Genetic Insights of Cerebral Palsy using Massively Parallel Sequencing

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TABLE OF CONTENTS

Introduction

Cerebral Palsy (CP) is an umbrella term used to address a complex phenotype, a combination of non-progressive everlasting disorders of the development, movement, and posture caused due to damage to the developing brain. Besides motor manifestations in these individuals, swallowing and feeding difficulties, speech and language discrepancies, epilepsy, gastrointestinal abnormalities, and intellectual disability are notable traits (Rosenbaum, Paneth, Leviton, Goldstein, Bax, Damiano & Jacobsson, 2007). The early signs that indicate cerebral palsy are poor head control, delayed and abnormal motor development, abnormalities of muscle tone and reflexes, and oro-motor/non-speech disturbances in mobility.

Consequent to the multiple problems, children with CP face numerous difficulties in their day to day life, which can affect their quality of life. Mortality reported in this clinical population is firmly connected with functional impedance and is also associated with non-motor impairments. Mortality rate increases with associated disabilities such as intellectual disabilities, hearing, and vision function (Mutch, Alberman, Hagberg, Kodama & Perat, 2008).

Among the several disorders that seriously cause disabilities of motor functioning in the younger generation, cerebral palsy is the most reported. The incidence of CP in western nations is reported as 2-2.5/1000 live births (Reddihough & Collins, 2003). In the Indian scenario, the incidence estimates are nearly the same. The recent statistics reported by the Indian Academy of Cerebral Palsy (IACP, 2011) revealed that the occurrence of CP is ~ 3 cases per 1000 live births, making it the most frequent reason for childhooddisability.

CP can be caused by several damages to the brain at different stages, starting from weeks after conception, during birth, or early childhood. The major prenatal causes include insult during the first trimester due to maternal viral infection such as rubella, influenza, toxoplasmosis, etc., ingestion of teratogenic drugs, radiation exposure, and chromosomal abnormalities so on. The major peri-natal causes include birth complications during labor and delivery, and the post-natal causes include birth asphyxia, neonatal jaundice, and sepsis (Denhoff & Robinault, 1960; Batshaw & Perret, 1986).

Various studies have revealed that only 10%–15% of individuals with CP may be due to delivery complications (Blair & Stanley, 1988). The additional reasons for CP are congenital malformations, prematurity, gestational age, intrauterine infections, atypical intrauterine growth abnormalities, ischemic stroke, and multiple gestation complications. However, most individuals with CP born at 36 weeks of gestation have no recognized birth asphyxia, and only ∼40% have a potential cause. As per Gustavson, Berg, and Sanner, 1969, nearly 60% of CP cases may have rare genetic pathogenic mutations. In 2% of the cases from Sweden and the United Kingdom, there is a genetic mechanism operating as the causation of CP (Bundey & Griffiths, 2008). Most cases have noticeable signatures as per brain imaging and have no identifiable pre or postpartum events. In half of the cases of both ataxic and athetoid forms of CP, no predisposing events are likely to have genetic mutations. According to Denhoff and, Robinault, 1960, the most common nervous system anomalies found, such as anencephaly, hydrocephaly, microcephaly, and spina bifida, imply that they are genetically related. Although genetic forms account for only 1%–2% of all cases, they provide a unique idea into the causes of the disease and neuronal development (Mchale, Mitchell, Bundey, Moynihan, Campbell, Woods,…Markham, 1999). Further, the previous studies on genetics and epidemiology show that approximately 8% of the population identified with

congenital anomalies has a genetic disorder before adulthood (Baird, Anderson, Newcombe & Lowry, 1988).

The diagnostic process in some children with CP is often long, complicated, and expensive without a conclusive molecular diagnosis due to its complexity, CP, hosting itself with multiple heterogeneous genetic disorders. The diagnosis of CP is based on attained motor milestones, tone of the muscle, and intellectual disability level. When the etiology has not been established, and if brain imaging and clinical reports are not conclusive, this hints a possible metabolic or geneticcause.

When it comes to genetic causes of CP, several studies support the theory of CP's risk, which is higher in consanguineous families than the risk in outbreeding families where compound genetic factors contribute to causing the condition. Reports from certain studies have reported monozygotic twins showing a higher concordance rate for CP when compared to dizygotic twin pairs (Petterson, Nelson, Watson & Stanley, 1993). The genetic form of CP (spastic) with other co-morbidities like Intellectual disability and microcephaly is reported in the literature (Bundey & Griffiths, 2008). Although no gene is responsible, an autosomal recessive form of inheritance is observed. Rajab, Yoo, Abdulgalil, Kathiri, Ahmed, Mochida…Walsh, 2006, reported a narrative of a large consanguineous family pedigree, in which affected individuals were spastic CP with additional notable traits like microcephaly and intellectual disability. Additional notable conditions include hyper reflexes, inappropriate gait, drooling, and speech problems. Their study revealed that phenotypic variability among individuals of their cohort, but diplegia (spastic), microcephaly, and intellectual disability were present in all individuals of the cohort. Their analysis provided support that a genetic factor causes these abnormalities in the family studied.

Growing evidence towards the CP genetics approach probably suspects the involvement of various genetic aspects, similar to other neurodevelopmental disorders like autism and mental retardation. There is no specific causal mechanism for CP besides a large number of proposed causes. In the recent past, CP is clinically viewed as a group of heterogeneous neurodevelopmental disorders with co-occurring motor impairments and medical disorders. To date, the genetic contribution towards CP studied is only 2% with rare genomic abnormalities. Most of the clinicians conclude their opinion that 70-80% of cases were due to parental issues, prematurity, birth labor, asphyxia, and more. CP, amid other brain disorders that result from multiple rare genetic variations, were rarely identified in genetic studies. This makes it difficult to conclude the exact genetic causes responsible for CP (Iyengar & Elston, 2007).

With the advancement of DNA studies in diagnosis, affordable Next-Generation Sequencing research completely overturned the research on genetics in CP. The focus of these genetic studies in CP has transformed from single or multiple gene studies to identifying responsible variant (disease-causing mutation) using Massively Parallel Sequencing (MPS). The NGS strategies like Whole Exome sequencing (WES) (*Refer to Glossary for details on exome sequencing*), Whole Genome Sequencing (WGS), targeted re-sequencing not only revolutionized but also shortened the time for genetic diagnosis. The results of these serve as a base approach for clinical neurology practice and diagnostic testing. Higher probability of detecting de novo mutations causing genetic disorders can be achieved using WES, which facilitates the diagnosis and treatment, and rehabilitation of individuals with thesedisorders.

DNA sequencing is determining the order of nucleotides. This order determination of DNA nucleotides was carried out by Sanger sequencing using Capillary Electrophoresis (CE). A limitation of this method is a maximum of 1Lakh DNA nucleotides can be sequenced by this method. Using this method, decoding the entire protein-coding region of the DNA from a single person takes decades to accomplish. With mounting evidence that mutation in the protein-coding regions is responsible for alterations in the phenotype, there is pressure on the scientific community to scale up the sequencing throughput. This revolutionized sequencing in terms of throughput and turnaround time by inventing massively parallel sequencing (MPS). MPS, also known as next-generation sequencing or deep/ultra-deep sequencing, can sequence the entire genome or protein-coding regions of the DNA in 10-48 hours and generate nearly about 12GB 1 TB of data choice of chemistry and platform used. This robustness brought a paradigm shift in the diagnosis of neurological diseases by ending the diagnosticodyssey (Applied BiosystemsSeqStudio Genetic Analyzer: Sanger Sequencing Guide, 2016)

Izatt, Nemeth, Meesaq, Mills, Taylor, and Shaw (2004), taking ataxia as a model, evaluated NGS's induction into regular clinical evaluation. They sequenced 58 genes, mutations in which were earlier reported to cause ataxia in 50 ataxia individuals whose phenotype is highly heterogeneous. These cases had been thoroughly investigated but failed to establish the diagnosis with usual methods. All cases had undergone invasive, various biochemical, genetic tests. After sequencing and subsequent bioinformatic analysis of suspected mutations, 13 mutations from 8 genes were pathogenic, and the genes are PRKCG, TTBK2, SETX, SPTBN2, SACS, MRE11, KCNC3, and DARS2. The study concluded that NGS based genetic testing is an efficient, low cost and can establish a molecular diagnosis for the cases who failed to receive a diagnosis by routine laboratory investigations. According to Yang, Muzny, Reid, Bainbridge, Willis, Ward, ABraxton, Beuten, Xia, Niu, and Hardison, 2013, the use of WES will eliminate diagnostic odyssey for individuals with no specific etiology or unique disease manifestations and also for individuals who have received a diagnosis of a genetic disorder which is heterogeneous.

The NGS has brought a remarkable change in identifying the causation of mutations in Mendelian disorders, orphan, and rare diseases.

If all the congenital anomalies are seen as genetic load, 8% of the population is identified as having a genetic disorder before adulthood. The majority of the individuals with genetic disorders remain without specific diagnosis having a significant adverse effect on the patient population and their families, including failure in identifying potential therapeutics, failure in estimating the risk for subsequent pregnancies, and failure in giving proper prognosis guidance. A few examples include CP and its spectrum disorders, various single-gene disorders, and chromosomal abnormalities. CP is one of the significant reasons for childhood disability. CP and its spectrum is a clinical diagnosis that comprises a diverse group of neurodevelopmental anomalies that compromise movement and posture observed throughout life. CP is characterized mainly basing on the motor and postural impairment as spastic, ataxic, dystonic/athetoid, and mixed. As per limbs affected, it is characterized as monoplegic, hemiplegic, diplegic, or quadriplegic. William John Little first described CP as peri-natal asphyxia with poor neurological outcomes in later life. Sigmund Freud basing on the observations made on persons with CP that included epilepsy, visual disturbances, and intellectual disability and proposed that CP's progression may start at very early days of life, probably during brain development in the uterus. Regardless of Sigmund Freud's hypothesis, it was widely accepted by common man, scientific and medical fraternities that CP is a result of complications during labor, and Freud's notion was only accepted after extensive scale population studies concluded that only a subset of CP cases is due to birth asphyxia.

Innatespasticparaplegiasarehighlyheterogeneousneurodegenerativedisorderswith additional neurodevelopmental conditions and usually follow inheritance patterns like

autosomal dominant, autosomal recessive, or X-linked. Hereditary mutations have been recognized among 40 different genes. Complex latent spastic paraplegias have been frequently connected with mutations in the genes SPG11, ZFYVE26/SPG15, SPG7, and many other rare mutations in the genes; however, numerous cases remain hereditarily vague (Ishiura, Takahashi, Hayashi, Saito, Furuya, Watanabe…Tsuji, 2014). Eleanna Kara et al., 2015, explored a progression of 97 cases with spastic paraplegia. Cutting edge sequencing technologies uncovered eleven rare variants in known involved spastic paraplegia genes, variants were identified in genes for spastic paraplegia and in the Parkinson's disease-related genes ATP13A2, neuronal ceroidlipofuscinosis TPP1, and the motor and sensory neuropathy DNMT1 gene, highlighting the genetic heterogeneity of spastic paraplegia (Ishiura et al., 2014).

The majority of the patients with genetic disorders remain without a specific diagnosis (Moreno-De- Luca, Helmers, Mao, Burns, Melton, Schmidt,…Martin, 2010), having a significant adverse effect on patient population and their families which include failure in identifying potential therapeutics, failure in estimating the risk for subsequent pregnancies, and failure in giving proper prognosis and guidance. Collectively, to address the challenges in the appropriate diagnosis of the CP, which is one of the most commonly seen childhood disability with delayed speech, language, and other co-morbidities, the present study adopted state of the art tool, i.e., Exome Sequencing (ES) using cutting edge massively parallel sequencing technology to identify the genetic determinants of CP and other congenital anomalies that mimic CP.

Why massively parallel sequencing?

Discovery of genetic determinants of cerebral palsy genes during the early days of linking the genotype with phenotype employed linkage analysis (Papatheodorou, Oellrich & Smedley,

2015). A linkage study requires large multigenerational families with at least one affected individual in each generation. The success rate of identifying a gene for a trait or phenotype through linkage analysis is subjected to limitations like multiple affected family members, availability of families with the same disorder, and appropriately targeting the microsatellites in the genome. The second wave of studies employed case-control and candidate gene strategies to overcome limitations of linkage studies, which were achieved using microarray and/or Sanger sequencing methods. These methods were not successful in genetic dissection of complex phenotype like cerebral palsy as these studies require multiple individuals with the same phenotype. Given high genetic heterogeneity behind cerebral palsy and whole-genome and exome sequencing methods, the third wave of studies employed state of art tools i.e., whole genome and exome sequencing techniques to dissect the genetics behind the complex phenotype of cerebral palsy. Whole-exome sequencing decodes entire 3.3 gigabases, whereas exome sequencing decodes approximately 35 million DNA bases that code proteins. Whole-genome sequencing is not cost-efficient and time-efficient, comparatively exome sequencing is rapid and cost-effective. It is estimated in 2011 that by 2015 molecular basis of nearly 7000 Orphan disorders which follow the Mendelian fashion of inheritance disorders can be established by using these technologies and notably, several genes for cerebral palsy were discovered by exome sequencing (may refer OMIM for genes/variants of spastic paraplegia and cerebellar ataxia). While it is evident that WES can identify the genetic determinants of neurological disorders, exome sequencing now has a presidential status in diagnosing complex neurological disorders. Proven its ability to detect mutations in the monogenic forms of cerebral palsy in the most cost-effective ways and by less Turnaround time, the present study employed cutting-edge genomic technology i.e., sequencing of the exome by massively parallel sequencing.

Aim of the study

With mounting evidence, the CP and its spectrum are genetic in a considerable amount of cases; the present study aims to use cutting-edge genomics technology, i.e., massively parallel sequencing technology, to identify the *de novo* mutations in typical CP and well as CP spectrum patient population with the following objectives.

1. Analysis of suspected genes in 100 CP cases and its spectrum.
2. Exome Sequencing in 100 individuals with CP and its spectrum to identify the de novo (unknown/ not reported) mutations.

Review of Literature

Communication is vital for biological and social existence and involves exchanging information between two or more individuals. Communication is defined as an act of conveying intended meanings from one entity or group to another through the use of mutually understood signs and semiotic rules. This involves two modes, namely verbal and non- verbal communication. Non-Verbal communication intents to the process of conveying meaning in the form of non-word messages such as hand gestures, eye gaze, facial expressions, touch, posture, facial behavior, physical appearance, non-verbal vocalizations, and smell (Argyle, 1988).

Speech and language form the two important components of communication. Speech, the act of speaking, is a verbal mode of communication, which involves precise coordination of oral neuromuscular movements in order to produce sounds and linguistic units. Language refers to the ability to communicate through speech by delivering and receiving meaningful messages. As soon as the child is born, he/she communicates with the world through a cry, which sets the platform for further speech and language development which continues through several years of life.

Any impairment in this ability to receive, send, process, and comprehend concepts of non-verbal, verbal and graphic symbol systems leads to communication disorder (ASHA, 1982). Communication disorder affects an individual's capacity to comprehend, detect, or apply language and speech to engage in discourse effectively with others (Collins, 2011). The delays and disorders can range from simple sound substitution in speech to the inability to understand or use their native language. Neurodevelopmental conditions, brain deformities/injury/infection, hearing impairment, intellectual disability, cleft lip and palate, emotional or psychiatric disorders, developmental delay etc. could lead to communication disorders. Cerebral palsy is one among the childhood

communication disorder which severely impairs the motor, psychological, speech and language and social aspects of development.

Explaining Cerebral palsy

CP is a broad diagnostic term used to refer to a cluster of anomalies which affects the movement, posture and balance due to brain damage during fetal development or at neonatal stage. William Little in 1862 for the first time documented medical description of this disorder that affects children during infancy. The term cerebral Palsy was later coined by Phelps in 1941.

One of the earliest definitions of CP was given by Cruickshank and Raus (1955), who observed CP as a single component with brain damage and include of neuromotor abnormalities, mental retardation and epilepsy. In 1964, Bax described CP as 'a disorder of posture and movement due to a fault or injury in the immature brain'. A summary of several conclaves which was held in developing countries between 1987 and 1990 resulted in a further revised definition to underline the heterogeneity of the condition which described CP as: 'broad diagnostic term, where the phenotype is cumulative result of non-progressive, but frequently altering, motor impairment disorders, which arise due to brain anomalies that took place in the early developmental stages'.

In 2005, a committee of the American Academy for Cerebral Palsy and Developmental Medicine (AACPDM), led by Peter Rosenbaum, defined CP as "a group of disorders of the development of movement and posture, causing activity limitation, that are attributed tonon-progressive

disturbances that occurred in the developing fetal or infant brain. The motor disorders of cerebral palsy are often accompanied by disturbances of sensation, cognition, communication, perception, and/or behavior, and/or by a seizure disorder." This definition was later on revised by Rosenbaum et al., (2007) which states that CP "includes a collection of permanent disorders of posture, development, movement and non-progressive disturbances that occurred in the developing foetal brain or during infancy. The motor disturbances of CP often associate with sensory issues, perception, communication, behavior, epilepsy and cognition,".

Incidence and Prevalence of Cerebral Palsy

According to the birth cohorts obtained from developed countries and as per the study conducted by Rosen &Dickinson (1992) approximated the occurrence of CP between 2 to 2.5 per 1000 live births. Novak, Hines, Goldsmith &Barclay (2012) and Australian Cerebral Palsy Register (2013) estimated worldwide incidence of CP as 1 in 500 births and also stated that 17 million individuals are present globally who received a diagnosis of CP

According to the estimation of WHO, globally 10% of the population has some type of anomaly and in India, 3.8% of the population is estimated to have one or another form of disability, out of which 15-20% of physically disabled children carries diagnosis of CP.

In India, National sample survey Organization (NSSO, 1991) records that CP is the cause for locomotor disability in 48% of the rural population and 43% of urban population. According to the National Sample Survey Organization (NSSO, 2002), for every 1, 00,000 number of persons with disability, there are 1008 individuals afflicted with a locomotor disability. The recent statistics reported by Indian Academy of Cerebral Palsy (IACP, 2011) revealed that the incidence of CP is ~3 cases per surviving making it one of the most frequent disability causes. According

to Vyas, Kori, Rajagopala&Kalpana, (2013), in India, it is estimated that around 3/1000 live births is the CP incidence.

Classification of Cerebral Palsy

According to Rosenbaum et al. (2007), any classification system is supposed to meet the purpose of description regarding the nature and severity of the problem, predict signs and symptoms to other health care individuals assist in comparison between series of CP cases and evaluate the changes across time. Below mentioned are a few examples of classification system for children with cerebralpalsy:

Based on severity level (Palisano, Rosenbaum, Walter, Russell, Wood & Galuppi, 1997):

a) Mild CP: is when the individual moves with no help; their routine daily actions are not restricted.
b) Moderate CP: is when the child requires medications, braces and adaptive technology to carry out activities of daily living.
c) Severe CP: is when the child is confined to wheel chair and faces significant difficulties in carrying out daily living activities.

This classification is very general and does not involve any specific criteria. Hence can be used when an accurate classification is not critical

Based on muscle tone:

The neuromuscular tone is defined as the strength and tension of the muscles. The type of movement depends on the severity of brain injury which has in turn impacted the muscle tone (Sankar & Mundkur, 2005):

a) Hypertonia: Sometimes used synonymously with spastic is a condition marked by increase in muscle tension leading to reduced ability of the muscle to stretch. Any damagetothemotorpathwaysofcentralnervoussystemwhichcarriesinformationfrom

the brain to the muscle and control postures, muscle tone and muscles is said to cause this condition. Untreated hypertonia can lead to loss of function and deformity.

b) Hypotonia: Hypotonia is due to inadequate amount of tension in the muscle and reduced resistance to stretch of the muscle. This condition eventually leads to loss of strength and firmness. Hypotonia is usually coupled with other neurological conditions such as CP, brain and spinal cord injury, meningitis, encephalitis, muscular dystrophy, myasthenia gravis, and few genetic disorders such as down syndrome, Praderwilli syndrome, Tay-sachs disease.

Based on location of movement problems, CP can be classified as (Graham, 2005):

a) Paresis: "Weakening of muscles of the body part which has been affected".
b) Plegic: "Paralysis of the muscles of the body part which has been affected".
c) Monoplegia: "Only one limb's movement is affected. This type of movement problem is veryrare".
d) Diplegia: "Two limbs, usually the legs, are affected. Often, those with diplegia have mild movement problems in the upper body as well".
e) Hemiplegia: "One side of the body is affected. The arm is usually more involved than other limbs, distinguished by a rigidly flexed wrist or elbow".
f) Triplegia: "Three limbs are affected".
g) Quadriplegia: "All four limbs are involved. The legs usually are severely affected than the arms. There may be limited control over the facial muscles as well".
h) Double hemiplegia: "Almost similar to quadriplegia where all four limbs are affected, but the upper limbs are more affected than legs".
i) Pentaplegia: "All four limbs affected, along with muscles of head and neck".

Based on neuromuscular symptoms, CP can be classified as (Stanley, Blair, & Alberman, 2000):

a) Spastic cerebral palsy: Spastic CP is the most frequently seen type of CP accounting for 70% to 80% of overall CP cases (Stanley, Blair, & Alberman, 2000), there is exaggerated muscle tone and release of postural stretch reflexes, which tends to vary from mild hypertonicity to extreme rigidity depending on the site of lesion and extent of involvement of pyramidal system. The indicators for spastic CP are exaggerated movements, hypertonia, contractures and unusual postural reflexes.

b) Dyskinetic CP: Dyskientic CP exhibits slow and involuntary writhing movements usually affecting the feet, arms, hands and/or legs and in few individuals may also involve the facial muscles causing griming and drooling. Periods of emotional stress often aggregates the involuntary movements which are otherwise absent during sleep. The dyskinetic movements are further classified into dystonia, chorea and athetosis (Stanger & Budney, 2010). Dystonia is mainly characterized by involuntary muscle contractions which result in slow twisting or repetitive movements, or abnormal sustained postures, that are triggered by attempts to move. Whereas chorea involves involuntary movements that are abrupt, brief, irregular and unpredictable. Around 10% of children with cerebral palsy are diagnosed with athetoid type which is also otherwise referred as non-spastic CP. This type of CP is characterized by a mixture of hypertonia and hypotonia, which causes muscle tone to fluctuate leading to involuntary movements of face, torso and limbs. Athetoid CP is caused due to damage in the basal ganglia and/or cerebellum. The basal ganglia are responsible for regulating voluntary motor function and eye movements, while the cerebellum controls balance and coordination. The notable hallmarks of athetoid CP include stiff or rigid body, involuntary movements, tremors, and unsteadiness, twisting of torso, slow- writhing movements, and floppiness in the limbs, improper posture, and swallowing difficulty.

c) Ataxic CP: Ataxia is a state that describes difficulties in balance and coordination. IndividualswithataxicCPhavedifficultyincarryingoutvoluntarymovements.This

form of CP is primarily caused due to the damage in the cerebellum. Individuals with ataxic CP may experience tremors. Notable features of ataxic CP include inaccurate motor skills, difficulty in walking and balancing leading to unsteady gait, tremors, difficulty in speaking, over-correcting movements, difficulty in repetitive movements.

d) Mixed cerebral palsy: Persons who exhibit movement problems that fall into two or more of these categories are classified as mixed cerebral palsy when the damage to the developing brain is in multiple regions. The most common variation of mixed CP is combination of spastic and athetoid. The signs and symptoms of this form of cerebral palsy are exaggerated, jerky movements, abnormal reflexes, poor posture, tremors, issues with coordination and crossed legs or abnormal gait.

According to Singhi (2002), out of 1000 cases of CP in India, 61% comprised of spastic quadriplegia, which was followed by 22% of diplegic CP. Shankar, and Mundkur (2005) also noted that diplegia was the most commonly occurring form of CP (30%-40%) followed by hemiplegia (20%- 30%) and quadriplegia accounting for 10%- 15%.

Associated problems

Cerebral palsy is caused due to an insult in the developing brain which reasons out to many other co-existing problems which commonly co-occur with cerebral palsy. The severity and location of brain damage plays a major role in the manifestation of CP and consequently the effect of CP varies from one child to another. The effects can range from complex and severe physical and cognitive involvement to a barely noticeable limp. The magnitude of these associated problems tends to affect the quality of life of the child having cerebral palsy. Ashwal, Russman, Blasco, Miller, Sandler, Shevell, and Stevenson (2004) described various associated conditions that co-occur with CP.

Epilepsy: Epilepsy is known to be the most common associated problem, occurring among 35% of children with CP as per Center for Disease Control and Prevention (CDC). Studies have shown that spastic quadriplegia and hemiplegia have a higher incidence of epilepsy compared to diplegia or ataxictype.

Mental retardation: It is noted that neuropsychological and cognitive functions in children with CP are commonly affected. Approximately around 66% of individuals with CP are found to have some degree of mental retardation. Children with spastic hemiplegia are usually seen to have lesser degree of intellectual disability than compared to individuals with spastic quadriplegia.

Ophthalmologic impairments: Around 28% of individuals with CP may have some form of ophthamologic impairments. Of these, strabismus, amblyopic (lazy eye, visual field defects nystagmus, optic atrophy, and refractive errors are frequently seen (Schenk-Rootlieb, Nieuwenhuizen, Graaf, Wittebol-Post, & Willemse, 2008).

Speech and language disorders: Dysarthric speech and other speech related impairments are observed in individuals with CP due to the dysfunction of oro-motor structures and bilateral corticobulbar region. Sankar and, Mundkur (2005) in their study stated that articulation disorders and impaired speech intelligibility were present in nearly 38% of individuals with CP. Language deficits were also noted to be in concurrence with verbal intellectual limitations associated with intellectual disability. Nutrition and growth, oral health, respiration, and self- esteem are seen to be seriously compromised due to oral-motor problems including feeding difficulties, swallowing dysfunction, and drooling.

Hearing impairment: Hearing impairment is observed to be present among 20% of children with CP. Involvement of etiological risk factors such as very low birth weight, kernicterus, neonatal meningitis, or severe hypoxic-ischemic insults are generally associated with hearing loss. Abnormal neuroimaging studies or mental retardation also pose as a greater risk for hearing impairment for children with CP.

Dysphagia: Children with CP generally present with a complaint of difficulty in swallowing due to oro-motor dysfunction in the muscles and nerves of the oral cavity, pharyngeal and esophageal sphincter. Symptoms may include pain when trying to swallow and/or inability to swallow, unusual weight loss, regurgitation, heartburn, hoarse voice, food getting stuck in the chest area and/or throat, gagging and coughing when attempting to swallow, drooling, delayed (or sometimes absent) swallowing reflex and sore throat. If not treated, dysphagia can lead to serious complications such as achalasia (dysfunction of the lower oesophageal or cardiac sphincter), aspiration or respiratory issues, dehydration, malnutrition and pneumonia.

Oral health issues: Cerebral palsy leads to oral issues, such as excessive gagging, gingivitis, problems with drinking and eating, involuntary cheek and tongue biting, and more. Other conditions such as tooth cavities and decay, teeth grinding while sleeping, drooling, abnormal alignment in the lower teeth and upper teeth are also known to be associated with CP.

Respiratory issues: Although CP does not cause respiratory distress, the associated conditions can often lead to life threatening respiratory issues. Some of the respiratory complications that are observed in children with CP are difficulties in coordination between swallowing and breathing, blocked airways, excessive drooling, low activity levels, and the inability to cough. Other respiratory conditions that are also associated with cerebral palsy include Bronchitis,

Respiratory distress syndrome (RDS), Pneumonia, Chronic lung disease, Bronchopulmonary dysplasia, Asthma, Aspiration, Pneumonia, and more.

*Behavioral and Emotional problems:*25% of the children with CP have been noted to experience some form of behavioral and/or emotional problems. The severity of the other associated disorders increases the intensity of these issues. Weber, Bolli, Heimgartner, Merlo, Zehnder, and Kättere, 2016 have recorded inattention, restricted social interaction, abnormal prosocial behavior, and difficulty in carrying out daily living activities, inadequate communication skills, withdrawing from social activities, short temper, depression, and anxiety in these children.

Quality of life

Individuals with CP have multiple problems due to which they face numerous difficulties in their day to day life. These can lead to shortage in different areas such as self-care, speech, communication, learning, mobility and independent living. Moreover, the severity of motor impairment and the associated cognitive communicative and behavioural impairments are different for each child with CP. Therefore, some of them need long-term care, treatment and rehabilitation and are usually entirely dependent for daily living and communication. Because the level of severity differs, their level of participation in everyday activities will vary greatly (Rosenbaum, et al., 2007). These disabilities can limit the individual's activities and participation and can cause a decrease in the quality of life (QoL). The higher the disability level of the child and more severe the presence of motor deficits, the higher is the reduction in the child's overall Quality of Life (Elbasan, Duzgun & Oskay, 2013).

The majority of children with CP live long, productive lives with the appropriate management approach and social care. However, life expectancy for children with cerebral palsy will also

greatly depend on how severe other associated conditions such as feeding issues, seizures, cognitive issues, limited mobility, vision problems etc. Mortality is reported in this clinical population which is firmly connected with both the level of functional impedance and additionally associated with non-motor impairments. Mortality rate increases with growing number of cerebral disabilities, including intellectual disability, limb, hearing, and vision function (Mutch, Alberman, Hagberg, Kodama, & Perat, 2008). These conditions when progress over time impacts the patient's quality of life.

Elbasan, Duzgun, and Oskay, 2013, studied the differences in QoL in children with different disabilities (cerebral palsy, mental retardation, and hearing loss), in terms of their self-care and social function in their daily life activities. The findings revealed that children with CP were more dependent in the areas of self-care and mobility activities that children in the other disability category.

Assessment

Shankar and Mundkar, 2005, extensively worked on CP and their classification, etiology and early diagnosis. According to them, the clinical diagnosis should be made with an awareness of risk factors, usual developmental screening of children born with high risk factors, a systematic approach focusing on maternal, obstetric and peri-natal histories, review of developmental milestones, and a thorough neurological assessment and examination of the child in various positions such as supine, sitting, standing, walking and running was considered mandatory. Delay in the developmental milestone, abnormal muscle tone, persistence of abnormal neonatal reflexes, asymmetrical movements like asymmetrical crawl and hyperreflexia, delay in the emergence of protective and postural reflexes are noted to be one of the earliest signs that are observed in a child suspected with cerebral palsy. Among the most clinically useful primitive reflexes are Moro, Tonic

labyrinthine and Asymmetric Tonic Neck Reflex (ATNR), which generally diminish by 6 months of age in non CP population, but are observed to remain persistent for long time even in children with CP. Studies have suggested repeated examinations and observations over a period of time in mild cases before a firm diagnosis is be made (Ellison, Horn, Browning, 1985; Ashwal et.al., 2004). In the further evaluation of a child with CP, an EEG is obtained if there is history of epilepsy. Neuroimaging studies are carried out if they have not been done in the neonatal period which provides insight towards the etiology of CP. MRI studies is preferred to CT scans. Genetic and metabolic tests are carried out if there is evidence of deterioration or metabolic compensation, family history of childhood neurological disorder associated with CP. The General Movements Assessment given by Morgan, Crowle, Goyen, Hardman, Jackman, Novak, and Badawi, 2015, can be used to predict cerebral palsy for children below 5 months of age, particularly when slight abnormalities are present in the MRI findings. However, the test does not indicate the severity of CP in the child. Complete evaluation of the spectrum of conditions for a child with CP should include assessment of the reproductive health factors of the parents, reviewing paternal health records, examination of pregnancy, labor and delivery records, considering the newborn screens conducted at birth, reviewing score, reviewing baby birth, medical, developmental and growth records, performing a physical examination of baby, conducting neuroimaging tests to determine the extent of brain damage, performing electroencephalography (EEG) or electromyography (EMG) to analyze nervous system function, carrying out lab tests (blood work, urinalysis or genetic testing).

An assessment of associated deficits like speech and hearing, vision, mobility and gait, sensory profile, feeding and digestion, oromotor evaluation, epilepsy, cognitive functioning and

rehabilitation needs are also necessary. Orthopedic evaluation is a must as muscle imbalance and spasticity cause subluxation/dislocation of the hips, contractures, and scoliosis. Team of experts that generally involve in the identification, assessment and management of children with cerebral palsy are Pediatrician, Neurologist, Radiologist, Ophthalmologist, Geneticist, Orthopedic Surgeon, Otologist, Speech and language pathologist, Audiologist, Dietician, Special educator, Physiotherapist and Occupational therapist. For those with milder symptoms, a diagnosis may not be rendered until the brain is fully developed at three to five years of age. For example, the average age of diagnosis for a child with spastic diplegia, a very common form of Cerebral Palsy is 18 months. The lengthy and detailed process can help rule out or confirm Cerebral Palsy. A formal diagnosis is usually made once the brain is fully developed between 2 to 5 years of age.

Etiology

CP has been noted as a commonest physical disability among the children. Also being a heterogeneous condition with multiple clinical manifestations; multiple associated developmental co-morbidities, the pathogenic causative factors behind the development of CP is still unknown (Maclennan, Thompson, &Gecz, 2015). The etiological factors can be classified into hereditary factors, prenatal, peri-natal and post-natal factors. As per the study conducted by National Institute of Neurological disorder and stroke (2013), the following are the types of various brain damage that results in characteristic symptoms of cerebral palsy:

I. White matter abnormalities (periventricular leukomalacia or PVL): The transmission of signals within the brain and to the rest of the body is carried out in the white matter of the brain. Any damage to this area will impair the conduction of signals. Researchers have

identified a period of selective vulnerability in the developing brain of the foetus, i.e., between 26 and 34 weeks of gestation, in which periventricular white matter is particularly sensitive to insults and injury.

II. Brain abnormal development (cerebral dysgenesis): Any retardation/interruption of the normal development of the brain during the gestation can cause brain malformation/deformities which interfere with the transmission of the signals. Mutations in the genes responsible for the development of the brain are noted as a potential cause for the abnormal development of the brain. Other conditions such as infections, trauma or any other condition that causes unhealthy environment inside the womb are also said to lead to malformation of the brain.

III. Bleeding in the brain (intracranial haemorrage): Also known as fetal stroke is caused due to bleeding in the tissues of the brain. This can be caused to the fetus due to blood clots in the placenta which restricts the blood flow to the brain or due to malformed and/ weak blood vessels or by blood clotting abnormalities. Maternal high blood pressure is proved to be a high risk for fetal stroke (Kattah & Garovic, 2013).

IV. Severe lack of oxygen in the brain: Also known as asphyxia is caused due to deprivation of oxygen to the brain due to the stress of the labor and delivery. Prolonged duration of insufficient supply of oxygen leads to a condition known as hypoxic ischemic encephalopathy which damages the tissues of cerebral motor cortex and other parts of the brain. This may be caused due to several conditions such as problems involving the umbilical cord, severe maternal hypotension, detachment of placenta, rupture of the uterus, severe trauma to the head during labor and delivery.

Thus, causative factors for the damage of the various pathways and structures of the brain causing multiple clinical manifestations of cerebral palsy are plenty. Only one predominant cause or a ‘combination of events’ can lead to disruption of these neuronal pathways. As suggested by

MacLennan, Thompson, &Gecz, 2015, CP should be considered as a descriptive term for affected individuals, with each case receiving adequate consideration of an underlying etiology. These risk factors vary from genetic factors to congenital malformations to pre-post partum risk factors. The following are the risk factors known to cause cerebral palsy:

Antenatal risk factors

Maternal health condition: Mother's health condition especially during the first two trimesters is also likely to affect the development of the unborn baby. Maternal factors such as higher parity (>5), consanguinity, African-American mothers, higher maternal age (>40 yrs), smoking, mothers presenting with conditions such as chorio amniotitis, hypertension, epilepsy, hyperthyroidism, abruption placenta are marked as higher risk factors for children with CP. Centers for Disease Control and Prevention (2013) also considered infertility treatments, endometrial complications, intellectual disability, and excess protein in the urine as potential risk factors for CP.

Mothers with pre-gestational diabetes are considered to be at an increased risk of having child with congenital birth deficits (Yang, Cummings, O'connell, & Jangaard, 2006). Gestational diabetes which usually occurs in 18% of the pregnant women also puts the fetus at a greater risk of cerebral palsy (Norwitz, Edusa, & Park, 2005). Untreated gestational diabetes results in a condition known as macrosomia (birth weight >4kgs) which leads to birth asphyxia and cerebral palsy. Maternal thyroid dysfunction especially in the early gestation also poses as a greater risk in causing injuries to the unborn baby. Researchers have also noted that maternal seizures and maternal cognitive impairment as strong predictors of cerebral palsy (Grether, Nelson, Emery, & Cummins, 1996; Nelson, 1984).

Among various other potential indicators of CP, infections during gestation have recently been implicated as a risk factor (Grether, 1997). According to a research article published in the Journal of American Medical Association, Grether, 1997, investigated 47 children with spastic CP and found that there was marked increased risk of CP to children who were exposed to maternal infection during early gestational stage. They also stated that maternal infection majorly linked with low scores, and an important predictor for birth asphyxia. Studies on antenatal and intrapartum factors with relation to CP have recognized intrauterine infections and chorio-amnionitis as risk factors for brain white matter damage (Baud, Sprumont & Donkelaar, 2018). Infections such as toxoplasmosis, rubella (German measles), cytomegalovirus, and herpes, are also known to infect the womb and the placenta. Inflammation triggered by these infections is likely to damage the developing nervous system in an unborn baby. Maternal fever during pregnancy or delivery also has the potential to set off similar kind of inflammatory response. Similarly, Miller, Pedersen, Streja, Bech, Yeargin-Aallsopp, Braun and Olsen, 2013 noted Rubella, TORCH, Rh incompatibility, intake of toxins, exposure to toxic materials such as methyl mercury during, as high risk factors of having an infant with CP.

Infertility treatments: Children born post certain infertility treatments are usually at greater risk for CP compared to the infants born as a result of un assisted reproduction. A population based cohort study was conducted in Denmark by Hvidtjorn, Grove, Schendel, Vaeth, Ernst, Nielsen, and Thorsen, 2006, to study the incidence of CP in children born out of in vitro fertilization and various types of infertility drugs (9255 children) when compared to children born without any fertility treatment. Factors such as maternal age, gender, early conceptional age and social variables like education and economic status were also compared between the two groups. The conclusion of the study indicated that preterm delivery mainly in twins conceived after assisted

reproduction.

Multiple pregnancies: Multi fetal pregnancies are said to come up with their own set of complications such as abnormal presentation during delivery, umbilical cord accidents during delivery, premature rupture of membrane and caesarean deliveries. Many researchers have noted a direct correlation between the risk of CP and the number of fetal infants during multiple gestation (Odding, Roebroeck, & Stam, 2006). Nelson, 1984, reported that CP occurred 1.1 times per singleton pregnancies compared to 12 times per twin pregnancies. Similar study was conducted by Peterson, Nelson, Watson, and Stanley, 1993, where they concluded their study by stating that the risk increased 47 times in triplet pregnancy and 8 times in twin pregnancies compared to singleton pregnancies. Among the twin pregnancies, death of one of the twin increased the risk of the other twin having CP by 108 times, compared to singleton pregnancies. Monozygotic twins were likely to be at a higher risk when compared to dizygotic twins especially when one of the twins suffered a fetal or infant death (Pharoah & Cooke, 1996 ; Pharoah, 2002). Preterm delivery and low birth weight were the two major risk factors associated with multiple pregnancies which likely increased the incidence of CP.

Prematurity: CP is summative of various clinical observations like prematurity etc., Fetus prematurely delivered is exposed to extra risks during the labor, and the post neonatal environment. Individuals born prematurely are at greater risk of peri-natal brain damage and subsequent neurodevelopmental abnormalities (Kunugi, Nanko, & Murray, 2001). In a population based study from United States, found that the prevalence of CP as 44 per1000 for infants born before 27 weeks of gestation, 21 per1000 for those born between 28 and 30 weeks of gestation, and 0.6 per 1000 for those born at term (Cummins, Nelson, Grether, & Velie, 1993). Murphy, Johnson, Sellers, and MacKenzie, 1995, conducted a case control study of 59 pre-term babies who developed cerebral

palsy and noted that frequency of CP increased with decreased gestational age and birth weight. They also noted that chorioamnionitis, prolonged rupture of membranes and maternal infection were the other potential causes for cerebral palsy. In support of this study, Jacobsson and Hagberg, 2004, also concluded that 70%-80% of the CP cases were due to predominant prenatal factors compared to 10% of the cases due to birth asphyxia. The antenatal risk factors which were repeatedly observed to cause CP were low gestational age, multiple gestation, male gender, intrauterine viral infections, maternal thyroid abnormalities, intrauterine infection/ inflammation with a maternal response (consisting of chorioamnionitis) and fetal inflammatory response.

Low birth weight: Many studies have aimed to study the prevalence of cerebral palsy in children born with low birth weight (<1500 grms). A retrospective study considered 72 singletons born between 1983-91 who were diagnosed with neurological impairment at 20 months of age and compared with 72 singletons neurologically normal low birth weight children. Cerebral palsy was found to be the most prevalent neurological disorder in children born with low birth weight (Wilson-Costello, Borawski, Friedman, Redline, Fanaroff, & Hack, 1998). The most important risk factors for CP were noted to be low birth weight, intrauterine infections and multiple gestations (Odding, Roebroeck, & Stam,2006).

A typical intrauterine growth: Refers to the condition where the fetus fails to achieve the potential growth and also considered to have lifelong implication on the neurological outcome of the fetus. Chromosomal or genetic abnormalities are known to majorly contribute to the intrauterine growth restriction. Long term sustained growth retardation in the fetus is known to cause significant neonatal birth complications such as cerebral palsy, birth asphyxia and other CNS and cardiovascular abnormalities. According to Jocobsson and Hagberg, 2004 year not matching, the risk of CP is said to increase 8 folds in case of a term baby with intrauterine growth restriction.

Environmental toxins: On a global scale, the traditional environmental hazards such as biologically contaminated water, poor sanitation, indoor smoke from biomass burning, and rampant disease vectors (for example, malaria) have remained as the primary source of ill health especially in children (United Nations Environment Programme, United Nations Children's Fund, and World Health Organization. 2002). These environmental contaminations may either be from exclusively non-natural substances or from natural compounds that are concentrated by industrial processes. Non-natural toxic threats alone represent a major potential problem. Specific concerns about environmental pollutants and children's health broadly fall in these areas, with most concern about metals, and persistent organic pollutants (including pesticides) according to a report published by Department for Environment, Food and Rural Affairs, Advisory Committee on Hazardous Substance in 2003. The committee's concern, however, focused on the data linking dioxin and children's neurocognitive development. Patandin, weisglas-Kuperus, De Ridder, Koopman-Esseboom, Van Staveren, Van Der paauw, & Sauer, 1997, reported a cohort of healthy Dutch infants and mothers and assessed PCB (polychlorinated-biphenols) exposure. Children with increased PCB exposure exhibited a slight decrease in a neurodevelopment test at 7 months. Similarly, the effect of lead on children's health is also one of those most extensively studied cause and effect relationship. Children are more sensitive to the toxic effects of lead, compared to adults as it is more absorbed through the gastrointestinal tract. Hence, a greater proportion of systemically circulating lead reaches the brain (especially under 5 years), and the developing nervous system is highly vulnerable to damage (Lidsky, & Schneider, 2003). Mercury, similar to lead, is also a toxic to the developing brain which inhibits the neurocognitive development (Grandjean, Weihe, White, Debes, Araki, Yokoyama Jorgensen, 1997).

Natal risk factors

Breech presentation: This is characterized by birth of the baby in the feet first presentation. 4% of the babies are usually present in the breech position at the time of birth. Babies born this way tend to face difficulties while navigating through the birth canal and also are prone for brain injuries, cerebral palsy, asphyxia and other mental and physical defects (Thorngren-Jerneck & Herbst, 2006). Other injuries such as umbilical cord compression, nuchal cord, fetal distress and traumatic injury are also associated with breech presentation. Nelson, 1984, though noted that breech presentation was an important contributor for cerebral palsy, but not the breech delivery. 33% of the cases with cerebral palsy who were at breech position at the time of birth had major non cerebral malformation.

Complicated labor and delivery: Many studies have shown an association between obstetric complications and cerebral palsy. Newborns that had vascular or respiratory problems during labor and delivery are likely to have been suffered from brain damage or abnormalities.

Instrumental Delivery: Thorngren-Jerneck and Herbst, 2006, considered breech presentation of the baby during birth, instrumental delivery, forceps delivery and immediate caesarian delivery to be important predicting factors for cerebral palsy.

Post-natal risk factors

Birth asphyxia: Asphyxia also knows as hypoxia is termed as lower than normal level of oxygen in the blood supply to any part of the body. Hypoxia when occurs to a new born infant, due to distress during or right after the birth process usually affects the brain functioning. Fetal heart rate patterns, including prolonged fetal bradycardia, as well as meconium staining of the amniotic fluid and fetal acidosis, are markers of fetal stress (or distress) which is usually related to intrauterine asphyxia. Hypoxia in utero due to hypoperfusion, a fibrotic placenta, premature

placental separation, or problems with the umbilical cord, may be responsible for damage to the brain. Hypoxic-Ischemic Encephalopathy (HIE) is the injury to the brain caused due to oxygen deprivation. The manifestation of this encephalopathy varies with the severity and duration of the insult, with the degree of development of the brain and also with the presence of underlying deficits in the brain substrate. According to Minchom, Niswander, Chalmers, Dauncey, Newcombe, Elbourne,…Williams, 1987, seizures which occur within 48 hours of birth in babies born at or later than 37 completed week's gestation are likely to reflect intrapartum asphyxia. They considered a total of 54 cases of such seizures and were compared with 41 090 controls in a geographically defined population. It was also noted that 5 out of the 54 babies who developed seizures died within 28 days of birth and 11 of the 49 survivors had an impairment diagnosed by 3 years of age which was usually associated with some degree of cerebral palsy. The frequency of CP associated with birth asphyxia according to a birth cohort study conducted by Yudkin, Johnsont, Clover, and Murphy, 1995, was estimated to be 1 in 3700 full-term live births. They also assessed the impact of birth asphyxia on the overall rate of cerebral palsy. Of the 30 cases, birth asphyxia at term were only associated with 10% of all cases of cerebral palsy.

Low score: The score is a numbered rating that reflects a newborn's physical health. Doctors periodically score a baby's heart rate, breathing, muscle tone, reflexes, and skin color during the first minutes after birth. A low score at 10-20 minutes after delivery is often considered an important sign of potential problems such as CP.

Jaundice: More than 50 percent of newborns develop jaundice (a yellowing of the skin or whites Of the eyes) after birth when bilirubin, a substance normally found in bile build super faster than their livers can break it down and also has a slower mechanism of clearance of it from the body. While some infants present with mild jaundice which usually clears within 1-2 weeks, for others it may last little longer. Severe, untreated jaundice can lead to Kernicterus which is caused due to high

levels of bilirubin damaging the brain cells. Rosenbaum, Walter, Hanna, Palisano, Russell, Raina,,Galuppi, 2002, considered neonatal jaundice as an important indicator for athethoid form of cerebral palsy.

Seizures: Epilepsy also known as seizures are the changes in the behavior or physical findings which is caused due to abnormal electrical activity in the brain. Epilepsy is considered as the most singleton complication of cerebral palsy besides mental deficits. Probability of a child developing seizures and cerebral palsy depends on the area and the extent of damage to the brain especially in the areas of the frontal and temporal lobe. The incidence of cerebral palsy is usually directly proportional to the severity of epilepsy as noted by Knezevic-Pogancev, 2010. Many studies have shown that the incidence of epilepsy significantly increases with the cerebral cortical lesions (Krageloh-Mann, & Cans, 2009; Sugiura, Shiota, Ieshima, & Ohno, 2003). Cognition is also said to depend on the development of the gray matter of the brain. Children with cognitive impairment were seen to have a higher frequency of epilepsy than those without cognitive impairment. Associated disabilities such as mental problems were noted much more common in children with cerebral palsy and epilepsy than in those without seizures (Carlsson, Hagberg, &Olsson, 2003). Epilepsy was noted to commonly affect children with spastic tetraplegia and those with mental abnormality. According to Bruck, Antoniuk, Spessatto, Bem, Hausberger, and Pacheco, 2001, all the subjects with tetraplegic CP and about one-third of the children with other CP types developed epilepsy. Also in a study conducted by Peduzzi, Defontaine, and Misson, 2006, seizure was noticed less frequently in mild symmetric spastic diplegia and athethoid form of CP. Many researchers have noted risk factors such as low birth weight, neonatal seizures; seizures during the first year of life, and family history of epilepsy were related to significantly increased occurrence of epilepsy in children with cerebral palsy (O'shea, 2008).

Periventricular leukomalacia: According to Ikonen, Janas, Koivikko, Laippala, and kuusinen, 1992, periventricular leukomalacia was considered as the main predictor of CP in preterm infants. In addition to hypocarbia, hyperbilirubinemia may also be involved in the pathogenesis of extensive (severe cystic) periventricular leukomalacia.

Genetic strategies used in studying communication disorders

In general CD and rare Mendelian disorders have been studied using candidate gene approach through high-throughput sequencing, where single experiment sequences full exome screens thousands of genes (Bamshad, Ng, Bigham, Tabor, Emond, Nickerson &Shendure., 2011). Carrier sequencing in brief would be a successful approach when dealing with communication disorders. Trio sequence analysis will be of greater advantage in diagnosis, where there is identification of unaffected individuals who carry one copy of dysfunctional gene for a disease to be expressed (Kumar, Mattan&Vellis, 2006). For genome wide studies dealing with trio families in mutation identification, a combination of sequencing data and pedigree information will be analyzed. The entire class of computational tools incorporate information about coverage, sequencing error rate, variants, SNPs, heterogeneity and more. Bioinformatics programs and information from public databases are very helpful in filtering, evaluating the mutation screening. Recently, the number of genes associated with rare diseases has grown at a very notable rate every year using high throughput technology (Koboldt, Steinberg, Larson, Wilson, Mardis, 2013).

Fundamental premise of genetic research is to find out the mutations that determine variations in phenotypes which can be immensely outreached by next-generation sequencing approaches (Claudio, Koser, & Mark, 2014; McKusick, 2007). Vast data generation will set a baseline by which gene variants in a genetic disease cohort can be evaluated for the disease biology, disease

susceptibility and genetic risk factors involved. Superior medical intervention is only possible with efficient diagnosis and in case of rare genetic disorders entire genome sequence study reveals positive number of gene variants and mutations involved in provoking the disorder (Nickerson, Ravichandran, Lundahl, Rodolico, Dunlap, Trksak, & Lukas, 2011). The behavior of communication disorders and the genes responsible causing mutations vary from patient to patient and family history, depending on environmental as well as lifestyle pattern. These patients typically present with wide range of clinical features and will remain undiagnosed by tools that are built on the therapy purpose. Some examples include Cerebral Palsy, Stuttering, Mental retardation and much more. In such cases NGS is mainly of sound interest, as a tool to examine each patient's genome individually to find out potentially causative mutations and compare genome variants across these patient sequences, which may result in possibility of newer findings towards the genes involved in causing disorders. High end Bioinformatics support makes this research more obliging in diagnosis of rare genetic diseases. This is ideal for the investigation of high penetrance rare diseases, but it may also provide long awaited breakthrough to completely understand complexity of disease pattern (Need, Shashi, Hitomi, Schoch, Shianna, Mcdonald & Goldstein, 2012).

When it comes to genetic causes of CP, studies support the theory of the risk of CP which is higher in families with consanguineous mating than the risk in outbreed families, where compound genetic factors contribute to the cause of CP. Reports from certain studies indicate that monozygotic twins show higher concordance rate for CP when compared to dizygotic twin pairs (Petterson, Nelson, Watson, &Stanley,1993). Genetic association studies in relation to CP

Amid other developmental brain disorders that result from multiple damages are genetic variations that are infrequently detected in association studies, makes it difficult to conclude exact genetic role

in the causation of CP (Izatt, Nemeth, Meesaq, Mills, Taylor & Shaw, 2004). DNA which is responsible for hereditary and carries majority of genetic load makeup an individual, any changes in its duplication may result in rare to severe genetic disorders. WES serves as a tool in the identification of molecular defects in patients with suspected genetic disorders. The previous studies on genetics and epidemiology show that approximately 8% of populations are identified with congenital anomalies as having a genetic disorder before reaching adulthood (Baird, Anderson, Newcombe, & Lowry, 1988). Throughout the globe, substantial number are affected with genetic disorders, in conclusion, the use of whole-exome sequencing serves as standard "diagnostic odyssey" for long-term search in genetic diagnosis for patients with nonspecific or abnormal ailment introductions of conceivable hereditary reason and for patients with clinical judgments of heterogeneous hereditary conditions (Yang et al.,2013).

The advancement of DNA studies in diagnosis and affordable Next generation sequencing (NGS) has completely overturned the research related to genetics in CP. The focus of genetic investigations in CP has been undergoing extensive change from gene association to the identification of the likely causal variants. The NGS studies like Whole Exome Sequencing (WES), Whole Genome Sequencing (WGS), targeted sequencing have revolutionized and shortened the genetic load of diagnosis. The results of these serve as base approach for clinical neurology practice and diagnostic testing. Higher probability of detecting de novo mutations causing genetic disorders can be achieved using WES, which facilitates the diagnosis and treatment of these disorders. Identification of underlying precise causes of the disorder benefits the individuals and their families dealing with CP by providing better assessment, diagnosis, information on the risk of recurrence and early intervention. Elucidation of the pathways involved in the expression of these genes will undoubtedly provide insight in the understanding of the

pathophysiology of CP and lead to the path towards preventing CP (MacLennan, Nelson, Hankins &Speer, 2005). In some cases, trios study highlights the association of de novo mutations in parental generations and inheritance pattern to their progeny.

Molecular basis of genetic abnormalities in rare genetic disorders can be resolved using Whole exome sequencing (WES). Whole exome sequencing and trio's studies of the families involved in the individuals presenting with ID and muscle weakness and minor or major forms of Arthrogryposis multiplex congenital (AMC) disorder have revealed certain specific de novo mutations and gene alterations (Hirata, Nanda, Van Riesen, Mcmichael, Hu, Hambrock & Kalscheuer, 2013). They concluded that genetic testing using targeted capture followed by NGS will be proficient, financially savvy, and enabled molecular diagnosis in many refractory cases. A novel mutation in ATPase family AAA-domain containing protein 3A (ATAD3A) is identified to cause severe neurological syndrome with multiple syndromes. Familial sequencing studies using WES for molecular diagnosis was carried out in a family where inheritance of a dominant heterozygous variant in ATAD3A is inherited from mother to her son presenting dyskinetic CP.
Higher level of complexity in mapping entire genomes towards genetic testing procedure can be achieved by applying Next generation sequencing (NGS) technologies. By this approach most novel disease genes involved in genetically heterogeneous disorders can be identified. This high throughput technique can cope to provide insights into the underlying genetics of Mendelian traits. Next Generation Sequencing (NGS) has become an important approach not only in the discovery of disease –provoking genes in research but also a discretionary in clinical diagnostics
(Dello, Di-Giacomo, Mesoraca, Démidio, Iaconianni, Minutolo, Giorlandino,2014). In 25% of the diagnostic results in patients with Mendelian diseases were achieved by using whole exome

sequencing through NGS (Yang et al., 2013). With one experiment, through the use of a NGS platform it is possible to identify ample number of selected genes which are potentially associated with the disorder and responsible for pathological mutations. According to reports of NIH the utility of Whole exome sequencing and Whole genome sequencing has led to identification of two new disorders (Ng, Buckingham, Lee, Bigham, Tabor, Dent &Bamshad, 2009).

Wide range of clinical features may tend to present with undiagnosed patients, and should consider each patient's genome individually rather looking for common disrupted gene with a simple phenotype (Need et al., 2012). New Mendelian disease genes can be identified using Exome sequencing, and also this approach will contribute to complex disease genetics (Gilissen, Hoischen, Brunner & Veltman, 2012). The available online web-based databases records the newer findings using the NGS technologies on rare genetic disorders, the retrieval of molecular basis of inherited diseases from these databases is almost doubled in recent (McKusick, 2007). With use of whole genome sequencing and whole exome sequencing based studies, the cancer genetics discovery over the precedent years is unprecedented in the literature (Forbes, 2010). In a very short time frame NGS technologies made a greater impact on our understanding of human genetic diseases.

CP makes the diagnostic process often long, complex and costly without ever reaching a conclusive molecular diagnosis due to its complexity and hosting itself with multiple heterogeneous genetic disorders. The NGS studies like WES, WGS, targeted sequencing have revolutionized and shortened the genetic load of diagnosis. The results of these will serve as base approach for clinical neurology practice and diagnostic testing. Higher probability of detecting de-novo mutations causing genetic disorders can be achieved using Whole Exome sequencing, which facilitates the diagnosis and treatment of these disorders. In some cases, trios study highlights the

association of de novo mutations in parental generations and inheritance pattern to their progeny. The symptomatic procedure is regularly long and complex with most patients experiencing various intrusive and expensive examinations while never achieving a definitive molecular conclusion.

Genetics of Cerebral palsy

CP encompasses an overall 2% of genetic burden associated with heterogeneous non-progressive disorders and with additional phenotypic features (Moreno-De-Luca et al., 2011). Outsized risk factors majorly provoke the occurrence of CP and the response of these risk factors is influenced by its genetic makeup, with some genotypes creating susceptibility to cerebral damage (Gibson et al.,2008; Gibson, Maclennan, Goldwater, Haan, Priest, Dekkar, 2006). The involvement of many different types of cells in the brain makes CP a complex un reversible disorder. Genetically inherited cases are mostly autosomal recessive with glutamate decarboxylase-1 enzyme involvement however, causative pathways are poorly understood (O Callaghan, MacLennan, Gibson, McMichael, Haan& Broadbent, 2011; Colver, Fairhurst & Pharoah, 2014). Interleukin-6 (IL6) is a cell signaling molecule that has been associated with over 20 diseases, and also associated with increased risk of CP in large population studies (Moreno-De-Luca et al., 2011; AbouJamra, Philippe, Ras-Rothschild, Eck, Graf, Buchert,…Colleaux, 2011). Elevated IL-6levels within brain also predict cerebral lesions which is a major risk factor for CP in preterm infants (Duggan, Maalouf, Watts, Sullivan, Counsell, Allsop, & Edwards, 2001). Consanguinity contributes a major role of Genetic factors (Moreno-De-Luca et al., 2011 & 2012), implicating autosomal recessive mutations.

A single nucleotide polymorphism (SNP), rs1800795, in the promoter region of the interleukin-6

(IL6) gene has been implicated in the pathogenesis of CP by mediating IL-6 protein levels in amniotic fluid and cord plasma and within brain lesions. This SNP has been associated with other neurological, vascular, and malignant processes as well, often as part of a haplotype block. (Gibson et al., 2009). Khankhanian, Baranzini, Johnson, Madireddy, Nickles, Croen, Wu (2013) performed Sanger sequencing of the IL6 gene in a nested case control study of CP among term infants to identify IL6 haplotypes and mutations that predispose individuals to high risk of CP. To refine the regional genetic association with CP, sequenced (Sanger) the IL6 gene and part of the promoter region in 250 infants with CP and 305 controls. They identified a haplotype of 7 SNPs that includes rs1800795. In a recessive model of inheritance, the variant haplotype conferred greater risk (OR = 4.3, CI = [2.0-10.1], p = 0.00007) than did the lone variant at rs1800795 OR = 2.5, CI = [1.4-4.6], p = 0.002). The risk haplotype contains one SNP (rs2069845, CI = [1.2-4.3], OR = 2.3, p = 0.009) that disrupts a methylation site. They concluded that the risk haplotype identified in this study overlaps with previously identified haplotypes that include additional promoter SNPs. A risk haplotype at the IL6 gene likely confers risk to CP, and perhaps other diseases, via a multi-factorial mechanism.

Recent studies on de novo mutations in CP investigations found three different genes KCNC3, ITPR1 and SPTBN2 which had undergone partial gene deletions due to de novo mutations. They also found higher rate of rare inherited copy number variations, four autosomal and homozygous variations in the TNFRSF14, AP4M1, RGMA and NINL genes (Leemput, Chandran, Knight, Holtzclaw, Scholz, Cookson, & Singleton, 2007 and Parolin Schnekenberg, Perkins, Miller, Davies, DÁdamo, pessia,…Skehel, 2015). Ataxias are a highly heterogeneous group of neurological disorders. Four children were identified who had a working diagnosis of sporadic ataxic cerebral palsy. They also changes in three distinct genes: KCNC3, which encodes a voltage-

gatedpotassiumchannel(Kv3.3);ITPR1,whichencodesthereceptorforinositol1,4,5-trisphosphate(IP3R);and SPTBN2,which encodes b-III spectrin were found (Nemeth,Kwasniewska, Lise, Schnekenberg, Becker, Bera. & Ragoussis, 2013). The pathogenicity of the identified variants was affirmed by utilizing bioinformatics and electrophysiology. The identification of specific, pathogenic mutations recommends that a valuable method for researching CP patients be utilized such as entire exome or genome sequencing, which effectively identifies denovo changes in parents and children trio tests. Cerebral paralysis is a sporadic issue with numerous possible aetiologies, yet as often as possible thought to be caused by birth asphyxia. Four patients in the cohort with a diagnosis of ataxic cerebral paralysis were explored utilizing focused cutting edge sequencing and trio-based exome sequencing and were found to have mutations in three unique genes, all the mutations were de novo and related with expanded parental age. Some of the experiments using WES uncovered that mutations in AP4 subunits AP4S1, AP4B1, and AP4E1 alongside AP4M1 and AP4E1 is related to CP and certain development issue (Bauer, Leshinsky-Silver, Blumkin, Schlipf, Schroder, Schicks & Schols, 2012; Moreno-De-Luca et al et al., 2011). AP4-deficiency disorder shares numerous clinical highlights and assumes a key part in AP4-intervened trafficking in mental health and brain functioning (AbouJamra et al., 2011). An examination light from a consanguineous family with two children influenced by an unpredictable type of CP was conducted. The siblings had an onset at around a year of age and they were homozygous for a novel and truncating AP4M1 gene mutation related with particularly diminished AP4M1 mRNA levels, phenotype which results is complex CP. One of these variants is situated in exon 3 of the AP4M1 gene and comprised of a two base combine deletion c.194_195delAT bringing about a Frame shift mutation p.Y65Ffs*50. Sanger sequencing affirmed homozygosity for the AP4M1 mutation in the two influenced relatives. The unaffected guardians and one healthy sibling were heterozygous for the wild-typeallele. Findings from earlier reports state that

congenital abnormalities and CP are closely associated and share the genetic component in the expression of disease. Later investigations have affirmed that CP might be acquired as a Mendelian attribute caused by single gene mutations. A study conducted on set of families by segregating autosomal passive CP that is caused by a change in four domain connector protein 4 genes. The alterations in AP-4 protein complex resulted in the diverse changes in the affected individuals sharing clinical attributes like ID, difference in head sizes, short stature and spastic di-or paraplegia.

Genes associated with CP and also overlapping with different neurological disorders suggests a considerable inherent heterogeneity. This highlights the complexity of the genetic contribution to CP (Hirata, Nanda, Van Riesen, Mcmichael, Hu, Hambrock & Kalscheuer, 2013). High level heterogeneity is involved in the gene distribution pattern which reflects risk in identification of causative mutations across the CP umbrella of disorders. Functional evaluations of these variants are required to build up the contributing part of these putative pathogenic CP genes.

Whole Exome Sequencing towards Cerebral Palsy Research

Nemeth et al., (2013) taken up a pilot examine utilizing heterogeneous ataxias as a model neurogenetic issue to evaluate the presentation of cutting edge sequencing technology into clinical practice. Genetic testing cutting edge sequencing will be proficient, practical, and empowers molecular finding in many unresolved cases.

CP hosting itself with multiple heterogeneous genetic disorders making diagnostic process often long, complex and involves costlier lab investigations without a definite conclusion for the diagnosis. In such cases routine laboratory investigations may not yield the fruitful conclusions, Molecular basis of such genetic disorders need to be studied. The dawn of NGS studies involving WES, WGS, targeted sequencing revolutionized and shortened the genetic load of diagnosis;

These results will serve as a base approach for clinical neurology practice and diagnostic testing. In recent, Whole Exome sequencing has a higher incidence of detecting de novo mutations causing genetic disorders facilitating the diagnosis and treatment. In some cases, the trios study highlights the association of de novo mutations in parental generations and inheritance patterns to their progeny. Trios analyses of four patient-participants revealed three novel variants responsible for ataxic cerebral palsy; reported genes were KCN3, ITPR1, and SPTBN2 (Parolin Schnekenberg et al., 2015). Targeted capture technology using NGS resulted in nine DE novo mutations from eight different genes: PRKCG, TTBK2, SETX, SPTBN2, SACS, MRE11, KCNC3, and DARS2, from highly heterogeneous ataxia patient population (Nemeth et al.,2011).

In conclusion, recent evidence points to the genetic mechanisms as one of the pathogenic factors in other neurodevelopmental disorders, intellectual disability, and single-gene disorders. It is not uncommon for persons with a broad range of neurodevelopmental conditions to be diagnosed as CP. These are termed as the spectrum of cerebral palsy like syndromes, suggesting more than a single gene in the disease pathogenesis. The last two decades have witnessed the identification of 6 pathogenic variants responsible for monogenic forms of cerebral palsy. Owing to its prevalence and recent evidence of locus heterogeneity, CP's genetics and its spectrum disorders were poorly investigated. The present study aims to use cutting-edge genomics technology, i.e., massively parallel sequencing technology, to identify the de novo mutations in typical CP and well as the CP spectrum patient population.

METHOD

The present study aimed to identify the de novo mutations and perform the analysis of genes by genetically analyzing the blood samples of children with CP and its spectrum disorders. Children with CP in the age range of 2-15 years were considered for the study. The study was undertaken in the following three phases:

Phase I: Biological sample collection and DNA isolation,
Phase II: Library preparation and Exome Sequencing
Phase III: Data analysis

Phase I: Biological sample collection

Parents of 531 children were interviewed regarding the pre, peri and postnatal history, family history, and other related medical records and explained the study's purpose. Families were included in the study on a first-come, first-serve basis for up to a total number of 102 cases. The exclusion criteria followed are as follows.

Exclusion criteria:

1. Cases born to the mothers with intrauterine viral infections or inflammations during pregnancy or any other severe form of infections such as chorioamnionitis, meningitis, and orencephalitis.
2. Mothers with a history of alcohol consumption or using any form of tobacco during gestation.
3. Mother being exposed to any hazardous chemicals before or during the gestational period.
4. Mothers who had a history of diabetes and were under medication for the same and mothers who reported gestational diabetes anytime duringpregnancy.
5. Children born with Rhincompatibility.

6. Children born out of instrumental delivery such as forceps and ventouse delivery.
7. Any episode of trauma to the mother during the gestation period or trauma to the child's head during or after birth.
8. Any direct result of brain infections, bacterial meningitis, viral encephalitis, accidents, or injuries leads to acquired cerebral palsy.

Step A: Obtaining consent from the parents of cases considered

The Institutional ethical clearance committee cleared the study. Parents were informed and explained the study's purpose(s) and the project's identity confidentiality. Parents were informed that the 4-5 mL blood would be collected from them and their child and siblings' blood if available. Parents were requested to fill the consent form themselves and also on behalf of their children. Blood was collected from a total of 102 individuals (64 males and 38 females) who received a diagnosis of CP or its spectrum. Spectrum refers to those cases with motor deficits due to metabolic disorders and syndromic conditions.

Step B: Collection of blood and DNA Extraction

After informed consent, blood was collected from the participants through the vein puncture technique by professional phlebotomists using butterfly needles. The client was seated comfortably on the arm rested chair. In the case of a child, he/she was made to sit on the caregiver's lap or made to lie down in a supine position. Blood was collected into BD vacutainers EDTA coated (the potassium salt, or K_2EDTA), an anticoagulant, and helps prevent the blood from clotting. These Vacutainers containing blood were then stored at -20^0C until the Extraction of the DNA.

Step C: DNA isolation from blood

DNA isolation from collected blood samples was performed using commercially available DNA kits. DNA extraction Kits allow rapid and efficient purification of genomic DNA from the blood.

The isolated DNA is 20–50 kilobases in size and suitable for PCR and other downstream applications. DNA was stored in low Tris-EDTA buffer until further use at -20^{0}C.

Phase II: Library preparation and Exome sequencing

Phase II has the following steps:

A. Library and templatepreparation

B. Sequencing

Step A. Library and template preparation

The two objectives of the project need the construction of DNA libraries before sequencing. As per the first objective, a targeted panel for 67 was custom designed. (Table No: 1) The following table contains the list of the genes which are part of the targeted panel.

The Exome panel contains 13,000 genes, and all these 13,000 genes were analyzed in the present study in all 102 cases. A list of these genes was not presented in the table due to its large size. Approximately 30 ng of genomic DNA was used to prepare a custom targeted library. For whole-exome sequencing, the Ion Ampliseqexome RDY kit was used. Approximately 100ng of genomic DNA was used to construct an exome library. Library preparation is 8 hours protocol and has to be performed only by trained personnel. The detailed description of the library preparation the following link may be referred. (https://assets.thermofisher.com/TFS-ssets/LSG/manuals/MAN0017003_IonAmpliSeqLibaryKitPlus_UG.pdf). Constructed libraries were quantified using Qubit and then enriched on One Touch 2 (OT2) or Ion Chef. Ion Chef and OT2 are 12-15 hours protocols, and the detailed description of the protocol can be referred from the following links.

https://assets.thermofisher.com/TFS-assets/LSG/manuals/MAN0010857_Ion_PI_HiQ_OT2_200_Kit_UG.pdf

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0010968_Ion_PI_HiQ_Chef_Kit_QR.pdf

Step B: Sequencing

Enriched libraries were loaded onto the P1 v3 chip either manually or by automation using Ion Chef. These loaded chips were then transferred to the allocated slot on the Ion Proton Sequencer for sequencing. Sequencing is a multiple-step protocol that takes 2- 3 hours to initiate the experiment and additional 3 hours to complete the sequencing. A detailed description of these protocols can be viewed from the following link. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0010947_Ion_PI_HiQ_Seq_200_Kit_UG.pdf

After nearly 3 hours of massively parallel sequencing on Ion Proton, the generated data was transferred to the server to align the generated data to the Human Reference Genome 19 (hg19). Hg 19 is the reference human genome. All the DNA sequencing results will be aligned to hg19 to find and evaluate the mutations. The sequencing metrics are shown below from the present study are shown below. (Table No:2)

Phase III Data Analysis

A. Data analysis and Variant prioritization

Coverage analysis and variant calling were performed by the plug-ins that are present in the Software console of the server. In the method of Massively Parallel sequencing, to ensure quality and confidence of each DNA nucleotide that is sequenced, each DNA nucleotide is sequenced more than 60 times, and each time sequencing is mentioned as X. So if the coverage analysis result is 100x, this means each DNA nucleotide is sequenced 100 times. This feature is more critical in massively parallel sequencing as it tells the overall quality of the sequencing experiment. This is called coverage analysis and is performed by the coverage analysis plug-in present in the server's software console. After passing the quality coverage, i.e., minimum 60x, sequencing data was then subjected to variant calling. This was performed using a variant calling plug-in present in the software console of the server. A maximum of 39000 variants was yielded from each sample.

Variants are also called mutations, and not all mutations cause the disorder/disease. Hence to further identify which mutation causes the disorder among the identified mutations, variants were annotated to the reference genome. This process of annotation gives the physical location and functional consequence of the mutation. This process is achieved by uploading the variants file to the Ion Reporter(IR).

As the phenotype(s) under consideration follows Mendelian inheritance and mostly autosomal recessive and rarely autosomal dominant, only one or two mutations will be responsible for the cases studied' underlying pathophysiology. Whittling down approximately 39000 mutations from each sample to a single or two pathogenic or susceptible mutation was performed using various available filters present in the ion reporter cloud. Primarily two main filters were used to narrow down the variants. First, ClinVar filter was used to check how many pathogenic, probably pathogenic and variants of unknown significance were present in each case. After applying the ClinVar filter, Variants that were yielded were verified manually for their phenotype relevance

.

This filter will not report ClinVar filter sort's variants listed only as pathogenic, probably pathogenic and variants of unknown significance, novel variants not listed in ClinVar and dbSNP. To investigate whether such novel variants have a role in developing phenotypes(s) under investigation, the db SNP filter was customized to yield the variants that are not listed in db SNP. As mentioned above, variant prioritization on Ion reporter® is done using various filters available in the cloud; In the present study, the two filters used after their customization are the filter for ClinVar and filter for *De novo* variants. Variants were prioritized based on the outputs by these two filters. *De novo* variants were further analyzed for their pathogenicity based on their Grantham score if it is a missense mutation and analyzed for protein truncation in a frameshift mutation.

Effects of all established, i.e., already reported as pathogenic and hitherto unknown mutations with relevance to the phenotype, were presented and discussed in the results and discussion section. (Fig No: 1)

Instrumentation

One study highlights the instrumentation used for the current projects that are still rare in India. The technique and technology are states of the art technologies. Nearly 20 different types of instruments are used to accomplish the proposed methodology. Of all these, three instruments are of utmost importance in terms of their complex technology and the work they accomplish and are listed as follows.

- Thermal cycler
- 3500 Genetic analyzer
- Massively parallel sequencer

Thermal Cycler: Thermal cycler is the instrument that cycles the temperature with the highest accuracy in terms of heat and time and is famously known as polymerase chain reaction (PCR). After DNA isolation, DNA has to be replicated in vitro to produce a sufficient amount of copies of the desired DNA fragment in genomics as DNA is 3.3 Giga bases and cannot be duplicated under normal conditions. After the invention of Thermal cycler / PCR, now entire 3.3 billion DNA bases can be duplicated for further downstream analysis using PCR. It is a primary instrument but highly essential to perform genetic studies.

3500Geneticanalyzer: 3500 Genetic analyzer is the model name of the automated sequencer. It performs the DNA sequencing or determination of the four nucleotides (A, T, G, and C) present in the DNA. This instrument employs a method called chain termination method to sequence the DNA. This method was developed by Frederick Sanger, who received a noble prize for developing this method. The Human Genome Project was carried using the automated sequencers that work on the di-deoxy chain termination method developed by F. Sanger. This method of sequencing is also called a sanger sequencing and is considered a gold standard method.

Massively Parallel Sequencing: As indicated by its name, this method sequences the DNA massively compared to the gold standard Sanger sequencing. In Sanger sequencing, a maximum of 1000 bases per reaction can be sequenced, which is a drawback in the genomics era. With the induction of genetics in medical diagnosis, there is a need to sequence the DNA in the shortest time possible. This need innovated the massively parallel sequencing and is a massive success in medical genetics and molecular diagnosis. In the present study, Ion Proton used the MPS, which can sequence two individuals' entire protein-coding regions of the human genome (37 million base pairs x 2) in 3 hours with each DNA nucleotide read more than 100 times. In gene, protein-coding parts are called exons. The collection of all the protein-coding parts from an individual are called the exome. As mutations in the protein-coding parts (exons) of the gene cause disorders/ syndromes, this targeted sequence of the entire protein-coding part of the individual genome in a massively parallel way.

Notes

- ClinVar: ClinVar is a database of pathogenic mutations. A mutation is said to cause disease after functional studies. Such mutations were listed in the database of the Clin Var.https://www.ncbi.nlm.nih.gov/clinvar
- db SNP: db SNP stands for database Single Nucleotide Polymorphism.https://www.ncbi.nlm.nih.gov/projects/SNP/
- Library preparation, enrichment, chip loading, and sequencing protocols takes several hours with numerous steps involved. The step by step protocol is not presented in the present report as each protocol is more than 75 pages.

RESULTS AND DISCUSSION

CP is a highly heterogeneous disorder with multiple associated pathologies such as epilepsy, intellectual disability, and sensory impairment. In their expert review, MacLennan, Thompson, and Gecz (2015) opined that CP is better called as "The Cerebral Palsies" because of the apparent fact of the existence of a varying degree of disability and many pathways in its clinical spectrum, each developing into a non-progressive and specific disorder of movement and posture. Due to the broad range of clinical symptoms, there is a high chance that specific syndromes and clinical conditions mimic CP (Lee, Poretti, Cohen, Levey, Johnston, & Fatemi, 2014). When not differentially diagnosed, such conditions are considered as CP or one among its spectrum of disorders. With the recent developments in technology and medical genetics, clinicians worldwide can provide an appropriate diagnosis for a broad range of neurodevelopmental disorders, further planning relevant therapeutics. The state of the art technology, i.e., exome sequencing, is widely used in this process across the globe, opening windows for differential diagnosis for a broad range of congenital disorders. Based on disorder type and selection of the patient, it has been approximated that exome sequencing can yield a diagnostic rate of 30% to 50% for Mendelian disorders*;* Veeramah, Johnstone, Karafet, Wolf,Sprissler, Salogiannis & Hammer, 2013*;* Clark, Stark, Farnaes, Tan, White, Dimmock, & Kingsmore, 2018) and thus ending diagnostic odyssey and opening windows for appropriate therapeutics in individuals with rare disorders. There is a lack of literature from India, whether exome sequencing can be effectively used as a diagnostic tool in the CP population with delayed speech and language. Hence, the present study "Genetic insights of cerebral palsy using massively parallel sequencing" is first of its kind, taken up by any speech and hearing organizations that diagnose or provide rehabilitation for the cerebral palsy population.

Biological samples from 102 (64 males, 38 females) children with CP were collected on a first-come, first-serve basis who willingly agreed to participate after understanding the study's purpose. The Institutional ethical committee approved the study of AIISH, Mysuru. The two main objectives of the present study were

1. To analyze the known genes in children with CP and its spectrum.
2. To perform exome sequencing in children with CP and its spectrum to identify the de novo mutations.

As part of the first objective, nearly 80 known genes that fell in the neurobiology pathway were sequenced. The study could identify no mutations from the genes that contribute to the phenotypes in the cases analyzed, i.e., no pathogenic or *de novo* mutations that can be pathogenic.

As part of the second objective, Genomic DNA from 100 children with CP was subjected to exome sequencing covering ~13000 genes in each case. This process yielded

~38,000 mutations in each sample. These mutations were further subjected to prioritization to identify the underlying pathogenic mutations. The prioritization process is time-consuming, as mutations that are not listed as pathogenic or mutations in the genes that are so far not attributed to causing neurodevelopmental disorders have to review their possible pathogenicity. This review is performed studying the gene's biochemical properties, their involvement in the brain physiology pathways, and results of knockout studies of the genes available, if any. This process is manual, and approximately 100 to 200 genes have to be manually verified in each sample. The below table shows the total number of genes that were identified in each sample by exome sequencing. (Table No: 3)

Variant Prioritization

As mentioned, variant prioritization on Ion reporter® was done using various filters in the cloud. In the present study, the two filters mainly used after their customization were filters for ClinVar and filters for *De novo* variants. Variants were prioritized based on the outputs by these two filters. The identified mutations that were pathogenic or probably pathogenic in terms of mutation severity and their relevance to the phenotypes studied have been listed in table 4. (Figure No: 3) explains the flow of variant prioritization.

Using exome sequencing, nearly 29% of the diagnostic yield was achieved: The Study used information from the databases like online inheritance in man (OMIM) and database SNP (db SNP), and CLINVAR. Using information from these databases, in nearly 29 cases, the diagnosis can be further fine-tuned based on the mutations they possess. The 29 cases' details, the mutations identified, and the exact disorder identified have been described below.

Case No.1 CP018A

CP018A was a 4 years old male with an elder female sibling born out of consanguineous marriage. A detailed evaluation at the institute received was acquired microcephaly, ataxic CP, Global developmental delay (GDD), severe intellectual disability with normal hearing and vision. Brain imaging indicated white matter demyelination. Exome sequencing identified a missense mutation in the PYCR2 gene. Identification of mutation helped in the definitive diagnosis of the case from CP, GGD to LEUKODYSTROPHY HYPOMYELINATING type10.

Case No.2 CP014A & CP014A1

CP014A and CP014A1 were two male siblings aged 6 and 4 years diagnosed as the Dandy – walker variant. Their hearing was normal; muscle tone was average to hypotonic with inaccurate gait

pattern. Detailed valuation at the institute was ataxic CP below-average intelligence. Exome sequencing has identified a frameshift mutation in the gene APTX. Pathogenic mutations in the gene APTX will cause ATAXIA, EARLY-ONSET, WITH OCULOMOTOR APRAXIA AND HYPOALBUMINEMIA. Hence, the novel frameshift mutation identified in the APTX gene was suggestive of the condition ataxia, oculomotor apraxia with hypoalbuminemia in the siblings.

Case No.3 CP069A

In the case CP069A, the finding from MRI of the brain was suggestive of Joubert syndrome, which was diagnosed by a neurologist. The client also had bilateral severe to profound hearing loss. As reported in the literature in none of the Joubert syndrome cases, hearing loss was associated with pathology. Exome sequencing identified a missense mutation in the AH1 gene, which causes JOUBERT SYNDROME 3. Sequencing also identified a nonsense mutation in the gene GJB2, explaining the hearing impairment in the client. Both mutations are listed in db SNP and CLINVAR as pathogenic. This is a very peculiar case. If the exome sequencing had not been performed for this case, there is a high probability that the condition may be considered as a new syndrome or a new subgroup of Joubert syndrome with hearing impairment.

Case No.4 CP062A

CP062A is a three-year-old male born out of full-term normal delivery, however, kept in the neonatal intensive care unit due to unconjugated hyperbilirubinemia. Further biochemical analysis revealed G6PD deficiency. His intelligence is found to be below average with developmental delay and mild hearing loss with auditory neuropathy. Exome sequencing identified a pathogenic mutation in the gene G6PD, indicating the case condition as G6PD deficient hemolytic anemia. Sequencing also identified a possibly pathogenic mutation or mutation of unknown significance in the gene GJB2 clarifying the client's mild hearing loss and Auditory neuropathy.

Case No. 5 CP079A

CP079A is a female child born to parents whose marriage was consanguineous. A pregnancy scan in the eighth month had revealed delayed development and microcephaly. The evaluation at the clinical services department at the age of 8 years revealed the head circumference as 40.5 cm, which suggests severe microcephaly. Genetic analysis by exome sequencing revealed a novel frameshift mutation in the ASPM gene, which causes primary microcephaly type 5. In this case, the exome sequencing possibly indicated that the microcephaly was due to mutation identified in the gene ASPM.

Case No.6 CP036A

CP036A is one of the male monozygotic twins born to parents whose marriage was non-consanguineous with a clinically normal sibling. Delivery was premature and by C-section. The client had a history of NICU admission for 15 days for neonatal jaundice and breathing difficulties. The neurological impression after the detailed evaluation was diplegic CP with intellectual disability. The client's right eye had strabismus and was reported to be having bilateral severe hearing loss. One of the crucial NICU observations was severe hypoglycemia. Exome sequencing revealed a heterozygous nonsense mutation in the gene ABCC8, which suggests the client's condition as leucine sensitive hypoglycemia of infancy. Different treatments are available for this condition if differential diagnosis of the type of hypoglycemia (leucine sensitive in this case) may reduce further brain biochemistry damage.

Case No. 7 CP045A

Case CP045A was aged 3.5 years' female who received an initial diagnosis as a west syndrome. Her parent's marriage was non- consanguineous, and her younger female sibling has clinically normal speech and hearing skills. The very first episode of seizures was reported at the age of 8 months. After the detailed evaluation, the institute's diagnosis was seizures disorder with developmental delay and moderate intellectual disability. The types of seizures are myoclonic, and the client is under ACTH medication for the same since the first episode. Genetic analysis revealed a novel nonsense mutation in the gene ADRA2B, probably suggesting myoclonic epilepsy type 2. Exome sequencing has nearly confirmed that the epilepsy was due to a nonsense mutation in the geneADRA2B.

Case No. 8 CP093A

Case CP093A was a five-year-old girl with a history of NICU admission following preterm delivery complications, low birth weight, seizures, and neonatal jaundice. The impression made was spastic cerebral palsy following a detailed evaluation. The biochemical diagnostic analysis revealed Glutaricaciduria, type I. Exome sequencing identified one missense and one nonsense mutation in the gene GCDH, which the client inherited one from each parent, and the condition is called compound heterozygosity (disease/disorder caused by two individual mutations from the same gene).

Case No.9 CP077A

Case CP077A was a 3-year-old female child carrying a diagnosis of cerebral palsy (diplegic) with intellectual disability. Other notable pathologic features include hypertonic muscle tone,

bilateral moderately severe sensorineural hearing loss, and equinus deformity. Exome sequencing identified a novel heterozygous frameshift mutation in the DNMT1 gene suggesting CEREBELLAR ATAXIA, DEAFNESS, AND NARCOLEPSY.In this case, the exome sequencing results revealed a different ataxic CP diagnosis instead of spastic CP.

Case No.10 CP043A

Case CP043A was a three-year-old male child who carried a diagnosis of cerebral palsy (Spastic Diplegic) with hypertonic muscle tone, average intelligence, and normal hearing and visual sensitivity. He had a history of premature birth at six and a half months and has a NICU history of 5 days for neonatal jaundice. Exome sequencing identified a missense mutation in the GLI2 gene. This mutation was listed as pathogenic and is designated to cause HOLOPROSENCEPHALY 9.

Case No.11 CP066A

Case CP066A is a 4.8-year-old female child born FTND with normal birth weight. The child had NICU history due to congenital heart ailment. MRI showed cerebellar hypoplasia and postero inferior vermis hypoplasia. A detailed evaluation revealed quadriplegia, microcephaly, global developmental delay, and intellectual disability. An additional notable feature in the client was Bilateral hearing loss. Exome sequencing identified a frameshift mutation in the CASK gene, which is reported to cause MENTAL RETARDATION AND MICROCEPHALY WITH PONTINE AND CEREBELLARHYPOPLASIA.

Case No.12 CP102A

Case CP102A is 3.6 years old male child born to a couple out of consanguineous marriage. The client received multiple diagnoses like axonal neuropathy, infantile motor sensory neuropathy, and spastic CP. Exome sequencing identified a nonsense mutation in the gene ARSA mutation, which

is suggestive of METACHROMATIC LEUKODYSTROPHY. This nonsense mutation was identified as pathogenic in Clinvar; the client's diagnosis will be METACHROMATICLEUKODYSTROPHY.

Case No.13 CP051A

Case CP051A is a 3-year-old male child who has elder and younger siblings with clinically normal speech-language and motor skills. The client's diagnosis was dyskinetic cerebral palsy with mild intellectual disability. Other comorbidities included bilateral severe hearing loss. Exome sequencing identified a novel frameshift mutation in the gene NIPBL, which is likely to cause Cornelia de Lange syndrome1.

Case No.14 CP021A

Case CP021A is a 9 years old male with an elder brother having a history of seizures. The client was born out of full-term normal delivery, normal birth cry. However, he had the first episode of seizures at the age of 2 years. The client's diagnosis was a seizure disorder with spastic cerebral palsy with mild intellectual disability. On further evaluation, the seizure was categorized as febrile seizures. Exome sequencing identified a novel frameshift mutation in the gene MAG, causing SPASTIC PARAPLEGIA 75, AUTOSOMALRECESSIVE.

Case No.15 CP044A

CaseCP044Aisafour-year-oldfemalechildwhoreceivedadiagnosisofSpasticcerebralpalsy.

– Quadriplegia. EEG and MRI were abnormal, showing bilateral multifocal epilepsy and Hemorrhagic necrosis of white matter and thinning the corpus callosum. Other congenital anomalies included were Incontinentiapigmenti. Family history was positive for seizure as both father and paternal grandfather were reported to have a seizure disorder. Molecular genetics

evaluation identified a mutation in the CACNA1A gene, which causes EPILEPTIC ENCEPHALOPATHY AND SPINOCEREBELLAR ATAXIA 6, the autosomal dominant mode of inheritance.

Case No.16 CP032A

Case CP032A is an 11 years old female child born to parents of non- consanguineous marriage. The client had a NICU history due to premature delivery (low birth weight 772 grams) and delayed birth cry. The first episode of seizures was recorded at 2ndday of birth. She was diagnosed as Cerebral Palsy – Quadriplegia, moderate mental retardation, diplegic gait, and squint eye. Exome sequencing identified a frameshift mutation in the gene CACNA1G. Pathogenic mutations in this are known to cause SPINOCEREBELLAR ATAXIA 42, EARLY- ONSET, SEVERE, WITH NEURODEVELOPMENTALDEFICITS.

Case No.17 CP019A

Case CP019A was a four and half-year-old female with an initial diagnosis of CP and moderate intellectual disability. She was born out of full-term normal delivery and had a history of NICU admission due to birth asphyxia, stage II HIE, neonatal seizures (febrile). Exome sequencing identified multiple mutations that may play a potential role in the underlying Pathophysiology in the client. However, the most logical mutation relevant to the phenotype was identified in the gene NDUFAF6. Mutations in this gene are said to cause LEIGH SYNDROME DUE TO MITOCHONDRIAL COMPLEX I DEFICIENCY.

Case No.18 CP057A

CP057A was a 7-year-old male born to parents whose marriage was consanguineous. The client was diagnosed as CP (spastic), intellectual disability. He had normal sensory abilities.

The client's elder sibling had clinically normal speech and hearing skills. Exome sequencing identified a nonsense mutation in the gene DDHD2; pathogenic mutations were found to cause SPASTIC PARAPLEGIA 54, AUTOSOMAL RECESSIVE. The identified mutation was earlier reported in db SNP, Clinvar.

Case No.19 CP039A

CP039A was a 12-year-old female born to parents whose union was consanguineous; she had a premature delivery (8½ months). Her initial diagnosis was cerebral palsy with mild intellectual disability, inaccurate gait pattern, probably due to bilateral fixed knee. Hearing and vision were reported to be expected. Genetic analysis identified a missense mutation in the gene PMM2; pathogenic mutations reported to cause CONGENITAL DISORDER OF GLYCOSYLATION, TYPE IA, a metabolic disorder. Segregation analysis confirmed the autosomal recessive pattern of inheritance with both parents identified as carriers.

Case No.20 CP026A

Born to parents whose marriage was not consanguineous, Case CP026A was a 6-year-old male diagnosed as diplegic cerebral palsy and partial focal epilepsy. There was a history of premature delivery at 8^{th} month, with a low birth weight of 2.3kgs along with delayed birth cry and respiratory distress. The child also had a history of NICU for hyperbilirubinemia and pneumonia. Exome sequencing identified a missense mutation in the gene ACSF3; pathogenic variants reported to cause COMBINED MALONIC AND METHYLMALONIC ACIDURIA. The mutation identified in the case is listed as pathogenic in db SNP and clinvar, hence providing a definitive diagnosis for the client as COMBINED MALONIC AND METHYLMALONIC ACIDURIA, which is a metabolic disorder.

Case No.21 CP023A

CP023A was an 8-year-old female diagnosed with CP, seizures and moderate intellectual disability with positive family history. The child was born out of a full-time normal delivery with a normal birth cry. The first episode of seizures was at 7 months of age; hence, she was on antiepileptic drugs. Sequencing identified a missense mutation in the gene ATP1A3. This mutation identified was established mutation and listed in the db SNP and Clin-var as a pathogenic variant and reported to cause ALTERNATING HEMIPLEGIA OF CHILDHOOD 2?, CAPOS SYNDROME?, DYSTONIA-12?. After a genetic analysis, the case's most probable diagnosis could be ALTERNATING HEMIPLEGIA OF CHILDHOOD 2 as per theclin-var.

Case No.22 CP094A

Case CP094A was a 6-year-old born to parents whose marriage was consanguineous. The initial diagnosis made after a detailed evaluation was cerebral palsy (spastic) with moderate intellectual disability. The client had a history of delayed birth cry, and the client was under medication for seizures. Exome sequencing identified a missense mutation in the gene ATP1A3. The mutation identified was established mutation and listed in the db SNP and Clin-var as a pathogenic variant. Pathogenic mutations in the gene ATP1A3 are reported to cause ALTERNATING HEMIPLEGIA OF CHILDHOOD 2?, CAPOS SYNDROME?,DYSTONIA-

12?. After a genetic analysis, the most probable diagnosis for the case is ALTERNATING HEMIPLEGIA OF CHILDHOOD 2 as per theclin-var.

Case No. 23 CP034A

Case CP034A was a 3-year-old male diagnosed with cerebral palsy (diplegic)with

severe intellectual disability and global developmental delay. The client has a history of NICU admission due to neonatal jaundice. The client's vision and hearing were reported tobe normal.

Parents union was non-consanguineous. Exome sequencing identified a novel heterozygous nonsense somatic mutation in the gene SLC35A2; mutations reported to cause CONGENITAL DISORDER OF GLYCOSYLATION, TYPE IIM.

Case No. 24 CP074A

Case 074A was a 4-year-old male born to parents whose marriage was consanguineous. He received a CP diagnosis with mental retardation and developmental delay, hypotonic muscle tone, and club foot. Vision and hearing were reported to be normal. Exome sequencing identified a frameshift mutation in the gene DVL4, mutations in which cause ROBINOW SYNDROME.

Case No. 25 CP012A

CP012A was a 6-year-old female with clinically normal monozygotic twin. At the age of the eighth month, she received a diagnosis of developmental delay and microcephaly. Further neurologic and pediatric diagnosis is CP (spastic), GDD, microcephaly, and facial dysmorphism. Sequencing identified a novel frameshift mutation in the gene KMT2C, mutations in which cause KLEEFSTRA SYNDROME2.

Case No. 26 CP046A

CP046A was a 10-year-old male who received a diagnosis of CP (quadriplegia) with profound Intellectual disability. Radiological findings showed deformity over both hands and legs. The hearing was reported to be normal; however, the vision was reported to be poor. Exome sequencing identified a novel frameshift mutation in the gene HDAC6, mutations in which cause CHONDRODYSPLASIA WITH PLATYSPONDYLY, DISTINCTIVE BRACHYDACTYLY, HYDROCEPHALY, ANDMICROPHTHALMIA.

Case No. 27 CP016A

CP016A was a 4-year-old female diagnosed with CP (diplegic) with mild intellectual disability and bilateral severe hearing loss. Exome sequencing identified a nonsense mutation in the gene ATM and a nonsense mutation in the gene MYO6. Mutations ATM gene are known to Ataxia Telangiectasia, and nonsense mutations in MYO6 cause sensorineural hearing loss. This clarifies the existence of two independent phenotypes in the case each developed by a different gene.

Case No. 28 CP103A

CP103A was a 4-year-old male diagnosed with CP (Spastic diplegia), GDD, and delayed speech with fine motor and cognitive delay. The case was reported to be having ankle spasticity and contractures. Electrophysiological tests were suggestive of normal lower limb peripheral nerve conduction. His brain imaging by MRI was nearly normal, and also his plasma amino acids were normal. Exome sequencing identified a missense mutation in the gene SPAST, where pathogenic mutations in this gene are established cause for SPASTIC PARAPLEGIA 4.

From the above, it is evident that the genetic intervention by the state of the art exome sequencing was able to uncover the mutation responsible for the clinical manifestations in the subset of cases studied. The 26 cases discussed above have now been segregated into different categories, i.e., different neurogenetic disorders (and not CP and its spectrum alone) based upon the mutation they possess.

- **Metabolic disorders:** The present study could genetically identify six different metabolic disorders, i.e., Glutaricaciduria (CP093A), G6PD deficiency (CP062A), CongenitaldisordersofglycosylationtypeIA(CP039A),Congenitaldisordersof

glycosylation type IIM (CP034A), Combined malonic and methylmalonicaciduria (CP026A) and hypoglycemia of infancy leucine-sensitive (CP036A) in the particular children with CP. These are purely metabolic disorders and shall not be diagnosed as cerebral palsy. However, they are diagnosed as CP may be due to the assessment of only phenotypic characteristics but not biochemical (except CP062A). Except for the CP062A, whose condition is G6PD deficiency, the rest of the metabolic disorders are rare disorders. It is now clear that such rare metabolic disorders can be genetically diagnosed using exome sequencing, which can end a long diagnostic journey.

- **Syndromes:** CP encompasses a wide range of neurodevelopmental conditions, which are also often seen in syndromes. Among phenotypes that are generally shared among the classical CP and syndromes include spasticity, delayed speech and language, seizures, gait abnormalities, and few others. The present study identified five syndromes that phenotypically resemble CP, and they are as follows. Kleefstra syndrome 2 (CP012A), Robinow syndrome (CP074A), Leigh syndrome due to mitochondrial complex 1 deficiency (CP019A), Joubert syndrome 3 (CP069A), Cornelia de Lange syndrome 1 (CP051A). The above conditions/cases are often misdiagnosed as they do not exhibit unique clinical manifestations, which are generally observed among syndromes like Usher syndrome, Down's syndrome, and Klienfilters syndrome, Waardenburg syndrome, etc. Using exome sequencing, this study could identify the underlying mutations in five cases.

- **Leukodystrophies:** These are the group of disorders characterized by white matter degradation in the brain. Unlike CP, leukodystrophies have a life span of 2-8 years or slightly more. The present study identified two different types of leukodystrophies, one in

each case. CP018A and CP102A were earlier diagnosed as Quadriplegic CP and motor-sensory neuropathy with spastic CP, respectively. Interventions by molecular genetics technique have identified a pathogenic mutation in these cases, as Hyepmyelinating leukodystrophy-10 and metachromatic leukodystrophy, respectively.

- **Cerebral Palsy and its spectrum:** Twelve cases were segregated into this category after exome sequencing. Their phenotype nomenclature, as per the identified mutation, is as follows. Ataxia, early-onset with oculomotor apraxia and hypoalbuminemia (CP014A, CP014A1), Spastic paraplegia 75 (CP021A), Alternating hemiplegia of childhood (CP023A, CP094A), Spinocerebellar ataxia 42 early-onset severe with neurodevelopmental deficits (CP032A), Holoprosencephaly 9 (CP043A), Epileptic encephalopathy and spinocerebellar ataxia 6 (CP044A), Myoclonic epilepsy familial adult (CP045A), Spastic paraplegia 54 (CP057A), Mental retardation and microcephaly with pontine and cerebellar hypoplasia (CP066A), Cerebellar ataxia deafness and narcolepsy(CP077A).

- **Dysplasia:** Case CP046A is a single isolated case with multiple developmental pathologies. Exome sequencing delineated the case's diagnosis as Chondrodysplasia with platyspondyly, distinctive brachydactylic, hydrocephaly, and microphthalmia, which is distinct from CP.

UNEXPECTED FINDINGS

In a sample of 102 cases, 17 cases (16.6 %) were reported to have congenital hearing loss. From an Audiological perspective, these cases are usually considered syndromic hearing

loss due to associated comorbidities. Cases that qualify this syndromic hearing loss category are a) CP051A, whose diagnosis is Cornea de Lange Syndrome 1, and hearing loss is seen among 40% of this syndrome population. b) CP077A, whose diagnosis is Cerebellar ataxia deafness and narcolepsy. These two cases cannot be considered rare or unexpected outcomes, but the cases that drag the attention are CP069A, CP016A, and CP062A, where hearing loss is an independent phenotype. CP069A harbors a pathogenic mutation from the gene AIH1that responsible for Joubert syndrome and possesses a pathogenic mutation from the GJB2 gene addressing the hearing loss. Whereas in the case CP016A, exome sequencing identified a mutation in the gene ATM explaining the underlying neuropathology; simultaneously, exome sequencing also identified a mutation in the gene MYO6 that is responsible for hearing loss in the case. Further, in the case, CP062A exome sequencing identified a pathogenic mutation in the gene G6PD responsible for the case's neurodevelopmental phenotype and a mutation in the gene GJB2 explain hearing impairment. Hence, hearing loss in these three cases is not syndromic as hearing loss was contributed by entirely different genes that have no role in the neuropathology in these three cases. These three cases explain the robustness of exome sequencing in identifying the molecular cause of the monogenic disorders (Mostly CP and hearing loss are inherited in Mendelian fashion of inheritance). If exome sequencing was not performed, hearing loss among these two cases might be diagnosed as syndromic hearing loss. Whether hearing loss is syndromic or non-syndromic, the process of audiological assessment and planning suitable interventions to recuperate hearing may not mainly differ in such cases. However, these findings contribute to the science of audiology, genetic and pre-marital counseling for the families at risk for recurrence of these two phenotypes

(neurodevelopmental and hearing loss) in a single person that was contributed from two independent biological pathways.

CP is a highly heterogeneous condition having more than one clinically recognizable phenotype. These include epilepsy, variable severity of intellectual disability, impairment of vision or hearing, variable physical disability, and different brain imaging patterns concerning neuropathology. These multiple morbidities are not limited to CP and are seen as constellations findings across numerous syndromes and disorders. Especially when taken into account muscle tone, physical disability, hypotonicity, hypertonicity, spasticity, and ataxia, these traits are seen across many neurodevelopmental disorders. Using exome sequencing, the present study has identified pathogenic mutations in the genes that are already scientifically proven to cause different disorders related to metabolic, brain movement, and posture opening windows for differential diagnosis or qualified to receive an alternative diagnosis after the molecular analysis. To further discuss and clarify, CP 018A having notable phenotypes hypo tonicity, spastic quadriplegia, and severe intellectual disability. It is natural to be diagnosed as CP, as CP and myelin disorders exhibit overlapping traits like spasticity, quadriplegia, and intellectual disability. This case condition is a primary white matter disorder that falls under Leukoencephalopathies' class after a molecular genetic analysis. The same is the case for all the cases discussed in this section as they possess a genetic mutation explaining the underlying pathology. These cases received a diagnosis as CP purely based on clinically recognizable phenotypes, which are also seen among numerous other disorders.

SUMMARY AND CONCLUSIONS

Affecting nearly 1 in 500 children, cerebral palsy is one of the significant neurodevelopmental disorders. Environmental factors and birth asphyxia are generally attributed to be the reason behind CP until recent (Moreno-De-Luca et a., 2012), and a recent addition to one of the causative factors behind cerebral palsies are genetic factors. In the last decade, hundreds of rare pathogenic mutations were discovered that were either responsible for the causation of CP or developed into a phenotype that masqueraders as CP. Using state of the art tool, i.e., Exome sequencing, the present study aimed to identify pathogenic or possibly pathogenic variants from the human genome in 102 individuals who received a diagnosis as cerebral palsy or its spectrum and the present work could identify pathogenic or probably pathogenic mutations that are responsible for the underlying phenotype in twenty-seven cases opening windows for differential diagnosis. Pathogenic mutations were not found among 76 cases from the present study. This does not clarify that there are no genetic factors involved behind the neuropathology in these cases, as the study only reported mutations from the genes that have been reported so far. This study does not report mutations of unknown significance, a mutation in the gene which was not earlier identified as causative factors because these mutations are yet to be validated scientifically for their pathogenicity.

India has exceptional population diversity categorized into approximately 5000 well-defined population groups, the majority of which are endogamous communities. Especially in Southern India, founder effects, inbreeding, and consanguinity would have led to increased pathogenic mutations' frequency leading to a high carrier frequency of pathologic mutations and high occurrence of recessive forms of CP. In our present study, 30% of the parent’s marriages were consanguineous. Further, out of 26 cases in which mutation is identified in relevance tothe

phenotype, ~31% (8 out of 26) of the parents had a consanguineous union. Consanguinity and breeding increase homozygosity, genetic load, and disease burden (Fareed&Afzal, 2016). Two examples for this notion from the present study are CP018A, CP057A, and CP102A, who possess pathogenic mutations that cause Hyepermyelinating leukodystrophy-10 (HLD10), spastic paraplegia type 54 (SPG 54), and Metachromatic Leukodystrophy (MLD) whose incidence is extremely rare with only 6 individuals with HLD10 identified so far (Nayakama, Al-Maawali, El-Quessny, Rajab, Khalil, Stoler,…Mochida, 2015 ; Zaki, Bhat, Sultan, Issa, Jung, Dikoglu,…Gleeson, 2016), both MLD and SPG54 with incidences rate <1 per 1 million. Although extremely rare, these cases received two copies of the defective mutation, one from each parent and siblings identified as carriers. This explains the role of consanguinity in the incidence of rare disorders more often than bychance.

Progressive metabolic disorders, whether treatable (Leach, Shevell, Bowden, Stockler-Ipsiroglu , & Karnebeek, 2014) or untreatable, pose a significant challenge to speech-language pathologists, physiotherapists, and occupational therapists due to the oblivious fact that these disorders, unless managed medically, the therapy will be ineffective due to increasing neuropathology in the brain. Seizures, mental retardation, hypo/hyper tone are few among the clinical conditions exhibited by individuals with metabolic disorders. Such individuals generally receive a diagnosis as CP due to their phenotypic variability. There are nearly 500 inborn metabolic disorders (Jorde, Carey, Bamshad, & Whote, 2003; Baric, Fumic, & Hoffmann, 2001), and it is not always possible to biochemically analyze all of them. Analyzing by inclusion and exclusion criteria is not only expensive but is a diagnostic odyssey. In such instances, diagnosis by exome sequencing is a time and cost-effective (Shakiba & Keramatipour, 2018). The three extremely rare metabolic disorders that are identified in the present study are a congenital disorder of glycosylation, type Ia (CP039A),

combined malonic and methylmalonicaciduria (CP026A), Congenital disorder of glycosylation, type IIm (CP034A).

Similarly, syndromes with CP like clinical manifestations resulting from a single mutation will not be identified by regular chromosomal testing, i.e., conventional karyotyping or by advanced fluorescent technique, i.e., Fluorescent in-situ hybridization (FISH) and often receive a diagnosis as CP due to their phenotypic and trait resemblance with CP. This is because they may not be due to large chromosomal anomalies but arise because a single mutation and conditions consume a large amount of time before reaching an appropriate diagnosis. Two classic examples from the present study are Robinow syndrome (CP074A) and Kleefstra syndrome (CP012A). Similarly, the case CP046A (with deformed legs and hands), Chondrodysplasia, received Quadriplegic CP, and profound intellectual disability.

Only 12 out of 26 cases (46%) fall under the CP, and its spectrum after exome sequencing, and the remaining 54% of the cases possess a phenotype that mimics CP. The diagnosis of these 54% cases as CP cannot be probably considered as misdiagnosis, as a diagnosis is made with observed phenotype. With the recent developments in the area of genetics and massively parallel sequencing, now there is a provision of making a molecularly confirmed diagnosis promptly for better disease management, and exome sequencing is widely used in this process, which is evident from the literature (Veeramah et al.,2013; Clark et al.,2018). Adapting genetic testing as a first-line diagnostic test, i.e., performing molecular diagnosis parallel with phenotyping, is required for pediatric neurodevelopmental disorders. This work supports genotype first approach by employing exome sequencing as a first-line diagnostic tool parallel to clinical phenotyping among the neurodevelopmental disorders to provide appropriate information regarding the reason behind the

underlying pathology of the client to credentialed paramedical professional like speech-language pathologists who are concerned with speech, language development, human communication, and related disorders.

Limitations of the Study

- The sample analyzed is homogenous as the samples represent only a limited geographic area of Karnataka and are collected from a single-center, i.e., AIISH, Mysuru. Also, the sample size is minimal, as only 100 cases were analyzed. A large sample size may provide more insights into the genetics of CP and its spectrum of disorders.

- As per the data collected from the case files, it is noted that most of the cases did not undergo biochemical/metabolic investigation or brain imaging results. It was difficult to correlate the genetic test results in a subset of cases with the non- availability of medical investigation reports.

- The study used medium-throughput instruments, which consumed a considerable amount of time in processing samples as only two samples can be processed at the given time.

Clinical implications

- The study may not have any direct clinical implications. However, using new Exome sequencing as a tool, the study showed how unpredicted metabolic disorders could be detected in individuals with neurodevelopmental disorders.

The study also contributed to the delineation of phenotypes that are diagnosed as CP. Genetic diagnosis helps in separating metabolic disorders, progressive disorders, and syndromes from CP. Sensitizing SLPs, physiotherapists, occupational therapists about the available genetic tests, and the differential diagnosis outcome is significant to plan for appropriate speech, language, physio, and occupational therapy

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APPENDIX I

Abbreviations used in the report

ABCC8- ATP Binding Cassette, Subfamily C, Member 8

ACSF3- ACYL CoA Synthetase Family, Member 3

ADRA2B- Alpha-2b-Adrenergic Receptor

AHI1- Apnea

APTX- Aprataxin

ARSA- Arylsulfatase A

ASPM- Abnormal Spindle-like, Microcephaly-Associated

ATM- Ataxia Telangiectasia Mutated Gene

ATP1A3- ATPase, Na+/K+ Transporting, Alpha-3 Polypeptide

CACNA1A- Calcium Channel, Voltage-Dependent, P/Q Type, Alpha-1a Subunit

CACNA1G- Calcium Channel, Voltage-Dependent, T Type, Alpha-1g Subunit

CASK- Calcium/Calmodulin-Dependent Serine Protein Kinase

CNS- Central Nervous System

CNV- Copy number variants

CP- Cerebral Palsy

dbSNP: Single Nucleotide Polymorphism Database

DDHD2- DDHD Domain-Containing Protein 2

DNA- Deoxyribonucleic acid

DNMT1- DNA Methyltransferase 1

DVL1- Dishevelled 1

G6PD- Glucose-6-Phosphate Dehydrogenase

GCDH- Glutaryl-Coa Dehydrogenase

Genome Genetic material of organism

GJB2- Gap Junction Protein

GLI2- Gli-Kruppel Family Member

HDAC6- Histone Deacetylase 6

KMT2C- Lysine-Specific Methyltransferase 2c

MAG- Myelin-Associated Glycoprotein

Mutation: Change in the structure of a gene, caused by the alteration of single base units in DNA.

MYO6- Myosin VI

NDUFAF6- NADH Dehydrogenase (Ubiquinone) Complex I, Assembly Factor 6

NGS- Next generation sequencing

NIPBL- Nipped-B-Like

OMIM- Online Mendelian Inheritance in Man

PCR- Polymerase chain reaction

PMM2- Phosphomannomutase 2

PYCR2- Pyrroline-5-Carboxylate Reductase 1

RNA- Ribonucleic acid

SLC35A2- Olute Carrier Family 35 (Udp-Galactose Transporter), Member 2

SPAST- Spastin

WES- Whole Exome Sequencing

WGS- Whole Genome Sequencing

APPENDIX II

GLOSSARY

- **Autosomal dominant**: A pattern of inheritance in which an affected individual has one copy of a mutant gene and one normal gene on a pair of autosomal chromosomes.
- **Autosomal recessive:** A genetic condition that appears only in individuals who have received two copies of an autosomal gene, one copy from each parent.
- **Deoxyribonucleic acid**

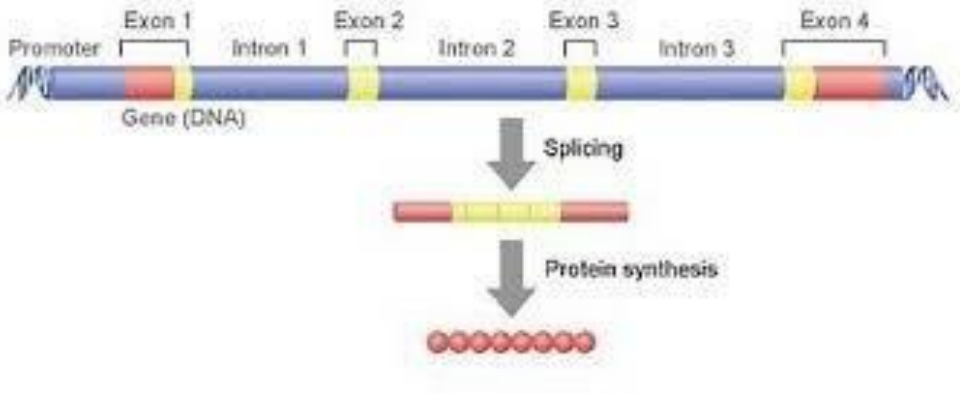
  (DNA): an extremely long macromoleculethat is the main component of chromosomes and is the material thattransfers genetic characteristics in all life forms.

- **Dizygotic:** "non-identical twins", will always have different genetic material on each chromosome, due to chromosomal crossover during meiosis.

- **DNA Library:** A DNA library is a collection DNA fragment of interest for the study.
  - **Epigenetic:**The study of heritable changes that occur without a change in the DNA sequence.
- **Exome*:*** the portions of a gene or genome that code information for protein synthesis; the exons in the human genome**.**

- **Exome sequencing:**

- Any gene is mainly divided into two parts, namely the *Intron* and *Exon*. During the process of protein synthesis from genes, all the intron of the genes will be removed and all the exons would join together to form proteins.



- So, exon is the part mainly used in the process of protein synthesis. In case of any mutations in the exons, the integrity of the protein is compromised and this leads to various disorders and syndromes. DNA sequencing means determining the order of nucleotides (ATGC) in a strand of DNA.
- DNA sequencing is a method to study the difference in the sequence of DNA nucleotides. To uncover the reason behind disorders where genetic reason is suspected, DNA sequencing will be performed.
- As majorly mutations in the exons will cause the diseases, usually exons are sequenced to identify the disease causing mutations. This is usually a very time consuming process. The best economic and time saving alternate process is massively parallel sequencing of all the exons using next generation sequencer.
- When an experiment is created that contains exons of thousands of genes, the collection of all these exons are called as exome. Exome sequencing means sequencing of all exons (protein coding regions) of thousands of genes in a single experiment. In our project under the exome sequencing experiment we sequenced exons from 13,000 genes.

- **Exons:** Any portion of an interrupted gene that is represented in the RNA product and is translated into protein.
- **Genetics:** Genetics is the branch of science concerned with genes, heredity, and variation in living organisms.
- **Genome:** A full set of chromosomes; all the inheritable traits of an organism.
- **Heredity:** The transmission of genetic characters from parents to offspring: it is dependent upon the segregation and recombination of genes during meiosis.
- **Heterozygous:** Having dissimilar pairs of genes for any hereditary characteristic of or relating to a heterozygote.
- **Homozygous:** Having identical pairs of genes for any given pair of hereditary characteristics of or relating to a homozygote.
- **Introns:** A noncoding segment in a length of DNA that interrupts a gene-coding sequence or nontranslated sequence, the corresponding segment being removed from the RNA copy before transcription.
- **Monozygotic:** Developed from a single fertilized ovum, as identical twins.

- **Mutation:** Sudden departure from the parent type in one or more heritable characteristics, caused by a change in a gene or a chromosome.
- **Next generation sequencing:** Massive parallel sequencing is any of several high-throughput approaches to DNA sequencing it is also called Next generation sequencing (NGS).
- **Nucleotide:** A group of molecules that form the building blocks of DNA or RNA.
- **Polymorphism:** The presence of two or more distinct phenotypes in a population due to the expression of different alleles of a given gene.
- **Sequencing:** DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule.
- **Sequencing:** To determine the order of (chemical units in a polymer chain), especially nucleotides in DNA or RNA or amino acids in a protein.
- **Single nucleotide polymorphism:** It is often abbreviated to **SNP** is a variation in a single nucleotide that occurs at a specific position in the genome.
- **Trait:** Which involve genes and characteristics of organisms
- **Variants:** Is a single nucleotide polymorphism that occurs at a specific position in the genome.

APPENDIX III

List of known genes that were included in the custom/ targeted panel

F5	SCN8A	TFPI	ALOX5AP
IL6	TUBA1A	THBD	AUTS2
F2	L1CAM	TLR4	GNB3
MTHFR	TENM1	PLAT	AP4B1
LTA	AGAP1	PTGS2	AP4S1
MBL2	CD99L2	CBS	GRIN3A
TNF	WIPI2	ANXA5	ACE
SERPINE1	JHDM1D	IL1B	AGT
F7	NAA35	IL4	AGTR1
ADD1	RFX2	MMP3	AP4M1
ADRB2	KCNC3	NPPA	VIP
PROCR	ITPR1	NRG1	SCNN1A
FGB	SPTBN2	ACADM	SELE
NOS2	NOS3	TOMM40	PDE4D
IL8	APOE	IL10	KANK1
SERPINB2	MAST1	IL18	GAD1
ITGB3	PAK3	AP4E1	

Shows sequencing metrics of the samples that were subjected to Exome Sequencing

S. No	Sample Identifier	Mapped Reads	Mean Read Length	On Target	Mean Depth
1	001A	959,94,277	180	94.8	279.2
2	002A II	625,51,637	180	92.64	179
3	003A	962,90,855	180	91.01	272.4
4	004A	934,13,308	178	95.04	268.6
5	005A	636,80,443	177	92.98	182.5
6	007A	884,02,679	190	95.27	272.2
7	008A	404,38,787	170	94.3	110.4
8	009A	142,79,466	185	95.69	42.5

9	009A1	772,66,630	180	94.71	224.8
10	010A	837,39,203	190	94.49	254.5
11	011A	496,65,026	181	94.65	145
12	012A	909,81,986	184	92.64	264.8
13	013A	903,40,181	190	94.23	274.7
14	014A SS	821,98,544	115	40.02	39.76
15	014A1	963,73,480	185	94.18	281.6
16	015A	289,12,202	164	94.97	76
17	016A	904,56,715	185	93.89	269
18	017A	665,27,823	172	90.95	181.7
19	018A	668,97,168	175	95.85	190.3
20	019A	439,50,842	173	94.29	122.1
21	020A	902,13,146	185	93.6	265.3
22	020A1	351,77,097	191	93.18	107.1
23	021A	362,67,704	186	91.55	110
24	022A	960,52,296	193	95.14	297.9
25	023A	427,01,281	191	92.97	129.5
26	024A	409,04,217	190	93.2	123.8
27	025A	372,14,830	186	94.6	111.2
28	026A	757,85,191	178	92.34	214.7
29	027A	868,30,607	181	94.5	252.6
30	028A	408,74,013	191	94.09	125.7
31	029A	463,26,687	183	93.12	134.8
32	030A	350,26,657	180	92.76	101.1
33	031A	324,78,247	190	93.99	98.57
34	031A1	389,92,349	181	92.62	112.3
35	031A2	360,86,990	183	93.81	105.6
36	032A	601,27,669	188	91.81	177.2
37	033A	438,25,024	187	94.66	131.7
38	034A	230,88,877	187	93.22	68.5
39	035A	839,17,278	190	94.34	254.7
40	036A	503,31,653	186	93.83	149.3
41	037A	189,29,221	179	N/A	N/A
42	038A	270,35,487	169	89.88	70.65
43	039A	805,23,806	186	94.53	241.2
44	043A	425,79,749	185	90.03	122.4
45	044A	251,85,861	181	89.59	71.12
46	045A	486,46,537	191	93.35	147.7
47	046A	385,01,668	191	94.43	118.3

48	047A	688,64,794	187	90.81	244.7
49	048A	287,95,941	172	90.09	76.8
50	049A	836,09,293	187	90.81	244.7
51	050A	611,89,561	188	91.43	179.6
52	051A	253,22,565	184	89.93	72.62
53	052A	435,92,151	184	90.48	125.6
54	053A	478,82,083	184	92.67	139.7
55	054A	552,22,996	188	94.61	166.7
56	055A	326,45,814	183	91.35	94.29
57	056A	566,37,239	185	92.79	166.9
58	057A	226,81,985	190	92.78	68.05
59	058A	255,47,343	183	89.85	72.05
60	059A	244,23,514	185	92.76	71.88
61	060A	390,45,840	184	91.35	113.2
62	061A	444,84,038	191	94.38	136.9
63	062A	557,09,873	191	94.86	171.9
64	063A	319,90,886	163	91.1	81.07
65	064A	386,74,555	189	95.13	117.9
66	065A	399,49,102	162	90.63	100.2
67	066A	353,99,677	187	92.99	106.5
68	067A	274,40,486	178	93.67	77.94
69	068A	502,11,975	176	91.09	137.9
70	069A	384,22,802	189	96.65	119.2
71	070A	518,59,165	189	95.34	159.2
72	071A	296,82,381	183	90.96	85.54
73	072A	456,85,469	185	90.56	132
74	073A	435,04,053	185	90.59	125.5
75	074A	333,76,490	183	93.87	98.71
76	075A	480,03,669	189	93.53	145.2
77	076A	542,53,997	N/A	N/A	N/A
78	077A	325,01,800	176	93.8	91.39
79	078A	340,10,221	N/A	N/A	N/A
80	079A	279,02,719	184	91.83	81.32
81	080A	414,41,905	188	93.59	125.4
82	081A	397,39,867	165	91.25	101.7
83	082A	250,00,365	167	92.38	65.43
84	083A	317,31,325	188	93.69	95.75
85	084A	484,34,684	189	92.33	144.8
86	085A	268,41,226	190	93.85	81.75

87	086A	322,61,085	186	95.11	97.07
88	087A	457,62,741	183	91.75	131.1
89	088A	340,09,107	184	92.42	92.42
90	089A	409,27,355	190	91.84	121.9
91	090A	281,58,236	181	90.1	85
92	091A	512,33,886	174	87.94	137.8
93	092A	481,75,417	189	91.49	142.7
94	093A	321,11,351	186	94.87	96.03
95	094A	414,46,985	189	91.96	123.5
96	095A	215,84,907	163	91.14	54.77
97	096A	307,26,707	163	90.66	77.68
98	097A	338,84,544	184	92.22	97.96
99	098A	412,68,227	182	92.34	119.3
100	099A	279,32,941	187	92.39	82.45
101	100A	454,05,134	190	92.96	136.6
102	101A	460,14,511	191	93.24	139.3
103	102A	459,10,799	177	90.45	117
104	103A	7,81,767	160	12	1.85

Sample identifier: unique sample id, Mapped reads: sequencing reads that mapped to the exome panel (bed file), read length: length of each sequenced read, uniformity: uniformity of the coverage in terms of percentage, coverage: average number of times each dna base is sequenced, N/A: not available (data not available)

Number of variants (mutations) identified in each sample sequenced.

SI. No	Identifier	Number of variant
1	CP001A	39342
2	CP002A	38893
3	CP003A	39540
4	CP004D	37105
5	CP004	38283
6	CP005	37845
7	CP007	37745
8	CP008	38684
9	CP009	34933
10	CP09A1	37090
11	CP010A	39477
12	CP011	38738
13	CP12A	36395
14	CP013A	36330
15	CP014	37845

16	CP015A	37593
17	CP016A	38684
18	CP017A	34588
19	CP018	37240
20	CP020A	39989
21	CP020A1	38945
22	CP021A	39124
23	CP022A	39254
24	CP023A	39078
25	CP024A	38989
26	CP025A	37941
27	CP026A	38809
28	CP27A	39465
29	CP028A	36772
30	CP029A	39214
31	CP030A	36566
32	CP031A1	38472
33	CP031A2	38350
34	CP32A	39278
35	CP033A	38823
36	CP034A	38128
37	CP35A	36760
38	CP36A	39201
39	CP037A	37823
40	CP038A	38410
41	CP039A	38796
42	CP040A	36974
43	CP041A	38052
44	CP042A	37545
45	CP043A	39102
46	CP044A	38397
47	CP045A	39334
48	CP046A	38711
49	CP047A	39318
50	CP048A	38622
51	CP049A	39481
52	CP050A	39362
53	CO051A	37174
54	CP052A	39127
55	CP053A	38585
56	CP054A	38775
57	CP055A	38470
58	CP056A	37956
59	CP057A	35668

60	CP058A	38035
61	CP059A	37383
62	CP060A	38849
63	CP061A	38742
64	CP062A	39226
65	CP063A	38321
66	CP064A	38238
67	CP065A	38741
68	CP066A	38551
69	CP067A	38288
70	CP068	39176
71	CP069A	38089
72	CP070A	38751
73	CP71A	38007
74	CP072A	39117
75	CP073A	38449
76	CP074A	37794
77	CP075A	37548
78	CP076A	39561
79	CP077A	38520
80	CP078A	37016
81	CP079A	38085
82	CP080A	38490
83	CP081A	38668
84	CP082A	38477
85	CP083A	38528
86	CP084A	39096
87	CP085A	38616
88	CP086A	37186
89	CP087A	38903
90	CP088A	38033
91	CP089A	39065
92	CP090A	37914
93	CP091A	38156
94	CP092A	38903
95	CP093A	38531
96	CP094A	39176
97	CP095A	37821
98	CP096A	38280
99	CP097A	39065
100	CP098A	39049
101	CP099A	37634
102	CP100A	38857
103	CP102A	38374

104	CP103A	39266
	Total number of Variants	39,85,765

Results of sequencing of 26 cases where a mutation was identified with relevance to phenotypes (CP and its spectrum) studied.

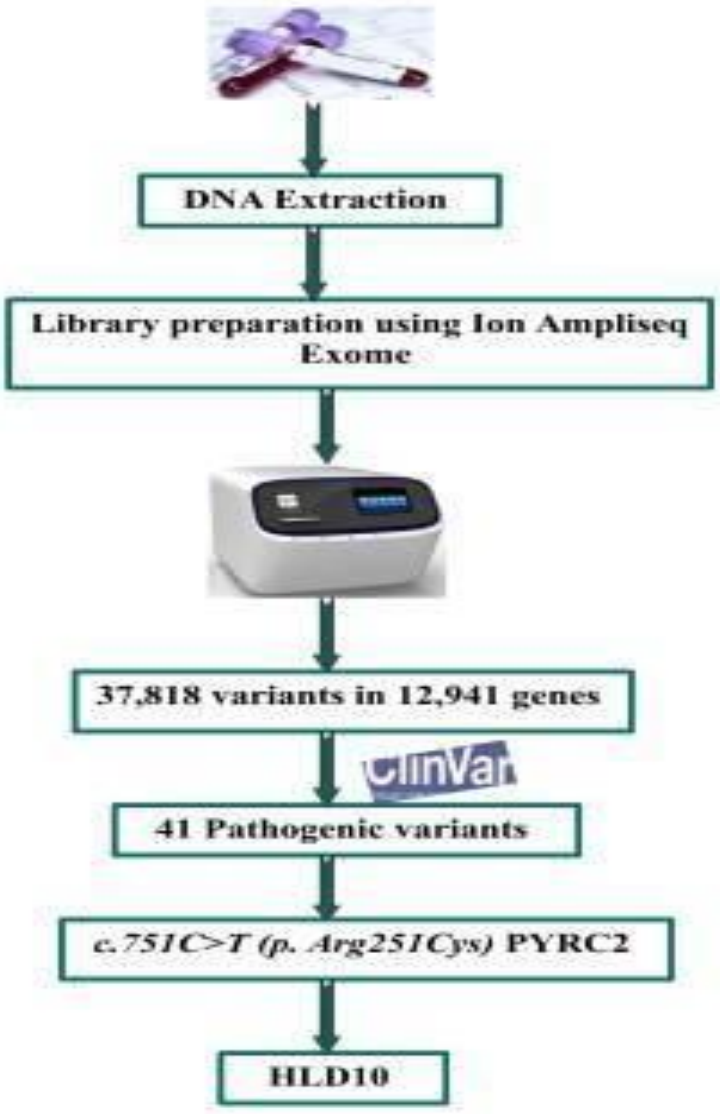
Sl. No	Case No.	Gene in which mutation is identified	Mutations / Amino acid change	Mutation Status
1	CP018A	PYCR2	ARG 251 CYS	rs876657403
2	CP014A	APTX	ARG213FS	*de novo*
3	CP014A1	APTX	ARG213FS	*de novo*
4	CP069A	AHI1	ASP675ASN	Established rs863225145
5	CP069A	GJB2	TRP24TER	Established rs104894396
6	CP062A	G6PD	SER218PHE	Established rs5030868
7	CP062A	GJB2	ARG127HIS	Established rs111033196
8	CP079A	ASPM	LYS1129TER	*de novo*
9	CP036A	ABCC8	TRP1246TER	*de novo*
10	CP045A	ADRA2B	GLU286TER	*de novo*
11	CP093A	GCDH	ARG386TER ARG402TRP	Established rs752127949 rs121434369
12	CP077A	DNMT1	MET734FS	*de novo*
13	CP066A	CASK	ALA249FS	*de novo*
14	CP102A	ARSA	ARG116TER	rs761860059

				Established
15	CP051A	NIPBL	LYS566FS	*de novo*
16	CP021A	MAG	GLY35FS	*de novo*
17	CP044A	CACNA1A	TRP1633TER	*de novo*
18	CP032A	CACNA1G	ASP1902FS	*de novo*
19	CP043A	GLI2	GLU629LYS	rs387907277 Established
20	CP019A	NDUFAF6	ASN162FS	Established rs774800472 or rs762093523
21	CP057A	DDHD2	ARG287TER	Established rs398122826
22	CP039A	PMM2	THR237MET	Established rs80338708
23	CP026A	ACSF3	ARG471GLN	Established rs387907119
24	CP023A	ATP1A3	ILE823PHE	Established rs606231440
25	CP094A	ATP1A3	GLY768ALA	rs606231434 Established
26	CP034A	SLC35A2	ARG342TER	*de novo*
27	CP074A	DVL1	ARG632FS	rs756990609
28	CP012A	KMT2C	P.LYS3793FS	*de novo*
29	CP046A	HDAC6	P. CYS621FS	*De novo*
30	CP016A	ATM	P. LEU2450TER	*De novo*
31	CP016A	MYO6	P.TYR1082TER	*De novo*
32	CP103A	SPAST	P.ARG499HIS	*rs878854991* *Established*

APPENDIX IV

List of figures

Schematic representation of work flow adopted in the present study for whole Exome sequencing



Instrumentation used in the present study.

1. Ion Chef
2. Ion Proton System for Next-Generation Sequencing
3. ABI 3500 Genetic Analyzer
4. ABI Quant Studio 6

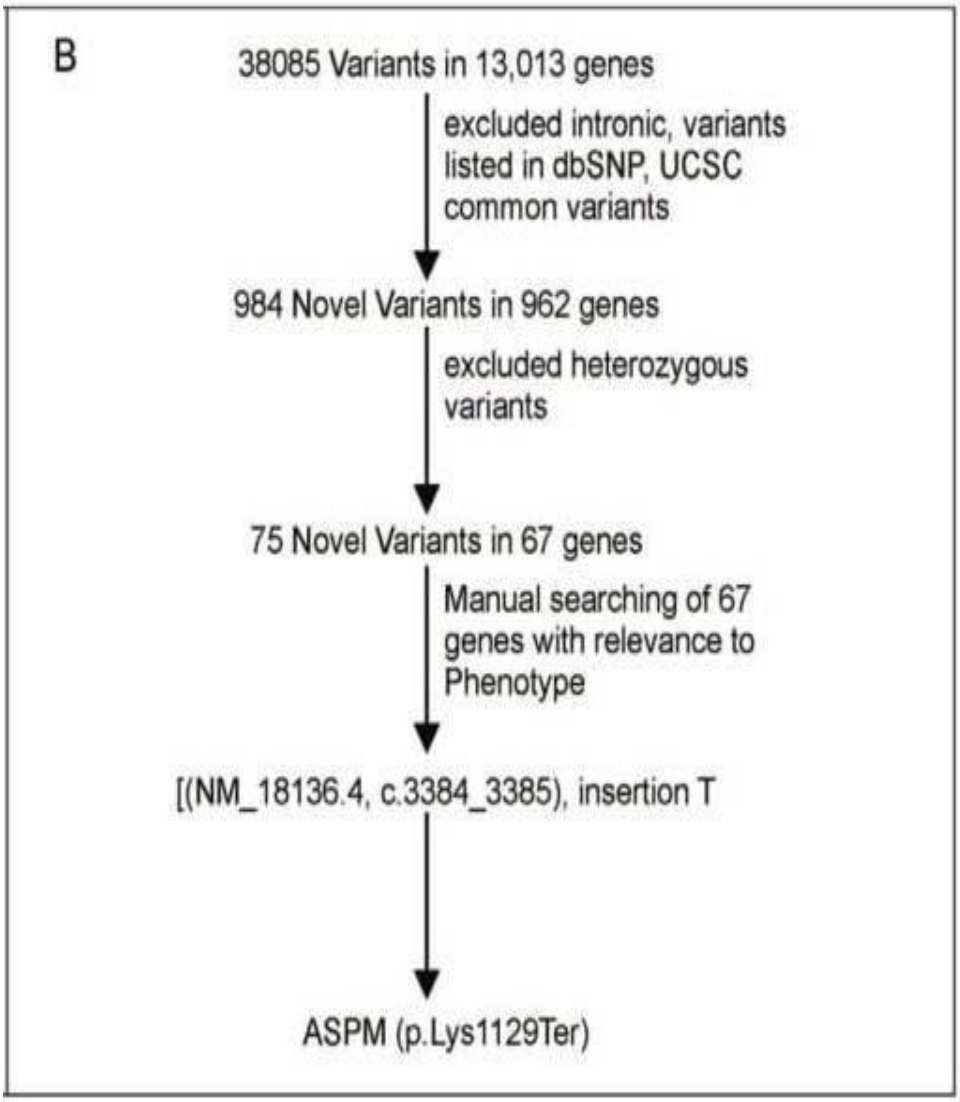
1. ION CHEF: Automation platform to carry out sample processing (Exome library). Also performs chip loading. Ion chef avoids human intervention and error.

2. Ion Proton: Ion proton is a massively parallel sequencer or next generation sequencer, which can produce 12-20 GB of data from each single run. Exome libraries were pre-processed using Ion Chef or OT2 were loaded on to a microchip, which is placed inside the sequencer for sequencing.

3. ABI 3500GA: 3500GA is also called as Sanger Sequencer. It uses di-deoxy nucleotides to determine the order or nucleotides in a stand of DNA.

4. ABI quant Studio 6: ABI quant studio 6 is a real time PCR which is generally used for expression profiling of genes and also for allelic discrimination (determining a mutation, determining the zygosity of the mutation).

Work flow of variant prioritization.



APPENDIX V

PARTICIPANT DETAILS

Sl.no	NAME	AGE	GENDER	TYPE	PROVISIONAL DIAGNOSIS
1	Ithihas	5	M	SPASTIC	Delayed speech and language
2	Sangam	6	M	CP NOT CLASIFIED	Delayed speech and language
3	Srinivas			SPASTIC DIPLEGIC	Delayed speech and language
4	Spoorthy	9	F	CP NOT CLASIFIED	Delayed speech and language
5	Dhanush	7	M	CP NOT CLASIFIED	Inadequate speech and language
6	Tharun	5	M	CP NOT CLASIFIED	Inadequate speech and language
7	Kiran	7	M	SPASTIC QUADRIPLEGIC	Delayed speech and language
8	Sharan Gowda	5	M	SPASTIC QUADRIPLEGIC	Delayed speech and language
9	Rachana	10	F	SPASTIC	Inadequate speech and language
10	Ranjitha	11	M	CP NOT CLASIFIED	Delayed speech and language
11	Shrish Kavalur	5	M	SPASTIC QUADRIPLEGIC	Delayed speech and language
12	Sanjana	5	F	SPASTIC	Delayed speech and language
13	Arman	3	M	SPASTIC QUADRIPLEGIC	Delayed speech and language
14	Harsha	7	M	CP NOT CLASIFIED	Delayed speech and language
15	Tribhuvan	4	M	CP NOT CLASIFIED	Delayed speech and language
16	Parinitha	4	F	HYPOTONIA DIPLEGIC	Delayed speech and language
17	Ajjaiah	8	M	CP NOT CLASIFIED	Inadequate speech and language
18	Yashas	5	M	CP NOT CLASIFIED	Delayed speech and language
19	Jevitha	4	F	CP NOT CLASIFIED	Delayed speech and language
20	Aavani	7	F	CP NOT CLASIFIED	Delayed speech and language
21	Ajmal Dilshad	9	M	CP NOT CLASIFIED	Inadequate speech and language
22	Ayesha	4	F	CP NOT CLASIFIED	Delayed speech and language
23	Niya	8	F	CP NOT CLASIFIED	Delayed speech and language
24	Vivesh Nath	4	M	ATHETHOID	Delayed speech and language

25	Farha	6	F	CP NOT CLASIFIED	Delayed speech and language
26	Athma Nandh	7	M	CP NOT CLASIFIED	Misarticulation with CP
27	Sayeed Marwan	6	M	CP NOT CLASIFIED	Delayed speech and language
28	Fathima Ziya	4	F	SPASTIC QUADRIPLEGIC	Delayed speech and language
29	Dinan.M.B	5	M	CP NOT CLASIFIED	Delayed speech and language
30	Vinay Kumar	4	M	SPASTIC QUADRIPLEGIC	Delayed speech and language
31	Salwa	3	F	CP NOT CLASIFIED	Global development and mental delay
32	Maya	11	F	CP NOT CLASIFIED	Delayed speech and language
33	Mohammed Izan	4	M	SPASTIC QUADRIPLEGIC	Delayed speech and language
34	Likith Raj	4	M	CP NOT CLASIFIED	Delayed speech and language
35	Noureen	4	F	SPASTIC	Delayed speech and language
36	Devdath	4	M	CP NOT CLASIFIED	Delayed speech and language
37	Aishwarya	4	F	SPASTIC	Delayed speech and language
38	Riza Fathima	5	F	SPASTIC QUADRIPLEGIC	Delayed speech and language
39	P.Meghana	12	F	SPASTIC	Inadequate speech and language
40	Sridhar.K	6	M	SPASTIC QUADRIPLEGIC	Delayed speech and language
41	Ayesha Ziya	5	F	SPASTIC	Delayed speech and language
42	Nida Fathima	5	F	SPASTIC	Delayed speech and language
43	Arya Adesh.M	3	M	SPASTIC	Delayed speech and language
44	Sreenandha	4	M	SPASTIC	Delayed speech and language
45	Sharmika	4	F	SPASTIC	Delayed speech and language
46	Mohammed Suhel	11	M	SPASTIC	Delayed speech and language
47	Neha.R.Binu	4	F	ATHETHOID	Delayed speech and language
48	Tanha Fathima	4.5	F	SPASTIC	Delayed speech and language
49	Mohammed Afsan	5	M	SPASTIC QUADRIPLEGIC	Delayed speech and language
50	Abhinav		M	CP NOT CLASIFIED	Delayed speech and language
51	Jaiveer		M	DYSKINETIC	Delayed speech and language
52	Ajnas Mohammed	8	M	SPASTIC	Inadequate speech and language
53	Ayan.P	3.5	M	SPASTIC	Delayed speech and language

54	Diganth.D	3	M	SPASTIC	Delayed speech and language
55	Miraz	7	F	SPASTIC	Delayed speech and language
56	Rafin Shan	6	M	SPASTIC	Delayed speech and language
57	Chandan.S	7	M	SPASTIC	Delayed speech and language
58	Rathan Raj	4	M	DEVELOPMENTAL DELAY	Delayed speech and language
59	Tanish.G	4	M	SPASTIC	Delayed speech and language
60	Kavin Ganesh	4	M	DEVELOPMENTAL DELAY	Delayed speech and language
61	Chetan	6	M	DEVELOPMENTAL DELAY	Delayed speech and language
62	Mohammed Razin			CP NOT CLASIFIED	Delayed speech and language
63	Lazza Fathima	4	F	SPASTIC	Delayed speech and language
64	Darshan	4	M	SPASTIC	Delayed speech and language
65	Nishan	5	M	SPASTIC QUADRIPLEGIC	Delayed speech and language
66	Ayisha Hanfa	4.8	F	GDD(GLOBAL DEVELOPMENTAL DELAY)	Delayed speech and language
67	Chirag Gowda	5	M	ATHETHOID	Delayed speech and language
68	Tamilselvan	4	M	SPASTIC	Delayed speech and language
69	Nuthan	3.5	M	SPASTIC	Delayed speech and language
70	Syed Safwan	8	M	SPASTIC	Delayed speech and language
71	Chetan.V	3	M	SPASTIC	Delayed speech and language
72	Mishab.T.K	5	M	SPASTIC	Delayed speech and language
73	Mohd.Shasbas.K.M	4	M	CP NOT CLASIFIED	Delayed speech and language
74	Behzad Rashim	3	M	SPASTIC	Delayed speech and language
75	Himani	4	F	SPASTIC	Delayed speech and language
76	Spoorthy	4	F	SPASTIC	Delayed speech and language
77	Fathima Shifa	5	F	SPASTIC	Delayed speech and language
78	Shiva Kumar	3	M	SPASTIC	Delayed speech and language
79	Traeesha	14	F	SPASTIC	Inadequate speech and language
80	Fathima Rafna	6	F	SPASTIC	Delayed speech and language
81	Dhanarved	5	M	SPASTIC	Delayed speech and language
82	Ziya Mariyam	3	F	SPASTIC	Delayed speech and language
83	Anshid /ALIN	3	M	SPASTIC	Delayed speech and language

84	Mohd. Musthafa	3	M	SPASTIC	Delayed speech and language
85	Md.Ameen	3.5	M	DD	Delayed speech and language
86	Darshini	3	F	SPASTIC	Delayed speech and language
87	B.D.Thanush	3	M	SPASTIC	Delayed speech and language
88	Jayath Kumar.B	5	M	DYSKINETIC	Delayed speech and language
89	M.D.Afthab	4	M	SPASTIC	Delayed speech and language
90	Meghana.V	3	F	SPASTIC	Delayed speech and language
91	G.D.Manjunatha	5	M	SPASTIC	Delayed speech and language
92	Medharth Charan	3	M	SPASTIC	Delayed speech and language
93	Aadya	5	F	SPASTIC	Delayed speech and language
94	Harshavardhan	6	M	SPASTIC	Delayed speech and language
95	Bhavish	4	M	SPASTIC	Delayed speech and language
96	Pavani Sri	3.2	F	HYPOTONIC	Delayed speech and language
97	Samanvi.S	4	F	SPASTIC	Delayed speech and language
98	B.Srujana	4	F	SPASTIC	Delayed speech and language
99	Rathna	8	F	CP NOT CLASIFIED	Delayed speech and language
100	Yuvraj.M.K	5.5	M	CP NOT CLASIFIED	Delayed speech and language
101	Gautham			CP NOT CLASIFIED	Delayed speech and language
102	Veera Bhadra	3.6	M	CP NOT CLASIFIED	Delayed speech and language
103	Vignesh	4	M	CP NOT CLASIFIED	Delayed speech and language