ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

UTILIZATION OF WHOLE AND CLINICAL EXOME SEQUENCING DATA IN A TURKISH COHORT OF CEREBRAL PALSY PATIENTS TO IDENTIFY NOVEL CANDIDATE GENES AND VARIANTS AND INCREASE DIAGNOSIS

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M.Sc. THESIS

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Date of Defence:	/ /

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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

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"Name Surname"

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PREFACE AND ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my thesis supervisors, Dr. Kaya Bilguvar and Prof. Ugur Ozbek, for their invaluable support and insightful guidance throughout this project. Their expertise and constructive criticism have been instrumental in shaping this research, and I am truly thankful for their mentorship, which has inspired my academic growth.

Secondly, I would like to express my gratitude to Assoc. Prof. Ozlem Akgun Dogan for providing the patients for this thesis and assisting us in the clinical evaluations. Her constant support and guidance have been invaluable to my personal and professional development.

I deeply thank all members of the Deep CP Project member, including Prof. Ugur Ozbek, Dr. Kaya Bilguvar, Assoc. Prof. Gunseli Bayram Akçapınar, Dr. Ozkan Ozdemir, and Prof. Ozden Hatırnaz for their valuable support, and TUBITAK (Project number: 221S889) for funding the Project. I also would like to especially thank Aybike Bulut and Merve Nur Koroglu for their friendship and for being great research partners, and Nazlıcan Ilhan for always being my best friend and supporter.

Last but not least, I would like to express my endless thanks and gratitude to my mother and to my father for providing all kinds of financial and moral support so that I could complete my education and this thesis, and I would like to dedicate to my parents and my brothers whom I always try to be a role model to them.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACMG	American College of Medical Genetics and Genomics
ACP	Ataxic Cerebral Palsy
ADHD	Attention-Deficit/Hyperactivity Disorder
ASD	Autism Spectrum Disorder
BAM	Binary Alignment Format
BCL	Binary Base Call
CFCS	Communication Function Classification System
CNV	Copy Number Variation
CP	Cerebral Palsy
DCP	Dyskinetic Cerebral Palsy
DNA	Dioxynucleicacid
EDACS	Eating and Drinking Ability Classification System
EEG	Electroencephalography
GATK	Genome Analysis Toolkit
GME	Great Middle East Project
GMFCS	Gross Motor Function Classification System
gnomAD	Genome Aggregation Database
HPO	Human Phenotype Ontology
HSP	Hereditary Spastic Paraplegia
MACS	Manual Ability Classification System
MAF	Minor Allele Frequency
MRI	Magnetic Resonance Imaging
NGS	Next Generation Sequencing
OMIM	Online Mendelian Inheritance in Man
SAM	Sequence Alignment Map
SCP	Spastic Cerebral Palsy
SCPE	Surveillance of Cerebral Palsy Europe
SV	Structural Variant
VCF	Variant Calling Format
WES	Whole Exome Sequencing

WGS	Whole Genome Sequencing

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ÖZET

Türk Serebral Palsi Kohortunda Yeni Gen ve Varyant adaylarını belirlemek ve Teşhisi Arttırmak için Tüm ve Klinik Exome Dizileme Verilerinin Kullanımı

Serebral Palsi (SP) dünya çapında yaklaşık 500 çocuktan 1'inde gözlenen, genellikle ilerleyici olmayan ve motor işlevleri ve duruş gelişimini etkileyen nörogelişimsel bir hastalıktır. SP için prematürite gibi prenatal ve perinatal çevresel risk etkenleri tanımlanmıştır. Bunun dışında, SP olgularının önemli bir kısmında çevresel etkenler saptanmamakta ve ilişkili bir konjenital anomali gözlenmekte veya genetik değişikliklerin varlığı öngörülmektedir. Klinik ekzom dizilemenin kohort tabanlı olması, de novo mutasyonlar da dahil olmak üzere nadir mutasyonları saptamak için etkili bir yöntemdir. Özellikle fenotipik çeşitliliği yüksek olan hastalık gruplarının genetik etiyolojisinin aydınlatılması için dizileme teknolojilerine ihtiyaç duyulmaktadır. Bu çalışma, önceden analizi yapılmış tanısız SP'li hastalarda ekzom verilerini kullanılarak tanı verimini artırmayı amaçlamaktadır. Çeşitli sağlık merkezlerinde kayıtlı tanısız hastalardan SP kliniğine benzer özellikler gösteren 58 hasta yeniden analiz için seçilip, hastalara ait klinik ve tüm ekzom verileri Acıbadem Biyobanka'ya entegre edildi. Veriler yeniden işlendi ve annotasyonu yapılıp belirli kriterlere göre varyant önceliklendirme yapıldı. Yeniden analiz sonucunda %27,5'i idiyopatik olarak sınıflandırılırken, reanaliz sonucu tanısız olguların %56'sında prematüre doğum ve polihidramnios gibi SP ile ilişkili risk faktörleri saptandı. Hastaların %38'i *SCN2A, WWOX, CTNNB1 ve EMD* genlerinde patojenik ve muhtemel patojenik varyantlara sahipti. Bir hastanın iki gende (*ASXL1 ve SETBP1*) patojenik varyant taşıdığı gözlenerek multimendelyen olarak değerlendirildi. SP kohortlarındaki genetik heterojenite varlığını destekler biçimde, SP benzeri semptomları olan hastaların seçilmiş olmasına rağmen birçok farklı genetik tanı saptandı ve çevresel risk faktörleri yanısıra Mendelyen varyantların da hastalık etıyolojisine önemli katkıları olduğu gösterildi.

Anahtar Sözcükler: serebral palsi , Ayırıcı tanı, etiyoloji, genetik, yeni nesil dizileme

ABSTRACT

Utilization of Whole and Clinical Exome Sequencing Data in a Turkish Cohort of Cerebral Palsy Patients to Identify Novel Candidate Genes and Variants and Increase Diagnosis

Cerebral palsy (CP) is a neurodevelopmental disorder that is observed in approximately 1 in 500 children worldwide and is non-progressive and affects motor functions and posture development. Prenatal and perinatal environmental risk factors have been defined but, environmental factors are not detected in a significant part of CP cases and a related congenital anomaly is observed or the presence of genetic changes is predicted. Cohort-based clinical exome sequencing is an effective method for detecting rare mutations, including de novo mutations. Especially, sequencing technologies are needed to elucidate the genetic etiology of diseases with high phenotypic diversity. This study aims to increase the diagnostic yield using exome data in undiagnosed CP patients. Among the undiagnosed patients registered in various health centers, 58 patients with CP-like characteristics were selected for reanalysis, and the clinical and whole exome data of the patients were integrated into Acıbadem Biobank. The data were reprocessed and annotated, and variant prioritization was performed with certain criteria. Result of the reanalysis, 27.5% of patients were classified as idiopathic, while CP-related risk factors were found in 56% of undiagnosed cases. 38% of patients had pathogenic and likely pathogenic variants in known genes like the *SCN2A*, *WWOX*, *CTNNB1*, and *EMD* genes. Supporting the presence of genetic heterogeneity in CP cohorts, although patients with CP-like symptoms were recruited, different genetic diagnoses were identified and Mendelian variants were shown to contribute significantly to the etiology of CP, as well as environmental risk factors.

Keywords: Cerebral palsy, differential diagnosis, etiology, genetics, next generation sequencing

1 INTRODUCTION AND AIM

Cerebral palsy (CP) is a common neurodevelopmental disorder in childhood with an incidence of approximately 1/500 live births worldwide (1). It is clinically heterogeneous, therefore difficult to diagnose, and a non-progressive group of disorders. Cerebral palsy affects the development of movement and posture which is attributed to nonprogressive disturbances which occurred in the developing fetal or infant brain (2). Although the initial neuropathological lesion is not progressive, children with CP may develop several secondary conditions over time (3). These secondary conditions/co-morbidities can cover intellectual disability, autism spectrum disorder, epilepsy, secondary musculoskeletal problems, speech and language deficits, and vision and hearing problems (4).

The underlying pathophysiology is brain damage that develops from the perinatal period to the neonatal period and can be classified according to functional ability, topographic involvement of the extremities, and neurological patterns (spasticity, dyskinesia, and ataxia). The most common type of cerebral palsy is spasticity, which can be classified into hemiplegia, quadriplegia, and diplegia. Gross Motor Function Classification System (GMFCS) makes the clinical definition by investigating functional motor ability at levels between I (walks without limitation) and V (transported in a manual wheelchair) (5, 6, 7).

A number of prenatal and perinatal risk factors for CP have been identified, including multiple births, prematurity, placental pathology, intrauterine infection, and intrauterine growth restriction, and male gender (male/female ratio 1.3:1) (4, 8, 9).

Also, recent studies of exome sequencing and copy number variant sequences have shown that between 10% and 31% of CP cases have a genetic cause (9). Although the etiology of cerebral palsy is attributed to various factors, the specific causal mechanism is not clear in many individual cases.

Cohort-based high-throughput sequencing of whole-exome sequencing (WES) is an efficient method for finding rare, causative mutations for diseases, including de novo mutations. In particular, sequencing technologies are necessary for the determination of the genetic etiology and diagnosis of these disease groups with high phenotypic diversity (10). Recently, many studies have been and continue to be done to reveal the genetic etiology of CP.

In 2020, a group from Yale University, Dr. Kaya Bilguvar who is my thesis project co-advisor is also involved in, performed whole exome sequencing of 250 parent-offspring triplets and identified candidate genes for CP that overlap with neurodevelopmental diseases. Eight genes had multiple damaging de novo mutations; of these, two (*TUBA1A* and *CTNNB1*) met genome-wide significance. Also, they identified two novel monogenic etiologies, *FBXO31* and *RHOB* (11).

In 2021, Moreno-De-Luca et al. evaluated the molecular diagnostic yield of exome sequencing in a patient cohort of 1526 patients with cerebral palsy. In this study, mutations were detected in 2 or more genes in *CTNNB1*, *KIF1A*, *GNAO1*, and *TUBA1A* genes in 2 or more unrelated cerebral palsy patients. Also, some patients with CP had variants in genes previously associated with cerebral palsy and hereditary spastic paraplegia, including *AP4E1*, *AP4M1*, *AP4S1*, *ATL1*, and *SPAST*. Although cerebral palsy and hereditary spastic paraplegia have different clinical features in terms of progression, common genomic findings have been observed many times (12, 13, 14).

All these studies shed light on the genetic etiology of cerebral palsy and provided strong evidence for the roles of genes in CP, particularly with the detection of mutated genes in more than one patient in large cohorts. In some cases, it has expanded the phenotype associated with known neurodevelopmental disorder genes to include cerebral palsy phenotypes (12).

Our study allowed reanalysis and diagnosis of undiagnosed patients and may contribute to the etiology of the disease. In this respect, it is unique and beneficial. In

addition, within the scope of our study, samples from the Turkey SP cohort integrated into the ACU biobank. This is another unique aspect of the study.

The Exome Negative Cohort consisted of volunteers in Acıbadem Healthcare Group and various health centers who were previously clinically undiagnosed with cerebral palsy, without age and gender restrictions. In this cohort, there are individuals whose exome sequencing and genome analysis were performed using standard methods and no CP-related variants were found (exome negative).

The data in FASTQ format of the cohort individuals whose raw data were obtained from clinical and whole exome sequencing used in all exome analyses. The re-processed of raw data was conducted with GENNEXT, an in-house pipeline created by Özkan Özdemir, Ph.D., and his team.

Here this study aimed to improve the diagnostic rate of clinical and whole exome sequencing data in individuals affected by CP-like symptoms with negative test results or with results that cannot clarify the phenotype.

2 BACKGROUND

2.1 Cerebral Palsy

2.1.1 Definition and history of cerebral palsy

Cerebral palsy (CP) is a common neurodevelopmental disorder that appears in childhood or infancy term, impairs early motor development, and is characterized by movement, muscle tone, and/or posture problems (15). Complicated and variable clinical signs and symptoms may be challenging to diagnose in cerebral palsy. The motor disorders of CP are usually accompanied by co-morbidities cognitive decline, seizures, structural abnormalities in the musculoskeletal system, and behavioral disturbances such as attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorder (ASD) (12, 16).

A consensual description for cerebral palsy was provided in 2006 by an international consortium under the direction of Rosenbaum and Bax. Cerebral Palsy represents a group of permanent disorders of the development of movement and posture, causing activity limitation, that are attributed to non-progressive disturbances that occurred in the developing fetal or infant brain. The motor disorders of CP are often accompanied by disturbances of sensation, perception, cognition, communication, behavior abnormalities, epilepsy, and musculoskeletal system disorders (2).

The mainstay of CP treatment is the management of symptoms, thus aiming to improve the quality of life because there is no known cure. Therapies aim to increase functionality and independence-related skills (17, 18).

The mummy of Pharaoh Siptah, who passed away in his 20s, is one of the most famous and the oldest case of CP owing to documented photographs of the Pharaoh's recognizable foot abnormalities including equinus and equinovarus deformities. (photographs by Elliot Smith (19); X-rays by E Harris & E Wente (20)).

However, it is more frequently mentioned that these deformities can develop from congenital defects, such as talipes equinovarus, which is caused by poliomyelitis or a linked neurological deficit that develops at the earliest stages of life (21, 22).

Hippocrates, widely regarded as the Father of Medicine in ancient Greece, wrote about the connection between prematurity, intrauterine infection, and prenatal stress, and their impact on brain damage. In his research "Of the Eight-Month Foetus," he observed that babies with intrauterine complications faced increased mortality rates. Hippocrates also linked unexplained fever or significant weight loss during pregnancy to risky births or terminations. He discovered the first correlation between eighth-month prenatal distress and birth defects like visual impairments or lameness (23).

William John Little was the first individual to be involved deeply with cerebral palsy it was until the first decade of the 19th century that there were only specific cases and descriptions of the condition (24). Little's lectures on the Physical Abnormalities of the Human Frame in 1843, reported later in Lancet, provided the first descriptions of cerebral palsy by referring to it as '*cerebral paralysis*' (25). Little hypothesized that the developing nervous system and brain are susceptible to injury at varying periods of prenatal development and linked the disorder to a traumatic birth, oxygen deprivation in the newborn period, and prematurity (26). By the end of the 19th century, Little had described the condition of spastic diplegia as "Little's Disease", associating it with premature birth and asphyxia (21). Then, Little argued his hypothesis that birth hypoxia could lead to irreversible damage or injury to the brain and nervous system in 1861 (27).

By the late 1800s, significant progress had been made in the development of SP diagnosis and classification, thanks in part to the contributions of two prominent figures in the field (24). William Osler is credited with coining the term 'cerebral palsy' and developing the first classification system for the condition (28). Following that, Sigmund Freud identified prenatal risk factors that played a role in the etiology of CP and led to the development of the most comprehensive classification system for spastic diplegia at the time (29).

Overall, the work of these three pioneers has inspired new avenues of research and fostered a greater understanding of cerebral palsy, marking a significant milestone in the history of neurology (23).

2.1.2 The epidemiology of cerebral palsy

The worldwide prevalence of CP is approximated to be around 2.0-2.5 out of every 1000 live births (30). The determined values of incidence and prevalence, and associated risk factors of CP have varied over time and by geographic region, based on the evolution of prenatal, antenatal, and postnatal pediatric care. There are significant regional differences in both reported rates and the most common causes of CP (31). Prematurity and low weight at birth complications are more common risk factors for CP in developed nations, whereas prenatal rubella, birth asphyxia, and neonatal hyperbilirubinemia are still challenges in developing countries (32).

To estimate the frequency of cerebral palsy, two approaches are commonly used. Prevalence, which is used to plan services, is the percentage of rate of a population that has the disease at a certain moment. Surveys can be used for estimation, but they can be time- and labor-intensive due to the necessary size for atypical cases. Incidence is the number of new cases in a particular population at a specific time (33). However, obtaining accurate prevalence estimates with this approach can also be challenging. This is due to data obtained from fatalities in prenatal terms with/without a cerebral palsy diagnosis from birth certificates or newborn hospital discharges may undercount the precise amount of CP cases (34).

2.1.3 Etiology of cerebral palsy

Since William John Little's first description of cerebral palsy, it has been widely accepted that prematurity or birth asphyxia is the primary cause of almost every case. Sigmund Freud, on the other hand, thought that these factors were not completely causative (8).

Today, approximately 92% of cerebral palsy cases are traced back to the perinatal period. Apart from this, around 8% of cerebral palsy patients are caused by head trauma or infection at a later age. Despite the discovery of risk factors, the cause of disease in 80% of cases, is referred to be idiopathic (8).

Studies on the etiology of CP have also shown that these causal pathways may be multiple and the etiology may be multifactorial. (35). To date, some of the identified risk factors are summarized in Table 1.

Table 1. Identified risk factors of cerebral palsy (25, 26)

Preconceptional	Prenatal	Perinatal	Postnatal
• Systemic illness of the mother • Use of drugs and stimulants • Immune system abnormalities • Spontaneous abortions • Socioeconomic factors • Poisoning • Infections • Impaired fertility & treatment of Fertility • Maternal age • Genetic factors	• Oligohydramnios, polyhydramnios • Intrauterine infections • Intrauterine Hypoxia • Multiple gestations • Pregnancy complications • Genetic abnormalities • Malformations of brain structures	• Prematurity and low birth weight • Birth asphyxia • Complicated labour and delivery • Placental abruption • Intrauterine growth retardation	• Non-accidental injury • Head trauma • Meningitis and encephalitis • Hyperbilirubinemia • Sepsis • Cardio-pulmonary arrest • Asphyxia • Respiratory distress • Seizures within 48 hours of birth

Risk factors that may increase the susceptibility of the developing brain to injury have been categorized into groups based on when they occur: before conception, during pregnancy, and after delivery (36).

2.1.3.1 Preconceptional causes

Preconception is defined as the health conditions of the mother before conception, and involves factors such as maternal age, poisoning during pregnancy, the medications used by the mother, and systemic illness she may have (36).

Research findings indicate that the health status of the mother before conception can impact the development of the developing fetal nervous system throughout gestation, potentially leading to cerebral palsy (37).

Another study found a substantial association between cerebral palsy risk and a previous maternal diagnosis of seizures, intellectual disability, or thyroid disorders (including hypo- and hyperthyroidism). The cerebral palsy risk was elevated by several other maternal medical conditions, but the effect was less significant for term infants. Additionally, all studies consistently identified maternal age as a risk factor for cerebral palsy, particularly maternal age above 40. In contrast, low maternal age did not consistently correlate with risk (38).

2.1.3.2 Prenatal causes

The term "prenatal" refers to the gestational period, from conception to birth. Prenatal risk factors involve intrauterine infections, multiple gestations, pregnancy complications, etc. A study conducted in Europe has revealed that multiple births are associated with a significantly higher risk of cerebral palsy (CP), nearly four times higher than in singletons (39). Interestingly, the study also found that while 10% of singleton births are premature, a staggering 58% of twin births occur three or more weeks before the expected delivery date, underscoring the potential risks of multiple pregnancies (40). Multiple pregnancies are correlated with a range of adverse outcomes, including preterm delivery, intrauterine growth restriction, birth defects, and intrapartum complications (41).

Preeclampsia, which is one of the complications of pregnancy, is characterized by hypertension and proteinuria after 20 weeks of gestation, either with or without pathologic edema (42). It is a risk factor for cerebral palsy in term infants, but there is a debate that the incidence of cerebral palsy following preeclampsia is lower than other causes of preterm birth (43). Many studies have found that preeclampsia has a protective effect against cerebral palsy (44).

Possible reasons for this include the planned nature of the majority of pregnancies with follow-up preeclampsia, and the potentially harmful other causes of preterm labor, such as abruption, chorioamnionitis, and cord complications, as opposed to pregnancies complicated by preeclampsia that allows for appropriate treatment and intrauterine transfer (44).

2.1.3.3 Perinatal causes

A substantial causative risk factor for cerebral palsy during pregnancy is premature birth. Despite being relatively uncommon among all births, preterm and very preterm births are responsible for at least half of all cases of preterm cerebral palsy (45). In general, premature babies are more likely to have CP. Cases with CP are generally reported to occur at a rate of 1.2 in term infants and 86 per 1000 in extremely preterm infants (44).

Additionally, a unique form of CP has just been obvious to clinicians as postnatal treatment has improved and the survival of newborns with extremely low birth weight has increased (46). Neuroimaging data from numerous studiehaveas revealed unexpected findings that point to a specific susceptibility of the lower regions of the brain that are correlated with gestational age. Cerebral palsy in extremely preterm infants often appears as spastic diplegia, and neuroimaging frequently detects white matter abnormalities as the underlying brain pathology (47).

2.1.3.4 Postnatal period causes

Postnatal trauma, infections, meningitis and encephalitis, respiratory distress, and newborn seizures have been identified as postnatal risk factors and are estimated to account for 10% to 25% of cases of cerebral palsy(48). Neonatal sepsis was also demonstrated to be a reasonably significant risk factor in the Han et al. (2002) study. Follow-up research revealed that 16.7% of preterm infants who had respiratory distress syndrome acquired cerebral palsy (49).

Different risk factors were examined in a study Elmagid and his colleague did in 2021 on a thousand cases of cerebral palsy. Out of these risk factors, 30.7% were identified during the perinatal and post-natal periods, 21% during the prenatal period, and 17.1% during the post-neonatal period. According to the study, the most significant predictor of cerebral palsy is the presence and severity of hypoxia ischemic encephalopathy during the newborn stage. Furthermore, with 97.7% of the infants being full-term, the study noticed that 8.7% (n=87) of the babies had neonatal encephalopathy. Sepsis has also been noted as a risk factor for cerebral palsy, particularly in premature infants. According to the study, sepsis affected 50 patients in the first month of life, 38 of whom were preterm (50).

2.1.4 Clinical presentation of cerebral palsy

The term 'cerebral palsy' refers to an umbrella term of abnormalities in posture, motor ability, and muscle tone that are non-progressive but not unchangeable (51). Depending on the etiology, there are various degrees of severity, types of motor disorders, and functional abilities (52).

Even though CP largely affects motor function, children with this disorder also have many comorbid conditions resulting from brain injury that are associated with CP and additional abnormalities. Along with seizures and musculoskeletal abnormalities, impairments in motor function in CP frequently come with sensory, perceptual, cognitive abilities, and behavioral problems (53). The comorbidities associated with CP are intellectual disability, micro-/macrocephaly, delayed speech and language development, and visual and hearing impairments (54). Autism spectrum disorders and social problems are also common (55). These various phenotypes contribute to CP's clinical heterogeneity.

2.1.4.1 Early signs of CP

Cerebral palsy is typically diagnosed until around the age of 2, and in more than half of cases, it occurs during the first 12 months of life by revealing the most common

symptoms, like delayed primitive reflexes and delayed developmental milestones (Table 2).

The diagnosis can be determined by considering clinical and neurologic signs, even though not all signs and symptoms are present. The diagnosis of cerebral palsy does not require abnormal brain imaging, and CP can not be evaluated by abnormal brain imaging in the absence of motor signs. When there is a delay in achieving expected developmental milestones, especially in babies with risk factors, a clinical evaluation for CP is conducted by (56).

Table 2. Early signs and symptoms of cerebral palsy (47)

Clinical Characteristics	- Hand preference, with between 6 and 24 months representing the earliest appropriate period. - Irritability signs and symptoms - Decreased level of consciousness including lethargy, and fluctuating sleeping behavior - Oromotor dysfunctions involve drooling, and feeding difficulties
Developmental Milestones	- Delay in volitional rolling and paradoxial early rolling - Delayed ability to walking and sitting
Neurological Assessment	- Retained of primitive reflexes involve Moro, asymmetric tonic neck reflex (ATNR) - Hypotonia and hypertonia - Abnormal muscle tone and muscle imbalances

2.1.4.2 Comorbidities associated with CP

Comorbidity, as described by Brown et al., refers to the presence of any other disorder that is connected to CP, but can also occur as a separate disorder in individuals who do not have CP (57). Comorbidities in people with CP were categorized by Brown et al. into three categories: co-occurring or comorbid, causal, and complications. Since some conditions, like epileptic seizures and cognitive impairment, may result from the

same type of brain damage that caused CP, while others, like scoliosis and contractures, can be seen as CP complications (57, 58).

Based on statistics from the Norwegian Patient Registry from 2008 to 2017, among the various phenotypes, musculoskeletal and connective tissue diseases represent a prevalent complication, with epilepsy standing out as the most frequently observed comorbidity. The distribution of some phenotypes associated with cerebral palsy is shown in Figure 1 (36).

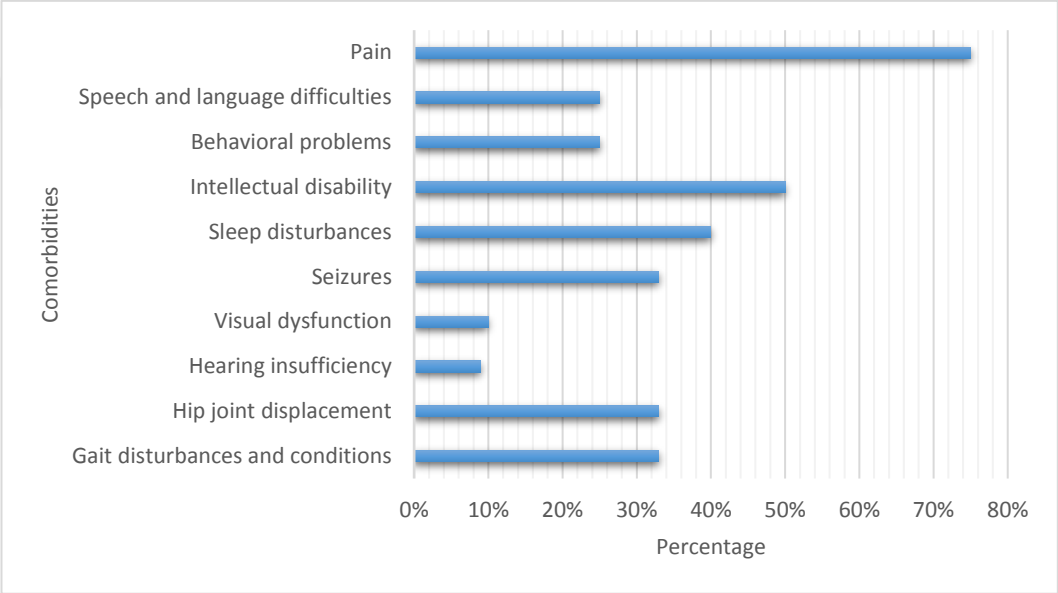

Figure 1. Prevalence percentages of comorbidities in children with CP (25)

2.1.5 Classification of cerebral palsy

Cerebral palsy (CP), predominantly seen in childhood, encompasses non-progressive disorders that affect movement and posture development. It is frequently accompanied by various comorbidities. Due to the varied clinical presentations and activity limitations, categorizing individuals with CP into specific groups is essential for effective management (59).

2.1.5.1 Functional classification systems for cerebral palsy

In recent decades, the field of cerebral palsy research and clinical practice has experienced the emergence of classification systems aimed at providing a standardized framework for assessing functional capacity. These systems utilize a straightforward ordinal grading approach, facilitating clearer communication and understanding among healthcare providers. By employing a shared language, these classification systems offer a means to describe and evaluate functional abilities in individuals with CP (60).

The Gross Motor Function Classification System (GMFCS), The Manual Ability Classification System (MACS), The Communication Function Classification System (CFCS), and The Eating and Drinking Ability Classification System (EDACS) are the four common functional classification systems developed to date in CP assessment (60).

The main objective for developing the Gross Motor Function Classification (GMFCS) was to provide a simple, five-level, system for grouping CP-diagnosed children under the age of 12 based on their functional abilities and limitations (61). The GMFCS describes independently initiated movements as well as the use of wheelchairs or scooters, walker sticks, forearm crutches for assistance, and other walking devices for mobility throughout a person's daily activities. The GMFCS was subsequently enlarged and modified in 2007, including the 12- to 18-year-old age range and increasing descriptors and differentiations for the levels depending on the child's age while considering developmental milestones and the usage of various supporting walking devices (60).

GMFCS is an age-related five-level system that provides a framework for classifying individuals based on their gross motor function limitations. Level I represents the slightest restriction, while level V represents the most severe limitation At level 1, individuals can walk without difficulty, although they may have limitations in more complicated gross motor skills. Level 2 indicates walking, but with limitations

when walking outdoors and in the community. At level 3, children still have obstacles when walking outside and in their neighborhood and need to use assistive mobility devices. Level 4 defines self-mobility with limitations, where children may require assistance or use power devices for motility when getting around outdoors and in their neighborhood. Finally, Level 5 is the lowest possible level of self-mobility, even with the aid of technologies that assist (62).

The Manual Ability Classification System (MACS), constructed by Eliasson et al. in 2006, has considerable similarities in approach with the GMFCS. The MACS is a five-tier classifier system that focuses on assessing between the ages of 4 and 18 CP children's fine motor skills. It focuses on their self-directed in handling objects in a variety of situations including their homes, schools, and communities (63).

Level 1 represents the ability to properly manage objects with ease and achieve successful outcomes. At Level 2, children can handle most objects, albeit with slightly reduced efficiency in terms of quality or speed. Level 3 indicates difficulties in object manipulation, requiring assistance in preparing and adapting activities. Level 4 involves the ability to handle a limited selection of easily manageable objects within specific contexts. Finally, Level 5 signifies a significant limitation in object handling and a severe impairment in performing even simple actions, necessitating complete assistance (64).

Approximately 31% to 88% of individuals with CP are estimated to have a co-occurring communication disorder. The Communication Function Classification System (CFCS), developed by Hidecker et al. in 2011, is a valid assessment for CP that focuses on the evaluation of functional communication skills rather than emphasizing ideal or perfect communication. The CFCS utilizes a five-point ordinal classification system that is intended to complement and work in conjunction with the GMFCS and MACS, creating a cohesive framework for classification (65).

The descriptions of the CFCS levels can be summarized as follows. Level 1 people with CP demonstrate expert communication abilities with both familiar and unfamiliar

people. Level 2 signifies people who can effectively communicate with both familiar and unfamiliar people, albeit more slowly. People who operate at Level 3 are those who communicate effectively only with people they know. Level 4 people are those who occasionally communicate effectively with familiar connections. Even with close individuals, Level 5 people rarely exhibit strong communication abilities (66).

Together with the challenges associated with gross motor function, fine motor function, and communication, individuals with CP may also have difficulty with eating and drinking because of motor control problems. It is estimated that between 27% and 90% of people with CP face varying degrees of difficulties including eating or drinking (60). The Eating and Drinking Ability Classification System (EDACS), introduced by Sellers et al. in 2014, is a reliable assessment for evaluating the capability of children diagnosed with CP, ages 3 and older, to eat and drink (67).

The descriptions of EDACS levels can be summarized as follows, level 1 represents individuals who consume food and beverages safely and efficiently. Level 2 indicates individuals who consume food and beverages safely but with some limitations in terms of efficiency. Level 3 describes individuals who consume food and beverages with some limitations in terms of safety, and there may also be limitations in efficiency.

Level 4 refers to individuals who consume food and beverages with significant limitations in terms of safety. Level 5 represents individuals who are unable to consume beverages safely, and tube feeding may be considered as an option for supplying nutrition (68).

2.1.5.2 Types of cerebral palsy

Typically, both the motor type and the topographical distribution have been used to categorize CP. Spastic, hypotonic, ataxic, and dyskinetic are examples of motor types. The topographic classifications, such as diplegia (or diparesis), tri-, tetra-, quadri-, or hemiplegia, specify the affected limbs (36).

According to the topographical classification of Surveillance of Cerebral Palsy Europe (SCPE), monoplegia refers to the involvement of a single limb, typically affecting the lower limb more frequently. Hemiplegia, on the other hand, affects one side of the body, with the upper limb usually more affected than the lower limb. All of the limbs are afflicted with diplegia; however, the lower limbs are substantially more severely affected than the upper limbs, which frequently only show fine motor deficits. Triplegia generally involves only one upper limb and one lower limb, with the lower limb being more severely afflicted on the same side as the upper limb involvement. Quadriplegia, sometimes known as tetraplegia or "whole-body paralysis," refers to the involvement of all four limbs and the trunk (Figure 2)(69).

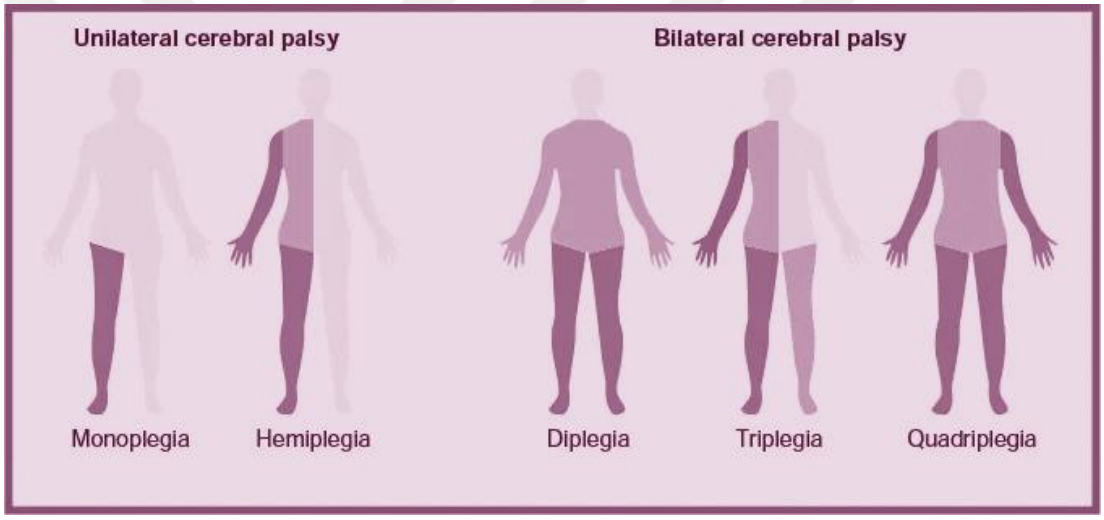

Figure 2. Topographical classification of CP (69)

In the study conducted by Pfeifer LI et al., in 2009 the relationship between age and different types of cerebral palsy (CP), both in terms of topographical distribution and motor type, was examined in a cohort of a hundred participants (70).

The findings revealed that approximately fifty-two children exhibited quadriplegia, thirty-three had diplegia, and fifteen had hemiplegia, indicating a dominant prevalence of quadriplegia in Figure 3. Furthermore, all children with hemiplegia and diplegia were classified as having spastic CP, highlighting spasticity as the predominant phenotype within these motor types. Notably, spastic CP was

consistently more prevalent across all age groups, demonstrating a significant association between motor type and age group (Figure 4) (70).

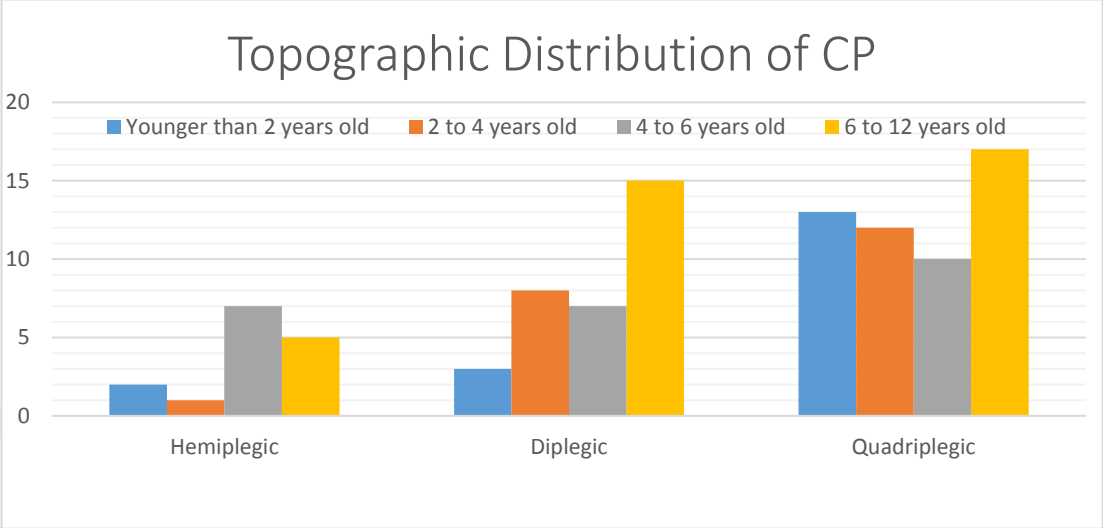

Figure 3. Distribution of participants by age and CP topographical classification

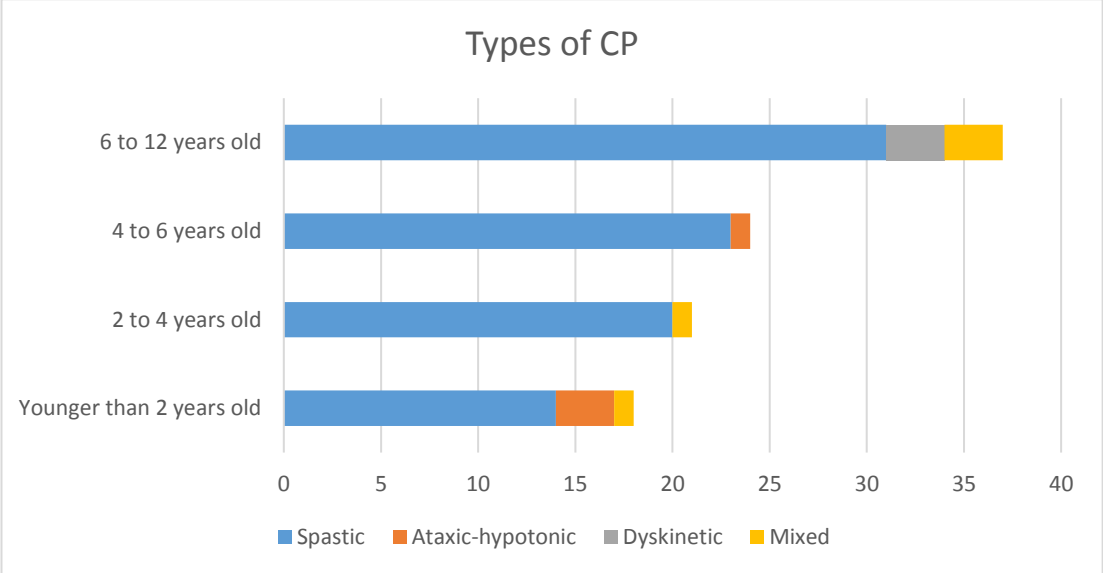

Figure 4. Distribution of CP types by age group

Spastic CP (SCP) affects the neck, the trunk, and both upper and lower limbs, and involves spasticity, weakened muscles, and abnormal reflexes including plantar reflex. The degree of symptom severity may differ, with lower limb problems typically being more severe than upper limb symptoms. Spastic cerebral palsy can also be unilateral

(hemiplegia) or bilateral (diplegia or quadriplegia), which further affects how motor deficits are distributed (71).

Spastic CP tends to arise from a variety of risk factors, such as asphyxia, hemorrhagic or ischemia-related stroke throughout the early stages of pregnancy or neonate period, prenatal or neonatal infection, cerebral malformation, incidents of trauma and, non-progressive genetic diseases (56).

Dystonia and choreoathetosis are the two main movement-related conditions that represent dyskinetic cerebral palsy (DCP), which is characterized by aberrant postures or motions linked to unable muscle tone management, control movement, and coordination. The concept of DCP can also encompass extrapyramidal, choreoathetotic, or choreoathetosis cerebral palsy. DCP is the second most prevalent type of cerebral palsy after SCP, taking into account up to 15% of cases (72).

Advanced neuroimaging techniques, such as magnetic resonance imaging (MRI), have improved our ability to visualize and date brain lesions in CP. In the case of DCP, these lesions are frequently discovered in the basal ganglia and thalamus. They are often associated with perinatal hypoxic-ischemic episodes or neonatal shock, particularly in newborns at or at the end of gestation (73).

Truncal and gait ataxia are hallmarks of ataxic cerebral palsy (ACP), which occurs in about 5% of children with CP. These signs can be seen, such as a wide gait, or they can be induced through examination. Intention tremor is commonly present, and fine motor skills are significantly impaired. Hypotonia is typically observed in this type of cerebral palsy. Hypotonia is frequently seen in this type of CP (74).

The mixed type of CP is characterized by a combination of neurologic abnormalities, with at least 10% of children exhibiting signs of more than one form. The most common combination observed is dyskinesia with spasticity. Identifying the 'dominant' motor abnormality is determined from a clinical perspective, as it plays the most significant role in activity limitation. In cases of mixed-type CP, the damage can

occur different regions of the brain, resulting in more widespread damage and often leading to quadriplegia in children with CP (74).

2.1.6 Management and prevention of cerebral palsy

Cerebral palsy, a condition without a cure, necessitates a comprehensive approach that considers the entire lifespan of the individual. The primary goal of management strategies is to enhance the amount of activity and encourage participation. Children with CP may exhibit various impairments, including uncomfortableness with gait and balance that may only become noticeable in later stages of childhood. To address all these problems, personalized approaches to management are essential, involving collaboration among healthcare experts, social care providers, and families (75).

To effectively manage cerebral palsy, encouraging efficient communication is essential. Customized home programs should be created using toys, boards of conversation, and the proper technical tools to match the child's cognitive skills. To maximize the advances in the development of affected children and allow their full participation in social interactions, adaptive apparatus integration is essential. Movement devices are frequently used as adaptable instruments to improve independence and their range of motion (76).

Management of spasticity is substantial to avoid and cure bone and joint abnormalities, relieve pain, and maintain functionality, it is essential to control spasticity. To receive expert advice on choosing appropriate therapies, such as nerve blocks, soft tissue lengthening, tendon transfers, and joint stability, primary-care doctors regularly recommend patients to surgical professionals. Depending on how severe the problem is, different referral timeframes apply (77).

Several widely used treatments include:

- Intramuscular administration of botulinum toxin (Botox) has been widely utilized to decrease spasticity, enhance mobility, and alleviate pain in children with CP (78).

- Pharmacological therapies for systematic antispasticity involve baclofen (lioresal) and diazepam (valium), which momentarily relax the affected muscles. However, these drugs also have adverse effects such as low seizure levels and depressive symptoms of the central nervous system. (79).
- A neurosurgical operation called selective dorsal rhizotomy identifies and selects certain roots of nerves to lessen stiffness and improve motor function. It is generally a recommended treatment for children with CP who have ambulatory spastic diplegia at GMFCS II or III levels. (80).

Professional physical and occupational therapies for developing movement and balance, as well as posture control, have been the mainstays of treatment for children and adults with CP. These therapies cover a range of techniques and approaches, consisting of muscle stretching and conditioning exercises, weight-supported activities, stability exercises, neuromuscular electrical stimulation, treadmill training, and aerobic exercises (77).

Children with hemiplegia frequently experience seizures, with rates being higher in those who are born with it or develop it postnatally. Children with CP can exhibit cognitive deficits as a result of uncontrolled seizures. CP-affected individuals are more likely to experience seizures in the first year of life, have a history of newborn seizures and epileptic status, and need second-line treatments compared to children lacking neurological impairments who develop seizures. Additionally, they have a lower probability of remaining to be seizure-free after taking antiepileptic drugs. Due to the complexity of the circumstances, it is advised to parents transfer children with CP and epileptic seizures to a neurological physician for therapy (81, 82).

Prevention strategies for CP involve addressing risk factors, implementing medical management that targets the underlying disease analysis, and providing interventions for neonates at risk. The literature outlines numerous methods aimed at avoiding brain damage during the period of pregnancy and delivery (83).

Except for those who suffer from newborn encephalopathy, most infants with CP had unnoticeable neonatal periods. Intrapartum hypoxia of the brain and ischemia, which may end up in cerebral palsy and permanent brain damage, typically induce neonatal encephalopathy.

Some newborns' potential for neurodevelopmental conditions, such as cerebral palsy, is minimized through hypothermia therapy, whether it is administered to only the head or the entire body. However, only a small percentage of children who acquire CP can use this approach (84).

Preterm birth is prevalent in about 12% of births worldwide, and it contributes significantly to infant mortality and morbidity. Progesterone use as a preventative measure has been demonstrated to lower the risk of preterm birth in women who have experienced past birth difficulties. For universal cervix screening, mid-trimester transvaginal ultrasonography is advised. In addition, controlling intrauterine growth restriction, supplying magnesium, and giving corticosteroids for fetal lung maturity are essential measures in avoiding premature birth and its related consequences (36, 85, 86).

2.1.7 Diagnosis of cerebral palsy

Cerebral palsy is diagnosed using an extensive method that comprises clinical signs, physical examinations, and neuroimaging. The mother's medical background and the child's motor abilities are crucial considerations in the differential diagnosis. Since the disorder has many components, extra evaluations such as psychological assessments, visual abilities evaluations, audiometric examinations, and electroencephalographic scans are carried out to acquire significant diagnostic details (36, 37).

Thoroughly monitoring of early signs and symptoms in the form of neurobehavioral signs, presence of developmental reflexes that did not disappear with

time, abnormal tone and posture, and missed milestones as well as associated comorbidities is essential to screen at-risk infants (36).

The American Academy of Neurology suggests a sequential approach to assist in getting a diagnosis of a child with CP. The first stage is diagnosing the condition through clinical history and physical evaluation, followed by investigating any potential comorbidities which may be relevant to (87).

The examination of perinatal medical histories, consisting of antenatal anatomical assessments and transcranial sonography of newborn babies, follows the next step. If there are no abnormalities, an MRI is advised to screen for intracranial anomalies. In addition, screening for genetic abnormalities or metabolic deficiencies is carried out if the test is nondiagnostic (87). Recent studies demonstrated that one of the underlying causes of CP can be genetic changes.

2.1.8 Genetics of cerebral palsy

To date, the etiology of cerebral palsy has been considered to result from a combination of environmental and genetic causes, and genetic abnormalities. However, some risk factors, such as prematurity and restricted intrauterine growth associated with CP, can represent genetic changes contributing to CP (16).

The latest research in this field has been correlated with genetics and/or genomics variables involving copy number variations (CNVs) and single nucleotide variants (SNVs), with both non- and progressive neurodevelopmental diseases involving CP. Several studies have aimed at discovering genetic variants in varied populations that are diagnosed with CP. While some studies recruited every participant with CP, others analyzed clinical characteristics such as unilateral, ataxic, or spastic CP with independent control groups (88).

According to up-to-date genetic research utilizing next-generation sequencing technologies or microarrays, 14% of sporadic cp cases have probably correlated

mutation in a single gene, and up to 31% have considered clinically significant copy number alterations including microdeletions and microduplications (3).

To date, *GAD1* (glutamate decarboxylase 1), *ADD3* (adducin 3), and *KANK1* (KN motif- and ankyrin repeat domain-containing protein 1) have been causally associated with CP, and biallelic variants in *AP4M1*, *AP4E1*, *AP4S1*, and *AP4B1* genes which generate up the adaptor protein complex 4, have identified a secondary syndrome is characterized with microcephaly and intellectual disability (89).

In another study done by Kruer et al., in 2020 de novo mutations have detected some genes involving *TUBA1A* (tubulin, Alpha-1A), *CTNNB1* (catenin, Beta-1), *ATL1* (atlastin GTPase 1), and *SPAST* (spastin) associated with CP (11).

In a patient with CP-like symptoms including spasticity, and various cortical brain malformations, monoallelic missense *TUBA1A* variants associated with lissencephaly 3 with autosomal dominant inheritance pattern (MIM #611603). Three loss of functions variants in the *CTNNB1* gene associated with exudative vitreoretinopathy 7 (MIM #617572), and neurodevelopmental disorder with spastic diplegia, and visual defects, in three unrelated patients, have speech and language problems, spasticity, cognitive impairment, behavior abnormalities, and microcephaly. In addition, one patient of them has pachygyria (11).

Also, autosomal dominant germline variants in other genes involving *ATL1* and *SPAST* were detected as correlated with spastic paraplegia, in patients who were affected and shared certain phenotypes like dystonia and spasticity (11).

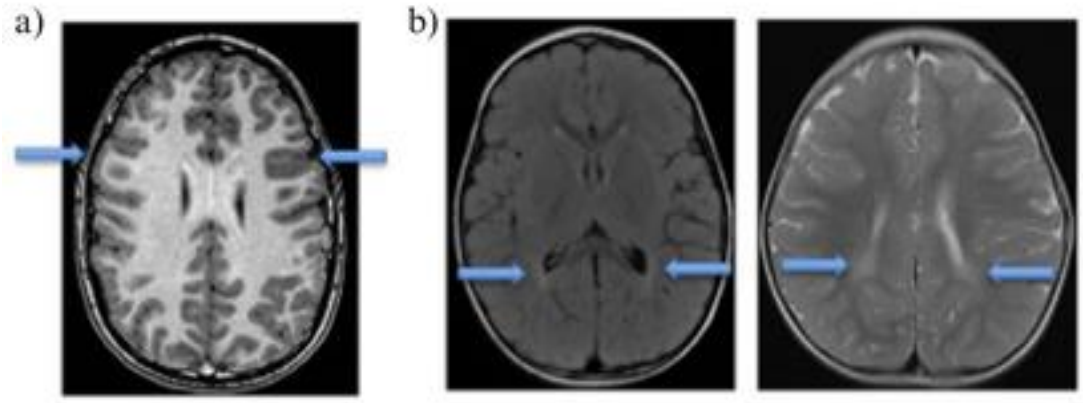

Figure 5. Brain MRI images (11)

a) The patient has a *TUBA1A* variant and shows signs of bilateral perisylvian pachygyria. b) Patients with *ATL1* variants exhibit evidence of mild periventricular T2 hyperintensity.

2.1.9 Development of next-generation sequencing methodologies

Sanger and colleagues, as well as Maxam and Gilbert, invented approaches to DNA sequencing employing the chain termination method in the decade of the 1970s (90, 91). This revolutionized biological sciences by making it possible to unlock whole genes and, later, the entirety of genomes.

The approach proposed by Sanger and collaborators, also known as Sanger sequencing, needed reduced handling of dangerous substances and radioactive materials compared to Maxam and Gilbert's technique, and consequently, it became the preferred DNA sequencing method for the 30 years that followed. A rising need for greater throughput resulted in the automation of laboratories as well as the use of parallelization which gradually reached the building of factory-like setups with numerous sequencing materials. Considering all these developments, the Sanger technology also referred to as first-generation sequencing was provided to complete the human genome project in 2004 (92).

On the other hand, the Human Genome Project utilized enormous amounts of time as well as finances, and it was obvious that more rapid, improved efficiency, and more

affordable technologies were needed. Because of this, the National Human Genome Research Institute announced a scheme to provide funding in the same year to decrease the price of genome sequencing in humans to a thousand dollars in the following ten years. This accelerated the evolution of next-generation sequencing (NGS) technologies. (93).

The launch of NGS technology has facilitated and accelerated the once extremely high labor of sequencing. NGS technologies, which are less time-consuming and less expensive, have gradually replaced their slower and more burdensome predecessors (94). Second-generation strategies, which include those available on the Illumina or Ion Torrent sequencers, typically begin with the process of DNA fragmentation, end-repair, adaptor ligating, surface attachments, and in-situ synthesis. Massive sequencing of short reads of clonally amplified DNA molecules, in which millions of different sequencing reactions occur in parallel, is a characteristic of "short-read" sequencing technologies (95).

Third-generation sequencers, which include those utilized by Pacific Biosciences or Oxford Nanopore, may reach read sizes of up to 10 kb, which is far greater than Sanger or short-read sequencing techniques. These "long read" approaches can overcome short-read challenges such as discovering genome-wide repetitive sequences and structural variants. Improvements to the third-generation sequencing, include minimal library preparation processes and real-time targeted of unfragmented DNA strands, with the synthesis of DNA with a high molecular weight being the limiting factor. The accuracy of reads was an early restriction of these third-generation methods compared to second-generation approaches, although this has since improved with software evaluation developments (96).

2.1.10 Application of NGS technologies

In many genetic centers, NGS technology is a standardized diagnostic approach for finding alterations to the genome in the germline and somatic. Whole exome, whole genome, targeted exome sequencing or clinical exome sequencing (CES) and

mitochondrial sequencing of DNA are examples of techniques that are appropriate to reveal germline changes in genetic disorders (97).

For different types of genetic disorders which include neurodevelopmental disorders, musculoskeletal and connective tissue diseases, and ciliopathies among others, targeted panel testing, focused on certain genes associated with the specific disease which differs between research centers, is an alternative (98).

For inherited genetic diseases, focused panels, for genes correlated with a particular clinical phenotype usually represent the initial evaluation method, which is also more cost-effective. Whole exome sequencing (WES) can be used in cases where the patient's diagnosis cannot be determined by targeted testing. To aid in interpreting variations and confirming variants, whole exome testing may require testing the child and both parents (99).

2.1.11 NGS analysis

The Illumina instrument outputs the sequencing of reads in a binary base call (BCL) data format as raw data. Because BCL is incompatible with most open-source analysis tools due to its large size. Therefore, the starting point of the analysis step involves converting BCL files into the widespread text-based FASTQ data format and also involves quality metrics of raw biological sequence (100).

The raw data FASTQ data format reads from any type of platform via a pipeline of bioinformatics processes to generate a variant call file format (VCF). Demultiplexing, quality control, read alignment to a reference genome, and variant calling/annotation are some of those processes.

3 MATERIALS AND METHODS

3.1 Patient Recruitment

In the CP Cohort, CES (n=52) and WES (n=6) data from 58 patients were recruited from Umraniye Training and Research Hospital and Acıbadem Healthcare Group hospitals. The collected raw data (FASTQ) from CES and WES, patient samples, and clinical information are stored indefinitely in ACU Biobank with patient consent.

The study had approval from Acıbadem University and Acıbadem Healthcare Institutions Medical Research Ethics Committee (ATADEK) with 2022-04/18 decision number. Written informed consent for this study, was provided from all participants. Figure 6 depicts how the demographical and clinical characteristics of the patients were distributed, and Table 3 contained a list of the specific clinical phenotypes obtained by deep phenotyping with Human Phenotype Ontology (101)

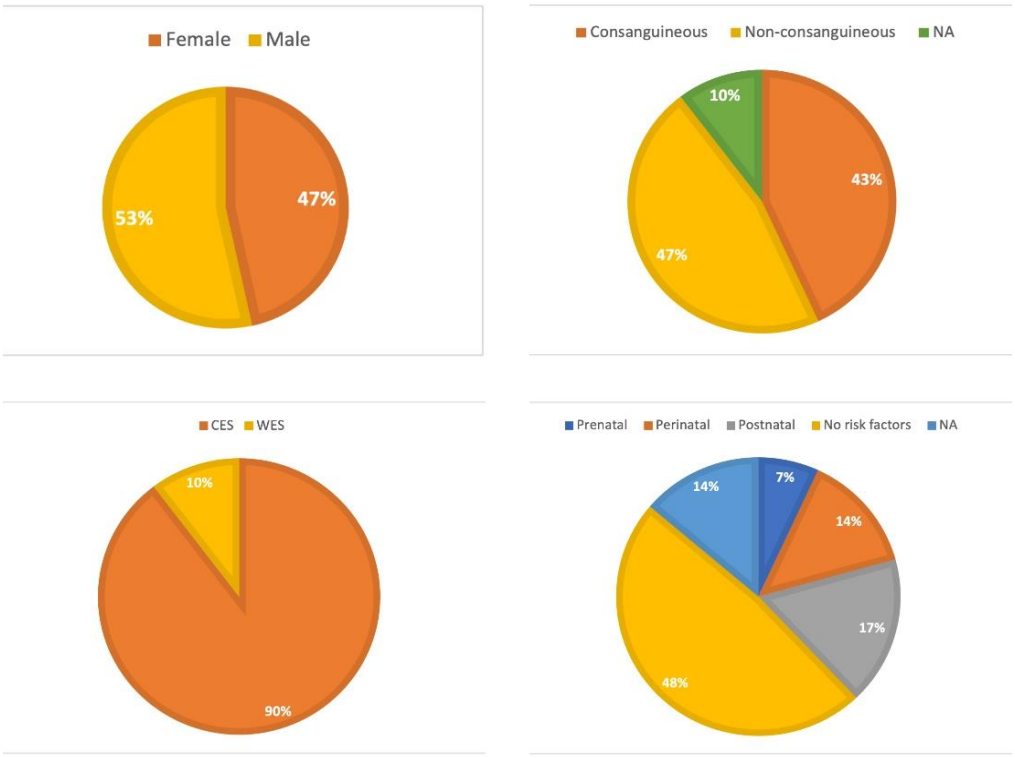

Figure 6. Distribution of cohort characteristics and data types

a, Sex distribution of the patients. b, Distribution of consanguinity. c, Distribution of reanalyzed data type. d, Distribution of the presence of risk factors associated with CP.

Table 3. Clinical details of patients in CP cohort

Patient ID	Sex	Age at Last Examination	Cons.	Phenotypic Features
CES-1	F	4 years 8 months	Yes	Facial dysmorphism, global developmental delay, ataxia, cerebellar vermis hypoplasia, periventricular white matter hyperdensities and ectopic posterior pituitary
CES-2	M	9 years 1 month	No	Gastroesophageal reflux, global developmental delay, seizures, dysphagia, hypoplasia of the corpus callosum, ventriculomegaly, frontotemporal cerebral atrophy, and calcification of the globus pallidus
CES-3	F	11 years 3 months	NA	Scoliosis, global developmental delay, hypotonia, and intellectual disability
CES-4	M	23 years 5 months	No	Emery-Dreifuss muscular dystrophy
CES-5	M	25 years 5 months	No	Emery-Dreifuss muscular dystrophy
CES-6	M	11 years 2 months	Yes	Global developmental delay, motor delay and intellectual disability
CES-7	F	4 years 1 month	No	Global developmental delay, hypotonia, ataxia, cerebral atrophy, and drooling
CES-8	F	7 months	NA	Facial dysmorphism, nuchal translucency, biparietal narrowing, and neonatal hypotonia
CES-9	F	9 years 6 months	Yes	Facial dysmorphism, lower limb spasticity, myopathy, muscular dystrophy, distal prominence of the metatarsals, global developmental delay, hypotonia, seizures, dysphagia, dysplasia of the corpus callosum, and elevated serum creatine phosphokinase
CES-10	M	7 months	NA	Distal artrogripozis
CES-11	M	9 years	NA	Facial dysmorphism, neonatal asphyxia, scoliosis, spastic diplegia, pes planus, cryptorchidism, hyperreflexia and feeding difficulties
CES-12	F	4 months, exitus	Yes	The high palate, neonatal respiratory distress, talipes equinovarus, facial hypotonia, polyneuropathy, and periventricular white matter hyperdensities
CES-13	M	12 years	NA	Microcephaly, and intellectual disability
CES-14	M	2 years 6 months	NA	Coarse facial features, global developmental delay, seizures, widespread papulopustular rash on the anterior and posterior surfaces of the chest
CES-15	F	7 years 3 months	Yes	Slender build, macrodontia, frequent falls, global developmental delay, hypotonia, seizures, ataxia, intellectual disability, and inappropriate happy demeanor
CES-16	F	1 year 2 months	Yes	Short neck, hypertelorism, scoliosis, global developmental delay, hypotonia, seizures and spasticity
CES-17	M	2 years 10 months	No	Global developmental delay, absent speech, seizures, spasticity, hydrocephalus, intellectual disability, motor delay, cerebral atrophy, bruxism, and hypoplasia of the corpus callosum
CES-18	M	6 years 6 months	No	Facial dysmorhism, retrognathia, hypertelorism, talipes equinovarus, pes cavus, hypospadias, cryptorchidism, global developmental delay, delayed speech and language development, and agenesis of the corpus callosum
CES-19	F	12 years 9 months	No	Bilateral hearing impairment, poor head control, global developmental delay, delayed speech and language development, learning difficulties, motor delay, increased deep tendon reflexes, inguinal hernia, thrombocytopenia, proteinuria and hematuria

Table 3. Clinical details of patients in CP cohort (continue)

CES-20	M	5 years 3 months	Yes	Strabismus, global developmental delay, seizures, motor delay, neurodevelopmental delay, deficiency in independent sitting, chronic infection and continuous fever
CES-21	F	8 years 9 months	Yes	Microcephaly, intellectual disability, long palpebral fissures, macrodontia, global developmental delay, delayed speech and language development, and seizures
CES-22	F	7 months	No	Microcephaly, retrognathia, cataract, strabismus, prominent fingertip pads, global developmental delay and hypotonia
CES-23	F	2 years 4 months	No	Pes planus, global developmental delay, motor delay, epileptic encephalopathy (under follow-up with a diagnosis of CP)
CES-24	M	1 year 9 months	Yes	Neonatal hypotonia, muscle atrophy in skeletal muscles, and muscular dystrophy
CES-25	M	5 years 7 months	No	Retinal detachment, and hypotonia
CES-26	F	7 years, exitus	Yes	Microcephaly, hypertonia, nephrotic syndrome, global developmental delay, seizures, spasticity, and elevated serum creatine phosphokinase
CES-27	F	1 year 1 month	No	Global developmental delay, hypotonia, seizures and muscle spasms
CES-28	M	1 year 1 month	Yes	Microcephaly, facial dysmorphism, neonatal hypotonia, hypertonia, seizures, agenesis of the corpus callosum, hyperreflexia, dilation of lateral ventricles and neurodevelopmental delay
CES-29	F	NA	Yes	Microcephaly, pectus excavatum, seizures, motor delay and frontal cortical atrophy
CES-30	F	5 months, exitus	No	Retrognathia, respiratory distress, flexion contracture, limited knee extension, wide intermammillary distance, arthrogryposis multiplex congenita, and poor suck
CES-31	M	8 years	No	Retinitis pigmentosa inversa, hearing impairment, respiratory distress, arthritis, global developmental delay, delayed speech and language development, seizures, and ataxic gait
CES-32	M	11 years 9 months	Yes	Global developmental delay, autism, absent speech, seizures, motor delay, polyneuropathy, and neurodevelopmental delay
CES-33	M	6 months	Yes	Microcephaly, facial dysmorphism, visual impairment, tapered finger, global developmental delay, seizures, dystonia, motor delay, EEG abnormalities, diffuse cerebral atrophy, striatal T2 hyperintensity, dystonic gait, epileptic encephalopathy, and congenital hypothyroidism
CES-34	F	5 years 4 months	Yes	Poor suck, scoliosis, torticollis, neonatal hypotonia, kyphosis, hip dislocation, myopathy, muscular dystrophy, and joint hypermobility
CES-35	F	10 years 8 months	Yes	High narrow palate, strabismus, hypertelorism, difficulty walking, clinodactly, global developmental delay, delayed speech and language development
CES-36	M	8 years 8 months	Yes	Microcephaly, global developmental delay, motor delay and neurodevelopmental delay
CES-37	F	3 years 7 months	Yes	Microcephaly, global developmental delay, motor delay and neurodevelopmental delay
CES-38	M	2 months	Yes	Neonatal asphyxia, hypotonia and dysphagia
CES-39	M	13 years 2 months	Yes	Facial asymmetry, seizures, homocystinuria, abnormalities of glutamine and homocysteine metabolism
CES-40	M	9 years 6 months	No	Microcephaly, tracheomalacia, bronchomalacia, spastic tetraplegia, cryptorchidism, seizures, cerebellar atrophy, infantile spasms, and feeding difficulties

Table 3. Clinical details of patients in CP cohort (continue)

CES-41	F	7 years 8 months	No	Nystagmus, strabismus, difficulty walking, global developmental delay, delayed speech and language development, seizures and agenesis of the corpus callosum
CES-42	F	1 year 9 months	No	Retrognathia, facial dysmorphism, visual impairment, abnormal electroretinogram, neonatal hypotonia, seizures, motor delay, cerebral atrophy, increased deep tendon reflexes, global developmental delay, milk allergy and feeding difficulties
CES-43	F	7 years	Yes	Microcephaly, intellectual disability, global developmental delay, seizures and autism
CES-44	M	7 years	No	Facial dysmorphism, strabismus, prominent fingertip pads, global developmental delay, motor delay, polyhydramnios and prematurity
CES-45	M	1 year 8 months	Yes	Brachycephaly, atrial septal defect, ventricular septal defect, autism, hypotonia, motor delay, self-injurious behavior, and inappropriate laughter
CES-46	F	1 year 10 months	Yes	Global developmental delay, hypotonia, seizures, cerebellar vermis atrophy, dilation of lateral ventricles, agreesive behavior, inferior cerebellar vermis hypoplasia, abnormality of glycolysis and abnormality of creatine metabolism
CES-47	F	2 year 10 months	No	Global developmental delay, delayed speech and language development, seizures, and EEG abnormality
CES-48	M	5 yeas 1 month	No	Neonatal sepsis, microcephaly, seizures, delayed speech and language development, global developmental delay
CES-49	M	4 years 10 months	No	Cerebral palsy
CES-50	M	3 years 1 month	No	Growth retardation, strabismus, scoliosis, pes cavus, abnormality of toe proximal phalanx, global developmental delay, spasticity and cerebellar vermis atrophy
CES-51	M	8 years 4 months	Yes	Hypertonia, lower limb spasticity, global developmental delay, stereotypes, and EEG abnormality
CES-52	F	1 year 9 months	No	Macrocephaly, facial dysmorphism, hypotonia, hydrocephalus, tonic-clonic status epilepticus, dilation of lateral ventricles, and periventricular leukomalacia
WES-1	F	5 years 4 months	No	Microcephaly, facial dysmorphism, hypotelorism, bilateral hearing impairment, global developmental delay, seizures, cerebellar vermis hypoplasia, hypoplasia of the corpus callosum, ventriculomegaly, broad based gait, brainstem dysplasia, abnormality of the pons, and elbow flexion contractures
WES-2	M	10 years 8 months	No	Mild global developmental delay, seizures, cerebellar atrophy, agenesis of corpus callosum, reduced tendon reflexes, neonatal hypotonia, poor suck, cerebral atrophy, gait ataxia, hypoplasia of the corpus callosum, febrile seizures, dilation of lateral ventricles
WES-3	F	7 years 7 months	No	Macrocephaly, hypotonia, and learning difficulties
WES-4	M	2 years 10 months	No	Mild microcephaly, impaired mastication, laryngomalacia, pes planus, infantile axial hypotonia, delayed speech and language development, motor delay, reduced tendon reflexes, delayed myelination, and hyperintensity of cerebral white matter on MRI
WES-5	M	4 years 6 months	Yes	Hypodontia, strabismus, nystagmus, difficulty walking, global developmental delay, seizures, motor delay and EEG abnormality
WES-6	M	2 years 9 months	No	Delayed gross motor development, increased nuchal translucency, delayed speech and language development, nystagmus, astigmatism, multiple tooth decay, relative macrocephaly, and pectus carinatum

3.1.1 Case presentation of patients

CES-1: This girl has symptoms that started with complaints of tremors in her hands and gait imbalance at the age of 2.5 years. She was globally delayed in attaining early milestones. She was born with aortic valve stenosis. Cerebral atrophy, cerebellar vermis hypoplasia, and periventricular white matter hyperdensities were also visualized in brain MRI. Dysmorphic features included a broad forehead, anteverted nares, and a short nasal bridge. There is no other affected member in the family, consanguinity was present as the parents are first cousins.

CES-2: This boy had a diagnosis of cerebral palsy and global developmental delay neonatally. Brain MRI revealed corpus callosum hypoplasia, frontotemporal cerebral atrophy, and globus pallidus calcification. Also, he exhibited a delay in global development, gastroesophageal reflux, and difficulty feeding. Consanguinity was present since the parents are first cousins; no other family members are affected.

CES-3: The patient (girl) followed up in the pediatric metabolism department with heavy metal poisoning. She cannot provide personal care and hygiene. There is a slight inward pressure on the right while walking. There is retardation in motor functions. She can walk unassisted but needs support in climbing stairs. Her development is less than expected for his age. In the neurological examination, it was concluded that she has mild cognitive impairment.

CES-4 and CES-5: Two brothers, aged 23 and 25, are being followed up with the diagnosis of Emery-Dreifuss muscular dystrophy. There is no consanguinity between mother and father, and no other family members are affected.

CES-6: A male patient who has been followed up with mental motor retardation since birth. He also can't walk. No causal variant was detected in the clinical exome analysis and karyotype analysis. There are 4 healthy brothers, there is first-degree consanguinity between mother and father, and no other family members are affected.

CES-7: A female patient was born as G1Y1 at 36 gestational weeks, 2330 gr, and 42 cm from a 26yo mother, and she did not have any problems until the 34th gestational week. After 39 weeks, it was said that there was a problem with feeding. She was observed as small for her gestational age. Head control was done on time, but when she was 6 months old, it was noticed that there was a delay in sitting, and her other developmental milestones were also delayed. Her sleep is irregular, waking up frequently. She has ADHD and autistic behavior. While the cranial MRI performed in 2015 was normal, cerebral atrophy was detected in 2019. No causal variant was detected in CES and microarray analysis. Physical examination revealed that she has relative macrocephaly, tremors, and ataxic gait.

CES-8: The girl was born as G1Y1 at the age of 25 from her mother, at term 4135g. Prenatal follow-up pregnancy was found to be normal in the non-invasive screening test, which was performed due to the high nuchal thickness in the fetus in the double screening test. No complications were experienced during the perinatal period, and no follow-up was required in the intensive care unit. Hypotonicity was noticed at the postnatal 2nd week, and the child was followed up by neurology. No causal variant was detected in the clinical exome analysis and microarray analysis. There is no history of seizures, there is global developmental delay. She continues physical therapy and special education. There is no consanguinity between mother and father.

CES-9: A female patient whose hypotonicity was observed when she was 2.5 months old, and the patient has been monitored. Her seizures started when she was 5 months old but were controlled with anti-epileptics. Lower extremities showed a more severe increase in tone, and serum creatinine was often elevated. Distal tapering was observed in the hands. Also, she has dysmorphic facial features. Speech and language development is delayed. She is still not able to walk. No causal variant was detected in the CES and microarray analysis. There is second-degree consanguinity between mother and father.

CES-10: The patient is being followed up with distal arthrogryposis.

CES-11: The male patient was born G2P2Y2 at the age of 32 from the mother at 39 gestational weeks, weighing 3550 g. It was a pregnancy with prenatal follow-up and no complications were experienced in the prenatal period. She was exposed to asphyxia during delivery and needed to be followed up in the intensive care unit for 3 months. At the time of speaking, walking was 3 years old. He goes to special education and receives education as an inclusive student, thus he learned to read and write. Hospitalization was required frequently due to infection and reflux. He had an operation for cryptorchidism. There is no known consanguinity between the parents, but they come from the same villages. There are similar cases in the family with mental and physical disabilities.

CES-12: She was followed up with presenting complaints of polyneuropathy and hypotonia. During the perinatal period, she needed to be followed up in the intensive care unit for 2 months due to respiratory distress. She had a weak sucking reflex and difficulty swallowing. When the patient was 4 months old, she had exitus. There is a first-degree consanguinity between the parents. No causal change was detected in the CES and microarray analysis.

CES-13: Follow-up on the male patient for microcephaly and motor retardation. Neither the CES nor the microarray analysis revealed any causative variants.

CES-14: A male patient was born G2P2 from a 28-year-old mother, 2800 g at term. He started supporting his head when he was 4-5 months old, and started to sit without support after 1 year of age. His speech is in the form of a single word. Seizures started on postnatal day 1, but seizures were controlled using Kepra. He hasn't had a seizure in the last 1 year. No other known health problems. The father started to speak at the age of 6 and has no known history of seizures. There is no known consanguinity between the parents, but they come from the same villages.

CES-15: A female patient was born as G5Y5 with a cesarean section of 3500 gr from a 28-year-old mother. She did not need follow-up in the neonatal intensive care unit. Although he had hypotonia until the 6th month, it was not very pronounced. In

the 6th month, motor retardation was more pronounced. There was no feeding problem in the first 6 months. However, the mother stated that she was very quiet, she never cried, and was very calm. He has no history of knowing the mother or laughing.

Bruxism and hyperacusis are present. The first seizure was noticed at the age of 8, now it is under control with medication. An increase in signal was observed in T2 examination in the periventricular area in cranial MRI. On Electroencephalography (EEG), the local epileptiform anomaly was detected in the frontocentral region. On physical examination, spasticity was observed in all extremities, especially in the lower extremities, and there was a limitation of extension in the knees and elbows. No causal variant was detected in the CES and microarray analysis.

CES-16: She was born as G3P3 at term with vaginal delivery, 3620 gr. She had prenatal follow-up pregnancy and intrauterine growth retardation. There was no postnatal intensive care stay. At 2 weeks of age, tonic-clonic seizures started, and antiepileptic drugs have been started. She has no independent sitting ability yet, and there are spasticity and significant kyphoscoliosis are present. No causal variant was detected in the CES and microarray analysis. Also, the brother died of the diagnosis of Coffin-Siris. There is a first-degree consanguinity between the mother and father.

CES-17: He was born with vaginal delivery from a 40-year-old G4P4 mother at 38 weeks of age, 4000 g and 52 cm. Due to her contractions after birth, he needed to be followed up in the intensive care unit. He has no history of seizures. There is walking-assisted stepping. Head control started after 1 year of age, no talking yet. Cranial MRI revealed hydrocephalus, cerebral atrophy, and corpus callosum hypoplasia. On physical examination, spasticity was prominent in all extremities. No causal variant was detected in the CES and microarray analysis. There is no consanguinity between parents.

CES-18: He was born by cesarean section at 38 weeks from a 23-year-old mother as G3P1. Prenatal follow-up pregnancy and termination due to corpus callosum agenesis were recommended. In the postnatal period, he was monitored in the intensive

care unit. There is general growth retardation, delayed speech, and corpus callosum agenesis. No causal variant was detected in the CES and microarray analysis. There is no consanguinity between parents.

CES-19: As G2P2Y2, 1400 gr was born at 35+2 gestational week. In the postnatal period, follow-up was needed in the neonatal intensive care unit. There is a delay in developmental milestones. There are signs of sensorial hearing loss and mild mental retardation. A Pediatric Nephrology examination revealed proteinuria, thrombocytopenia, and hematuria. No causal variant was detected in the CES and microarray analysis. There is no consanguinity between parents.

CES-20: A 20-year-old G2Y2 mother was born as the first living cesarean section at 38 weeks, with 2470 g. He had frequent infections and recurrent fevers. Delayed sitting walking, and speaking. The patient was followed up for neuromotor developmental delay and epilepsy, and there was no similar case in the family.

CES-21: She was born at term G4P4 to a 30-year-old mother. She started having seizures when she was 5 months old and used antiepileptic for 3 years. No active seizures now. She is receiving special education. The other siblings were healthy, there is no consanguinity between the parents.

CES-22: She was born G2Y2 at the age of 26 from the mother at 39 weeks of age, 2450 gr. The patient was followed up due to microcephaly, hypotonicity, and growth retardation; she had no seizure history. She had head control, but still no support sitting. There is no consanguinity between parents.

CES-23: Patient referred with the diagnosis of cerebral palsy with neuromotor developmental delay. There are no dysmorphic features.

CES-24: A male patient followed up for congenital hypotonia. There is atrophy in the muscles, and intelligence is normal. There is no consanguinity between the parents. His older brother is 4 years old, he has the same clinical features.

CES-25: A male patient has been tracked for mental motor retardation, retinal detachment, and central hypotonia.

CES-26: A 7-year-old female patient with CP, diagnosed with nephrotic syndrome.

CES-27: She has presenting a complaint of severe hypotonia when she was 4 months old and has been monitored with a suspicion of metabolic disease since 7 months of age with convulsions. She has an atypical facial appearance, and no head holding and support yet. There is no consanguinity between the parents, the other siblings are healthy.

CES-28: A male patient followed up with a diagnosis of neonatal hypotonia. He had a microcephalic appearance and global developmental delay is present. He also has a medical history of seizures.

CES-29: The girl, who was followed up with the diagnosis of cerebral palsy, has also microcephaly, seizures, and motor retardation. There is a first-degree consanguinity between the mother and father.

CES-30: The female patient was born as G1P1 from a 26-year-old mother at 29 weeks of age by cesarean section of 1613 g. She had been under observation in the neonatal intensive care unit due to respiratory distress. She had no sucking reflex. There is neonatal onset joint contracture and hip dislocation. There are also facial dysmorphic findings, such as retrognathia.

CES-31: A male patient was born from a 28-year-old mother as G3P2 at 36 weeks and weighs 2770 g. Due to respiratory distress in the perinatal period, he needed follow-up in the neonatal intensive care unit. His gait and speech are delayed. Hearing impairment was detected in the neonatal period. He has had a balance problem since birth, he walks ataxic. Eye examination revealed retinitis pigmentosa inversa. There is

no obvious dysmorphic finding on the face. There is no consanguinity between parents, there is a healthy sibling.

CES-32: A male patient was born 3300 gr at term with a vaginal delivery as G3P2. He was followed up continuously in the prenatal period. No complications were experienced during the perinatal period, and no follow-up was required in the intensive care unit. Keeping head up is 1 year old, and walking is 4 years old. Still no speech. He has been receiving special education since he was 1 year old. He's been diagnosed with autism. He uses Depakin for epilepsy. There is a first-degree consanguinity between parents.

CES-33: The male patient was born from a 25y mother as G5P2, 2500 gr at term. There was no need for follow-up in the intensive care unit. He had neonatal hypotonia from birth and delayed head control. He had his first seizure when he was 4 months old. The seizures continued in tonic-clonic type, 5-6 times a day, and the seizures continue despite multiple antiepileptics. He bites his hand when he gets angry. Still no head control, no sitting and walking. There are no words, and it does not respond to its name. No causal variant was detected in the CES and microarray analysis. Cranial MRI revealed diffuse cerebral atrophy and striatal T2 hyperintensity. On physical examination, axial hypotonicity was prominent. He has a microcephalic appearance and facial dysmorphic findings including a narrow forehead, anteverted ears, long eyelashes, depressed nasal bridge, and high-arched eyebrows.

CES-34: She was born at term with a vaginal delivery of 3950 g. On the postnatal 1st day, she had a seizure in the form of convulsions without fever. He needed follow-up in the neonatal intensive care unit. Epileptiform changes were detected in EEG. She has started using antiepileptic drugs and had no seizures for 3 years. Head control was done when she was 1 year old, and her other developmental milestones were also delayed, not walking or sitting yet. She has a healthy sibling, there is first-degree consanguinity between her parents.

CES-35: The female patient was born as G2P2A0, 1400 g, 48 cm, with vaginal

delivery. It was not followed in the prenatal period. She was followed in the intensive care unit in the postnatal period. They went to the doctor with the patient's presenting complaints of developmental delay, inability to speak, and delayed walking. No causal variant was detected in the clinical exome analysis and microarray analysis. There is a first-degree consanguinity between the mother and father.

CES-36 & CES-37: Two siblings (girl and boy) presented with neuromotor developmental delay and microcephaly. No causal variant was detected in the clinical exome analysis and microarray analysis. There is a first-degree consanguinity between the mother and father.

CES-38: The male patient was born by cesarean section at 37+2 weeks. He was followed in the neonatal intensive care unit due to hypotonicity and respiratory problems in the postnatal period. He has a healthy sibling, there is first-degree consanguinity between his parents.

CES-39: A male patient followed up for mental retardation and epilepsy. He is being followed up in the pediatric metabolism department because of his glutamine and homocysteine metabolism abnormality.

CES-40: The patient was born with a 43-week vaginal delivery. There is no prenatal history. He had no sucking reflex and crying in the neonatal period. After birth, he was followed up in the neonatal intensive care unit due to feeding difficulties. He had an operation at the age of 6-7 for cryptorchidism. He is still followed with spastic tetraparesis, epilepsy, microcephaly, cerebellar atrophy, and ongoing respiratory problems.

CES-41: A female patient was born at 36 weeks of age and has no history of asphyxia. She was followed up in the neonatal intensive care unit due to her postnatal seizures, continuing as resistant seizures. Sitting without support was at the age of 1.5-2 years. She still does not walk, the words are 2-3 syllables. She also has visual

problems such as strabismus and nystagmus. There is no consanguinity between her parents.

CES-42: She was born as G3Y3, 3050 gr., from the mother at the age of 30. There was no problem in prenatal follow-ups. She was followed up in the neonatal intensive care unit due to postpartum feeding difficulties. She had his first seizure when he was 6 months old. No supported or unsupported sitting, unable to control head.

She has a history of neonatal onset hypotonicity. As a result of the cranial MRI, cerebral atrophy was detected. She has two healthy brothers and no family history of similar diseases. There is no consanguinity between mother and father.

CES-43: A female patient followed up with a pre-diagnosis of cerebral palsy with co-morbidities with autism and epilepsy.

CES-44: A male patient was born to a G2P2 mother at 34 weeks with 2160 g with vaginal delivery. He was tracking regularly in the prenatal period. In the prenatal period, the mother has a history of polyhydramnios and bleeding. He was followed up in the neonatal intensive care unit in the postnatal period. He has neonatal sepsis. Head control was done on time, but his walking and speech were delayed. There is no similar case in the family, there is no consanguinity between the parents.

CES-45: Male patient born as G1P1. He had an operation due to an atrial and ventricular septal defect. He can't walk or talk yet. He was receiving special education. There is a first-degree consanguinity between the mother and father.

CES-46: A female patient followed up for growth retardation and hypotonia. Her EEG was normal, but she has epileptic seizures. Cranial MRI findings revealed cerebral atrophy, inferior venous hypoplasia, dilation of the lateral ventricles, and width of the cortical fissures. There is consanguinity between mother and father.

CES-47: A girl was born with vaginal delivery at term, 2600 gr. She was breastfed

and had a developmental delay. She speaks as one word. She can walk with support, has EEG abnormalities, and has a history of febrile convulsions. There is no consanguinity between the parents.

CES-48: He was born at term with vaginal delivery, 2850 g, 52 cm. He received phototherapy for neonatal sepsis. It has been said that he has retardation in developmental stages. His speech is only two words.

CES-49: He was born at term by vaginal delivery and had no postnatal problems. He was being followed up with a preliminary diagnosis of cerebral palsy. There are no dysmorphic findings, there is laxity in the hand joints. There was no consanguinity between the mother and father.

CES-50: He was born as G1Y1 to a 26-year-old mother at 41 weeks, 4200 gr. The prenatal term was tracked, and screening tests were normal. There were no complications in the perinatal period. No history of seizures. Head control began when he was 4-5 months old, sitting without support was around 14 months. No gait, no clear words. There is spasticity in the lower extremities and scoliosis in the back. There is no similar disease in the family, there is no consanguinity between the parents.

CES-51: He was born at term by cesarean section, 3105 g, 49 cm. Prenatal follow-up pregnancy was not a problem in the prenatal period. No complications were experienced during the perinatal period, and no follow-up was required in the intensive care unit. Head control occurred when he was 2.5 months old. No crawling or walking. He speaks as one word. From the 5th month onwards, he had developmental retardation, slowness in movements, and hypertonicity. Tendon loosening operation was performed on the ankles. Epileptiform changes were detected in EEG. He's using Depakine. There is no similar case in the family, there is first-degree consanguinity between the parents.

CES-52: She was born at term 4350 gr, 51 cm by cesarean section. There was no postnatal problem, no jaundice. She had neonatal onset hypotonia and a history of

tonic-clonic convulsions. Head holding occurred at 5 months, sitting without support at 8 months, crawling at 10 months, and walking at 15 months. She speaks in one word. Cranial MRI findings are present. She has a 4-year-old brother with a diagnosis of epilepsy. There is no consanguinity between the parents.

WES-1: Female patient aged 5 was delivered vaginally at term weighing 2.5 kg and measuring 152 cm. From birth, she has severe microcephaly with 48,5 cm of measurement head circumference. An MRI of the brain at the prenatal term revealed corpus callosum hypoplasia and ventriculomegaly.

Several congenital brain anomalies were also seen on the MRI scan, including ventriculomegaly, corpus callosum hypoplasia, pons abnormality, and brainstem dysplasia. She delayed developmental milestones and has a global developmental delay. Dysmorphic facial features involve hypotelorism, up slanted palpebral fissures, bulbous nose, synophrys, and narrow forehead. She also is affected by bilateral conductive hearing loss. There is no consanguinity between parents and no family member has similar symptoms with the patient.

WES-2: A 10-year-old male patient was born at term G3P2 from a 38-year-old mother. At the age of 4-5 months, it was noticed that there was retardation in the motor development stages. His gait was 3.5 years old and his speech was delayed until 5 years old. His first seizure occurred when he was 2 years old, but it was controlled with anti-epileptic drug use.

Diffuse cerebral atrophy was detected in cranial MRI, and he was being followed up due to hypotonicity. He is currently studying as an inclusive student. In addition, pes planus was found in the physical examination findings. Among the dysmorphic findings are hypotelorism, prominent forehead, wide/flared eyebrows, prominent incisors, and synophrys.

WES-3: A 7-year-old female patient was born from a 39-year-old G3P3 mother, 1320 grams at 30 weeks of gestation. She was followed up in the prenatal period, and

there was no problem during the pregnancy period. A large head size was noticed in the neonatal intensive care unit after birth and the patient was followed up. There was no delay in his developmental milestones, and her speech was normal. Exome analysis was requested due to learning difficulties and macrocephaly. There is no consanguinity between the mother and father and there are healthy siblings.

WES-4: A 3-year-old male patient was born to a 34-year-old mother with a vaginal delivery of 3650 g at term as G2Y2. There were no problems after birth. Head control at 3 months, sitting after 8 months, but still no sitting without support. He has been walking with support since he was 1 year old, he has no independent walking. He has 15-25 words and knows colors and shapes. No history of seizures. The night's sleep is regular, there is no problem. On physical examination, spasticity was observed in all extremities. There was no obvious dysmorphic finding on the face. There is no consanguinity between the parents.

WES-5: Male patient was born as G3P2 from a 28y mother, at term, with vaginal delivery, 3950 gr. There was a history of seizures in the form of contractions twice in the first 24 hours after birth. He was followed in the neonatal intensive care unit because of his many seizures per day, controlled by using an anti-epileptic drug now. Epileptic activity continues in EEG. He has no words but can understand emotions. There is the behavior of grinding teeth and biting his hand when he is angry or excited. No walking, or sitting without support has just begun. (6.5-year-old) It has no chewing, it is fed in the form of puree.

Brain MRI revealed findings that a thin corpus callosum, bilateral symmetric periventricular white matter hyperintensities, mild ventriculomegaly, and sulcal enlargement.

Nystagmus, strabismus, myopia, and astigmatism were observed on physical examination. There is no obvious dysmorphic finding on the face. Widespread spasticity and deep tendon reflexes were increased in the upper and lower extremities.

WES-6: A 4-year-old male patient was born G2P2, 3020 grams, from a 30-year-old mother. Pregnant with prenatal follow-up, and increased nuchal transparency detected in the prenatal period. He had head control when he was 4-5 months old and still can't walk. At 6 months, hypotonicity became more pronounced. He started at the age of 1 and started speaking in the form of a few words.

Gross motor retardation was present in the neurological examination, but no cognitive retardation was found. Ocular examination revealed nystagmus and astigmatism; physical examination revealed relative macrocephaly and pectus carinatum. There was no obvious spasticity. He had multiple tooth decay in his mouth. There is no consanguinity between mother and father.

3.1.2 Bioinformatic analyses

The re-processed of raw data was conducted with GENNEXT (https://github.com/GenivaInformatics/gennext-workflows), an in-house pipeline created by Özkan Özdemir, Ph.D., and his team. It involves FASTQC, Trim Galore, SNAP, elPrep, Samtools, Picard, Genome Analysis Toolkit (GATK), DeepVariant, and mosdepth as bioinformatics packages (Figure **7**).

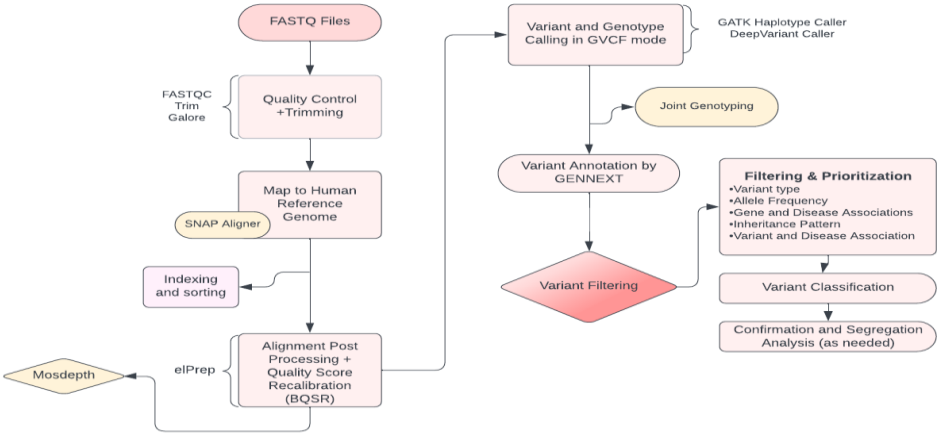

Figure 7. Flowchart of the GENNEXT pipeline

The workflow starts that the raw sequencing reads' quality was checked via FastQC (102). Then, raw FASTQ data were trimmed with Trim Galore to remove sequence adapters (103). The FastQ sequences were aligned to the human reference genome (GRCh38) using SNAP, generating a sequence alignment map file (SAM) (104). Elprep was then utilized to align after-processing and recalibrate the quality score (105). GVCFs are produced in two different variant callers GATK haplotype caller and deep variant caller, and joint calling was done by GATK's GenotypeVCFs tool (106, 107).

As reporting tools, MultiQC, and mosdepth are used to analyze the statistics and results obtained from bioinformatics tool outputs.

3.1.3 Variant annotation and prioritization

GENNEXT developed by Özkan Özdemir, PhD and his teams, was used for the variant annotation process. Data from the ClinVar database, the Human phenotype ontology (HPO), and the Online Mendelian Inheritance in Man (OMIM) were utilized to find causal variants in known CP-associated genes, building a gene set (101, 108, 109).

Then, variants were evaluated according to the inheritance pattern and filtered out the rare variants with minor allele frequency (MAF) less than 5 in 1000 genome for an autosomal recessive inheritance pattern, and less than 5 in 100000 for autosomal dominant inheritance model according to The Genome Aggregation Database (gnomAD), and Turkish Variome data (110, 111).

In the beginning, we also focused on the variants whether they were coding region variants and/or splice region variants or predicted as damaging according to most of the in-silico prediction tools, such as SIFT (112), CADD (113), PolyPhen-2 (114), Splice AI (115), and GERP (116) as a conservation score.

We categorized filtered variants based on recommendations provided by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) (117). To categorize a variant as pathogenic, likely pathogenic, uncertain significance, likely benign, or benign, it is necessary to evaluate the variant according to the specified criteria. A conclusion is reached by combining all the criteria.

Minor allele frequency information in population databases, the prevalence of a variant in affected individuals, segregation data, functional research about the variant or gene, the consequence of mutation and its predicted impact, similarities of the changes to known variations, predictive models of effect, and inheritance aspects are some of the criteria to consider (117).

		Population data	Computational and predictive data	Functional data	Segregation data	De novo data	Allelic data	Other database	Other data
Benign	Strong	MAF is too high for disorder BA1/BS1 OR observation in controls inconsistent with disease penetrance BS2		Well-established functional studies show no deleterious effects BS3	Nonsegregation with disease BS4				
	Supporting		Multiple lines of computational evidence suggest no impact on gene /gene product BP4 Missense in gene where only truncating cause disease BP1 Silent variant with non predicted splice impact BP7 In-frame indels in repear w/out known function BP3				Observed in trans with a dominant variant BP2 Observed in cis with a pathogenic variant BP2	Reputable source w/out shared data = benign BP6	Found in case with an alternate cause BP5
Pathogenic	Supporting		Multiple lines of computational evidence support a deleterious effect on the gene /gene product PP3	Missense in gene with low rate of benign missense variants and pathogenic missenses common PP2	Cosegregation with disease in multiple affected family members PP1			Reputable source = pathogenic PP5	Patient's phenotype of FH highly specific for gene PP4
	Moderate	Absent in population databases PM2	Novel missense change at an amino acid residue where a different pathogenic missense change has been seen before PM5 Protein length changing variant PM4	Mutational hot spot or well-studied functional domain without bening variation PM1		De novo (without paternity & maternity confirmed) PM6	For recessive disorders, detected in trans with a pathogenic variant PM3		
	Strong	Prevalence in affecteds statistically increased over controls PS4	Same amino acid change as an established pathogenic variant PS1	Well-established functional studies show a deleterious effect PS3		De novo (paternity and maternity confirmed) PS2			
	Very strong		A predicted null variant in a gene where LOF is a known mechanism of disease PVS1						

Figure 8. ACMG standards and guidelines for variant interpretation (117)

3.1.4 Exome sequencing-based analysis of copy number variation (CNV)

Although, exome sequencing data has limitations for detecting large CNVs, but also a cost-effective and reliable technique for detecting small deletions or duplications within the coding and splice site regions.

The Parliament2 tool was used for CNV and SV calling of WES data. This tool provides a combination of different callers, including Breakdancer, Breakseq2, CNVnator, Delly2, Manta, and Lumpy (118). For the annotation process of these variants, AnnotSV was used (119).

4 RESULTS

4.1 Characteristics of the CP Cohort

A total of 58 patients who have CP-like syndrome with an average age of 6.2 years, (27 females, 31 males), analyzed between the years of 2008 and 2021, were included in the cohort. Global developmental delay, seizures, and muscular tone abnormalities were among the most prominent phenotypes, as generally observed in CP cases. Figure 9 summarized the distribution of prominent phenotypes that the participants in our cohort have.

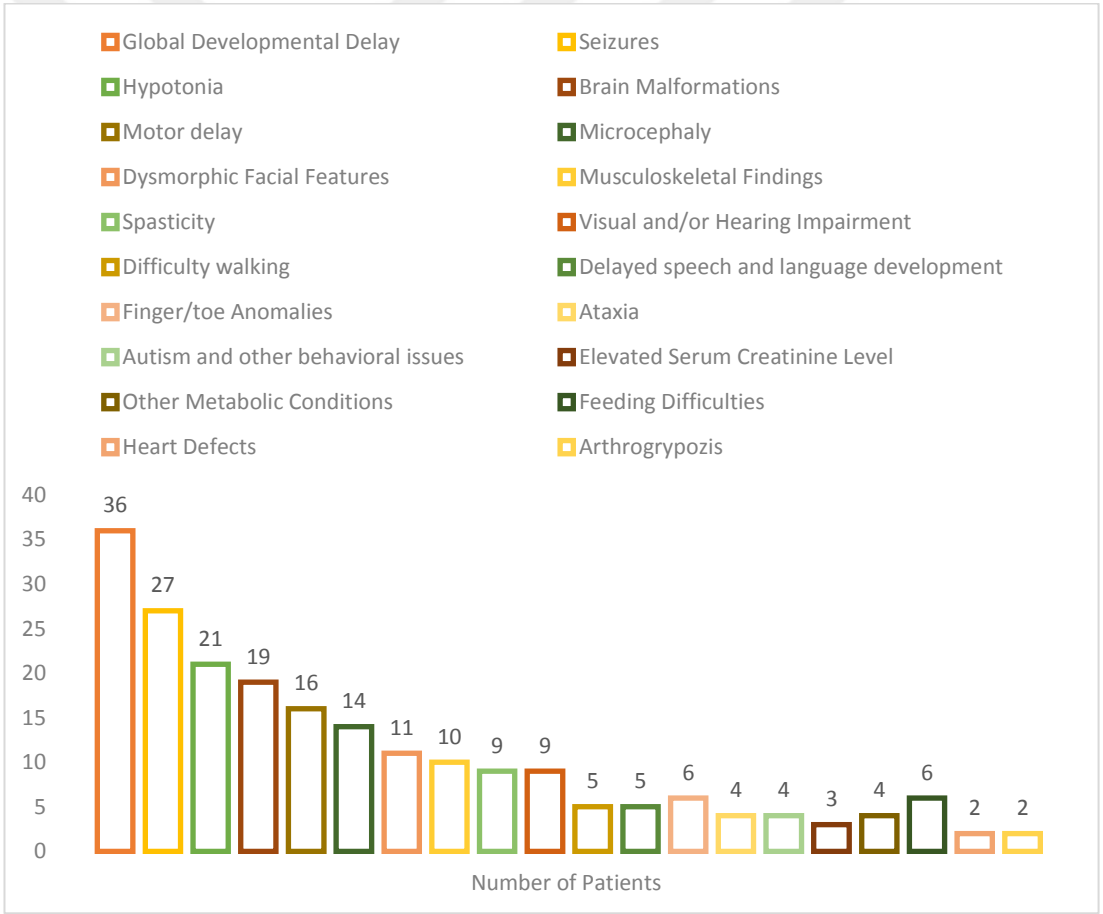

Figure 9. Distribution of patients and prominent clinical features

Across the 58 participants, 22 (38%) had identified risk factors including prematurity, small for gestational age, birth asphyxia, postnatal respiratory distress,

polyhydramnios, vaginal bleeding in the mother, breech presentation in delivery, neonatal sepsis, and intrauterine growth retardation, associated with their CP, and 36 (62%) had no or unknown identified risk factors (Figure 10).

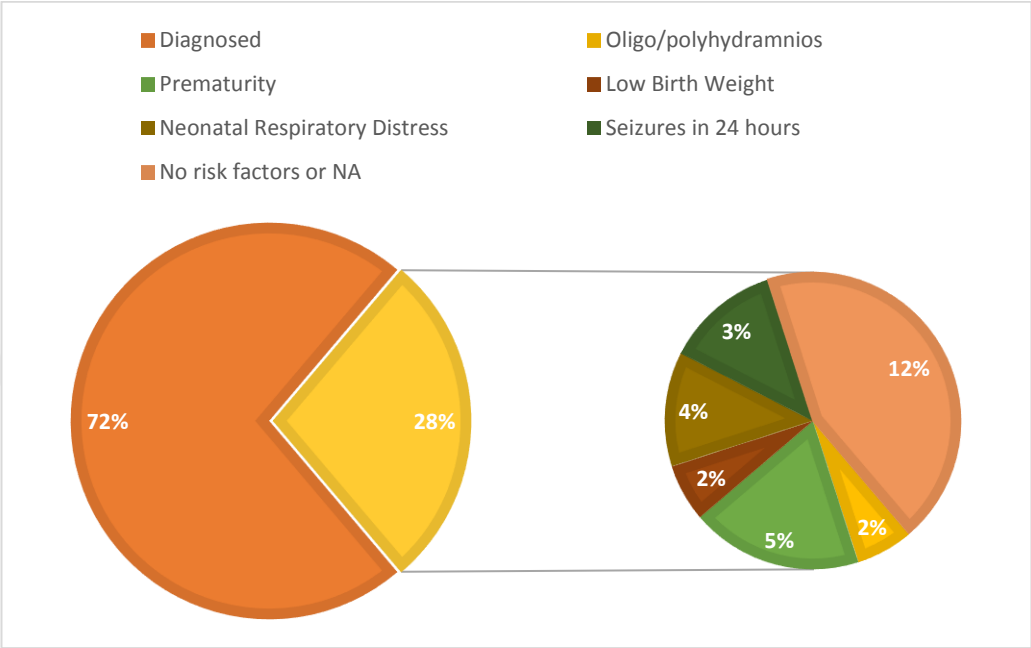

Figure 10. Distribution of risk factors in exome-negative patients

4.2 Clinical- and Whole Exome Sequencing Data

Among 58 cases, 52 CES and 6 WES data were reanalyzed, and 42 of them (approximately 72.4%) have a candidate variant that may be related to the CP phenotype. As a result of reanalysis, candidate variants compatible with the phenotype were detected in 42 patients, while no variant that could explain the phenotype was found in 16 patients. These cases resulted as exome negative.

Among the candidate, variants, 22 have been classified to be both likely pathogenic or pathogenic, while the remaining variants were classified to be of unknown significance. The genetic variations are presented in detail in Table 4 given along with an ACMG classification for each of them.

Table 4. Candidate variants discovered in the results of the reanalyzed CP cohort

Patient	***Gene***	**Variant**	**Variant Type**	**Zygosity**	**ACMG Classification**
CES-1	*SCN2A*	NM_001040142.2:c.5726C>T (p.Ala1909Val)	Missense	Het	VUS
CES-2	*PLA2G6*	NM_003560.4: c.1772G>A (p.Arg591Gln)	Missense	Hom	Pathogenic
CES-3	*GATM*	NM_001482.3: c.1081A>G (p.Asn361Asp)	Missense	Hom	VUS
CES-4	*EMD*	NM_000117.3: c.83-2A>G	Splice site variant	Hem	Likely Pathogenic
CES-5	*EMD*	NM_000117.3: c.83-2A>G	Splice site variant	Hem	Likely Pathogenic
CES-6	*GRM1*	NM_001278064.2: c.1124G>A (p.Arg375Gln)	Missense	Hom	VUS
CES-7	*NEGATIVE*				
CES-8	*SCN8A*	NM_014191.4: c.3352G>C (p.Asp1118His)	Missense	Het	VUS
CES-9	*ABHD14A-ACY1*	NM_001316331.2: c.1360C>T (p.Arg454Cys)	Missense	Hom	Pathogenic
CES-10	*COL6A1*	NM_001848.3: c.1693C>T (p.Arg565Ter)	Stop gain	Hom	Pathogenic
CES-11	*SAMD9*	NM_017654.4: c.3317C>A (p.Ala1106Glu)	Missense	Het	VUS
CES-12	*NEGATIVE*				
CES-13	*SLC9A6*	NM_001379110.1: c.1983G>A (p.Met661Ile)	Missense	Hem	VUS
CES-14	*ARID1B*	NM_001374828.1:c.1391_1400del (p.Gly464fs)	Frameshift Truncation	Het	Pathogenic
CES-15	*NEGATIVE*				
CES-16	*WWOX*	NM_016373.4: c.716T>G (p.Leu239Arg)	Missense	Hom	Likely Pathogenic
CES-17	*CLIC2*	NM_001289.6 : c.103C>T (p.Arg35Cys)	Missense	Hem	VUS
CES-18	*NEGATIVE*				
CES-19	*ASXL1 / SETBP1*	NM_015338.6: c.2077C>T (p.Arg693Ter) NM_015559.3: c.2612T>C (p.Ile871Thr)	Stop gain / missense	Het /Het	Pathogenic Pathogenic
CES-20	*PNPT1*	NM_033109.5:c.2212C>T (p.Arg738Cys)	Missense	Het	VUS
CES-21	*NEDD4L*	NM_001144967.3:c.1116G>T (p.Glu372Asp)	Missense	Het	VUS
CES-22	*ABL1*	NM_005157.6:c.910G>A (p.Val304Ile)	Missense	Het	VUS
CES-23	*NTNG1*	NM_001113226.3:c.67T>C (p.Tyr23His)	Missense	Het	VUS
CES-24	*DMD*	NM_004006.3: c.4071+2T>C	Splice site variant	Hem	Pathogenic
CES-25	*CTNNB1*	NM_001904.4: c.1923dup (p.Glu642ArgfsTer6)	Frameshift Elongation	Het	Pathogenic
CES-26	*DEAF1*	NM_021008.4:c.62C>G (p.Ala21Gly)	Missense	Hom	VUS
CES-27	*ATP1A2*	NM_000702.4:c.736A>G (p.Asn246Asp)	Missense	Het	VUS
CES-28	*NEGATIVE*				
CES-29	*KCNT1*	NM_020822.3:c.2756C>T (p.Thr919Met)	Missense	Het	VUS
CES-30	*NEGATIVE*				
CES-31	*NEGATIVE*				
CES-32	*NSUFS3*	NM_004551.3:c.252A>T (p.Leu84Phe)	Missense	Hom	VUS
CES-33	*NEGATIVE*				
CES-34	*COL6A1*	NM_001848.3: c.811C>T (p.Arg271Ter)	Stop gain	Hom	Pathogenic
CES-35	*SAMD9*	NM_017654.4:c.2159del (p.Asn720ThrfsTer35)	Frameshift Truncation	Het	Pathogenic
CES-36	*NEGATIVE*				
CES-37	*NEGATIVE*				
CES-38	*COL1A2*	NM_000089.4:c.3275dup (p.Gly1093TrpfsTer17)	Frameshift Elongation	Het	Likely Pathogenic
CES-39	*NEGATIVE*				
CES-40	*KIF1A*	NM_001244008.2: c.2494C>T (p.Pro832Ser)	Missense	Hom	VUS
CES-41	*PACS1*	NM_018026.4:c.1775G>A (p.Cys592Tyr)	Missense	Het	VUS
CES-42	*ST3GAL5*	NM_003896.4: c.722G>C (p.Arg241Thr)	Missense	Hom	VUS
CES-43	*CACNA1G*	NM_018896.5: c.1211G>A (p.Arg404Gln)	Missense	Het	Likely Pathogenic
CES-44	*PPM1D*	NM_003620.4:c.1262C>A(p.Ser421Ter)	Stop gain	Het	Pathogenic
CES-45	*LINS1*	NM_001040616.3:c.1870del (p.Gln624LysfsTer4)	Frameshift Truncation	Hom	Likely Pathogenic
CES-46	*NEGATIVE*				
CES-47	*PIK3R2*	NM_005027.4: c.1117G>A (p.Gly373Arg)	Missense	Het	Pathogenic
CES-48	*SMARCA4*	NM_003072.5:c.886A>C (p.Thr296Pro)	Missense	Het	VUS
CES-49	*NEGATIVE*				
CES-50	*NEGATIVE*				
CES-51	*CACNA1E*	ENST00000367573.7:c.5365-3_5365-2del	Splice site variant	Het	Likely Pathogenic
CES-52	*PTEN*	NM_000314.8:c.275A>T (p.Asp92Val)	Missense	Het	Likely Pathogenic
WES-1	*TUBA1A*	NM_006009.4:c.514T>C (p.Tyr172His)	Missense	Het	Likely Pathogenic
WES-2	*L1CAM*	NM_001278116.2:c.414>A (p.Trp138Ter)	Stop gain	Het	Likely Pathogenic
WES-3	*NEGATIVE*				
WES-4	*SPAST*	NM_014946.4:c.1537-3C>G NM_014946.4:c.131C>T (p.Ser44Leu)	Intron variant missense	Het / Het	VUS/VUS
WES-5	*NEGATIVE*				
WES-6	*MFN2*	NM_014874.3: c.743T>C (p.Leu248Pro)	Missense	Het	Likely Pathogenic

5 DISCUSSION

Despite the existence that plenty of risk factors related to CP have been defined, many people may find it tough to identify the cause of their CP or to decisively diagnose the differential diagnosis. Because the degree of severity of the disease can vary, and CP symptoms might take longer to appear. Since the disease's signs and symptoms can vary from patient to patient, it can be misdiagnosed for other conditions. For this reason, the patient's clinic mustn't be lacking in terms of differential diagnosis or not causing misdiagnosis. Therefore, deep phenotyping is essential. Recent studies have shown that a significant proportion of CP cases can be caused by damaging genomic variants.

Clinical- and whole exome sequencing are the most prevalent NGS technology for diagnosing the genetic etiology of rare diseases. While clinical exome sequencing covers only known disease-associated genes, WES covers coding regions across the whole genome. Although CES is a more constrained analysis compared to WES, it is a more concentrated evaluation and might be more effective in identifying differential diagnoses (120).

However, in undiagnosed patients, WES is more suited when reanalysis needs to be performed, so reprocessing and interpreting raw data provides a re-evaluation of the diagnosis with new disease associations and up-to-date research. Therefore, it is quite important to store this data.

While WES is more comprehensive than CES data, it is still insufficient for the identification of structural variations (SVs), which involves copy number variants (CNVs). Poor WES's sensitivity to SVs makes detection challenging. While WES may detect some CNVs, which include indels and duplications, others are likely to go overlooked due to technical restrictions (121).

Another essential point of NGS analysis is using an appropriate and well-design bioinformatics pipeline for pre-processing of the raw data. GENNEXT pipeline which

was formed with optimized various bioinformatics tools for short-read NGS data, was used for the analysis. In the study, joint calling was performed by using two different callers, as explained in the methods, capturing all of the variants that might be causative. The joint genotyping method processes data from the entire set of genomes to increase statistical accuracy, minimize false positive calls, and enhance the sensitivity of genotype determination across each genotype (122).

This study demonstrated that a significant number of patients with CP or individuals who develop symptoms similar to CP may have an underlying monogenic disorder. The gene-based results in our study cohort were heterogeneous, which is possibly an aspect of the evidence that several genes have been identified as causally associated with CP signs and symptoms.

Based on cases with determined pathogenic or likely pathogenic mutations, the total diagnostic yield of clinical and whole exome sequencing data was 37.93%. The yield rate would be 72.4%, however, if variations of uncertain significance (VUS) that are accounted for disease-related genes or candidate genes that have significant biological candidates were taken into account. 27.58% of the total accounted for negative results (undiagnosed cases).

In our cohort, 28/58 patients were from inbreed population had definite consanguinity between their parents, or the parents were individuals from the same villages. 67.85% (19/28) had a diagnosis, and 32.15% (9/28) were exome-negative patients. 51.72% (30/58) patients were from the unbreed population, and 76.6% of them had a causative variant that was predicted as disease-causing. In the unbreed population, compound heterozygous variants were examined, but there were no candidates associated with CP.

Eight causal variants were detected in known genes that have been reported as associated with CP, involving *TUBA1A*, *CTNNB1*, *L1CAM*, *SPAST*, *KIF1A*, *SCN2A*, and *SCN8A* (4, 11, 12, 51, 123, 124, 125).

Two patients (CES-1 and CES-8) have heterozygous missense variants in sodium voltage-gated channels genes including *SCN2A* and *SCN8A*.

CES-1 patient has c.5726C>T variant which has been reported in reputable databases as a variant of uncertain significance and associated with developmental and epileptic encephalopathy 11 (MIM #613721), episodic ataxia, type 9 (MIM #618924) and seizures, benign familial infantile 3 (MIM #607745) in OMIM. The patient has facial dysmorphism, ataxia, global developmental delay, cerebellar vermis hypoplasia, and periventricular white matter hyperdensities.

CES-8 patient has a novel variant (c.3352G>C) in the *SCN8A* gene. Although in the phenotypes associated with this gene, seizures typically begin infancy or are seen between 6 and 12 months of age, our patient did not have a history of epilepsy at the time of the clinical evaluation, which was at the age of 7 months. The patient's presentive complaint was neonatal hypotonia, and she had a developmental delay from birth. No CP-related risk factors were observed.

Sodium-channel genes are generally associated with developmental and epileptic encephalopathies, but recent studies point to the phenotypic extension described in sodium channelopathies. Although these genes may explain the phenotype of patients, it is uncertain whether they can explain the CP phenotype, because of characteristics of CP.

CES-25 is a male patient with mental motor retardation, retinal detachment, and central hypotonia, but detailed clinical data could not be provided for the patient. Result of reanalysis, a heterozygous frameshift elongation variant, c.1923dup in *CTNNB1* gene, associated with the neurodevelopmental disorder with spastic diplegia and visual defects (MIM #615075) and exudative vitreoretinopathy 7 (MIM #617572).

The variant is classified as pathogenic, with PVS1, PM2, and PP5 (single submission as pathogenic in Clinvar) evidence. The variant was considered to have the potential to explain the patient's phenotype in light of all of this information.

WES-1 is a 5-year-old female patient with severe microcephaly (<-3SD), seizures, hearing impairment, contractures, and various congenital brain malformations including brainstem dysplasia, ventriculomegaly, hypoplasia of corpus callosum and cerebellar vermis, and abnormality of pons. *TUBA1A* c.514T>C heterozygous missense variant identified in WES-1 has not previously been reported before as a novel variant. This variant is classified as likely pathogenic with PM1, PM2, PP2, and PP3 criteria. However, there is no affected family member, and there is no consanguinity between parents, therefore segregation analysis was needed. Segregation analysis revealed that the variant is de-novo. (the process has been carried out at an external center)

The primary diagnostic features of the *TUBA1A*-related disorders which are called tubulinopathies comprise congenital brain anomalies, microcephaly, delayed development, and seizures. In OMIM, *TUBA1A* (MIM #611603) is cataloged with a Lissencephaly with an autosomal dominant inheritance pattern. It is also known that the majority of the variants relevant to the disorder were discovered as de-novo. Due to the compatibility of the cases in the literature with the case in this study and the estimated damaging effect of the variant, it was decided that the given variant was causative.

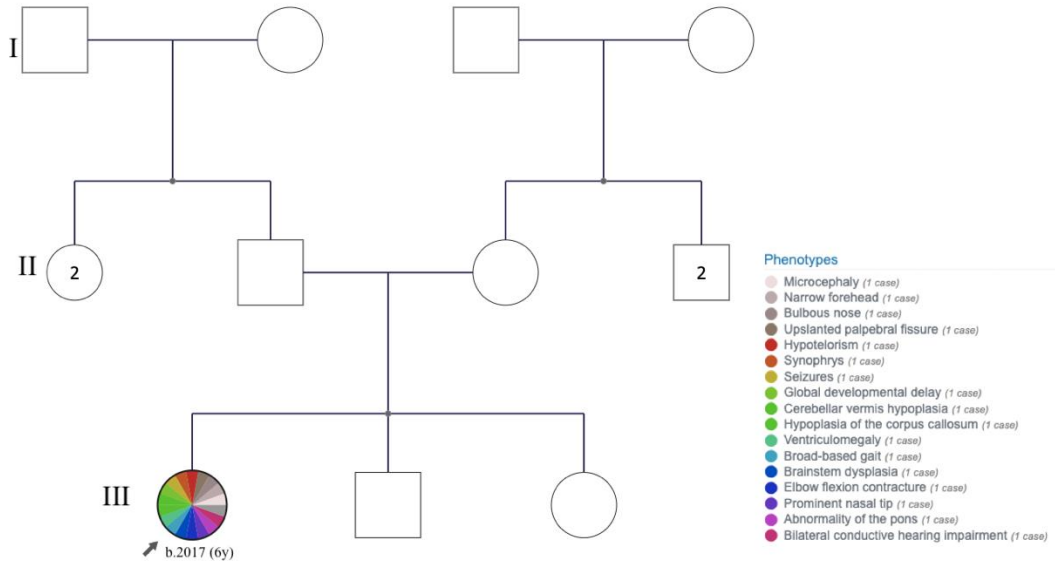

Figure 11. The information on the clinical features and pedigree of WES-1

WES-2, a male patient aged 11 was admitted and has developmental delays and postnatal hypotonicity. At the age of 2.5, he experienced his first seizure and began using medicines for antiepileptic purposes. In addition, family members have a history of epilepsy too. His older brother is 21 years old and healthy. He had a febrile seizure at the age of 7-8 years and took antiepileptic drugs for 4 years. The father has a diagnosis of epilepsy. A cranial MRI was performed when the patient was 2 years old in 2014, with cerebellar atrophy, partial agenesis and hypoplasia of the corpus callosum, and enlargement of lateral ventricles.

Considering the patient's phenotypes, a hemizygous stop gain variant c.414G>A was given priority in *L1CAM*, which is one of the genes known to be associated with CP. Segregation analysis was conducted at an external center and the result of the analysis the variant is detected as de-novo, and the variant is reclassified as pathogenic with PM2, PVS1, and PS2 criteria.

Although this variant is not intended to explain the epilepsy of the older brother and father, it does explain the gait ataxia, seizures, and multiple brain malformations in the proband. Additionally, because it is a novel variant, it will contribute to the literature.

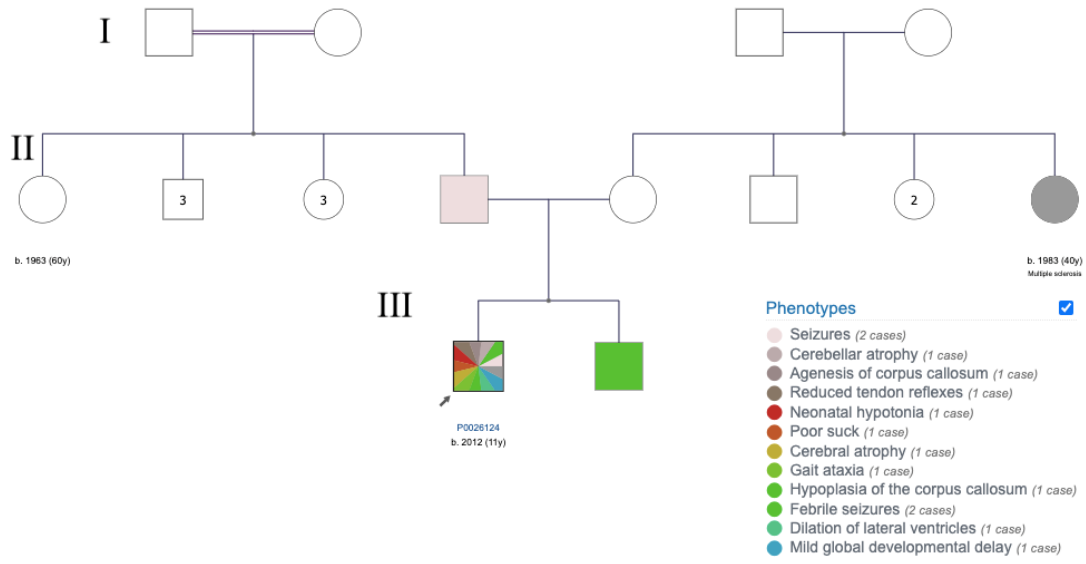

Figure 12. The information on the clinical features and pedigree of WES-2

WES-4 patient, a 3-year-old male, presented with complaints of sitting and walking with support and inability to take independent steps since the age of 1 year. On physical examination, spasticity was detected in all extremities. There is no consanguinity between the parents, also no family member who is affected with similar symptoms. Therefore, heterozygous variations were thoroughly investigated. Following the variant filtering two candidate variations were identified in the *SPAST* (also called *SPG4*) gene associated with hereditary spastic paraplegia (HSP), with an autosomal dominant inheritance pattern (MIM #182601).

The age of onset and degree of severity of SPG4 are known to be widely varied. *SPAST* variant presents a phenotype diagnostic of HSP, indicating elevated but not complete. Around six percent of those diagnosed with *SPAST* mutation have no symptoms at all.

Result of the reanalysis, two heterozygous variants, c.1537-3C>G (intron variant) and c.131C>T (missense) in the *SPAST* gene were detected. c.131C>T (p.Ser44Leu) is a common variant with an allele frequency of 0.5 in the gnomAD database and has been previously submitted in ClinVar as a pathogenic risk factor with several publications. In the cases reported in the studies, it was determined that the onset of symptoms was earlier in the presence of this risk allele (126, 127, 128).

Although intronic variants and polymorphisms (because of above the MAF threshold) were not prioritized among the filtering criteria, we were able to detect the risk factor because variants classified as pathogenic in ClinVar were evaluated in this analysis. In this way, we discovered the existence of a second variant.

Despite the mean age of onset, reported as 29±17 (129), representing a wide age range from early infancy to late adulthood, the early onset of symptoms in this study was associated with the presence of a genetic modifier which has been reported before as a risk factor in the patient. However, considering the risk of developing the disease with a late onset of the other allele without a risk factor, it was recommended that other family members remain under neurology follow-up.

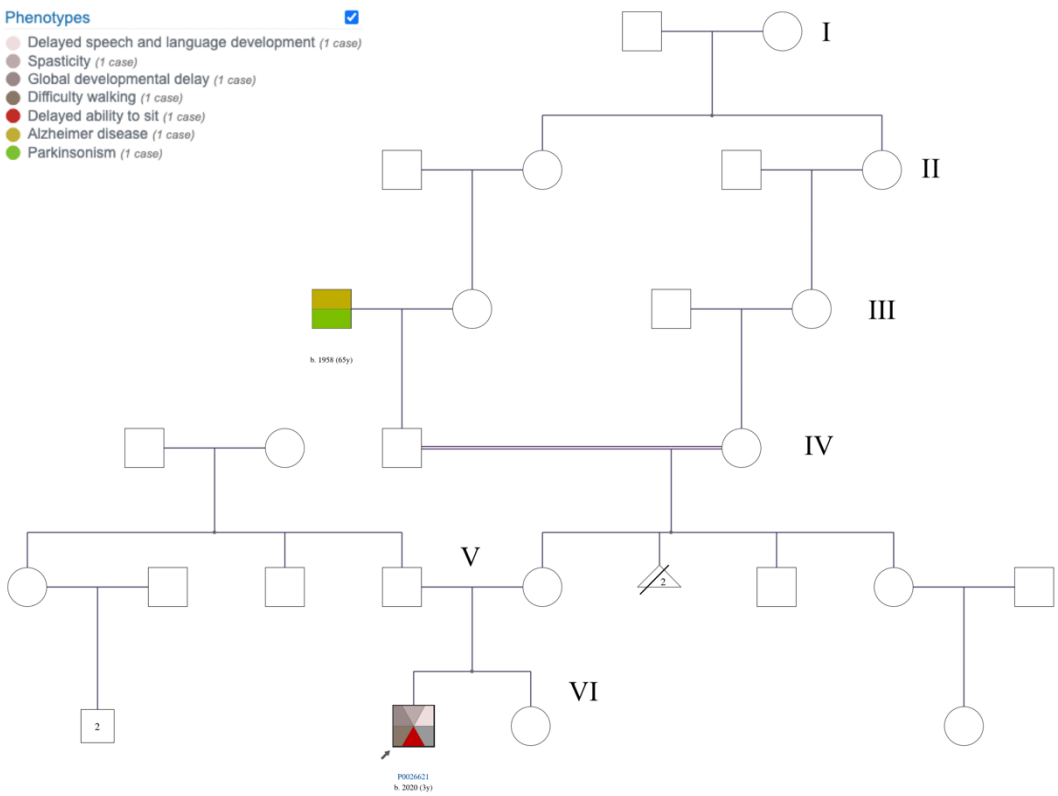

Figure 13. The information on the clinical features and pedigree of WES-4

Several genes were mutated in two or more related or unrelated patients in the cohort, including *EMD, COL6A1, and SAMD9* genes. Detected mutation in *EMD* gene, previously reported as likely pathogenic significance in ClinVar in a patient with neuromuscular disease. In our cohort, 23 and 25-year-old (CES-4 and CES-5 patients) two brothers who are affected by Emery-Dreifuss muscular dystrophy shared a hemizygous likely pathogenic splice site variant (c.83-2A>G) in the *EMD* gene. It was thought that the detected variants can explain the clinic of both of our patients.

Two patients (CES-10 and CES-34) have homozygous stop gain variants (c.1693C>T and c.811C>T, respectively) in one of the collagen genes, the *COL6A1* gene cause Bethlem Myopathy 1 (MIM #158810) and Ulrich Congenital Muscular Dystrophy 1 (MIM #254090). One of them has distal arthrogryposis and the other patient has presentive complaints including hypotonia, muscular dystrophy, scoliosis, torticollis, kyphosis, hip dislocation, and joint hyperextensibility. The mutations

detected in the *COL6A1* gene have ClinVar submission as pathogenic, and c.811C>T variant reported in a patient with Bethlem myopathy previously. It was thought that the detected variants were compatible with the clinic of both of our patients.

Two patients (CES-11 and CES-35) have heterozygous missense and frameshift truncation variants (c.3317C>A and c.2159del, respectively) in the *SAMD9* gene which codes for a protein implicated in the endosome fusion pathway. CES-11 patient has c.3317C>A variant, which is a novel variant classified as Uncertain significance with PM2 criteria. The male patient has scoliosis, spastic diplegia, pes planus, hyperreflexia, feeding difficulties, cryptorchidism, and neonatal asphyxia.

The c.2159del variant has been previously reported as likely pathogenic and has uncertain significance associated with MIRAGE syndrome (MIM #617053) by multiple submissions. CES-35 patient has a global developmental delay, speech delay, walking difficulties, clinodactyly, and visual impairment including strabismus. Since both patients had phenotypes compatible with the MIRAGE syndrome clinic, it was thought that this syndrome, which is an autosomal dominant inheritance pattern, could explain the clinic.

Also, a female patient (CES-19) was evaluated as having multi-Mendelian inheritance, carrying pathogenic variants in *ASXL1* and *SETBP1*. The patient was born as small for the gestational week by 1400 gr at 35+2 gw. Some developmental milestones like head control, walking, and speech development were delayed. There are signs of sensorial hearing loss and mild mental retardation. A Pediatric Nephrology examination revealed proteinuria, thrombocytopenia, and hematuria. She had surgery for an inguinal hernia at 5 years old.

End of the reanalysis, two heterozygous pathogenic variants, c.2077C>T (stop gain variant) in *ASXL1* gene associated with Bohring Opitz syndrome (MIM #605039) and c.2612T>C (missense variant) in *SETBP1* gene related to Intellectual Developmental disorder (MIM #616078) and Schinzel-Giedion Midface Retraction syndrome (MIM #269150) were detected. Although the developmental delay of the

patient is generally explained by Bohring Opitz syndrome, the *SETBP1* variant was thought to explain hearing loss and other metabolic findings such as thrombocytopenia and hematuria (130). As far as is known, there was no consanguinity between the parents, no miscarriage, or affected individuals in the family.

It was challenging to identify risk factors in patients with CP-like symptoms for several reasons in our study. One of the primary obstacles was the lack of comprehensive hospital records for thorough analysis, limiting our ability to conduct a detailed examination. Additionally, not all infants necessitate monitoring in the neonatal intensive care unit (NICU), leading some families to seek medical attention at a later stage. In such cases, relying on family accounts or medical reports becomes essential in identifying potential risk factors. However, this reliance on secondary sources can result in incomplete data, particularly in developing countries where not all pregnancies receive adequate monitoring, and documentation during childbirth and the postnatal period might be insufficient.

In this study, we classified risk factors into prenatal, perinatal, and postnatal categories. In 7% of cases, a prenatal risk factor was identified, while 14% had a perinatal risk factor, and 17% experienced postnatal risk factors, such as the development of seizures on the first day after birth. In 14% of cases, a reliable risk factor could not be determined. Among the risk factors studied, premature birth and small weight for gestational age were observed more frequently, accounting for approximately 32% of cases identified with a risk factor.

When cases with identified risk factors were examined, it was observed that in approximately 60% of these cases, a genetic variation that could explain their clinical condition was detected, while in the remaining approximately 40% of cases, no identifiable causative variants that could explain their condition were found. The lack of a significant ratio of identified causative variants in this distribution could be attributed to several factors.

Firstly, our sample size may not have been sufficiently large or powerful enough

to detect statistically significant associations. Secondly, despite having CP-like symptoms, the patients might have various genetic diagnoses, which could contribute to the complexity and the absence of a clear pattern in the results.

Our study also had other constraints. First, the sample size of the cohort is inadequate, as previously mentioned, because it is still expanding. For stronger statistics, a greater number of participants is necessary for a group of conditions like cerebral palsy, whose etiology is unknown and may be multifactorial.

Although our cohort consisted of patients with phenotypes similar to CP phenotypes, there were also patients with various brain malformations and other neurological findings. The differential diagnosis of the patients required deep phenotyping, but clinical examination or detailed clinical data could not be provided for every patient.

Finally, this study was performed using only clinical and whole-exome sequencing data. The majority of patients were reanalyzed on clinical exome data. Therefore, some patient diagnoses may have been missed, and more advanced and comprehensive NGS technologies were not used at this stage.

6 CONCLUSION

In this study, the diagnostic yields in a phenotypically wide spectrum of patients in Turkey with CP-like symptoms were evaluated. According to ACMG-AMP recommendations, 38% of patients have pathogenic or likely pathogenic variants of clinical relevance.

Our findings in the result of reanalysis, comprise genetic alterations that contribute to an extensive number of genetic disorders. The genetic burden of CP may be influenced by variable penetration or onset age observed in pathogenic alterations in genes in our data, and it may contribute to the elucidation of the etiology of CP.

Despite the cohort's inclusion of patients with CP-like symptoms, several other genetic diagnoses were found, supporting the existence of genetic heterogeneity. As such, these diagnoses are significant when they indicate an unusual presentation of a genetic condition.

At the end of the reanalysis, 16 patients (or around 27.5%) remained classified as undiagnosed. To determine the differential diagnosis, further NGS technologies will be utilized, along with deep phenotyping.

In conclusion, our investigation has beneficial clinical outcomes. Specific genetic findings can be informative for families and can assist existing health services. These medical services can help with issues such as counseling for recurrence risk and family pregnancy planning.

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