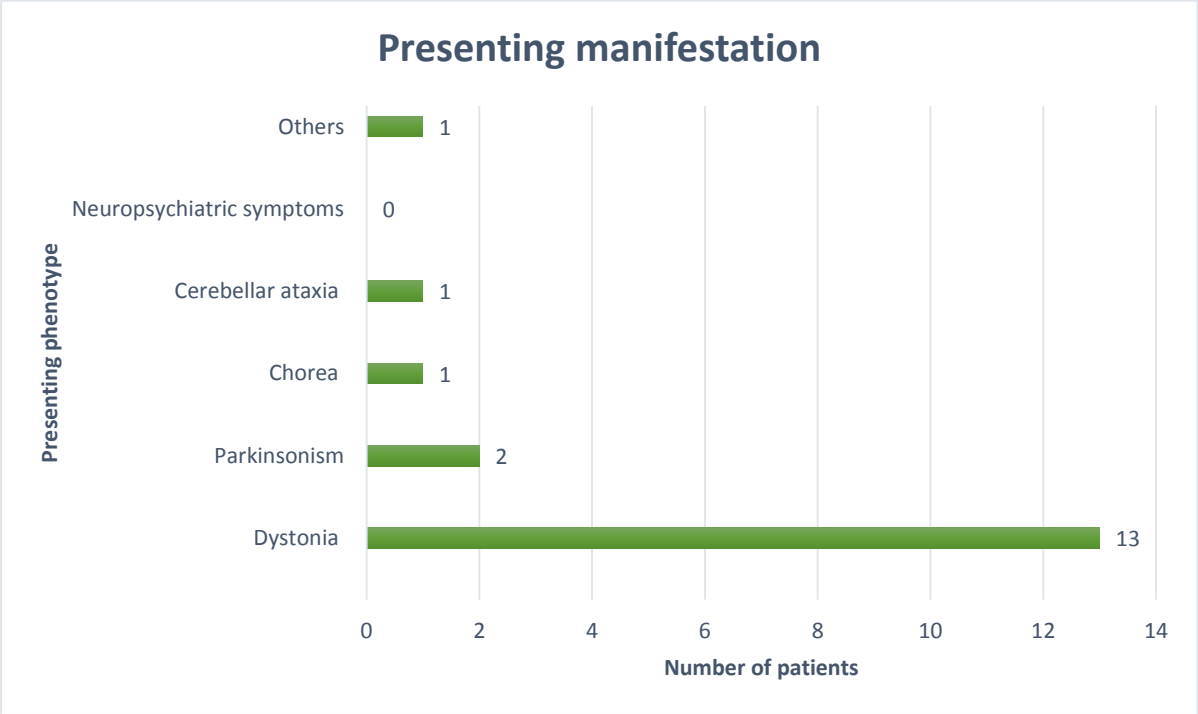


Out of 18 patients, 9 patients (50%) had their disease onset either in childhood or adolescence (5 patients in childhood, and 4 in adolescence). While, 9 patients (50%) had their onset in adulthood.

Table 4: Age distribution (age at onset) by age groups

Age groups (Years)	Number of patients (n=18)
0-10	5
11-20	4
21-30	2
31-40	4
41-50	1
51-60	1
61-70	1

Figure 3: Presenting clinical manifestation at onset (phenotype)

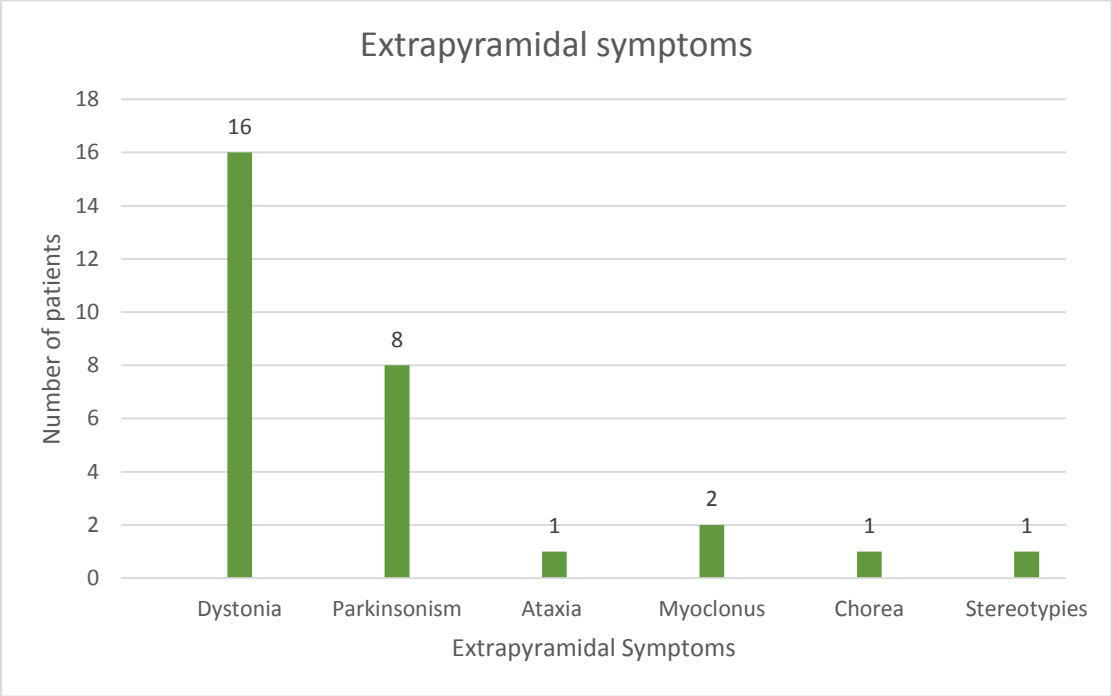


Dystonia was the most common presenting manifestation at the onset.

Out of 18 patients, 13 (72.2%) patients had dystonia as their first manifestation. Two patients presented with parkinsonism, one patient presented with chorea, and one with cerebellar ataxia as their first symptom. One patient had gait initiation difficulty (freezing of gait) as his first symptom (included as others).

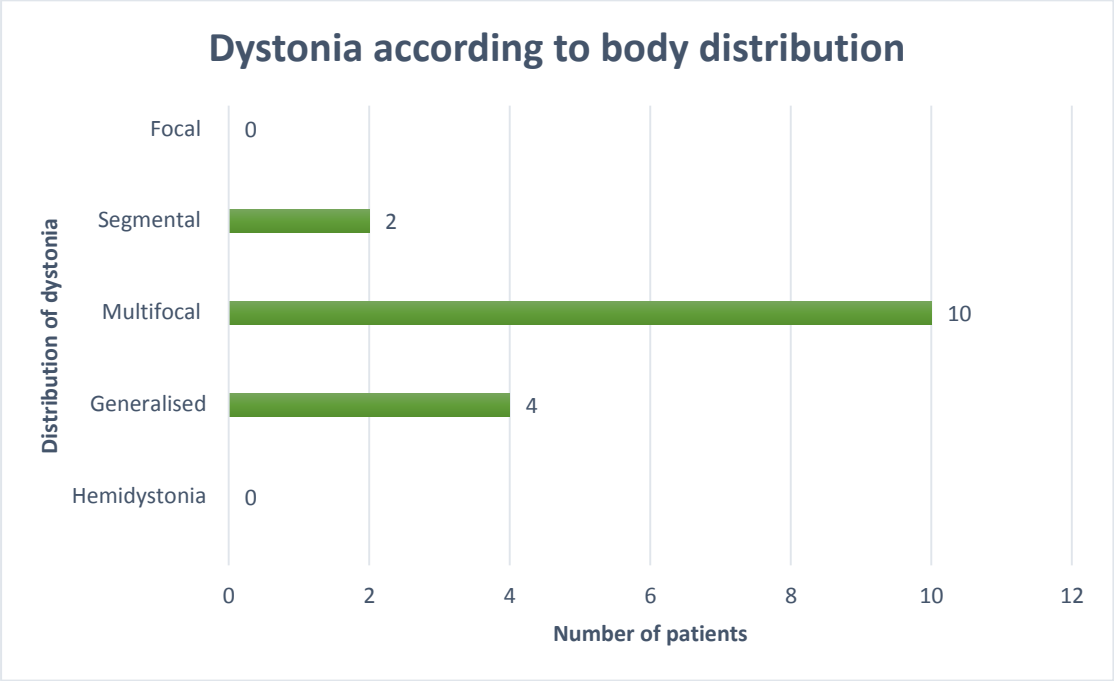
Out of 13 patients with dystonia as the presenting manifestation, 11 patients (84.61%) presented with limb dystonia (single extremity) as their first manifestation, while 1 patient had cervical dystonia and one had lingual dystonia (dysarthria) as the presenting symptom.

Figure 4: Overall frequency of extrapyramidal symptom



Overall, dystonia was the most frequent extrapyramidal manifestation, present in 16 patients (88.9%) over the course of illness, followed by parkinsonism in 8 patients (44.4%). Other extrapyramidal findings were less common (myoclonus in 2 patients, and cerebellar ataxia, chorea and hand stereotypies in 1 patient each).

Figure 5: Dystonia according to body distribution



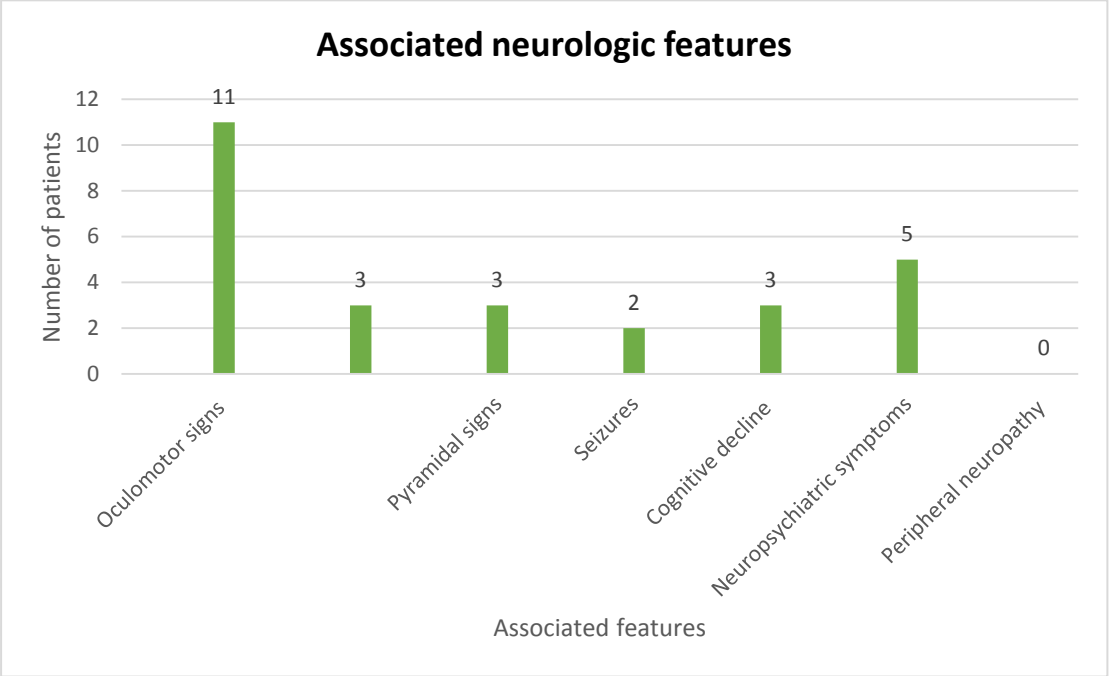
Multifocal dystonia was the commonest form of dystonia according to body distribution, which was present in 10 patients (62.5%). Four patients (25%) had generalised dystonia, and two patients had segmental dystonia. No patients had focal or hemidystonia. Oromandibular dystonia was seen in 7 patients (43.75%). Two patients with suspected PKAN had characteristic dystonic opisthotonus.

Table 5: Isolated v/s combined dystonia

Type of dystonia	Number of patients (n=16)
Isolated dystonia	7
Combined dystonia	9

Out of 16 patients with dystonia, 9 patients had combined dystonia (with other movement disorder/s), and 7 patients had isolated dystonia. 8 out of 9 patients with combined dystonia had parkinsonism.

Figure 6: Associated neurological features



Oculomotor abnormalities were the most common associated neurological features which were present in 11 patients (61.11%), most common being slow velocity of saccades (10/11 patients). One patient had oculomotor apraxia and apraxia of eyelid opening.

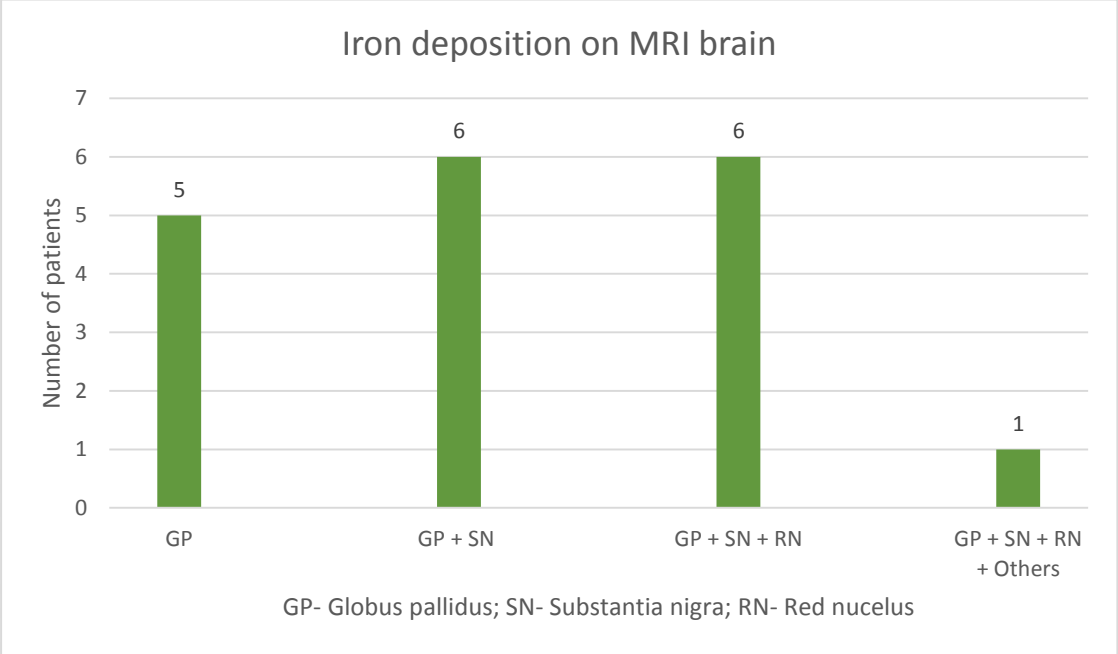
Neuropsychiatric symptoms were present in 5 patients (three had depression, one had obsessive-compulsive behaviour and one had bipolar mood disorder) during the course of illness.

Two patients had optic disc pallor and one had retinal pigmentary degeneration.

Three patients had pyramidal sign and two had seizures.

Cognitive decline was present in 3 patients.

Figure 7: Pattern of iron deposition pattern on MRI brain



All patients showed neuroimaging evidence of iron deposition in globus pallidus, detected on SWI images in MRI. The most common pattern of iron deposition was a combination of globus pallidus, substantia nigra and red nucleus blooming on SWI images, seen in 6 patients (33.3%). While, 5 patients had isolated globus pallidus iron deposition. Five patients had iron accumulation in globus pallidus, substantia nigra and red nucleus, and 2 patients had more diffuse pattern of iron deposition involving red nucleus, dentate nucleus, and striatum (Figure 8).

Two patients with PKAN showed classical “eye-of-the-tiger” sign (Figure 8).

Table 6: Other MRI brain findings

MRI findings	Number of patients
White matter hyperintensities	2
Partially empty sella and/or small pituitary gland	2
Caudate atrophy	2
Generalised cerebral and cerebellar atrophy	1

Two patients (one patient with Woodhouse-Sakati syndrome) showed bilateral white matter hyperintensities in T2-weighted and FLAIR images, and 2 patients with Woodhouse-Sakati syndrome showed partially empty sella. Two patients also had caudate atrophy, while one patient with BPAN had generalised cerebral and cerebellar atrophy.

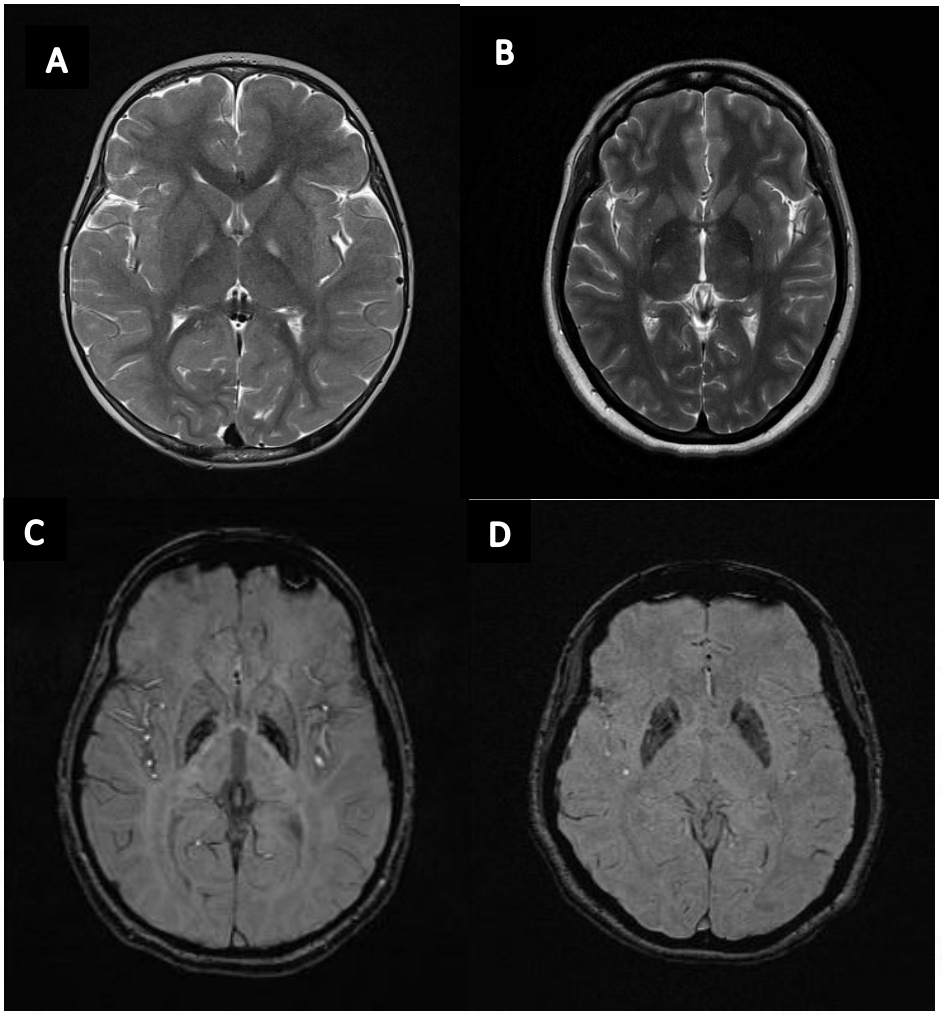
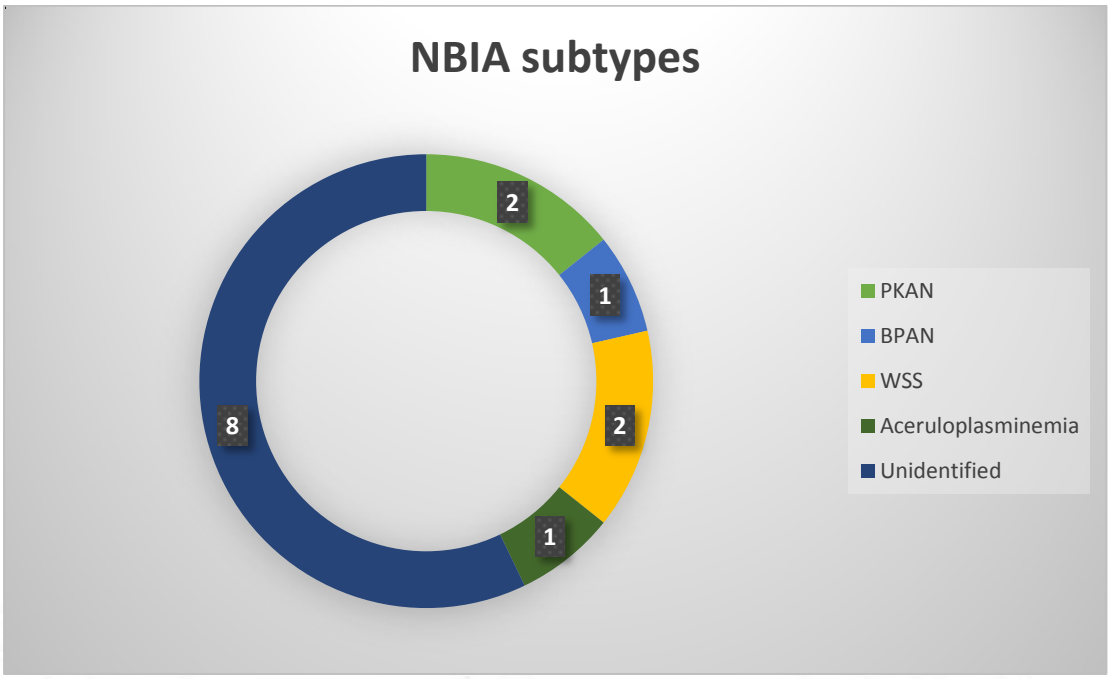


Figure 8: A) T2-weighted image showing classical “eye-of-the-tiger” sign in globus pallidus classical of PKAN, B) T2-weighted image shows hypointensity of bilateral globus pallidus interna, C & D) Susceptibility weighted images (SWI) show blooming in globus pallidus, caudate, and putamen (diffuse pattern of deposition in image D)

Figure 9: NBIA subtypes (according to genetic results)



Genetic testing results were available for 14 patients. Out of 14 patients, 2 patients were positive for *PANK2* mutations, 1 patient had a mutation in *WDR45* causing BPAN, 1 had a heterozygous mutation in *CP* gene, and 2 siblings with Woodhouse-Sakati syndrome had an identical mutation in *DCAF17* gene. In contrast, 8 patients had negative genetic testing by targeted gene panel. As these patients were negative, further genetic testing such as clinical exome sequencing will be planned for genetic confirmation.

DISCUSSION

Discussion

We studied clinical, radiological, and genetic profile of patients with NBIA. The diagnoses of NBIA were made on the basis of a combination of a progressive extrapyramidal syndrome with or without other neurological features, and iron accumulation in brain MRI. Eighteen patients with a clinico-radiological suspicion of NBIA were recruited in the study, and blood samples were sent for targeted gene panel for 10 known NBIA genes. To best of our knowledge, this is the largest single centre case series of NBIA patients from India. The only case series from Indian subcontinent till date included 6 patients with NBIA(32).

Clinical & Genetic Profile:

NBIA syndromes demonstrate marked phenotypic heterogeneity, not only between different subtypes but also within a single subtype. It remains an important differential diagnosis both in paediatric and adult-onset progressive extrapyramidal syndromes with or without other neurological features(28). Majority of NBIA subtypes have their disease onset in childhood, adolescence or early adulthood. The two major exceptions are neuroferritinopathy and aceruloplasminemia, which usually present in mid to late adulthood(33). Consistent with the literature, half of our patients had their disease onset before 20 years of age, and more than 3/4th of patients had their disease onset before the age of 40 years which was consistent with previous Indian case series(32).

Dystonia was the most common presenting manifestation in our study, particularly limb dystonia at onset. Overall, dystonia and parkinsonism were the commonest

extrapyramidal manifestations, while other extrapyramidal manifestations were less common. According to the body distribution, multifocal dystonia was the commonest pattern (in 2/3rd of patients). In our study, combined dystonia was more common than isolated dystonia. Approximately 60% of patients had combined dystonia (with other movement disorder/s), most commonly in combination with parkinsonism. Patients with NBIA who present with dystonia have predilection to involve oromandibular and bulbar region from the beginning, with predominant axial involvement in advanced stages. The presence of prominent oromandibular dystonia (particularly early in the disease course) is a characteristic feature of PKAN, neuroferritinopathy, and aceruloplasminemia. Oromandibular dystonia was seen in 1/3rd of our patients. All 3 suspected PKAN patients (1 genetically proven) and one patients with suspected neuroferritinopathy had prominent oromandibular dystonia.

The most common associated clinical feature was oculomotor abnormalities (particularly slow velocity of saccades), which was present in 2/3rd of patients, followed by neuropsychiatric symptoms, cognitive decline, and pyramidal signs.

Out of 3 clinically suspected patients with PKAN, one patient had classic PKAN with age at onset at 2 years with rapid progression, while 2 had atypical PKAN with their disease onset in adolescence and relatively slower progression, consistent with previous case series of PKAN patients(12). The patient with typical PKAN had generalised dystonia (with prominent axial dystonia- dystonic opisthotonus) with parkinsonism, cognitive decline and slow eye movements. She became bedbound and dependent on caregiver by the age of 10 years. Both patients with atypical PKAN presented with isolated dystonia syndrome. One patient had generalised dystonia with

characteristic dystonic opisthotonus, while the other had multifocal dystonia (oromandibular and both upper limb). One patient also had spasmodic dysphonia as well as optic disc pallor. All 3 patients had prominent oromandibular dystonia (two patients had jaw opening and one had jaw closure dystonia). No patients had retinal pigmentary degeneration. Genetic study of one patient with atypical PKAN revealed compound heterozygous missense mutations (c.1561G>A; p.Gly521Arg and c.1432A>G; p.Lys478Glu) in exon 6 and 5 of *PANK2* gene respectively. This mutation has been reported previously in patients with PKAN(99). In previous case series by Hayflick et al(12), almost all patients with atypical PKAN had missense mutations indicating possible residual pantothenate kinase 2 activity.

Two siblings were suspected to have Woodhouse-Sakati syndrome based on childhood onset alopecia (frontotemporal), hypogonadism, underdeveloped secondary sexual characteristics, endocrinopathy (diabetes mellitus in both and hypothyroidism in younger sibling), and extrapyramidal symptoms in form of isolated non-task specific dystonia of upper extremities. One patient had segmental dystonia and other had multifocal dystonia. Both siblings had facial dysmorphism (depressed nasal bridge and hypertelorism in both patients, and triangular face in elder sibling), and the elder sibling had mild sensorineural deafness detected on audiometry. ECG abnormalities were seen in neither of the two siblings. As reported by Bohlega et al(84), alopecia and hypogonadism is seen in all patients with WSS, with dystonia (focal or segmental in $2/3^{rd}$ of patients) as the most common neurological manifestation, as seen in both our cases. Other previous studies(81,82,100) have also reported childhood onset facial dysmorphism, alopecia, endocrinopathy, and childhood to adolescent onset neurological manifestations as the most common

manifestations of WSS. Genetic testing of both siblings showed a pathogenic homozygous nonsense mutation (c.85C>T) in exon 1 of *DCAF17* gene.

One patient was suspected to have BPAN based on characteristic biphasic course of illness, in form of childhood onset seizures and poor scholastic performance followed by early adulthood onset multifocal dystonia, symmetrical parkinsonism, and cognitive decline(52,101,102). The parkinsonism was levodopa responsive, and patient developed early motor fluctuations and dyskinesia requiring reduction of levodopa dose, as reported in the literature(52,102,103). Genetic study showed a pathogenic heterozygous 5' splice site mutation (c.830+1G>A) in intron 10 of *WDR45* gene, reported previously(52).

One patient had late adulthood onset pancerebellar syndrome with autonomic symptoms (urinary urge incontinence), and slow vertical and horizontal eye movements. He did not have evidence of retinal degeneration. His blood sugar, ferritin, and ceruloplasmin levels were within normal limits, but MRI brain showed iron deposition in bilateral globus pallidus and substantia nigra. His genetic study showed a novel heterozygous missense mutation (c.2592A>C) in exon 15 of *CP* gene, which was shown to be damaging by MutationTaster2 and PolyPhen-2. Previous studies have shown heterozygous cases presenting with incomplete clinical phenotype, as seen in this case(71,97,104).

Radiological profile:

All patients showed neuroimaging evidence of iron deposition in globus pallidus, with or without iron accumulation in other areas of brain and other additional findings(64),

consistent with the literature. The most common pattern of iron accumulation in our study was a combination of globus pallidus (GP), substantia nigra (SN) as well as GP, SN, and red nucleus. The patterns of iron deposition as well as presence of other imaging findings varied according to different subtypes of NBIA.

Two of 3 suspected PKAN cases had classical “eye-of-the-tiger” sign on T2-weighted images, while one patient with atypical PKAN lacked this hallmark sign but had iron deposition in globus pallidus and substantia nigra, consistent with previous case series(12) Both patients with WSS showed characteristic imaging findings of a small pituitary gland, partially empty sella, and iron deposition in globus pallidus and substantia nigra. The elder sibling also showed bilateral frontoparietal white matter hyperintensities on T2 and FLAIR images. These MRI findings are characteristic of WSS, as reported by Abusrair et al(87).

The patient with BPAN showed characteristic imaging features in form of prominent iron deposition in substantia nigra as compared to globus pallidus, along with characteristic substantia nigra hyperintense halo on T1-weighted images. These imaging findings are hallmark of BPAN(38,52).

Strengths of the study:

1. This is one of the largest current Indian study to assess clinical as well as imaging profile, and genetic patterns of patients with different NBIA syndromes.
2. The genetic mutations were identified in six patients and showed genotype-phenotype correlation as in previous studies.

Limitations of study:

1. Patients without the evidence of iron deposition on MRI were not included in the study. Certain NBIA subtypes (such as PLAN, Kufor-Rakeb syndrome) can have normal MRI initially or throughout the course of illness and may be missed out in NBIA cohorts based on the clinico-radiological diagnosis.
2. Targeted gene panel was used as the method of genetic testing, which includes 10 most commonly identified NBIA genes. This would miss out on deletions as well as intronic and splice site mutations in the previously identified genes. Also as the field of genetics is evolving, there may be many causative genes which are yet to be identified and needs further studies.

Conclusions and Summary

1. This study presents the clinical, radiological, and genetic profile of 18 patients with clinico-radiological suspicion of NBIA syndromes.
2. Dystonia was the most common presenting symptom. According to body distribution, majority of patients had either multifocal or generalised dystonia.
3. Overall, dystonia and parkinsonism were the most common extrapyramidal symptoms. The most frequent associated neurological features were oculomotor abnormalities, and neuropsychiatric disturbances.
4. All patients showed imaging evidence of iron deposition in globus pallidus, and majority of patients also showed substantia nigra iron accumulation.
5. Genetic profile of patients revealed *PANK2* mutations in 2 cases, *DCAF17* mutations (causing WSS) in 2 cases, *WDR45* mutation (causing BPAN), and heterozygous *CP* mutation (causing aceruloplasminemia) in one case each. Rest of the cases were negative for commonly identifiable gene mutations (genetically undefined cause).

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1. ABBREVIATIONS:

ACP: Aceruloplasminemia

ADC: Apparent diffusion coefficient

aNAD: Atypical neuroaxonal dystrophy

BPAN: Beta-propeller protein-associated neurodegeneration

CoA: Coenzyme A

COASY: CoA synthase

CoPAN: Coenzyme A synthase protein-associated neurodegeneration

CP: ceruloplasmin

CT: computed tomography

DBS: Deep brain stimulation

DMT1: Divalent metal transporter 1

DPCK: Dephospho-CoA kinase

DWI: Diffusion weighted images

EEG: Electroencephalography

ER: endoplasmic reticulum

FAHN: Fatty acid hydroxylase-associated neurodegeneration

FA2H: Fatty acid 2-hydroxylase

FPN1: Ferroportin

FTL: ferritin light chain

GP: Globus pallidus

GPI: glycophosphatidylinositol

GPi: Globus pallidus interna

GRE: Gradient echo

HSP: Hereditary spastic paraparesis

HSS: Hallervorden–Spatz syndrome

INAD: Infantile neuroaxonal dystrophy

KRS: Kufor-Rakeb syndrome

MPAN: Mitochondrial membrane protein-associated neurodegeneration

NBIA: Neurodegeneration with brain iron accumulation

NFT: Neuroferritinopathy

PANK2: Pantothenate kinase 2

PKAN: Pantothenate kinase-associated neurodegeneration

PLAN: Phospholipase A2-associated neurodegeneration

PPAT: 4'-PP adenyltransferase

QSM: Quantitative susceptibility mapping

RN: Red nucleus

ROS: Reactive oxygen species

SENDA: Static encephalopathy with neurodegeneration in adulthood

SN: Substantia nigra

SWI: Susceptibility-weighted images

TFRC: Transferrin receptor

WSS: Woodhouse-Sakati syndrome