Clinical Significance of Rare Copy Number Variations in Neurodevelopmental Disorders

PhD thesis

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Budapest
2023

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Abbreviations

AC	amniocentesis
ACM	arteria cerebri media
ACMG	American College of Medical Genetics and Genomics
AD	autosomal dominant
ADHD	attention deficit/hyperactivity disorder
array CGH	array comparative genomic hybridization
AR	autosomal recessive
AS	Angelman syndrome
ASD	autism spectrum disorder
atrial SD	atrial septal defect
BAC	bacterial artificial chromosome
B	benign
BMI	body mass index
bp	basepair
BP	break point
CA	congenital anomaly
CALM	café au lait macule
CCA	corpus callosum abnormality
CCR	complex chromosomal rearrangement
CHD	congenital heart disease
CMA	chromosomal microarray analysis
CNV	copy number variation
CT	computer tomography
CVS	chorionic villi sampling
DCD	developmental coordination disorder
DD	developmental delay
DECIPHER	Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources
DGV	Database of Genomic Variants
DMR	differentially methylated region
DOC	depth of coverage

DSB	double-strand break
DSM	Diagnostic and Statistical Manual of Mental Disorders
EEG	electroencephalogram
EMG	electromyogram
FISH	fluorescent *in situ* hybridization
FoSTeS	fork stalling and template switching
GDD	global developmental delay
GERD	gastroesophageal reflux disease
GH	growth hormone
GI	gastrointestinal
HC	head circumference
HCMP	hypertrophic cardiomyopathy
HPO	Human Phenotype Ontology
HT	hypertension
ID	intellectual disability
IEM	inborn errors of metabolism
IG-DMR	intergenic differentially methylated region
indel	insertion/deletion
IU	intrauterine
IUGR	intrauterine growth restriction
IVS	intervening sequence
JH	joint hypermobility
Kb	kilobase pair
LB	likely benign
LCR	low copy repeat
LGA	large for gestational age
LOH	loss of heterozygosity
LP	likely pathogenic
LRS	long read (third-generation) sequencing
LTR	long terminal repeat
LINE	long interspersed nuclear element
m	month

MA	minor anomaly
mat	maternal
Mb	megabase pair
MCR	minimal critical region
MI	mitral valve insufficiency
MIR	mammalian-wide interspersed repeat
MLPA	multiple ligation-dependent probe amplification
MMBIR	microhomology-mediated break-induced replication
MRI	magnetic resonance imaging
MS-MLPA	methylation-specific MLPA
MuHy	muscular hypotonia
NAHR	non-allelic homologous recombination
NDD	neurodevelopmental disorder
NH-CSS	Netchine-Harbinson Clinical Scoring System
NHEJ	non-homologous end joining
OMIM	Online Mendelian Inheritance in Man
P	pathogenic
pat	paternal
PCR	polymerase chain reaction
PDA	patent ductus arteriosus
PI	pulmonary valve insufficiency
PS	pulmonary valve stenosis
PVL	periventricular leukomalacia
PWS	Prader-Willi syndrome
QMPSF	quantitative multiplex PCR of short fluorescent fragments
ROH	regions of homozygosity
SD	segmental duplication
SGA	small for gestational age
SMS	Smith-Magenis syndrome
SNP	single nucleotide polymorphism
SNV	single nucleotide variation
SRS	Silver-Russell syndrome

STPC	single transverse palmar crease
TAD	topologically associating domain
UPD	uniparental disomy
US	ultrasound
ventricular SD	ventricular septal defect
VUS	variant of uncertain significance
WBS	Williams-Beuren syndrome
WES	whole exome sequencing
WGS	whole genome sequencing
XLD	X-linked dominant
XLID	X-linked intellectual disability
y	year

1. Introduction

1.1. Neurodevelopmental disorders

1.1.1. Definition

Neurodevelopmental disorders (NDDs) are chronic medical conditions characterized by deficits in one or more developmental domains due to altered neural development (Ismail & Shapiro, 2019; Savatt & Myers, 2021; Thapar et al., 2017). NDDs include intellectual disability (ID), global developmental delay (GDD), autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADHD), specific learning disorders, motor disorders (developmental coordination disorder, stereotypic movement disorder, tic disorders, etc.), and communication disorders (language disorder, speech sound disorder, etc.) (Savatt & Myers, 2021). While the above listed are distinct diagnoses recorded within the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5), they show wide phenotypic overlap and often present as comorbidities (Morris-Rosendahl & Crocq, 2020; Savatt & Myers, 2021). Widening the scope – and certainly if NDDs are viewed through the lens of genetic etiology - conditions such as cerebral palsy, epilepsy and schizophrenia can also be grouped as NDDs (Ismail & Shapiro, 2019; Savatt & Myers, 2021).

NDDs are usually diagnosed in the pediatric setting; and as their overall prevalence is high [~17% in children 3-17 years old in the United States, (Zablotsky et al., 2019)], they pose a considerable challenge in everyday pediatric practice. Affected children face difficulties in adaptive functions, social interactions and self-care, which may translate to entire families under serious emotional (and often financial) stress. Early and accurate diagnosis is therefore not only important for optimal care of the affected child, but for alleviating the burden and guilt on the parents as well.

1.1.2. A short historical overview

Common NDDs have been chronicled in historic texts well before clinical entities were realized. The symptoms of ADHD were first described as “attentio volubilis” in 1775 by Melchior Adam Weikard (Barkley & Peters, 2012). According to psychiatric historians, the concept of developmental disorders was first mentioned in a textbook published in 1820, authored by Étienne Jean Georget (Morris-Rosendahl & Crocq, 2020) and defined as a “lack of development of intellectual faculties”. Dr. J. L. Down

described Down syndrome in 1862. He also described patients with symptoms we would now consider autistic traits. Down recognized three forms of ID: congenital, accidental and developmental (Down, 1887), and also described developmental regression and behavioral stereotypies. Jean Itard, along with his student Édouard Séguin, are considered the forefathers of special needs education (Seguin, 1866). Autism was first recognized as a diagnostic category by Leo Kanner in 1943 (Harris, 2018).

In the era of modern medicine, the term "developmental disorders" was first included in the DSM III (published in 1980), wherein it mostly referred to autistic disorder. As mentioned above, the DSM-5 (published in 2013) included the category of NDDs as we think of them today, with the previously listed subcategories.

1.1.3. Neurodevelopmental models

To facilitate understanding of NDDs, researchers have earlier adopted models from adult neuropsychology. This neuropsychological approach postulates that the brain is subdivided into distinct domains, which function separately from each other. Therefore, if one domain malfunctions, the other modules are not necessarily affected; e.g. localized brain injuries in adults can lead to very specific cognitive deficits, while other functions remain normal (D'Souza & Karmiloff-Smith, 2017; Ismail & Shapiro, 2019). Even in adults, this view is somewhat controversial, as there is convincing functional evidence of large-scale interconnectivity within the fully developed human brain (Bressler & Menon, 2010). The static neuropsychological model is even less satisfactory in the context of a developing child. This led to the description of developmental approaches, which view development as a self-organizing process resulting from interaction between several systems, influenced by intrinsic and extrinsic factors, genes and environment, etc. (D'Souza & Karmiloff-Smith, 2017). One such model is neuroconstructivism (Westermann et al., 2007), which characterizes the plastic child brain as an atypical system developing under altered conditions. Due to its complex and interactive nature, an initial impairment will have cascading effects on the overall development of the brain. Neuroconstructivism draws our attention to the importance of these dynamic interactions and highlights the need to track developmental trajectories (D'Souza & Karmiloff-Smith, 2017).

An increasing amount of evidence shows that NDDs have common genetic risk alleles, also shared by certain psychiatric disorders (schizophrenia, bipolar disorder) (Morris-Rosendahl & Crocq, 2020; Singh et al., 2017). The burden of genetic variants is positively correlated with the severity of the childhood NDD (Girirajan et al., 2011), and is greater than the burden in schizophrenia (Kirov et al., 2014). The model of a neurodevelopmental continuum has thus been proposed, which suggests pediatric NDDs and adult psychiatric disorders lie on an etiological continuum and have overlapping pathomechanisms, rather than being distinct disorders. This approach aims to reconcile the high rate of comorbidity seen in the neurodevelopmental and adult-onset neuropsychiatric disorders, and suggests the possibility of shared therapeutic options down the line.

1.1.4. Etiologic overview

The etiology underlying NDDs is exceedingly complex, primarily including conditions that interfere with brain development and/or functioning. Well defined causes are environmental factors such as teratogens (maternal alcohol consumption, maternal hypothyroidism, ionizing radiation, mercury, lead, certain antiepileptic drugs, e.g. valproic acid and phenytoin, recreational drugs etc.), infections (toxoplasma, rubella, cytomegalovirus, herpes simplex, varicella, syphilis, parvovirus B19, etc.), malnutrition, perinatal asphyxia, intracranial hemorrhage, and neurologic disorders (epilepsy, structural brain abnormalities, etc.).

The most common etiologic factors are hereditary conditions, and thanks to the technological advances in the diagnostic field, genetic underpinnings show increasing heterogeneity (Rauch et al., 2012). Major groups include the following:

- Chromosomal abnormalities are microscopically visible numerical and structural alterations of the chromosomes. These account for up to 15% of cases with ID (P Chiurazzi & Oostra, 2000), including Down syndrome [Online Inheritance In Man (OMIM) #190685], the most common genetic disorder. The majority are also associated with neurodevelopmental disorders.

- Microdeletion/microduplication syndromes or genomic disorders caused by copy number variations (CNVs). These will be the main focus of this dissertation, and will be detailed below.
- Monogenic disorders are caused by pathogenic variations in a single gene. Over 800 genes are linked with syndromic or non-syndromic ID (Pietro Chiurazzi & Pirozzi, 2016). Inheritance can be autosomal dominant (AD), in which cases the associated ID is often quite severe. Autosomal recessive (AR) disorders are caused by biallelic variants in a gene, and include most inborn errors of metabolism (IEM). Over 100 genes [a notable ~10% of chromosome X vs. ~4% genomic average, (Pietro Chiurazzi & Pirozzi, 2016)] are associated with X-linked ID (syndromic or non-syndromic), a common cause of NDDs in males; females are much less frequently, and usually less severely affected. Important examples are the fragile X (OMIM #300624) and Rett syndromes (OMIM #312750).
    - Mitochondrial disorders are a subgroup of IEM caused by mutations of nuclear genes or pathogenic variation of the mitochondrial DNA. Affected patients usually have very complex phenotypes.

Finally, a proportion of children with NDDs have a multifactorial etiology (https://www.uptodate.com/contents/intellectual-disability-in-children-evaluation-for-a-cause).

1.2. Copy number variations

CNVs are submicroscopic [a few dozen basepair (bp) to several megabase pair (Mb) large] structural variations in the genome (Pös et al., 2021). CNVs are common in healthy individuals - in which cases they are termed copy number polymorhisms (Iafrate et al., 2004; Sharp et al., 2005), represent susceptibility loci in complex diseases, and have been identified as one of the most common causes underlying NDDs and congenital anomalies (Cooper et al., 2011; Kaminsky et al., 2011). Disorders caused by CNVs tend to have variable expressivity, and many have been shown to have incomplete penetrance (Kaminsky et al., 2011; Smajlagić et al., 2021). CNVs can be either recurrent or non-recurrent based on the molecular mechanisms by which they are formed: approximately 75% occur as individually rare, non-recurrent alterations,

whereas the remainder are recurrent rearrangements associated with genomic hotspots (Deshpande & Weiss, 2018; Kaminsky et al., 2011; Shaikh, 2017; Sharp et al., 2005).

CNVs can lead to disease by altering the dosage of one or more dosage-sensitive (haploinsufficient or triplosensitive) genes, disrupting a gene in a specific location leading to its inactivation, creating fusion genes, disrupting the expression of a gene by altering upstream or downstream regulatory elements or topologically associating domains (TADs), or they may unmask point mutations associated with recessive disorders on the other allele (Gu et al., 2008; Shaikh, 2017).

1.2.1. Recurrent CNVs

In the past 15 years many genomic regions have been identified that are predisposed to both deletions and duplications (also referred to as recurrent reciprocal CNV regions (Smajlagić et al., 2021). These genomic regions are flanked by repetitive sequences called segmental duplications [SDs, also known as low copy repeats (LCRs)]. SDs are highly homologous (with at least 90% sequence identity) blocks of DNA that flank unique genomic segments, occur repeatedly in the genome and are usually 10-400 Kb in size. SDs comprise a notably large portion of the human genome (~5-10%) due to the evolutionary expansion that occurred during primate evolution, and they frequently cluster around centromeric and subtelomeric regions of chromosomes. SDs associated with known genomic disorders are usually larger than 100 Kb and have sequence identity over 96%. This high level of identity predisposes SDs to misalignment, and subsequent recombination may then lead to the formation of reciprocal CNVs; a molecular mechanism referred to as non-allelic homologous recombination (NAHR) (Antonacci et al., 2010; L. Chen et al., 2014; Linardopoulou et al., 2005; Marques-Bonet & Eichler, 2009; Shaikh, 2017; Shaw & Lupski, 2004; Stankiewicz & Lupski, 2002; Tai et al., 2016).

Several human chromosomes are enriched with SDs (e.g. 7, 15, 16, 17, 22), and multiple CNV disorders have been associated with these hotspot regions (Girirajan et al., 2010), including the earliest and most well-known: the Prader-Willi and Angelman syndromes (PWS/AS, deletions in 15q11-q13) (Driscoll et al., 1998 [Updated 2023]), Williams-Beuren syndrome (WBS, deletions in 7q11.23) (Morris, 1999 [Updated 2023]) and the 22q11.2 deletion syndrome (previously referred to as

DiGeorge/Velocardiofacial syndromes) (McDonald-McGinn et al., 1999 [Updated 2020]). These disorders are most commonly *de novo* alterations, show near complete penetrance, and tend to be more traditionally "syndromic", but with marked variable expressivity. Other similar recurrent syndromic CNV disorders are e.g. the Smith-Magenis syndrome (SMS, deletion of 17p11.2) and the Sotos syndrome (deletion of 5q35) (Girirajan et al., 2012). In contrast – as stated previously – many recurrent genomic disorders have decreased penetrance and even greater variability of symptom presentation and severity. An interesting observation regarding such variants, first described in relation to the 16p12.1 recurrent deletion, is the so-called two-hit model (Girirajan et al., 2010). While the 16p12.1 microdeletion is an independent causative factor for NDDs, Girirajan and colleagues also found a significant enrichment of second hit CNVs amongst carriers of the microdeletion. Furthermore, in cases where the 16p12.1 deletion associated with a previously described genetic disorder, the patients who carried both CNVs presented with more severe phenotypes. The two-hit model thus states that a second insult is necessary to result in more severe clinical manifestations of NDDs, and may help explain their high comorbidity (Girirajan et al., 2010). Subsequent studies of other recurrent reciprocal CNVs corroborated the generalization of the two-hit model (Girirajan et al., 2011, 2012). An inverse correlation was identified between the proportion of *de novo* cases and the prevalence of double hits, i.e. very few second hits were associated with syndromes such as WBS, SMS or the 22q11.2 deletions, probably owing to negative selection (these syndromes are severe on their own, therefore an additional hit would likely be lethal; and surviving individuals are unlikely to reproduce). Notably, the second-hit model very possibly applies to variants of uncertain significance (VUS) as well: the presence of two clinically uncertain variants over 500 Kb in size was eight times more likely to occur in an NDD patient as in a control sample. Qualitative phenotypic assessment revealed that a higher burden of second hits correlated with deficits in more domains, i.e. a more severe clinical presentation. Overall, these observations suggest that the majority of recurrent reciprocal rearrangements are either subject to substantial modification or are themselves important genetic modifiers. The two-hit model is consistent with a basic genetic concept: additional (and/or larger) variations – even if they have not yet been associated with human disease - in an individual's genome increases the number of

affected genes, and thus can have an additive or synergistic effect on neurodevelopmental pathways (Girirajan et al., 2010, 2011, 2012). More recent studies reveal a multidimensional effect of rare variants in the "genetic background" of an individual. A primary pathogenic variant "sensitizes" the patient towards a phenotypic trajectory, which is then significantly influenced by additional rare variation. The ability to analyze this genetic background will facilitate patient management and genetic counselling (Iyer et al., 2018).

1.2.2. Non-recurrent CNVs

Non-recurrent CNVs are individually rare dosage changes of unique segments of the genome. They vary in size and have distinct breakpoints, but can overlap to define a minimal critical region (MCR), which defines the phenotype and is often useful in identifying disease-causing genes (Pös et al., 2021). Non-recurrent CNVs are often large subtelomeric deletions or duplications but can be interstitial or pericentromeric as well. In many patients they result from parental balanced translocations, in which cases two subtelomeric chromosomal regions are affected simultaneously (Nowakowska et al., 2012).

The molecular mechanisms underlying non-recurrent CNVs are not fully understood. In rare instances, NAHR mediated by highly homologous repetitive sequences (for example *Alu*, LINE) has been shown to cause unique rearrangements (Gu et al., 2008). In most cases however, breakpoint analyses have suggested two main mechanisms: non-homologous end joining (NHEJ) and replication based models (Pös et al., 2021; Shaikh, 2017).

- NHEJ is a four step mechanism utilized by human cells to repair double-strand breaks (DSBs). Detection of the DSB occurs first, then molecular bridging of the broken DNA ends, then modification of the end to make them ligatable, and finally ligation of the break. NHEJ has been proven as a forming mechanism in deletions in the DMD gene causing Duchenne muscular dystrophy, in atypical cases of SMS, and as a major rejoining mechanism of translocated chromosomes in cancer (Gu et al., 2008; Shaw & Lupski, 2005).

- Fork stalling and template switching/microhomology-mediated break-induced replication (FoSTeS/MMBIR): the FoSTeS model is based on the proposition that DNA replication can stall. The lagging DNA strand then disengages from the original template and swithches to a new replication fork, where microhomology allows the restarting of DNA synthesis (Zhang et al., 2009). This process can result in erroneous replication leading to deletions or duplications depending on whether the new replication fork is downstream or upstream of the original fork. FoSTeS is therefore independent of DSBs, in contrast to the MMBIR model, which is break-induced. The classic break-induced replication model is a homologous recombination-based repair mechanism and requires long stretches of homology, which are not present in human rearrangement events. In the MMBIR model the breakage of the replication fork induces an aberrant replication process. Single strand 3' tails anneal on nearby single strand DNA with microhomology (Hastings et al., 2009; Zhang et al., 2009).

Breakpoints of rearrangements formed by NHEJ are often near or localized within repetitive sequences of the DNA (SD, LTR, LINE, *Alu*, MIR elements). SDs might also be able to bring replication forks together, facilitating template switching. These observations suggest that although these mechanisms are not dependent on the genomic architecture like NAHR is on SDs, they are possibly stimulated and/or regulated by repetitive elements (Gu et al., 2008; Shaw & Lupski, 2005; Zhang et al., 2009).

1.3. Diagnostic possibilities

The first described genetic disorder caused by a submicroscopic deletion was the cri du chat syndrome [partial deletion of distal 5p, reviewed in Lengyel et al., 2018, discovered by J.R. Lejeune in 1963]. Chromosomal G-banding became possible in 1971, which allowed the detection of aneuploidies and large (over 5-10 Mb) partial deletions and duplications of the chromosomes, as well as structural rearrangements. Routine G-banded karyotyping has been the gold standard clinical test for genetic imbalances up until the 2010s, and is still in use today (Miller et al., 2010; Pös et al., 2021). The diagnostic yield of karyotyping is approximately 5% in the ID patient

population, or less than 3% if Down syndrome (trisomy 21) cases are excluded (Miller et al., 2010; van Karnebeek et al., 2005).

1.3.1. Targeted methods – FISH and PCR-based techniques

Targeted testing became possible in 1982 with the description of fluorescent *in situ* hybridization (FISH), and was then followed by polymerase chain reaction (PCR) based methodologies in the mid 1990s (Pös et al., 2021). Subtelomeric FISH uses fluorescent probes [usually 100 kilobase pairs (Kb) or larger] targeting all subtelomeric chromosome regions, and is capable of identifying ~2.5% of genetic causes of ID/DD (Miller et al., 2010). Targeted FISH probes are commercially available for common genomic disorders. An important "disability" of FISH is the targeted nature itself, as this limits its usefulness in many of the aforementioned disorders with significant clinical variability. Multiple ligation-dependent probe amplification (MLPA) is a PCR-based method. As suggested by the name, multiple targeted regions – recurrent disorders and other common microdeletions/microduplications as well - can be tested simultaneously with MLPA (Schouten et al., 2002). Resolution is a great advantage, as it is capable of detecting intragenic deletions. MLPA furthermore is applicable to methylation analyses, but is still incapable of genome-wide CNV detection.

1.3.2. Chromosomal microarray

Today, the recommended gold standard techniques for diagnosing disease-causing CNVs (excluding those that cause clinically recognizable syndromes, for which FISH or MLPA are still time and cost effective) are chromosomal microarray analyses (CMA). These methods allow genome-wide detection of CNVs at a higher resolution compared to G-banding. The resolution of CMA varies as it is dependent on probe size and genomic coverage. Early versions utilized in clinical settings used bacterial artificial chromosome (BAC) clones; targeted for critical regions associated with known disorders, and peri- and subtelomeric regions; and had a diagnostic yield of 7-11% in patients with negative G-banding (Miller et al., 2010). Later, additional probes were added, the so-called "genomic backbone" coverage, which allowed for detection of novel rare CNVs.

1.3.2.1. Types of CMA

1) Array comparative genomic hybridization (array CGH) measures the quantity of genomic DNA in a sample and compares it to a control. The sample and the control DNA are fragmented, labeled with different fluorescent colors, and mixed in equal proportions on an array, which contains oligonucleotide probes representing the target sequences and the genomic backbone. The patient and the control DNA competitively hybridize with the probes on the array. Fluorescent signals are measured using digital imaging software and normalized, allowing direct comparison between patient and control copy number (Levy & Burnside, 2019). Clinical CGH arrays usually contain a few hundred thousand probes; the minimal recommended resolution of array CGH platforms is 400 Kb (Miller et al., 2010).

2) Single nucleotide polymorphism (SNP) arrays use DNA probes that derive from SNPs, regions in the genome that show differences between individuals at a single base pair site (Levy & Burnside, 2019). The patient sample is compared to a combined reference set of multiple controls. SNP arrays provide additional information, most notably the detection of regions of homozygosity (ROH)/loss of heterozygosity (LOH), i.e. identical chromosomal segments. LOH are commonly associated with uniparental disomy (UPD), therefore identification of certain LOH enables the diagnosis of UPD syndromes (Savatt & Myers, 2021; Shaikh, 2017). Other supplementary information clinicians can derive from SNP arrays include consanguinity, potential unmasking of recessive disorders, parent of origin, and several other phenomena relevant in the prenatal setting (e.g. maternal cell contamination, complete hydatiform mole, etc.) (Levy & Burnside, 2019).

Most CMA in use today are hybrid arrays, combining oligonucleotide probes for better CNV detection and SNP arrays for the plethora of additional genotypic data listed above.

1.3.2.2. Diagnostic yield of CMA

These methodologies have elevated the diagnostic yield of traditional cytogenetics from approximately 5% to 15-20% (van Karnebeek et al. 2005; Miller et al. 2010; Chaves et al. 2019; Wayhelova et al. 2019). Table 1 shows an overview of the

diagnostic yield of international studies with relatively small patient cohorts (comparable to the cohort presented in this study).

Table 1. Overview of CMA studies and their diagnostic yield

ID: intellectual disability; NDD: neurodevelopmental disorder; CA: congenital anomaly; CCA: corpus callosum abnormality; CHD: congenital heart disease; MA: minor anomalies; n.a.: not available

Study	Cohort	Sample size	Diagnostic yield	CMA platforms
Bartnik et al., 2014	ID	112	21.4%	60K array CGH (Agilent and Cytosure)
Kashevarova et al., 2014	ID	79	27.8%	44K and 60K array CGH (Agilent)
Coutton et al., 2015	Mild ID	66	21.2%	180K Human Genome CGH Microarray Kit (Agilent)
Chan et al., 2018	ID	138	11.6%	60K PerkinElmer CGX™ v2 (Agilent)
Nassir et al., 2022	NDD	98	15.3%	n.a.
Borlot et al., 2017	ID and epilepsy	143	16.1%	180K Oligonucleotide Array (Agilent Technologies) and CytoScan HD SNP Array (Affymetrix)
Kessi et al., 2018	ID and epilepsy	100	28.0%	CNV_01 Affymetrix aGGH+SNP Microarray, Illumina HumanCytoSNP-12 BeadChip, and low-depth whole-genome sequencing
Bruno et al., 2009	ID and CA	117	15.4%	250K Nsp arrays (Affymetrix)
Lay-Son et al., 2015	ID/DD and CA	40	25%	CytoScan HD SNP Array (Affymetrix)
Mihaylova et al., 2017	DD and CA	81	38.3%	44K CytoChip ISCA oligo microarray
Heide et al., 2017	ID and CCA	149	13.4%	370CNV-Quad, cytoSNP-12, or HumanOmniExpress-24 microarrays (Illumina)
Hussein et al., 2018	CHD and ID/CA	82	18.3%	400K array CGH and 400K CGH/SNP microarray (Agilent)
Wu et al., 2017	CHD and ID/CA	104	27.9%	CytoScan HD SNP Array (Affymetrix)

1.3.3. Short read sequencing (CNV analysis inclusive WES and WGS) and beyond

Whole exome sequencing (WES) is the first-tier method used to detect single nucleotide variations (SNVs) and small insertion/deletions (indels). Whole genome sequencing (WGS) is a universal test capable of identifying almost any type of variation (Quenez et al., 2021); currently, however, due to limitations in data processing, interpretation, cost and accessibility, WGS is primarily implemented in research settings. Over the past decade several bioinformatic algorithms have been developed to enable CNV detection from WES and WGS data, including depth of coverage (DOC), discordant sequence read pair, split-read and assembly-based methods (Hehir-Kwa et al., 2015; Quenez et al., 2021). The various methods vary in breakpoint accuracy and detectable CNV size ranges. DOC methods are most effective, and most commonly used in CNV detection from exome data, as they are relatively tolerant for repetitive regions (which present difficulties for short read sequencing in general), but have lower breakpoint accuracy, and cannot identify CNVs affecting less than three exons. DOC-based tools predict deletions/duplications by the decrease/increase of DOC of the test samples compared to reference samples (Hehir-Kwa et al., 2015). CNV analysis inclusive WES is quite sensitive and very specific. A recent study (74 NDD patients and their parents were tested by trio WES incorporating CNV calling) revealed an overall diagnostic yield of 54% - 35.1% for SNV/indel analysis and 18.9% for CNV analysis (Zhai et al., 2021). To counteract certain disadvantages of WES and WGS, primarily the abovementioned difficulties of interpreting repetitive elements and highly homologous regions in the DNA, third-generation sequencing/long read sequencing (LRS) methods have also been developed (Amarasinghe et al., 2020; Mantere et al., 2019). CNV analysis inclusive WES, WGS and LRS-based tests are not yet readily accessible in Hungary.

1.3.4. Diagnostic algorithm

Professional recommendations for genetic diagnosis of NDDs have evolved over the past decade and a half (Savatt et al., 2021). All consensus statements agree that evaluation must begin with thorough phenotyping and data collection, followed by targeted testing if a specific etiology is suspected. In the early part of the 2010s G-banded karyotyping and testing for fragile X syndrome were recommended in unspecific cases, followed by CMA. Soon however, CMA became the first-tier genetic

test, and many guidelines recommended it before traditional cytogenetic methods; while *FMR* repeat analysis lost some of its relevance. Later in the decade, WES became more and more available, and eclipsed even CMA in diagnostic yield. A recent paper collected and compared current testing guidelines from various medical organizations, and suggested an optimized approach to genetic testing in NDDs (Savatt & Myers, 2021).

1. Complete physical examination and data collection (developmental, medical and family history).
    - Appropriate targeted testing, if warranted based on phenotype.
2. Genome-wide examination for causal exonic sequence variants and copy number variants.
    - CNV analysis inclusive WES, if available.
    - If stepwise testing is necessary due to certain restrictions, WES should be performed first, whenever possible.
    - Trio WES should be performed, if possible.
3. Periodic reanalysis of reported variants and exome data.
    - Additional genetic testing should be considered (e.g. genome sequencing, *FMR1* repeat analysis if not already done, cytogenetic testing, if warranted).

At our Unit we follow a stepwise diagnostic approach due to financial limitations that exist in Hungary, and CMA testing still often precedes WES (and nearly always preceded WES between 2010-2020, which is the chosen time interval of the current study).

2. Aims

The primary goal of my research was the systematic analysis of CMA results obtained from patients referred to the Tűzoltó street Unit of Semmelweis University's Pediatric Center with neurodevelopmental disorders and/or congenital anomalies in the ten-year period between 2011 and 2020. The aims of this study were the following:

1) To reevaluate clinically uncertain CNVs identified in the patients.
2) To determine the diagnostic yield of CMA in the cohort.
3) To delineate clinical features associated with definitive CMA results.
4) The genotype-phenotype association of patient subgroups carrying recurrent/functionally identical CNVs and comparison with the literature.
    a. Recurrent rearrangements of the short arm of chromosome 16.
    b. Overlapping microdeletions of the long arm of chromosome 14 including *SUPT16H* and *CHD8* genes.
5) The analysis and discussion of the genetic alterations identified in a phenotypic subgroup – patients with clinical features reminiscent of Silver-Russell syndrome.
6) To highlight potentially pathogenic VUS, and the dissemination of phenotypic data of all discovered VUS to facilitate genetic counselling in the future.

3. Methods

3.1. Patients and clinical data

During the period between 2011 and 2020, 88 patients of the Tűzoltó street Unit of Semmelweis University's Pediatric Center underwent CMA testing. Children were selected for investigation if they had idiopathic DD/ID or a major congenital anomaly, and at least one additional suggestive feature (other NDDs, characteristic facies, multiple congenital anomalies, etc.), and the family consented to further genetic testing. At our Unit, the typical DiGeorge syndrome (proximal A-D 22q11.11 deletion, including *TBX1* gene), Williams syndrome and the deletion form of Prader-Willi syndrome are diagnosed by FISH, therefore these cases are not included. 78 patients underwent CMA after negative routine cytogenetic evaluations, while 10 patients were tested to refine the findings of previous tests. As one of the main aims of this study was to determine the diagnostic yield of CMA in our cohort, unconfounded by previous positive test results, the patients with microscopically visible rearrangements have been excluded from further statistical analysis; an overview of their genetic data is listed in Table 2.

Clinical data up to the point of genetic diagnosis/negative CMA result were retrospectively gathered and organized. The collected phenotypic data included epidemiological data (sex, date of birth, age at first genetic consultation), pre- and perinatal data (spontaneous conception/assisted reproduction, parental ages, teratogenic factors, prenatal genetic testing, fetal movement, intrauterine (IU) diagnosed abnormalities, family history, birth parameters, perinatal adaptation), postnatal somatic developmental parameters, craniofacial minor anomalies, minor anomalies of the hands, feet and other regions, neurology and neurodevelopment (seizures, muscular abnormalities, brain imaging studies, other specific neurologic problems, ID/DD, ASD, ADHD, stereotypies, other neuropsychologic features), major congenital anomalies and other diseases (cardiovascular, ear-nose-throat and respiratory, gastrointestinal, urogenital, endocrinological, immunological, malignancies).

Table 2. Genotypic data of the excluded patients

Kb: kilobase; OMIM: Online Mendelian Inheritance in Man; P: pathogenic; LP: likely pathogenic; VUS: variant of uncertain significance

Patient SEG2_1	
Karyotype	47,XY,+mar[60] /46,XY[40].ish idic r(22)(q11.1.q11.21)x4
Copy Number Variation, Size, Classification and Related Disorder	arr[GRCh37] 22q11.1q11.21 (17435645_18656678×3(17598642_17799783) × 4dn; 1.221 Mb and 201.1 Kb; LP; Cat eye syndrome [OMIM#115470, (Haltrich et al., 2014)]
Patient SEG2_4	
Karyotype	46,XY, SRY+ (phenotypic female)
Copy Number Variation, Size, Classification and Related Disorder	arr[GRCh37]16p11.2(29591326_30190029)x1; 598.7 Kb; P; 16p11.2 microdeletion (OMIM#611913)
Patient SEG2_10	
Karyotype	46,XY with complex chromosomal rearrangement affecting chromosomes 1,2,4,5,10,17
Copy Number Variation, Size, Classification and Related Disorder	arr[GRCh37]2q14.3(123431180_124854926)x1; 1.424 Mb; LP; 2q14.3 microdeletion (Lengyel et al., 2021b)
Patient SEG2_18	
Karyotype	46,XX, SRY- (phenotypic male)
Copy Number Variation, Size, Classification and Related Disorder	arr[GRCh37]17q24.3(69511806_69666935)x3; 155.1 Kb; P; 17q24.3 (*SOX9* regulatory region) microduplication (Pinti et al., 2019)
Patient SEG2_19	
Karyotype	46,XX, SRY- (phenotypic male)
Copy Number Variation, Size, Classification and Related Disorder	arr[GRCh37]17q24.3(69577001_69618000)x3; 41.0 Kb; P; 17q24.3 (*SOX9* regulatory region) microduplication (Pinti et al., 2019)
Patient SEG2_22	
Karyotype	46,XX,t(X;10)(p21;p12)
Copy Number Variation, Size,	arr[GRCh37]7q31.1(107692546_107849992)x3; 157.5 Kb; VUS

Classification and Related Disorder	arr[GRCh37]Xp22.11(22000870_22167026)x3; 166.2 Kb; VUS
Patient SEG2_43	
Karyotype	46,XX,der(X)t(X;19)(p11;p13)
Copy Number Variation, Size, Classification and Related Disorder	CMA negative
Patient SEG2_58	
Karyotype	47,XXX, SRY-
Copy Number Variation, Size, Classification and Related Disorder	CMA negative
Patient SEG2_83	
Karyotype	46,X,der(X)dup(X)(p10p22.1)[9]/45,X[6]
Copy Number Variation, Size, Classification and Related Disorder	arr[GRCh37]7p22.3p21.3(42976_12448132)x3; 12.41 Mb; P; 7p22.3p21.3 microduplication (Chaves et al., 2019) arr[GRCh37] Xp22.33(61091_4062749)x1; 4.001 Mb; P; Xp22.33 microdeletion (OMIM#300830) arr[GRCh37]Xp22.23p11.22(4078736_53762693)x3; 53.76 Mb; P; Xp22.23p11.2 duplication (Kokalj Vokac et al., 2002)
Patient SEG2_86	
Karyotype	46,X,rec(X)del(X)(p22.3pter)ins(Y)(q11.21q12)mat
Copy Number Variation, Size, Classification and Related Disorder	arr[GRCh37]Xp22.33(61091_2676167)x1; 2.615 Mb; LP; Xp22.33 microdeletion (D'Ambrosio et al., 2019) arr[GRCh37]Yq11.2q12(14619835_59335913)x1; 44.72 Mb; LP; Xp22.33 microdeletion (D'Ambrosio et al., 2019)

3.2. Genetic testing

Karyotypes (at standard band resolution of 450-550) were determined by analysis of 20 Giemsa-stained metaphases each from standard 72-hour peripheral blood lymphocyte cultures. The platforms and analysis software used for CMA were NimbleGen Array (CGX 1.4 M) with NimbleGen MS 200 Microarray Scanner (30 patients; Roche NimbleGen Inc., Madison, WI, USA), Agilent qChip Post (60K; 5 patients) and Agilent 180K oligo-array (18 patients) with Agilent Genomic Workbench 7.0 (Agilent Technologies, Santa Clara, CA, USA), Affymetrix CytoScan Optima (300K; 5 patients), Affymetrix CytoScan 750K (17 patients), and Affymetrix CytoScan HD (3 patients) with Affymetrix Genechip Scanner or Chromosome Suite Ananlysis (ChAS) 4.0 (Thermo Fisher Scientific, Inc.; Waltham, MA, USA). Relevant CNVs were validated using FISH or quantitative multiplex PCR of short fluorescent fragments (QMPSF) analysis. Parental studies were possible in 10 families: parents of three families and a mother of a fourth child underwent CMA, two sets of parents and two additional mothers were tested using QMPSF, and targeted FISH testing was performed for two parent pairs (see Table 3 in Results).

3.3. CNV reevaluation

Each VUS [and likely benign (LB) variant, if reported] was reevaluated according to the latest recommendations of the American College of Medical Genetics and Genomics [ACMG (Riggs et al., 2020)] as a primary guideline. In practice, this was conducted in two steps, starting with a first round of manual classification of the variants. Taken into consideration were the following factors: size, genes/other functionally important elements contained within the CNVs, and a search of case and control databases (see below). The second round of evaluation was performed with the help of the recently published SVInterpreter TAD-based tool (Fino et al., 2021). Finally, the CNVs were assigned to three groups for further study: 1) disease-causing variants (variants classified as P and LP), 2) VUS, and 3) benign variants [classified as benign (B) and LB].

3.3.1. Public databases and online sources

Literature searches were conducted with the help of PubMed (https://pubmed.ncbi.nlm.nih.gov/) and Google Scholar (https://scholar.google.com/).

Gene-related information was collected from the Online Mendelian Inheritance in Man database (OMIM; https://omim.org) and GeneCards (Safran et al., 2010). The former offers a comprehensive and up to date overview of over 16.000 human genes and currently known Mendelian disorders, including exhaustive literature references and external links; the latter is an integrated database of gene-specific data, obtained from possibly the highest number of outside scientific sources. I primarily called upon the following genomic variation-specific resources to further facilitate CNV reclassification efforts (de Leeuw et al., 2012):

- The Database of Genomic Variants [DGV; (MacDonald et al., 2014)] is a database of systematically reviewed structural variants (genomic alterations larger than 50 basepairs) in the human genome, identified in healthy control samples. The DGV is a useful tool in filtering out B variants, and in the interpretation of VUS. DGV Gold Standard Variants are a curated set of variants from multiple high quality studies. However, one must keep in mind the possibility of false positives in the database (due to e.g. technical differences between studies and/or the utilized CMA platforms, the possibility of inaccurate variant boundaries, the merging of variants from different studies, reliability of older studies, etc.), as well as the fact that the presence of a variant in the DGV does not conclusively exclude a similar variant as a possible disease-causing alteration in one's own patient cohort.

- ClinVar database (Landrum et al., 2018) contains reports of variants identified in patient samples with additional information regarding associated phenotypes, classifications and other supporting data. Data submitted to ClinVar is archived, then experts review the evidence surrounding a variation and assign levels of confidence to each submission. Conflicting interpretations and uncertainty is thus transparent, greatly informing reevaluation.

- The Clinical Genome Resource or ClinGen database (https://clinicalgenome.org/) is dedicated to "building an authoritative central resource that defines the clinical relevance of genes and variants for use in precision medicine and research". ClinGen aggregates, curates and disseminates genomic data related to human health issues. The database includes gene-validity curations, clinical actionability and dosage sensitivity curations. The latter is a particularly useful tool in

the evaluation of CNVs, and contains an up to date list of recurrent rearrangements associated with disease.

- The Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources [DECIPHER; (Firth et al., 2009)] is an interactive database aggregating data from various other resources, therefore allowing easy and integrated analysis of genomic regions. DECIPHER includes a genome browser tool where the queried position can be visualized and compared to normal and pathogenic variation at the same locus, and extensive data regarding the encompassed genes is displayed as well. A community of clinical geneticists and researchers upload variations with associated phenotypic data to the database, which opens up the possibility of more straightforward genotype-phenotype correlation of rare variants.

- SVInterpreter (Fino et al., 2021) is a free online application allowing for comprehensive evaluation of the genomic region in question, including gene function and dosage-sensitivity data, an automatic variant overlap search of several case and control databases (DGV, 1000 Genomes, ClinGen, ClinVar, Deletion/Duplication syndromes, GnomAD) and selected developmental delay publications (Chaisson et al., 2019; Coe et al., 2014; Collins et al., 2017; Cooper et al., 2011), calculation of phenotype similarity score when applicable, an overview of available animal studies, potentially altered genomic architectural elements, that in turn could have a position effect on gene regulation. The output of SVInterpreter also links to visualization on the UCSC Genome Browser (Speir et al., 2016).

3.4. Statistical analyses

The main phenotypic features of the patients with disease-causing CNVs were compared to the patients with negative CMA results using the chi squared test. If any cell of the contingency table had an expected value less than five, the Fisher exact test was applied. Statistical analyses were performed using Microsoft Office 365 Excel.

4. Results

4.1. Overview

The final investigation cohort consisted of 78 individuals, of which 47 were males and 31 were females (male:female ratio 1.52:1). The average age at first clinical genetics consultation was 4.17 years (median 2.00 years, range from 4 days old to 20 years 7 months old). Overall the patients are known to carry 172 CNVs (Lengyel et al., 2022).

Inheritance was investigated in 12.8% of patients; including three families where only the mother agreed to testing (neither mother carried the CNV of the respective child). Samples were available from both parents for seven patients. These seven children carried in total 9 CNVs, three proved to be *de novo* alterations, three were maternally, and three were paternally inherited. As patients SEG2_26 and 27 are brothers, overall we identified two carrier mothers and three carrier fathers. One of the carrier parents was healthy, while the other four showed mild symptoms (history of learning difficulties and/or pediatric obesity, behavioral disorders, etc., see Table 3 and Lengyel et al., 2022).

Table 3. Inheritance of the CNVs in the presented patient group

Kb: kilobase; Class.: classification; P: pathogenic; LP: likely pathogenic; VUS: variant of unknown significance; LB: likely benign; mat: maternal; pat: paternal; QMPSF: quantitative multiplex PCR of short fluorescent fragments; FISH: fluorescent *in situ* hybridisation; CMA: chromosomal microarray; [#]: GRCh38; all other genomic coordinates are according to GRCh37; unk.*: unknown, maternal inheritance was ruled out;

[a]: Cytocell Technologies, Ltd., Cambridge, UK; [b]: Agilent Technologies, Santa Clara, CA, USA

Patient	Copy Number Variation Size (Kb)	Class.	Inheritance	Method	Parental phenotype
SEG2_5	[#]16p13.3(3263725_4309863)x1 1046.1	P	de novo	FISH Probe: Rubinstein Taybi[a]	Healthy
SEG2_15	19p13.3(753219_1477508)x3; 724,3 5p15.33p15.32(4260205_5088435)x3; 828.2	LP VUS (LB)	de novo pat	CMA	Healthy
SEG2_26	16p11.2(29624765_30199351)x3 574.6	P	mat	CMA	Mat: Learning, social and behavioral difficulties
SEG2_27	16p11.2(29624765_30199351)x3 574.6	P			
SEG2_53	2q37(242855645_243030854)x1; 175.2 16p11.2(28824802_29040571)x1; 215.8	LB P	pat mat	FISH Probes: ATXN2L/SH2B1[b]; subtel 2q	Pat: learning difficulties, early obesity Mat: early obesity
SEG2_77	16p11.2(31980001_33825000)x1 1845.0	VUS	de novo	QMPSF	Healthy
SEG2_81	20p11.21(24554628_24708699)x3 154.1	VUS	pat	QMPSF	Pat: neuropsychiatric symptoms

SEG2_17	16p11.2(29620689_30190568)x3 569.9	P	unk.*	QMPSF	Healthy
SEG2_39	16p11.2(29656684_30190568)x1 533.9	P	unk.*	QMPSF	Healthy
SEG2_57	4q24(102058416_102443207)x3 384.8	VUS	unk.*	CMA	Healthy
	20p11.23(19240620_19745197)x3 504.6	VUS	unk.*		
	Xq25(124088718_124169834)x0 81.1	VUS	unk.*		

The noteworthy results of our reevaluation efforts are listed in Table 4 (variants originally classified as LB and reclassified as B are not included).

Table 4. Results of reevaluation in the presented patient cohort

*All genomic locations are according to GRCh37.

Kb: kilobase; LP: likely pathogenic, VUS: variant of uncertain significance, LB: likely benign

Patient	CNV*	Size (Kb)	Originial classification	Final classification
SEG2_49	14q11.2(21414942_21966929)x1	552.0	VUS (LP)	P
SEG2_68	14q11.2(21438704_22101647)x1	662.9	VUS (LP)	P
SEG2_89	14q11.2(21511829_22131455)x1	619.6	VUS (LP)	P
SEG2_61	Xp22.33(566719_807207)x3	240.5	VUS (LP)	P
SEG2_32	16p12.2(21953152_22480514)x3	527.4	VUS	LP
SEG2_20	2q37(242855645_243028377)x1	172.7	VUS	LB
SEG2_53	2q37(242855645_243030854)x1	175.2	VUS	LB
SEG2_65	2q37(242855645_243033605)x1	177.9	VUS	LB
SEG2_47	17p11.2(18933772_19128870)x1	195.1	VUS	LB
SEG2_42	17q12(34437475_34475514)x4	38.0	VUS (LB)	LB
SEG2_81	Yq11.221(15421662_ 15734785)x2	313.1	VUS (LB)	LB

Of note, we believe that of one variant presented in our previous publication on 16p CNVs (Lengyel et al., 2020) might be considered clinically relevant. Patient SEG2_32's (P2 in the previous article) 16p12.2 duplication (chr16:21953152_22480514) was originally classified as a VUS. We argued for its potential pathogenic/predisposing role in the patient's phenotype. Our current reevaluation efforts led to the finding that this CNV has been listed as a pathogenic CNV in peer-reviewed scientific literature on multiple occasions (Kendall et al., 2017; Kushima et al., 2018).

4.2. Disease-causing variants

Disease-causing variants (n=30) were identified in 29 patients (15 males and 14 females), which translates to an overall diagnostic yield of 37.18% (Lengyel et al., 2022). The disease-causing CNVs were on average 3.481 Mb large (median 1.124 Mb); 19/30 were deletions and 11/30 were duplications (Table 5).

Table 5. Size comparison of disease-causing and clinically uncertain CNVs

Kb: kilobase; Mb: megabase; VUS: variant of uncertain significance

	Disease-causing variants	VUS
Percent of deletions	63.3%	50.0%
Average size	3481 Kb	586 Kb
Median size	1124 Kb	228 Kb
< 300 Kb	4 (13.3%)	12 (37.5%)
300 Kb – 1 Mb	10 (33.3%)	7 (29.2%)
1-5 Mb	10 (33.3%)	5 (20.8%)
5-10 Mb	3 (10.0%)	0
>10 Mb	3 (10.0%)	0

One child (patient SEG2_82) carries multiple disease-causing CNVs, specifically a terminal duplication of chromosome 19 (57243585_58445449; GRCh38) and a terminal deletion of chromosome 22 (49368551_50759338; GRCh38). These rearrangements originated from a paternal balanced translocation, and subsequent targeted FISH analyses revealed the CNVs in the patient's younger sister as well. Of further note is patient SEG2_68 whose parents are first cousins, which resulted in the identification of LOH of 21 regions on 12 chromosomes (overall 196.8 Mb of the genome).

Chromosome 16 was most frequently affected by disease-casuing CNVs (9/30; 30.0%; note: as the sample size is small, percentages throughout the text are meant to enhance comparability), followed by chromosome 22 (4/30; 13.3%), and chromosomes 14, 17 and X (3/30; 10.0% each) (Figure 1). Six of the nine chromosome 16 CNVs corresponded to recurrent microdeletion/microduplication regions on the short arm, and thus were the consequence of NAHR. Amongst the 16p alterations, a noteworthy four were gains or losses of the proximal 16p11.2 region, which corresponds to 5.1% of the entire cohort, and 13.8% of children diagnosed (Lengyel et al., 2020, 2022).

Microdeletions of one non-recurrent region, specifically within chromosome region 14q11.2, were enriched in our study. 3/78 patients (3.8% of the studied cohort, 10.3% of diagnosed patients) carried approximately 500 Kb large deletions of 14q11.2 (Lengyel et al., 2022).

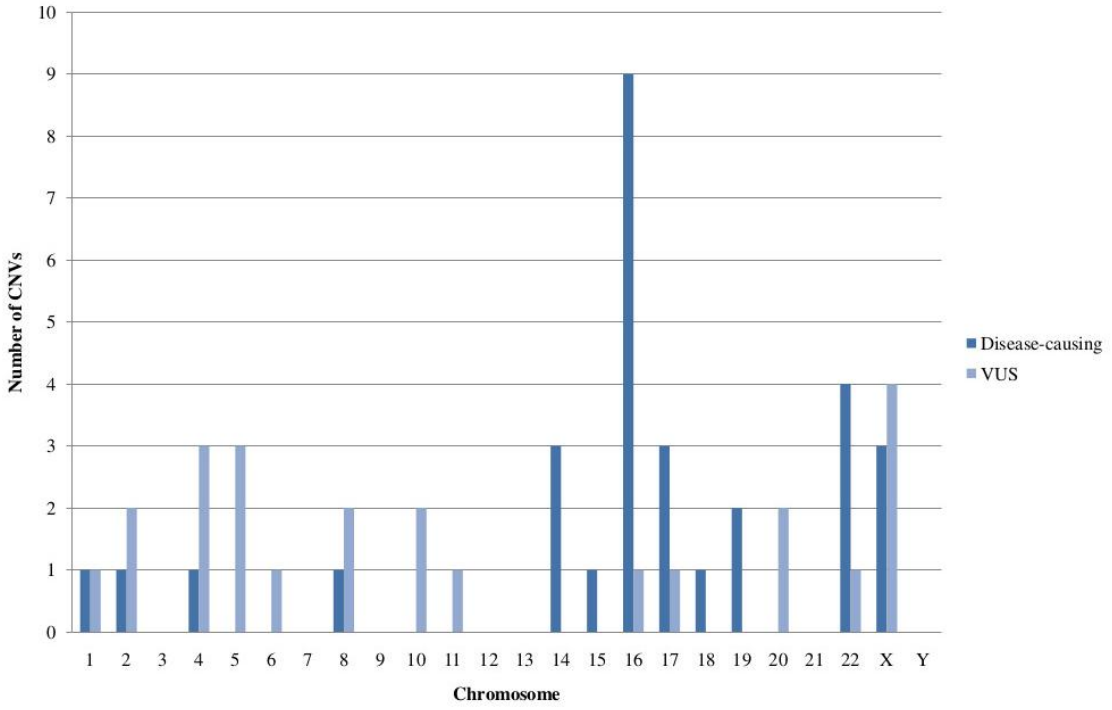

Figure 1. Chromosomes affected by disease-causing and clinically uncertain CNVs

Two patients in the cohort, who presented with phenotypes reminiscent of Silver-Russell syndrome, underwent CMA after negative MLPA testing of the typical causative region. For patient SEG2_6 SNP array identified a large loss of heterozygosity (LOH) on chromosome 14 (20052038_106871264 Mb; GRCh37) along with an incidental pathogenic CNV (see Table 6 and the Discussion). This finding was followed up by methylation analysis and maternal uniparental disomy 14 (Temple syndrome, OMIM #616222) was confirmed, which was ultimately the underlying cause of his primary phenotype (Table 7). For patient SEG2_37, CMA revealed a 10 Mb large deletion of chromosome 16q: arr[GRCh37]16q22.2q23.3(72155844_82148404)x1 (Table 6 and Lengyel et al., 2021a). An additional SRS-like patient (identified as 2021.1), though not a part of the original cohort, is discussed due to relevance of CMA results, which revealed a 77 Kb deletion: arr[GRCh37]8q12.1(57079399_57155945)x1; *de-novo* occurrence was proven by quantitative PCR of the parental DNA samples (Baba et al., 2022).

Data regarding the disease-causing CNVs are detailed in Table 6, the accompanying phenotypes of the patients are elaborated in Table 7.

Table 6. Disease-causing CNVs identified in the presented patient group

Kb: kilobase; DECIPHER: Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources; OMIM: Online Mendelian Inheritance in Man; ORPHA: Orphanet

Patient	DECIPHER ID	Copy Number Variation	Size (Kb)	Syndrome/disorder
SEG2_5	517746	arr[GRCh38]16p13.3(3263725_4309863)x1	1046.1	OMIM#610543
SEG2_7	517745	arr[GRCh37]22q11.21q11.22(21934268_22336871)x3	402.6	OMIM#608363
SEG2_12	517747	arr[GRCh37]22q13.33(50971316_51224252)x3	252.9	OMIM#615538
SEG2_15	517749	arr[GRCh37]19p13.3(753219_1477508)x3	724.3	ORPHA:447980
SEG2_17	411596	arr[GRCh37]16p11.2(29620689_30190568)x3	569.9	OMIM#614671
SEG2_21	414308	arr[GRCh38]18q22.1q23(69071896_80256240)x1	11184.3	OMIM#601808
SEG2_26	517820	arr[GRCh37]16p11.2(29624765_30199351)x3	574.6	OMIM#614671
SEG2_27	517821	arr[GRCh37]16p11.2(29624765_30199351)x3	574.6	OMIM#614671
SEG2_30	517822	arr[GRCh37]17p13.3(7_2084490)x1	2084.5	OMIM#247200
SEG2_32	517744	arr[GRCh37]16p12.2(21953152_22480514)x3	527.4	Kushima et al., 2018
SEG2_33	517823	arr[GRCh37]15q11.2q13.1(22765628_29060493)x1	6294.9	OMIM#105830
SEG2_37	414204	arr[GRCh37]16q22.2q23.3(72155844_82148404)x1	9992.6	OMIM#614541
SEG2_39	517824	arr[GRCh37]16p11.2(29656684_30190568)x1	533.9	OMIM#611913
SEG2_44	517825	arr[GRCh37]2q23.1(149060704_149313819)x3	253.1	Chung et al., 2012
SEG2_49	517827	arr[GRCh37]14q11.2(21414942_21966929)x1	552.0	OMIM#613457
SEG2_50	517830	arr[GRCh37]8p23.1(7117851_11969155)x1	4851.3	ORPHA: 251071
SEG2_52	517831	arr[GRCh37]1p36.33p36.22(820001_9348000)x1	8528.0	OMIM#607872
SEG2_53	411578	arr[GRCh37]16p11.2(28824802_29040571)x1	215.8	OMIM#613444
SEG2_59	517832	arr[GRCh37]17p11.2(16727490_20433502)x1	3706.0	OMIM#182290
SEG2_61	517833	arr[GRCh37]Xp22.33(566719_807207)x3	240.5	OMIM#127300, #300582
SEG2_62	517835	arr[GRCh37]Xp22.31(6537108_8167604)x0; arr[GRCh37]14q11.2q32.33(20052038_106871264)hmz	1630.5 86819.2	OMIM#308100 OMIM#616222

SEG2_68	517850	arr[GRCh37]14q11.2(21438704_22101647)x1	662.9	OMIM#613457
SEG2_70	517853	arr[GRCh37]4q32.2q35.2(161869551_190790881)x1	28921.3	Rossi et al., 2009
SEG2_78	517854	arr[GRCh38]Xq22.1q23(101597527_111626047)x1	10028.5	Hijazi et al., 2020
SEG2_80	517855	arr[GRCh37]22q11.21q11.22(21460640_22962962)x1	1502.3	OMIM#611867
SEG2_82	517856	arr[GRCh38]19q13.43(57243585_58445449)x3; arr[GRCh38]22q13.33(49368551_50759338)x1	1201.9 1390.8	OMIM#606232 J. Wang et al., 2020
SEG2_85	517857	arr[GRCh37]16q12.2q21(56340118_60294492)x1	3954.4	Yamamoto et al., 2016
SEG2_87	517858	arr[GRCh37]17q12(34835983_36243365)x3	1407.4	OMIM#614526
SEG2_89	517859	arr[GRCh37]14q11.2(21511829_22131455)x1	619.6	OMIM#613457

Table 7. Phenotypes of patients with disease-causing CNVs

y: years; m: months; d: days; OMIM: Online Mendelian Inheritance in Man; ORPHA: Orphanet; ACM: arteria cerebri media; ASD: autism spectrum disorder; CALM: café au lait macule; CCA: corpus callosum abnormality; DD: developmental delay; EEG: electroencephalogram; EMG: electromyogram; GERD: gastroesophageal reflux; ID: intellectual disability; LGA: large for gestational age; MRI: magnetic resonance imaging; PDA: patent ductus arteriosus; PI: pulmonary insufficiency; PS: pulmonic stenosis; SD: septal defect; SGA: small for gestational age; STPC: single transverse palmar crease

Patient	Sex	Age*	Syndrome/disorder	Detailed phenotype
SEG2_5	F	0y 4m	Proximal 16p13.3 microdeletion (OMIM#610543)	→ Decreased fetal movement, laryngospasm → Microcephaly, small forehead, retrognathia, hypotelorism, narrow and depressed nasal bridge, low-set ears, abnormal pinna morphology, high palate, enlarged thorax, pectus excavatum, duplication of thumb phalanx, broad thumb, hitchhiker thumb, broad 1st toes, low anterior hairline → Primary congenital glaucoma, atrial SD, left ventricular hypertrophy, PS

SEG2_7	M	1y 4m	22q11.2 microduplication [OMIM#608363]	→ Premature birth, complicated perinatal adaptation, neonatal hypoglycaemia, abnormal cry, maternal alcohol consumption during pregnancy possible → Growth delay, microcephaly, small forehead, unilateral narrow palpebral fissure and microphthalmia, hypoplastic philtrum, high palate, unilateral clinodactyly of the 5th finger, unilateral STPC, pectus excavatum, rectus diastasis, dystrophic toenails → ID/DD, hypotonia, brain MRI: CCA, hypoplastic hippocampus → PDA, atrial SD, inguinal hernia, hypospadias
SEG2_12	M	4y 9m	22q13 microduplication (OMIM#615538)	→ Relative macrocepahly, broad face and forehead, hypertelorism, high palate, widely spaced teeth, joint hypermobility, cutis laxa → Global DD/ID, severe speech and language delay, gait imbalance, autistic behavior
SEG2_15	M	2y 11m	19p13.3 microduplication (ORPHA:447980)	→ Feeding difficulties in infancy → Growth delay, microcephaly, micro- and retrognathia, low-set, prominent and simple ears, hypoplastic philtrum, narrow mouth, rib anomaly, pectus excavatum, joint hypermobility, coarse hair, low anterior hairline → Global DD/ID, generalized hypotonia, brain MRI: mild cerebral atrophy → Unilateral coloboma, lacrimal duct stenosis, bilateral sensorineural hearing impairment (familial), cryptorchidism, hypospadias, bicuspid aortic valve, duodenal atresia, cholestasis, splenomegaly, impaired liver function, decreased circulating cortisol level
SEG2_17	M	1y 11m	16p11.2 microduplication (OMIM#614671)	→ Facial asymmetry, prominent forehead, epicanthus, wide and depressed nasal bridge, low-set and prominent ears, high palate, clinodactyly of 3rd-5th toes → Global DD, severe speech and language delay, developmental regression, generalized hypotonia, brain CT: cerebral atrophy and ventriculomegaly, autistic

				behavior, short attention span, abnormal temper tantrums, abnormal eating behavior → Strabismus, cryptorchidism
SEG2_21	F	1y 2m	18q22.1q23 microdeletion (OMIM#601808)	→ SGA, feeding difficulties in infancy → Relative macrocepahly, broad forehead, micrognathia, epicanthus, depressed nasal bridge, overlapping toes, high anterior hairline → Global DD, generalized hypotonia, lower limb skeletal muscular atrophy, reduced tendon reflexes, nystagmus, brain MRI: ventriculomegaly, delayed myelination → Atrial SD
SEG2_26	M	3y 11m	16p11.2 microduplication (OMIM#614671)	→ Prominent forehead, micrognathia, down-slanted palpebral fissures, hypertelorism, absent medial eyebrows, low-set and prominent ears, downturned corners of the mouth → Global DD/ID, seizure, ASD, short attention span → Strabismus, carious teeth
SEG2_27	M	2y 1m	16p11.2 microduplication (OMIM#614671)	→ Prominent forehead, down-slanted palpebral fissures, hypertelorism, absent medial eyebrows, low-set and prominent ears, downturned corners of the mouth, joint hypermobility → Global DD/ID, generalized hypotonia, ASD
SEG2_30	F	1y 0m	17p13.3 microdeletion (Miller-Dieker syndrome, OMIM#247200)	→ Premature birth, large fontanelles, delayed closure of fontanelles → Growth delay, triangular face, high, broad and prominent forehead, microphthalmia, wide and depressed nasal bridge, low-set ears, thin upper lip vermilion, downturned corners of the mouth, short philtrum, high palate, pectus excavatum, small hands, brachydactyly, STPC, low posterior hairline → Global DD/ID, brain MRI: CCA, ventriculomegaly, aqueductal stenosis,

				arachnoid cysts, hydrocephalus, tethered cord → Strabismus, delayed skeletal maturation, atrial SD, anteriorly placed anus
SEG2_32	F	2y 11m	16p12.2 microduplication (Kendall et al., 2017; Kushima et al., 2018)	→ Epicanthus, sparse eyebrows and eyelashes, short neck, joint hypermobility, low posterior hairline → Global DD/ID, developmental regression, generalized hypotonia, ataxia, dyssynergia, brain MRI: abnormal myelination, abnormal repetitive mannerisms, bruxism → Strabismus
SEG2_33	F	1y 7m	15q11.2q13.1 deletion (Angelman syndrome, OMIM#105830)	→ Microcephaly, trigonocephaly, prominent forehead, prognathia, epicanthus, depressed nasal bridge, thin upper lip vermilion, high palate, short neck, shield chest, STPC → Global DD/ID, seizure, generalized hypotonia, ataxia, dyssynergia, incoordination, brain MRI: syringomyelia, Arnold-Chiari type I malformation, encephalopathy, tongue thrusting, abnormal repetitive mannerisms, sleeping disorder, abnormal social behavior → Strabismus, 1 CALM, hyperhidrosis and excessive salivation
SEG2_37	M	1y 3m	16q22.2q23.3 microdeletion (OMIM#614541)	→ Wide anterior fontanelle → Growth delay, relative macrocepahly, triangular face, high, broad and prominent forehead, micrognathia, wide nasal bridge, low-set and crumpled ears, high palate, joint hypermobility, STPC → Global DD, brain MRI: ventriculomegaly, focal hyperintensities in frontal lobe, sleeping disorder → Delayed eruption of teeth, 4 CALMs
SEG2_39	M	12y 2m	Proximal 16p11.2 microdeletion (OMIM#611913)	→ Childhood-onset truncal obesity, brachydactyly → Global DD/ID, abnormal repetitive mannerisms, abnormal emotion/affect

				behavior, short attention span, abnormal eating behavior, encopresis, impaired ability to form peer relationships → Unilateral renal agenesis
SEG2_44	M	12y 1m	2q23.1 microduplication (Chung et al., 2012; Hodge et al., 2014)	→ LGA, umbilical hernia → Postnatal overgrowth, relatively large facial skeleton, facial asymmetry, micro- and retrognathia, epicanthus, depressed nasal bridge, macrotia, abnormal pinna morphology, linear earlobe crease, thick lower lip vermilion, hypoplastic philtrum, high palate, flat occiput, hypermobility of finger joints, stiffness of other joints, deep palmar and plantar creases, long toes, sandal gap, high anterior hairline, down-sloping shoulders, scoliosis → Global DD/ID, generalized hypotonia, speech articulation difficulties, good memory, autistic behavior → Accelerated skeletal maturation, gynecomastia, naevus flammeus
SEG2_49	M	0y 6m	14q11.2 microdeletion (OMIM#613457)	→ Microcephaly, micrognathia, lacrimal duct stenosis, deep set eyes, depressed nasal bridge, bulbous nose, abnormal pinna morphology, open mouth, unilateral STPC, brachydactyly, bilateral cutaneous syndactyly of the 3rd-4th toes, scoliosis, flexion contractures of the fingers → ID/DD, axial hypotonia, limb hypertonia, incoordination, broad-based gait, cranial US: ventriculomegaly → Hearing impairment, strabismus, delayed eruption of teeth
SEG2_50	F	1y 8m	8p23.1 microdeletion (ORPHA: 251071)	→ Broad and prominent forehead, micro- and retrognathia, hypertelorism, depressed nasal bridge, low-set ears, high palate, pectus excavatum, kyphosis and lumbar hyperlordosis, congenital hip dislocation, broad 1st toe, joint hypermobility → Global DD, axial hypotonia, reduced tendon reflexes, normal brain MRI, short attention span → Atrial SD, strabismus, myopia, delayed eruption of teeth, sacral dimple

SEG2_52	F	0 (4d)	1p36 microdeletion (OMIM#607872)	→ Feeding difficulties in infancy, wide anterior fontanelle → Broad forehead, hypertelorism, low-set and simple ears, high palate, thin lip vermilions, short and hypoplastic philtrum, dorsalflexion of the feet, bilateral STPC, overlapping toes, clinodactyly of the toes, low anterior hairline, hirsutism → Global DD, generalized hypotonia, seizure, brain MRI: ventriculomegaly, frontotemporal polymicrogyria, CCA, hypoplasia of the brainstem and the cerebral white matter → Tetralogy of Fallot
SEG2_53	F	2y 9m	Distal 16p11.2 microdeletion (OMIM#613444)	→ LGA → Postnatal overgrowth, childhood-onset truncal obesity, genu varum, small hands and feet → Global DD, generalized hypotonia, polyphagia, abnormal social behavior → Strabismus
SEG2_59	F	0y 3m	17p11.2 microdeletion (Smith-Magenis syndrome, OMIM#182290)	→ Assisted reproduction (healthy twin) → Triangular face, broad and high forehead, wide nasal bridge, open mouth, genu valgum, calcaneovalgus deformity, broad 2nd fingers, clinodactyly of the 4th toes → Global DD, developmental regression, broad-based gait, ataxia, epileptiform EEG, brain MRI: arachnoid cysts, hemosiderin deposits, abnormal repetitive mannerisms, tongue thrusting, self-biting, aggressive behavior, abnormal eating behavior → Atrial SD, strabismus, 1 CALM, naevus flammeus, excessive salivation, spleen cysts, GERD
SEG2_61	M	1y 3m	Xp22.33 microduplication [Léri-Weill dyschondrosteosis (OMIM#127300),	→ Congenital stridor → Growth delay, mesomelia, wide and depressed nasal bridge, low-set and prominent ears, thin lip vermilions, short neck, flat occiput, small hands and feet,

			idiopathic short stature (OMIM#300582)]	brachydactyly, scoliosis → Global DD, generalized hypotonia, brain MRI: lateral ventricular asymmetry, tongue thrusting, impaired pain sensation → Tongue hemangioma, hepatomegaly, lymphangioma
SEG2_62	M	1y 1m	Xp22.31 microdeletion (X-linked ichtyosis, OMIM#308100) and UPD14mat (Temple syndrome OMIM#616222)	→ Decreased fetal movement → Growth delay, relative macrocepahly, high and prominent forehead, low-set ears, high palate, clinodactyly of the 5th finger, STPC → Global DD, EMG: myopathic abnormalities, generalized hypotonia, brain MRI: partial empty sella, thin pituitay gland stalk → Delayed skeletal maturation, micropenis, unilateral retractile testis, growth hormone deficiency, hypoglycemia → Generalized dry skin, white scaling skin on extensor surfaces, ichthyosis on ears and scalp
SEG2_68	F	9y 1m	14q11.2 microdeletion (OMIM#613457)	→ Parents: first cousins → Growth delay, short stature, turricephaly, long face, thick eyebrows, abnormal pinna morphology, hypodontia, long and slender fingers, talipes equinovarus → ID/DD, absent speech, inability to walk, spasticity, skeletal muscle atrophy, seizure, brain MRI: cerebral atrophy → Hirsutism, hypothyroidism
SEG2_70	M	Oy 8m	4q32.2q35.2 deletion (Rossi et al., 2009)	→ Feeding difficulties in infancy, weak cry → Growth delay, micro- and retrognathia, wide and depressed nasal bridge, low-set ears, preauricular skin tag, bilateral STPC, camptodactyly of 2^{nd}-5^{th} fingers, aplasia of the distal phalanx of the 5^{th} finger, overlapping toes, camptodactyly of 4^{th} toes → Global DD, generalized hypotonia, brain MRI: choroid plexus cysts

				→ Cleft palate, glossoptosis, tracheal stenosis, cardiomegaly, hypospadias, double meatus urethrae, unilateral retractile testis, contralateral cryptorchidism, unilateral severe hydronephrosis, nephrolithiasis, osteoarthritis (shoulder region), haemangioma
SEG2_78	F	0y 7m	Xq22 microdeletion (Hijazi et al., 2020)	→ Premature birth, polyhydramnios, LGA (gestational diabetes mellitus), small fontanelles, complicated perinatal adaptation, respiratory distress in infancy → Microcephaly, turricephaly, increased facial adipose tissue, bitemporal narrowing, micrognathia, upslanted and short palpebral fissures, hypotelorism, depressed nasal bridge, abnormal pinna morphology, high palate, webbed neck, low anterior and posterior hairlines, wide intermammillary distance, cutis laxa, joint stiffness, ulnar deviation of the hands, overlapping fingers, unilateral STPC, clinodactyly of the 5th fingers, overlapping toes, camptodactyly of toes, sandal gap, flexion contractures of the fingers, dorsalflexed feet, hypoplasia of the nails → Global DD, generalized hypotonia, poor visual behavior for age, reduced tendon reflexes, brain MRI: ventriculomegaly, cerebral atrophy, CCA → Atrial SD, PS, absent right superior vena cava, persistent left superior vena cava, double aortic arch, GERD
SEG2_80	M	10y 2m	22q11.21q11.22 microdeletion (OMIM#611867)	→ SGA, premature birth → Growth delay, microcephaly, long face, micrognathia, epicanthus, prominent nasal bridge, simple ears, high palate, crowded teeth, thin upper lip vermilion, brachydactyly → Speech articulation difficulties, enuresis nocturna → Atrial SD, submucous cleft palate, ankyloglossia, umbilical hernia, conductive hearing impairment, delayed skeletal maturation, dry skin, recurrent infections
SEG2_82	F	3y 6m	22q13.33 microdeletion (Phelan-McDermid	→ High palate, widely spaced teeth, conical teeth, broad thumbs and 1st toes, joint hypermobility

			syndrome, OMIM#606232) and 19q13.43 microduplication (J. Wang et al., 2020)	→ Global DD, generalized hypotonia, ataxia, frequent falls, normal brain MRI, reduced eye contact, hyperorality → Mitral regurgitation, PI, recurrent infections
SEG2_85	F	20y 7m	16q12.2q21 microdeletion (Yamamoto et al., 2016)	→ SGA → Short stature, obesity, narrow forehead, low-set ears, open mouth, macroglossia, high palate, brachydactyly → Global DD/ID, seizure, normal brain MRI → Coarctation of the aorta, hypertrophic cardiomyopathy
SEG2_87	F	0y 3m	17q12 microduplication (OMIM#614526)	→ High forehead, depressed nasal bridge, hypertelorism, low-set ears → Mild global DD → Atrial SD
SEG2_89	M	5y 7m	14q11.2 microdeletion (OMIM#613457)	→ Perinatal hypoxemia, polyhydramnios, complicated perinatal adaptation → Growth delay, short forehead, deep-set eyes, synophris, short and hypoplastic philtrum, high palate, broad 1st finger, brachydactyly tapered fingers, unilateral STPC, broad hallux, overlapping toes, cutis laxa, lumbar hyperlordosis, abnormal vertebral morphology, hip dysplasia → ID/DD, Trendelenburg sign, tongue tremor, brain MRI: ventriculomegaly, tongue thrusting → Umbilical hernia, abnormality of refraction, hypertrichosis, bilateral pyelectasis, cryptorchidism, GERD

4.3. Phenotypic comparison

We compared the main phenotypic features of the disease-causing CNV carrier patients and the negative CMA group (visualized on Figure 2). Due to the patient selection criteria, NDDs were common in both groups, as were congenital anomalies of the internal organs. Postnatal growth delay was the only symptom to approach significance (p=0.05564). Pectus excavatum (p=0.07484), brain imaging abnormalities (p=0.07848), global DD (0.08070), the sub-phenotype of speech and language delay (p=0.08070) and macrocephaly (p=0.08919) were more commonly, but non-significantly associated with disease-causing CNVs. Conversely, errors of refraction were more common in the negative group, the difference reached significance (p=0.02880) (Lengyel et al., 2022).

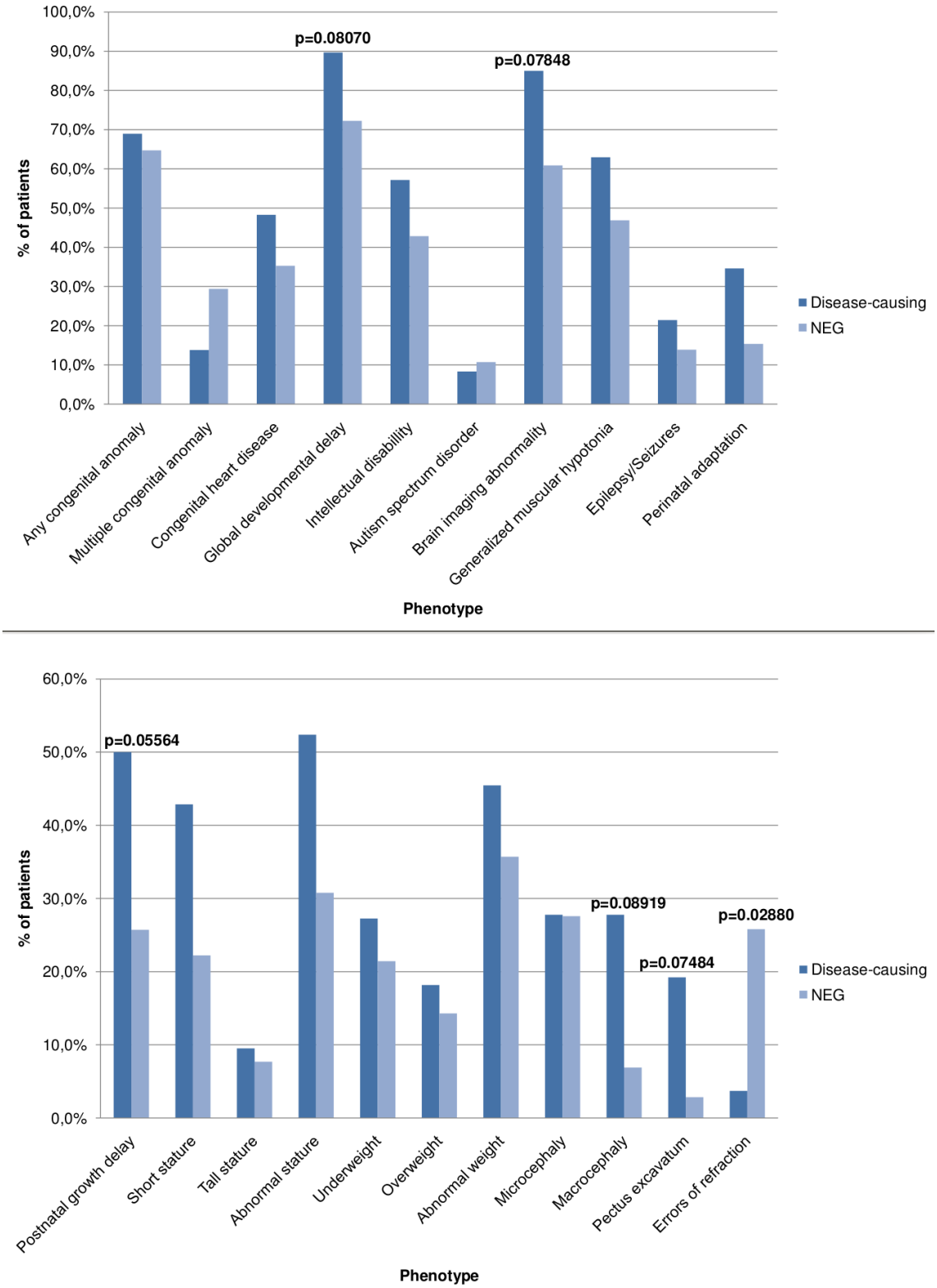

Figure 2. Phenotypic comparison of the patients with disease-causing variants and those with negative CMA results

4.4. Variants of unknown significance

We identified VUS in 17 children (21.79% of the cohort); five individuals carried two, while one individual carried three VUS simultaneously (Tables 8 and 9). Four children carried VUS in addition to disease-causing variants (Table 8). The average size of the VUS variants was 585.9 Kb (median 228.2 Kb), 12 were losses and 12 were copy number gains (Table 5, Lengyel et al., 2022).

Table 8. Patients carrying disease-causing CNVs and clinically uncertain variants simultaneously

Kb: kilobase; P: pathogenic; LP: likely pathogenic; VUS: variant of unknown significance

Patient	Copy Number Variation	Size (Kb)	Classification
SEG2_49	arr[GRCh37]14q11.2(21414942_21966929)x1	552.0	LP
	arr[GRCh37]8p23.1(38796051_39378051)x1	582.0	VUS
	arr[GRCh37]10q26.3(135104029_135254513)x3	150.5	VUS
SEG2_50	arr[GRCh37]8p23.1(7117851_11969155)x1	4851.3	P
	arr[GRCh37]Xq25(127030885_127510702)x1	479.8	VUS
SEG2_52	arr[GRCh37]1p36.33p36.22(820001_9348000)x1	8528.0	P
	arr[GRCh37]1p36.22(9348001_10824000)x3	1476.0	VUS
SEG2_53	arr[GRCh37]16p11.2(28824802_29040571)x1	215.8	P
	arr[GRCh37]22q11.21(18878343_19011099)x1	132.8	VUS

The relevant data obtained from our literature and database search regarding the VUS are summarized in Table 9. Table 10 lists the phenotypes of the patients who carry VUS (excluding those with simultaneous disease-causing CNVs, see Table 7).

Table 9. Overview of the available scientific data concerning variants of unknown significance identified in our cohort

*For larger CNVs containing over 10 protein coding genes, only the morbid genes (highlighted in bold) are listed.

DECIPHER: Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources; AD: autosomal dominant, AR: autosomal recessive, XLD: X-linked dominant, LP: likely pathogenic, VUS: variant of unknown significance, LB: likely benign, B: benign, CHD: congenital heart disease, GDD: global developmental delay, ID: intellectual disability, IVS: intervening sequence, NDD: neurodevelopmental disorder, TAD: topologically associating domain, XLID: X-linked intellectual disability

Patient	CNV	Relevant data
SEG2_6 DECIPHER 517864	arr[GRCh37]6q24.2(145089139_145133632)x1; 44.4 Kb	Genes: *UTRN* DECIPHER: 1 overlapping VUS (*316928: no phenotypes listed*) SVInterpreter: Affects consensus TAD; evidence for gene association weak (*EPM2A*, progressive myoclonic epilepsy, AR)
SEG2_9 DECIPHER 517865	arr[GRCh37]4q34.1(172264978_173419929)x3; 1155.0 Kb	Genes: *GALNTL6* DECIPHER: overlapping variants classified LP (*331524: ~20% overlap, no other variants, phenotypic overlap*)/VUS/unspecified (*270147: ~100% overlap, no other variants, phenotypic overlap, inherited from normal parent*)] DGV: nsv461838 (100% overlap, frequency: 0.001%) SVInterpreter: Variant overlap search: 1 VUS in ClinGen (nssv16206734, 76% overlap); 3 case entries (50-75% overlap) in Developmental Delay studies (Coe et al., 2014; Cooper et al., 2011) Affects several chromatin loops and clustered interactions, evidence for gene association is weak

SEG2_23 DECIPHER 517866	arr[GRCh37]5q13.3q14.1(75706732_78852164)x3; 3145.4 Kb	Genes*: ***PDE8B, AP3B1, ARSB, DMGDH*** DECIPHER: overlapping variants classified VUS/LB/B (*largest overlap <50%, NDD phenotypes*) SVInterpreter: Variant overlap search: negative Affects several chromatin loops and clustered interactions, evidence for gene association is weak
	arr[GRCh37]5q23.2q31.1(122319833_126403246)x3; 4083.4 Kb	Genes: ***PRDM6, CEP120, ALDH7A1, LMNB1*** (ClinGen triplosensitivity score: 3, Leukodystrophy, adult-onset, AD; Microcephaly 26, primary, AD) DECIPHER: overlapping variants classified LP (*401274: ~15% overlap, contains LMNB1, phenotype similar, but unspecific*)/VUS/LB/unspecified (*400622: ~80% overlap, no phenotypes listed*) SVInterpreter: Variant overlap search: negative Affects several chromatin loops and clustered interactions, evidence for gene association is weak
SEG2_24 DECIPHER 517867	arr[GRCh37]2q13(110779468_111141038)x3; 361.6 Kb	Genes: *MALL*, ***NPHP1*** (Nephronophthisis 1, juvenile, AR; Joubert syndrome 4, AR) DECIPHER: functionally similar variants classified as VUS *(NDD phenotypes in multiple cases, e.g. ID:346654, 366560)*/LB SVInterpreter: Variant overlap search: conflicting classifications ClinGen: triplosensitivity unlikely Affects several chromatin loops and clustered interactions, evidence for gene association is weak
SEG2_45 DECIPHER 411594	arr[GRCh37]Xq28(153409765_153520551)x0; 110.8 Kb	Genes: ***OPN1LW, OPN1MW*** (different forms of colorblindness, XL), *TEX28* DECIPHER: functionally similar variant classified LP (*327823:*

		craniofacial phenotype) SVInterpreter: Variant overlap search: conflicting classifications Breakpoint disrupts *OPN1LW* (autistic phenotype in animals) Affects several chromatin loops and clustered interactions, evidence for gene association moderate (*MECP2* gene, X-linked syndromic ID, susceptibiltiy to autism) Several other genes assiciated with XLID and/or susceptibility to autism within the disrupted TAD
SEG2_48 DECIPHER 517868	arr[GRCh37]8p23.1(11145007_11291512)x1; 146.5 Kb	Genes: *MTMR9, SLC35G5* DGV: nsv1033312 (73% overlap, frequency 0.003%) SVInterpreter: Affects several chromatin loops and clustered interactions, evidence for gene association is weak *GATA4* (important CHD gene) in vicinity of breakpoint, no evidence of position effect
SEG2_49 DECIPHER 517827	arr[GRCh37]8p23.1(38796051_39378051)x1; 582.0 Kb	Genes: *PLEKHA2, HTRA4, TM2D2,* ***ADAM9****, ADAM32* DECIPHER: functionally similar variant classified VUS (*306948: ~75% overlap, one other VUS listed, no phenotypic information*) SVInterpreter: Variant overlap search: 1 case entry (55% overlap) in Developmental Delay studies (Coe et al. 2014; Cooper et al. 2011) Affects several chromatin loops and clustered interactions, evidence for gene association is weak
	arr[GRCh37]10q26.3(135104029_135254513)x3; 150.5 Kb	Genes: ***TUBGCP2*** (disrupted in IVS 8; Pachygyria, microcephaly, developmental delay, and dysmorphic facies, with or without seizures; AR)*, ZNF511, CALY, PRAP1, FUOM,*

		ECHS1 *(*AR mitochondrial disorder), *PAOX, MTG1, SPRN* DECIPHER: functionally similar variants classified VUS (*345428: no other variants, no phenotypic information*)/unspecified SVInterpreter: Variant overlap search: 1 B in ClinGen (nssv15171977, 55% overlap), 1 case entry (80% overlap) in Developmental Delay studies (Coe et al. 2014; Cooper et al. 2011) Affects chromatin loops and clustered interactions, evidence for gene association is weak
SEG2_50 DECIPHER 517830	arr[GRCh37]Xq25(127030885_127510702)x1; 479.8 Kb	Genes: *ACTRT1* DECIPHER: functionally similar variants classified VUS (*407109: one other VUS, no phenotypic information*)/unspecified (*e.g. 254266: no other variants, minor anomalies and NDD phenotypes*) SVInterpreter: Variant overlap search: 1 B (nssv15175084, 99% overlap) and 1 VUS (nssv15136123, 99% overlap) in ClinGen No evidence of potential position effect
SEG2_52 DECIPHER 517831	arr[GRCh37]1p36.22(9348001_10824000)x3; 1476.0 Kb	Genes: ***PIK3CD, NMNAT1, KIF1B, PEX14*** DECIPHER: functionally similar variants classified VUS (*403542: no phenotypes listed; 307783: GDD*) SVInterpreter: Variant overlap search: 1 VUS (nssv15134911, ~60% overlap) in ClinGen Affects chromatin loops and clustered interactions, evidence for gene association is weak
SEG2_53 DECIPHER 411578	arr[GRCh37]22q11.21(18878343_19011099)x1; 132.8 Kb	Genes: *DGCR6, PRODH* DECIPHER: functionally similar variants classified P (example with no other variants - *332402: no phenotypic*

		information)/VUS (*317171: GDD; 433468: no phenotypic information)* /B/unspecified (*338643: microcephaly, ID*) SVInterpreter: Variant overlap search: conflicting classifications [*1 DGV entry (nsv4535813), 1 ClinGen benign (nssv15164041), 1 ClinGen VUS (nssv15606257), 1 ClinGen P (nssv15121171), present as both case and control in Developmental Delay studies* (Coe et al., 2014; Cooper et al., 2011)] Evidence for gene association is weak
SEG2_55 DECIPHER 517869	arr[GRCh38]5q14.1(80253904_80965738)x3; 711.8 Kb	Genes: *SPZ1, ZFYVE16, ANKRD34B,* ***DHFR***, ***MSH3***, *RASGRF2* DECIPHER: overlapping variants classified LP (*290543: ~40% overlap, NDD phenotype*)/VUS (*NDD phenotypes, e.g. 269277*) /unspecified SVInterpreter: Variant overlap search: negative Affects several chromatin loops and clustered interactions, evidence for gene association is weak/moderate (several genes linked with NDD phenotypes in animal models)
	arr[GRCh38]10q11.22(46321708_46780831)x4; 459.1 Kb	Genes: *ANTXRL, ANXA8L1, NPY4R, GPRIN2, SYT15* CN=3 is a LB variant SVInterpreter: no evidence of potential position effect
SEG2_57 DECIPHER 517870	arr[GRCh37]4q24(102058416_102443207)x3; 384.8 Kb	Genes: ***PPP3CA*** (Arthrogryposis, cleft palate, craniosynostosis, and impaired intellectual development, AD; Developmental and epileptic encephalopathy 91, AD) DECIPHER: 1 overlapping variant (*384473: classification unspecified, NDD phenotypes and short stature listed*) DGV: nsv1011351 (100% overlap, frequency 0.003%) SVInterpreter:

		Variant overlap search: 2 case entries (100% overlap) in Developmental Delay studies (Coe et al., 2014; Cooper et al., 2011) Proximal breakpoint disrupts IVS2 of *PPP3CA*, distal breakpoint disrupts IVS19 of *BANK1* Affects several chromatin loops and clustered interactions, evidence for gene association is weak (*SLC39A8*: Congenital disorder of glycosylation, type Iin, AR)
	arr[GRCh37]20p11.23(19240620_19745197)x3; 504.6 Kb	Genes: ***SLC24A3***, ***RIN2*** (Macrocephaly, alopecia, cutis laxa, and scoliosis syndrome, AR*)* SVInterpreter: Variant overlap search: negative Breakpoint disrupts IVS16 of *SLC24A3*, no disease association in human or animals, no evidence for triplosensitivity Affects chromatin loops/clustered interactions (*RIN2* gene)
	arr[GRCh37]Xq25(124088718_124169834)x0; 81.1 Kb	Genes: *TENM1* DECIPHER: overlapping variants classified VUS/LB/unspecified (*NDD phenotypes listed for all*) SVInterpreter: Variant overlap search: negative Breakpoint disrupts *TENM1* gene (no disease association in human, XLID in mice) Affects chromatin loops/clustered interactions (*STAG2* gene - Mullegama-Klein-Martinez syndrome, XL)
SEG2_71 DECIPHER 517871	arr[GRCh37]17q25.3(79989503_79992106)x1; 2.6 Kb	Genes: ***RAC3*** (disrupted in exon 6, Neurodevelopmental disorder with structural brain anomalies and dysmorphic facies, AD) SVInterpreter: Variant overlap search: negative

		No evidence for position effect
SEG2_72 DECIPHER 517872	arr[GRCh37]11p13(36062989_36291232)x1; 228.2 Kb	Genes: *LDLRAD3* DECIPHER: 1 overlapping variant (*371453: unspecified classification, no phenotypes listed*) SVInterpreter: Variant overlap search: 1 B entry in ClinGen (nssv15172015, 87% overlap, frequency 0.001%) Affects several chromatin loops and clustered interactions, evidence for gene association is weak
SEG2_75 DECIPHER 517873	arr[GRCh38]2p25.1(10617430_10795996)x3; 178.6 Kb	Genes: *NOL10, ATP6V1C2, PDIA6* DECIPHER: overlapping variants classified VUS (*337612, 288930: similar, but unspecific phenotypes*) DGV: nsv1000731 (99% overlap, frequency 0.003%) SVInterpreter: Variant overlap search: negative Affects several chromatin loops and clustered interactions, evidence for gene association is weak (*ODC1* gene - Bachmann-Bupp syndrome, AD)
	arr[GRCh38]4q13.3(70295481_70415836)x1; 120.4 Kb	Genes: *UGT2B4* DECIPHER: functionally similar variants classified VUS *(e.g. 293676, 306115, 314279: NDD phenotypes*)/LB/unspecified (*e.g. 299798, 299923: NDD phenotypes*) SVInterpreter: Variant overlap search: negative Breakpoint disrputs IVS2 of *CLNK* (no disease association) Affects several chromatin loops and clustered interactions, evidence for gene association is weak (*WDR1* gene - Periodic fever, immunodeficiency, and thrombocytopenia syndrome, AR)
SEG2_77	arr[GRCh37]16p11.2(31980001_33825000)x1;	Genes: *TP53TG3*

DECIPHER 411577	1845.0 Kb	DECIPHER: overlapping (~50-60%) variant LP (*328130: moderate phenotypic overlap*); functionally similar variants classified VUS/LB SVInterpreter: Variant overlap search: conflicting classifications Affects several chromatin loops and clustered interactions, evidence for gene association is weak/moderate (*SETD1A* - Neurodevelopmental disorder with speech impairment and dysmorphic facies, AD; *KAT8* - Li-Ghorgani-Weisz-Hubshman syndrome, AD)
	arr[GRCh37]Xq28(153409765_153520551)x0; 110.8 Kb	Genes: ***OPN1LW***, ***OPN1MW*** (different forms of colorblindness, XL), *TEX28* DECIPHER: functionally similar variant classified LP (*327823: craniofacial phenotype*) SVInterpreter: Variant overlap search: conflicting classifications Breakpoint disrupts *OPN1LW* (autistic phenotype in animals) Affects several chromatin loops and clustered interactions, evidence for gene association moderate (*MECP2* gene, X-linked syndromic ID, susceptibiltiy to autism) Several other genes associated with XLID and/or susceptibility to autism within the disrupted TAD
SEG2_81 DECIPHER 517874	arr[GRCh37]20p11.22(24554628_24708699)x3; 154.1 Kb	Genes: *SYNDIG1* (disrupted in IVS2) SVInterpreter: Variant overlap search: 1 entry in 1000Genomes (esv3645553, 50% overlap, frequency 0.01%) Affects several chromatin loops and clustered interactions, evidence for gene association is weak

Table 10. Phenotypes of patients with variants of unknown significance

*: age at first genetic consultation; y: years; m: months; AC: amniocentesis; ADHD: attention deficit/hyperactivity disorder; ASD: autism spectrum disorder; CALM: café au lait macule; CCA: corpus callosum abnormality; CVS: chorionic villi sampling; GERD: gastroesophageal reflux disease; GH: growth hormone; HT: hypertension; ID/DD: intellectual disability/developmental delay; IUGR: intrauterine growth retardation; LGA: large for gestational age; NDD: neurodevelopmental disorder; PDA: patent ductus arteriosus; PS: pulmonic stenosis; PVL: periventricular leukomalacia; SD: septal defect; SGA: small for gestational age; STPC: single transverse palmar crease; US: ultrasound

Patient	Sex	Age*	Detailed phenotype
SEG2_6	M	10y 11m	→ LGA → Macrocephaly, low-set and protruding ears, thick lower lip vermilion, pectus excavatum, pes planus, sandal gap → ID/DD, absent speech, hypotonia, brain MRI: intracranial cystic lesions, abnormal repetitive mannerisms, inappropriate laughter

SEG2_9	M	2y 6m	→ AC: 46,XY → Growth delay, relative macrocephaly, trigonocephaly, scaphocephaly, long face, tall forehead, retrognathia, epicanthus, wide and depressed nasal bridge, low-set ears, hypoplastic philtrum, overlapping toes, clinodactyly of the 2nd and 4th toes → ID/DD, hypotonia, seizures (normal EEG), brain MRI: corpus callosum atrophy, suspected PVL, left amygdala larger, aggressive behavior → Ventricular SD
SEG2_23	F	6y 5m	→ SGA, prenatal US: nuchal fold, CVS: 46,XX → Growth delay, low-set ears, high palate, clinodactyly of the 5th fingers, syndactyly of the 2nd-3rd toes, joint hypermobility → ID/DD, hypotonia, brain MRI: ventriculomegaly, short attention span → Ventricular SD, GH deficiency, Kawasaki syndrome, strabismus, abnormality of refraction, bronchial asthma
SEG2_24	M	2y 7m	→ Microcephaly, downslanted palpebral fissures, depressed nasal bridge, low-set and prominent ears, abnormal pinna morphology, high palate, overlapping toes → Delayed speech and language development → Atrial SD, PS, cryptorchidism
SEG2_45	M	2y 9m	→ Oligohydramnios, weak and high-pitched cry → Small and low-set ears, abnormal pinna morphology, blepharophimosis, STPC, joint hypermobility, genu valgum, pes planus, polydactyly → ID/DD, hypotonia, gait imbalance, impaired social interactions → Mild conductive hearing impairment
SEG2_48	M	1y 5m	→ IUGR, neonatal respiratory distress → Growth delay, microcephaly, high anterior hairline, prominence of the premaxilla, retrognathia, hypertelorism, exophthalmus, wide nasal bridge, short ears, conical teeth, tapered fingers,

			clinodactyly of 3rd toes, lymphedema of the feet, pes planus → ID/DD, axial hypotonia, limb hypertonia, brain MRI: CCA, speech articulation difficulties → Atrial SD, delayed skeletal maturation, strabismus, midnasal stenosis, retractile testis, recurrent infections (often severe, including e.g. osteomyelitis – immunological evaluations normal), unilateral inguinal hernia, ekzema
SEG2_55	M	1y 0m	→ LGA, neonatal hypoglycemia (gestational diabetes mellitus) → Postnatal overgrowth, facial asymmetry, triangular face, high and broad forehead, micrognathia, retrognathia, epicanthus, high palate, misalignment of teeth, bilateral partial cutaneous syndactyly of the 2nd-3rd toes, abnormality of the temporal hairline → ID/DD, hypotonia, cranial US: intracranial cystic lesions → Unilateral hydronephrosis and hydroureter, inspiratory stridor
SEG2_57	M	1y 4m	→ Complicated pregnancy: seizures, hypertension, otitis media, premature rupture of membranes, prolonged labour, feeding difficulties in infancy, poor suck → Macrocephaly, bitemporal narrowing, prominent forehead, hypertelorism, low-set ears, high palate, complex syndactyly of all 4 extremities, bilateral talipes equinovarus → ID/DD, absent speech, axial hypotonia, limb hypertonia, inability to walk, cerebral atrophy, postoperative hypoxic-ischaemic encephalopathy and cytotoxic edema, cortical necrosis, ventriculomegaly, seizures, abnormal repetitive mannerisms, inappropriate laughter → Hyperhidrosis, atrial SD, coarctation of the aorta, left ventricular hypertrophy, visual impairment, abnormality of refraction, increased serum lactate
SEG2_71	F	2y 1m	→ Oligohydramnios, IUGR, weak cry → Growth delay, high forehead, micrognathia, prognathia, hypertelorism, depressed nasal bridge, wide and bulbous nose, low-set, small and prominent ears, abnormal pinna morphology, overfolded helix, bifid uvula, clinodactyly of the 5th fingers, broad fingertips, syndactyly of the toes (left: 1st-2nd and 3rd-5th; right: 4th-5th), beaked nails, scoliosis, abnormal vertebral morphology, abnormal hip bone

			morphology → Global DD, normal intelligence, hypotonia, incoordination, speech articulation difficulties, nasal voice, enuresis nocturna → Bilateral sensorineural hearing impairment, abnormality of refraction, atrial SD, PDA, pulmonary HT, gallbladder cysts, ectopic kidney, bilateral inguinal hernia
SEG2_72	M	4y 6m	→ Facial asymmetry, epicanthus, downslanted palpebral fissures, narrow and open mouth → ID/DD, skeletal muscle atrophy, abnormal repetitive mannerisms, excessive salivation
SEG2_75	F	1y 4m	→ Defined zygomatic region, hypertelorism, epicanthus, depressed nasal bridge, high palate, widely spaced teeth, joint hypermobility, scoliosis, genu valgum, calcaneovalgus deformity, pes planus → ID/DD, hypotonia, poor gross and fine motor coordination, cranial US: ventriculomegaly, autistic behavior, short attention span → Sensorineural hearing impairment
SEG2_77	M	6y 2m	→ LGA → Wide nasal bridge, clinodactyly of the 5^{th} fingers, syndactyly of the 2^{nd}-3^{rd} toes, joint hypermobility, scoliosis → Motor DD, learning difficulties (due to behavioral problems, normal intelligence), speech articulation difficulties, ASD, ADHD, abnormal temper tantrums → Sensorineural hearing impairment, tracheal and anal stenosis
SEG2_81	M	3y 8m	→ ID/DD, absent speech, autistic behavior, complex NDD

5. Discussion

5.1. Overview and diagnostic yield

Reevaluation of VUS is recommended every 2-3 years due to the fast accumulation of scientific data (Riggs et al., 2020). I have conducted reevaluation according to the most recent ACMG guidelines. These efforts led to the reclassification of VUS in eleven patients, five of whom now have clinically significant results (Table 4). Including these five cases, CMA enabled an etiologic diagnosis in 29 children in a cohort of 78 patients with NDDs and/or congenital anomalies (Lengyel et al., 2022). Despite the rigorous reevaluations, a relatively high proportion of CNVs remained VUS.

The diagnostic yield (37.18%) of this study is quite high compared to the estimated 15-20% yield of CMA methods, albeit these estimates are often based on studies where selection criteria were rather broad and CMA resolution was relatively low (Miller et al., 2010). Our diagnostic yield is comparable to several studies with smaller patient groups and/or similarly strict selection criteria (Ho et al., 2016; Iourov et al., 2012; Kashevarova et al., 2014; Kessi et al., 2018; Lay-Son et al., 2015; Liu et al., 2022; Mihaylova et al., 2017; Perovic et al., 2022; R. Wang et al., 2019; Werling et al., 2020; Wu et al., 2017).

5.2. Phenotypic analysis

Said rigorous patient selection also impacted our statistical observations (keeping in mind the limitations of the small sample size as well). In contrast to our results, congenital heart defects and other major congenital anomalies are often observed in significantly higher frequencies in patients with definitive CMA results (Chaves et al., 2019; Shoukier et al., 2013; Wu et al., 2017). The association of postnatal growth delay, global DD, speech and language delay and brain imaging abnormalities is in line with literature data (Chaves et al., 2019; Heide et al., 2017; Shoukier et al., 2013). Macrocephaly and pectus excavatum are well-known suggestive features/minor anomalies, and are therefore not unexpected results. Only one child had an error of refraction in the disease-causing CNV group, in contrast to 7 patients in the negative group (Lengyel et al., 2022).

5.3. Recurrent reciprocal CNVs identified in the studied patients

Chromosomes 16, 22, 14, 17 and X were most frequently affected by disease-causing imbalances (Lengyel et al., 2022). Six of the nine chromosome 16 CNVs, two of the four imbalances affecting chromosome 22, two of three on chromosome 17, one on chromosome 15 and one on chromosome X corresponded to recurrent microdeletion/microduplication regions, and thus are the consequence of NAHR mediated by segmental duplications (Golzio & Katsanis, 2013). Chromosome 16 will be discussed in detail below; the other recurrent CNVs identified in the presented cohort are listed in Table 11.

Table 11. Recurrent CNVs identified in the presented patients

Excluding CNVs affecting chromosome 16; *All coordinates according to GRCh37; OMIM: Online Mendelian Inheritance in Man

Copy Number Variation*	Size (Kb)	Patient	Syndrome/Disorder
15q11.2q13.1(22765628_29060493)x1	6294.9	SEG2_33	Angelman syndrome (OMIM#105830)
17p11.2(16727490_20433502)x1	3706.0	SEG2_59	Smith-Magenis syndrome (OMIM#182290)
17q12(34835983_36243365)x3	1407.4	SEG2_87	17q12 microduplication (OMIM#614526)
22q11.21q11.22(21460640_22962962)x1	1502.3	SEG2_80	22q11.21q11.22 microdeletion (OMIM#611867)
22q11.21q11.22(21934268_22336871)x3	402.6	SEG2_7	22q11.2 microduplication (OMIM#608363)
Xp22.31(6537108_8167604)x0	1630.5	SEG2_62	X-linked ichtyosis (OMIM#308100)

5.3.1. Recurrent CNVs of the short arm of chromosome 16

Chromosome 16 has one of the highest percentages [(approximately 10% of its sequence, ~7.8 Mb (Martin et al., 2004)] of SDs amongst the human chromosomes, clustered along the short arm (16p). This predisposes 16p to rearrangements, and the

resultant recurrent CNVs are implicated in various genomic disorders (Cooper et al., 2011; Girirajan et al., 2012; Itsara et al., 2009; Kanduri et al., 2016; Sahoo et al., 2011; Sanders et al., 2011; Stefansson et al., 2014; Weiss et al., 2008). Figure 3 visualizes the 16p disease-causing variants identified in the presented cohort, as well as two additional CNVs (one VUS and one LB) worthy of discussion.

Figure 3. Recurrent copy number variations of the short arm of chromosome 16 and the rearrangements of the presented patients

A: Region 16p12.2. **B**: Recurrent CNV regions in 16p11.2. **C**: Patient SEG2_77's deletion at proximal 16p11.2. The purple bars denote known recurrent CNV regions;

red bars indicate deletions and blue bars indicate duplications identified in the patients. Disease-causing OMIM genes are in dark green.

5.3.1.1. Region 16p12.2

Region 16p12.2 (Figure 3A; previously mapped to 16p12.1), mentioned in the Introduction in relation to the second-hit theory, is a well-known NDD predisposition region. The affected, approximately 500 Kb large chromosomal segment contains four OMIM genes: the cancer related *EEF2K* (*606968) and *CDR2* (*117340) genes, the *POLR3E* (*617815) gene with DNA dependent *RNA* polymerase activity and the disease causing *UQCRC2* gene (*191329). The latter encodes a protein that is part of the ubiquinol-cytochrome c reductase complex, and its mutations are responsible for AR mitochondrial complex III deficiency. The distinct recurrent 16p12.2 microdeletion region was first identified in association with ID/DD and congenital malformations (Girirajan et al., 2010; Itsara et al., 2009). The CNV is phenotypically variable: ID/DD, speech delay, craniofacial and skeletal abnormalities are common features; less frequent are muscular hypotonia, growth delay, microcephaly, congenital heart disease, seizure disorders and psychiatric and behavioral abnormalities (Geng et al., 2014; Girirajan et al., 2010).

Patient SEG2_32 (DECIPHER ID: 517744) carries a 526.9 Kb large duplication of 16p12.2 (chr16:21953152_22480514), which is reciprocal to the NDD predisposition microdeletion (Kendall et al., 2017; Kushima et al, 2018). In the study led by Kushima, the 16p12.2 duplication (ClinVar accession number: VCV000545195.1) was identified in one ASD and one schizophrenia case, and in no controls. Detailed phenotypic information is available for the ASD case, including ADHD, motor delay, sensory hypersensitivity, mood disorders, stereotypic arm waving and depressive symptoms. DECIPHER catalogues 25 duplication cases with similar breakpoints and nearly identical gene content. At least one NDD is listed in 13 of the 18 entries with available phenotypic information (IDs: 251934, 253837, 270602, 272962, 277148, 300770, 303553, 304664, 305863, 322284, 338128, 389569, 412250). There is a nearly identical Gold Standard Variant (gssvG14362) listed in the DGV with an overall allele frequency of only 0.03%. ClinVar database lists two LP, 18 VUS and four LB variants corresponding to this duplication. ClinGen Dosage Sensitivity curations give the region

a haploinsufficiency score of 2 (some evidence for dosage sensitivity) and a triplosensitivity score of 0 (no evidence for dosage sensitivity).

SEG2_32 carries an additional large LB deletion of chromosome 5q13.2 (1.824 Mb; chr5:68830621_70654255), including 6 OMIM genes: *OCLN* (*602876), *SERF1A* (*603011), *GTF2H2* (*601748), and spinal muscular atrophy (#253400) associated *SMN1* (*600354), *SMN2* (*601627) and *NAIP* (*600354). Of the 13 DECIPHER cases listed above (the duplication carriers with NDD phenotypes), four patients carry additional CNVs.

Although it is still debated whether the reciprocal 16p12.2 duplication is a similar NDD risk factor as the microdeletion, SEG2_32's case provides further evidence towards its potential predisposing effect (Lengyel et al., 2020). Based on the NDD cases listed above, I would argue that the reciprocal 16p12.2 microduplication could be a low-penetrance NDD risk factor variant, but likely not solely responsible for the phenotype. Further evidence is needed to reach a final conclusion regarding this complex issue.

5.3.1.2. Distal 16p11.2 region

Another recurrent region is the distal BP2-BP3 16p11.2 region (Figure 3B), which is approximately 220 Kb spanning ~28.8-29.0 Mb. Phenotypes associated with the microdeletion include obesity, generalized overgrowth, various NDDs (ID, GDD, delayed speech and language development, ASD, seizures, ADHD), and less often congenital anomalies of other organ systems (Bachmann-Gagescu et al., 2010; Barge-Schaapveld et al., 2011; Bochukova et al., 2010). A tendency towards increased head circumference (HC) was also observed, along with mirroring body mass index (BMI) and HC reduction in the reciprocal duplication (Loviglio et al., 2017b).

The gene suspected to play a role in the obesity phenotype associated with the distal microdeletion is *SH2B1* (OMIM *608937), encoding a cytoplasmic adaptor protein for various members of the tyrosine kinase receptor family. The protein is postulated to enhance hypothalamic leptin sensitivity, thus regulating energy balance, body weight, peripheral insulin sensitivity and glucose homeostasis. Alterations of *SH2B1* lead to leptin and insulin resistance, polyphagia, obesity and type 2 diabetes mellitus in mice and humans alike (Ren et al., 2007; Rui, 2014). *LAT* (OMIM *602354) - encoding a protein that is part of a complex required for T-cell development and signalling, and

likely has an important role in neurogenesis - was proposed as a major driver gene of the mirror HC phenotype in the BP2-BP3 rearrangements.

Patient SEG2_53's (DECIPHER ID: 411578) phenotype is consistent with the distal 16p11.2 microdeletion syndrome (OMIM #613444). She also has a deletion overlapping the 2q37 microdeletion syndrome (OMIM #600430), characterized by mild-moderate ID/DD, brachymetaphalangy, short stature, obesity, hypotonia and characteristic facies (which SEG2_53 does not resemble). However, her CNV (chr2:242855654_243030845; GRCh37) is much smaller than the deletions of previously reported patients and does not include the *HDCA4* gene (OMIM *605314), proposed to be a critical gene within the microdeletion (Aldred et al., 2004; Williams et al., 2010), therefore I reclassified the 2q37 deletion as LB (Table 4). SEG2_53's phenotype was predominantly caused by the 16p11.2 deletion (inherited from her mother, who is obese, but mentions no history of NDDs).

Patient SEG2_45 (DECIPHER ID: 411594) also has a 16p11.2 deletion, which is a LB variant, but serves as corroborating evidence for the pathogenicity of the distal BP2-BP3 region on 16p. SEG2_45's alteration encompasses one non-disease associated OMIM gene, *EIF3C* (*603916). A search of ClinVar database yields three smaller deletions involving *EIF3C*, classified as benign. The DGV lists a gold standard variant (gssvL43531) involving only this gene (approximately 40% overlap with SEG2_45's CNV) with a frequency of 8.82%. All other ClinVar records that overlap SEG2_45's deletion, and overlapping DECIPHER cases as well, either include the BP2-BP3 pathogenic region, or are much larger in size. SEG2_45's proximal breakpoint is virtually identical with SEG2_53's distal breakpoint (~28824800). Phenotypic comparison of the two patients shows reverse findings regarding BMI (Figure 4A and 4B).

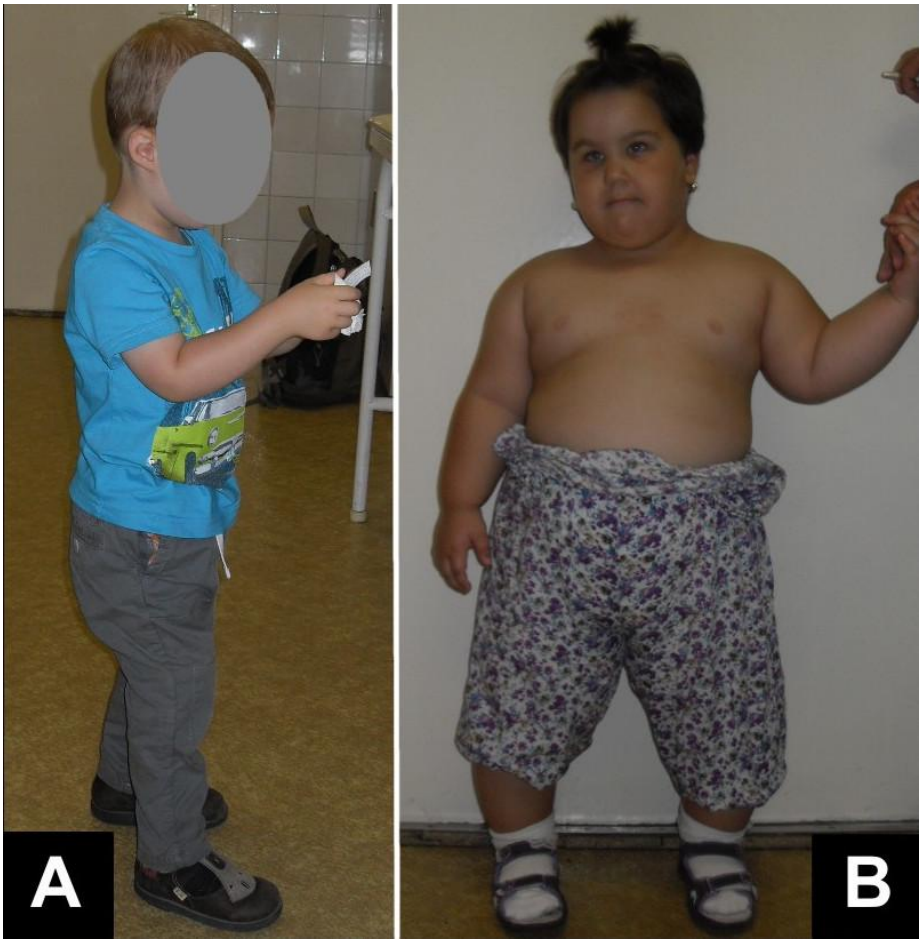

Figure 4. Phenotypes of Patients SEG2_45 and SEG2_53

A: SEG2_45's phenotype is dominated by speech delay, dyslalia, cognitive disability and conductive hearing loss. His somatic development is normal. **B**: SEG2_53's phenotype is consistent with the distal 16p11.2 deletion: obesity, polyphagia and developmental delay (speech and social interactions are most severely affected). She also has strabismus, relatively small hands and feet, and a tibia varus deformity. Identifiable photographs are published with the written informed consent of the parents.

The CNV seen in SEG2_45 is directly upstream of the recurrent BP2-BP3 microdeletion and *SH2B1* gene, and thus his lack of the typical obese phenotype further consolidates the role of the distal 16p11.2 region and the genes it encompasses; and suggests that the upstream region does not confer similar risk through position effects (Lengyel et al., 2020).

5.3.1.3. Proximal 16p11.2 region

The most frequently affected CNV region is the proximal (BP4-BP5) region of 16p (Figure 3B), associated with a recurrent microdeletion (OMIM #611913) and a reciprocal recurrent microduplication (OMIM #614671). Amongst the 16p alterations, a noteworthy four were gains or losses of the proximal 16p11.2 region, which corresponds to 5.1% of the entire cohort, and 13.8% of children with a definitive diagnosis.

The region is approximately 600 Kb in size, located from genomic position ~29.6 to ~30.2 Mb (GRCh37). In close proximity lies a 95 Kb segment encompassing *BOLA2* (OMIM *613183; a gene suspected to be relevant in early embryonic development), which has undergone *Homo sapiens*-specific expansion relatively recently in human evolution, and has been suggested to predispose the BP4-BP5 region to recurrent rearrangement (Nuttle et al., 2016). The CNVs are associated with a wide variety of neuropsychiatric phenotypes, growth abnormalities, skeletal abnormalities and other, less frequent congenital anomalies (Table 12). CNVs of this region were initially ascertained in ASD populations: an estimated 0.28-1% of patients carry BP4-BP5 CNVs (Fernandez et al., 2010; Hanson et al., 2015; Kumar et al., 2008; Walsh & Bracken, 2011; Weiss et al., 2008), and subsequent large studies identified the recurrent CNVs in 0.6-0.7% of patients with NDDs (Rosenfeld et al., 2010; Shinawi et al., 2010; Steinman et al., 2016). In large 16p11.2 CNV populations 90% or more of carrier children were found to have neuropsychiatric diagnoses; the prevalence of ASD has been reported between 15-25%, and in most cohorts the frequency of autistic-like features is even higher (Green Snyder et al., 2016; Hanson et al., 2015). The proximal CNVs are furthermore associated with developmental coordination disorder (DCD), specific speech disorders, epilepsy, and an increased prevalence of sacral dimples (often with atypical features, which may signify the presence of occult spinal dysraphism) (D'Angelo et al., 2016; Green Snyder et al., 2016; Hanson et al., 2015; Owen et al., 2018; Shinawi et al., 2010; Steinman et al., 2016; Zufferey et al., 2012). Comparing the reciprocal CNVs, duplication carriers are more likely to have tremor, ADHD and other behavioral problems (aggression, outbursts, etc.), and they have an increased risk for psychosis and severe ID. Deletion patients, on the other hand, have a higher incidence of ASD, as well as speech and language disorders (Giaroli et al., 2014; Green Snyder et

al., 2016; Hanson et al., 2015; Shinawi et al., 2010; Steinman et al., 2016). The prevalence of behavioral disorders shows consistent increase with age in both groups (Bernier et al., 2017). Furthermore, the reciprocal CNVs cause mirror phenotypes in terms of BMI, HC and brain volume: deletion carriers present with obesity, significantly increased HC and global increased brain size; meanwhile duplication carriers have a tendency towards being underweight and microcephalic, and they have reduced brain volume measurements (Bijlsma et al., 2009; Jacquemont et al., 2011; Qureshi et al., 2014; Shinawi et al., 2010; Steinman et al., 2016; Tabet et al., 2012; Walters et al., 2010). The deletion is more often associated with abnormally shaped, thicker corpora callosa, overgrowth in the posterior fossa, Chiari I malformations and tonsillar ectopia, whereas duplication carriers are more likely to have thinner corpora callosa, decreased white matter volume and ventriculomegaly (Owen et al., 2018).

Several genes within the region have been implicated in the associated phenotypes. A smaller, ~118 Kb deletion within the proximal recurrent region co-segregated with ASD/autistic features in a three generation family, refining a possible critical region for ASD that includes three candidate genes: *MVP*, *SEZ6L2*, and *KCTD13* (Crepel et al., 2011). *SEZ6L2* (OMIM *616667) has been suggested to have a role in the modulation of neuronal differentiation (Boonen et al., 2016). *MVP* (OMIM *605088) has been linked to ADHD through transcriptome-wide association and mRNA expression profile analyses (Qi et al., 2019), has been shown to have a role in synaptic plasticity (Ip et al., 2018), and is known to interact with *PTEN* (OMIM *601728), a gene implicated in ASD/ID with macrocephaly (Loviglio et al., 2017b; Yu et al., 2002). *KCTD13* (OMIM *608947) encodes a protein that is a substrate-specific adapter of a BTB-CUL3-RBX1 E3 ubiquitin ligase complex. The latter targets small GTPase RhoA for ubiquitination and degradation, and is necessary for normal synaptic transmission (Y. Chen et al., 2009). The gene has been suggested to drive the mirror microcephaly/macrocephaly phenotypes in a zebrafish study (Golzio et al., 2012). This experimental finding was not reproduced in a subsequent study; Kctd13 reduction did, however, lead to decreased functional synapse number and reduced synaptic transmission in mutant mice (Escamilla et al., 2017). Furthermore, reduced synaptic transmission correlated with increased levels of Ras homolog gene family, member A (RhoA - a substrate of the aforementioned ubiquitin ligase complex), and was reversed by RhoA inhibition, which

might prove therapeutically relevant in the future (Escamilla et al., 2017). Significantly, co-injection of *LAT* (the abovementioned gene postulated to drive the mirror HC phenotypes in the distal, BP2-BP3 region) and *KCTD13* seems to have an additive effect on zebrafish HC, providing evidence for genetic interactions between the distal and the proximal recurrent 16p11.2 CNV regions (Loviglio et al., 2017a; Loviglio et al., 2017b). Possibly relevant in regards to seizures is the *PRRT2* gene [OMIM *614386, (Scheffer et al., 2012)], which is linked to a benign epilepsy syndrome (seizures, benign familial infantile, 2; OMIM *605751).

SEG2_26 and SEG2_27 (DECIPHER IDs: 517820 and 517821), a pair of brothers, show typical features associated with the recurrent 16p11.2 duplication, although SEG2_26 presented a more severe phenotype with epilepsy. The children inherited the microduplication from their mother (Table 3), who never had formal neuropsychiatric evaluations, but was referred to have social and behavioral difficulties. This family exemplifies the interfamilial variable expressivity linked to recurrent rearrangements. SEG2_39's (DECIPHER ID: 517824) phenotype corresponds to the reciprocal chromosome 16p11.2 deletion syndrome (OMIM #611913) (Lengyel et al., 2020).

One microduplication carrier had noteworthy additional health problems. Patient SEG2_17 (DECIPHER ID: 411596) had normal birth parameters and perinatal adaptation. Bilateral cryptorchidism was observed upon birth. He had apnoeic episodes and feeding difficulties in infancy, but exhibited average psychomotor development until he was 5 months old, when he lost the ability to hold his head, roll over and crawl. He was transported to a hospital due to dehydration, generalized lymphedema, hepatomegaly, fluctuations in consciousness, horizontal nystagmus and mild facial asymmetry. Brain CT revealed cerebral atrophy and ventriculomegaly, but extensive examinations ultimately failed to explain his acute condition. He is currently 5 years old with global DD and absent speech. He has yet to undergo official psychological evaluation, but the mother reported a change in behavior around 3 years of age (he stopped reacting to his name, has abnormal temper tantrums, started presenting abnormal eating behavior, is not interested in toys and has a short attention span). The patient's parents are healthy, but the paternal grandmother and uncle have abnormal aggressive and impulsive behavior. SEG2_17's neurodevelopmental phenotype is in line with the microduplication syndrome, however it remains unclear if (or to what

extent) the CNV could be responsible for his acute multi-system disease (Lengyel et al., 2020).

Table 12 summarizes the phenotypic features of the proximal reciprocal CNVs, integrating the symptoms of patients from the presented cohort, and information from recent literature and online databases (Lengyel et al., 2020).

Table 12. Main phenotypic features of the proximal 16p11.2 (BP4-BP5) deletion/duplication

n.a.: not available; BMI: body mass index; EEG: electroencephalography; MRI: magnetic resonance imaging; CT: computer tomography. HPO: Human Phenotype Ontology (https://hpo.jax.org/app/); VF^{HPO}: listed as very frequent (83-99%) in HPO; F^{HPO}: listed as frequent (30-79%) in HPO; O^{HPO}: listed as occasional (5-29%) in HPO.

[a] : Steinman et al., 2016; [b] : D'Angelo et al., 2018; [c] : Green Snyder et al., 2016; [d]: Hanson et al., 2015

	Proximal 16p11.2 duplication				**Proximal 16p11.2 deletion**		
PHENOTYPE	**Frequency**	**SEG2_17**	**SEG2_26**	**SEG2_27**	**PHENOTYPE**	**Frequency**	**SEG2_39**
Growth							
Short stature/Reduced BMI/Failure to thrive in infancy	VF^{HPO}	-	-	-	Obesity	O^{HPO}	+
Head and Neck							
Microcephaly	13-17%[a]	-	-	-	Macrocephaly	17%[a]	-
Sparse eyelashes and/or eyebrows	VF^{HPO}	-	+	+	Hypertelorism	O^{HPO}	-
Hypertelorism	VF^{HPO}	-	+	+	Other minor abnormalities of the eyes	variable	-
Other minor abnormalities of the eyes	variable	+	-	-	Eye convergence difficulties	11%[a]	-
Eye convergence difficulties	20%[a]	+	-	-	Broad forehead	F^{HPO}	-
Microtia	VF^{HPO}	-	-	-	Malar flattening	F^{HPO}	-
Other minor abnormalities of the ears	variable	+	+	+	Other facial minor anomalies	variable	-
Thin upper lip vermilion	VF^{HPO}	-	-	-			
Other facial minor anomalies	variable	+	+	+			
Skeletal abnormalities							
Scoliosis	O^{HPO}	-	-	-	Scoliosis	O^{HPO}	-

Arachnodactyly	VF[HPO]	-	-	-	Hand polydactyly	O[HPO]	-
Other abnormalities of the fingers and/or toes	variable	+	+	-	Other abnormalities of the fingers and/or toes	variable	+
Neurology and Behavior							
Intellectual disability	30.5%[b] / 40%[c]	+	+	+	Intellectual disability	10%[d]	+
Motor Delay	VF[HPO]	+	+	+	Global Developmental Delay	VF[HPO]	+
Speech Delay	32%[c]	+	+	+			+
Speech articulation difficulties	30%[a] / 19%[c]	-	n.a.	n.a.	Speech articulation difficulties	79%[a]	-
					Phonological Processing Disorder	56%[d]	-
Developmental Coordination Disorder	47%[c]	-	-	-	Developmental Coordination Disorder	58%[d]	-
Abnormal agility	25%[a]	-	-	-	Abnormal agility	47%[a]	-
Muscular hypotonia	~40%[a]	+	+	+	Muscular hypotonia	~50%[a]	-
Muscle weakness	23-32%[a]	+	-	-	Muscle weakness	17-22%[a]	-
Tremor	18-43%[a]	-	-	-	Tremor	5-8%[a]	-
Hyperreflexia	32%[a]	-	-	-	Hyperreflexia	13%[a]	-
Hyporeflexia	31%[a]	-	-	-	Hyporeflexia	48%[a]	-
Seizures/Epilepsy	26-29%[a]	-	-	+	Seizures/Epilepsy	22-27%[a]	-
Abnormal EEG and Neuroimaging (CT/MRI)	40/31/55%[a]	+	n.a.	n.a.	Abnormal EEG and Neuroimaging (CT/MRI)	54/28/26%[a]	-
Reduced brain volume		+	n.a.	n.a.	Overgrowth in posterior fossa		-
Thinner corpora callosa		-	n.a.	n.a.	Thicker corpora callosa		-
Ventriculomegaly		+	n.a.	n.a.	Chiari I malformation		-
Attention Deficit Hyperactivity Disorder	24%[c]	-	-	-	Attention Deficit Hyperactivity Disorder	19%[d]	-
Autism Spectrum disorder	19%[c]	-	+	+	Autism Spectrum disorder	26%d	-

Anxiety Disorder	18%[c]	-	-	-	Anxiety Disorder	6%d	-
Mood Disorder	O[HPO]	-	-	-	Mood Disorder		+
Additional features							
Café au lait macules	31%[a]	-	-	-	Café au lait macules	30%[a]	-
Sacral dimple	28%[a]	-	-	-	Sacral dimple	34%[a]	-
Congenital diaphragmatic hernia	O[HPO]	-	-	-	Congenital diaphragmatic hernia	O[HPO]	-
Heart defects	infrequent	-	-	-	Heart defects	O[HPO]	-
Developmental differences of the urinary tract	infrequent	-	-	-	Developmental differences of the urinary tract		+
Abnormalities of the digestive system	infrequent	+	-	-	Abnormalities of the digestive system	O[HPO]	-

5.4. Non-recurrent CNVs - Microdeletions of 14q11.2 involving SUPT16H and CHD8 genes

Three patients were identified as carriers of an overlapping microdeletion on the long arm of chromosome 14. Microdeletions of 14q11.2 have been associated with NDDs since the first report of three children with similar NDD phenotypes and minor anomalies in 2007 (Zahir et al., 2007). The three patients carried deletions of varying sizes, but the authors defined a minimal critical region (MCR) of 35 Kb encompassing two genes: *SUPT16H* (OMIM*605012) and *CHD8* (OMIM*610528). Since then, several other cases with deletions containing the MCR have been reported in the scientific literature and online databases (Drabova et al., 2015; Paderova et al., 2018; Prontera et al., 2014; Thygesen et al., 2018). The established clinical picture includes ID/DD, ASD, other neuropsychologic disorders, macrocephaly and characteristic facial features (hypertelorism, down-slanting palpebral fissures, broad nose, long philtrum, prominent Cupid's bow and abnormalities of pinna morphology) (Yasin et al., 2019).

CHD8 belongs to the chromodomain-helicase-DNA binding family, the encoded protein interacts with the chromatin insulator-binding protein CTCF and is involved in transcription, epigenetic processes, promotion of cell proliferation and regulation of RNA synthesis (Ishihara et al., 2006). Furthermore, it acts as a negative regulator of the Wnt signaling pathway by regulating beta-catenin (*CTNNB1*) activity (Caracci et al., 2021). SNVs in *CHD8* have been identified as a cause of ASD in 0.4% of cases in a study of 3730 children (Bernier et al., 2014), establishing the gene as one of the most common genetic drivers of the disorder. Furthermore, there seems to be a specific "ASD subtype" consisting of macrocephaly, facial minor anomalies and gastrointestinal (GI) problems (Bernier et al., 2014), which aligns with data from animal studies (knockout mice show NDD phenotypes, and have increased brain volume and GI defects (Katayama et al., 2016). Deletions and duplications encompassing *CHD8* have also been identified in adults with ID and comorbid psychiatric disorders - schizophrenia and bipolar disorder for the duplication carriers, and ASD for the deletion carrier (Thygesen et al., 2018). Recent data also suggests that *CHD8* is a principal causative gene in patients with overgrowth and NDDs (Tatton-Brown et al., 2017; Yasin et al., 2019), and a prognostic indicator for certain cancers (Yasin et al., 2019).

SUPT16H encodes the 140 kDa subunit of the FACT (facilitates chromatin transcription) complex, which interacts with histones to enable nucleosome disassembly and transcription elongation (Belotserkovskaya et al., 2003). Until recently, *SUPT16H* was considered a modifying factor in 14q11.2 microdeletions, as no reports of its individual role in disease have been available. Then, in 2020, a new study emerged detailing five individuals with de novo variants in *SUPT16H* (four missense variants and one large CNV) associated with DD/ID, ASD, seizures, precocious puberty, craniofacial minor anomalies, and corpus callosum abnormalities, but not macrocephaly (Bina et al., 2020). The authors concluded that variations of the gene are likely enough to cause abnormalities of the corpus callosum, and their observations likewise strengthen the role of *CHD8* in macrocephaly.

We compared our patients' phenotypes (detailed in Table 7) to those accessible from the literature. Table 13 and Figure 5 compile 12 patients carrying overlapping deletions up to approximately 1 Mb, including the three current patients [SEG2_49 (DECIPHER ID: 517827), SEG2_68 (DECIPHER ID: 517850), SEG2_89 (DECIPHER ID: 517859)], three patients from DECIPHER database (D976 is patient Vancouver 5566 from Zahir et al's original 2007 report), four further patients from the literature (Drabova et al., 2015; Paderova et al., 2018; Prontera et al., 2014; Thygesen et al., 2018), and two entries from other databases (detailed clinical features are not available for the latter two patients).

Table 13. Phenotypic overview of 14q11.2 microdeletion patients

Bold: most common features according to scientific literature; D: DECIPHER; STPC: single transverse palmar crease

Phenotype \ Patient	SEG2_49	SEG2_68	SEG2_89	D976	D126	D383504	V4444	Prontera	Drabova	KLS4	All	nssv577456	nssv577457	All	%
ID/DD	+	+	+		+		+	+	+	+	**8/10**	+	+	**10/12**	**83**
Autism/ASD					+	+	+	+	+	+	**6/10**			**6/12**	**50**
Abnormal pinna morphology	+	+		+				+		+	5/10			5/12	42
Macrocephaly				+			+	+	+		**4/10**			**4/12**	**33**
Microcephaly	+										1/10			1/12	8
Muscular hypotonia	+			+	+			+			4/10			4/12	
Brachydactyly	+		+				+			+	4/10			4/12	33
High palate			+					+	+	+	4/10			4/12	
Micrognathia	+			+				+			3/10			3/12	
Syndactyly	+			+			+				3/10			3/12	
Abnormal repetitive mannerisms			+				+	+			3/10			3/12	25
Prominent Cupid’s bow				+					+	+	3/10			3/12	
Irregularity of dentition								+	+	+	3/10			3/12	

Growth delay/short stature		+	+								2/10			2/12	
Complicated perinatal adaptation			+						+		2/10			2/12	
Muscular hypertonia	+	+									2/10			2/12	
Ventriculomegaly	+		+								2/10			2/12	
Strabismus	+				+						2/10			2/12	
Deep-set eyes	+		+								2/10			2/12	
Hypertelorism									+	+	2/10			2/12	
Abnormality of refraction			+						+		2/10			2/12	
Broad forehead						+				+	2/10			2/12	
Prominent forehead							+	+			2/10			2/12	17
Large ears									+	+	2/10			2/12	
Depressed nasal bridge	+			+							2/10			2/12	
Short nose				+					+		2/10			2/12	
Short and hypoplastic philtrum			+							+	2/10			2/12	
STPC	+		+								2/10			2/12	
Prominent finger pads								+		+	2/10			2/12	
Clinodactyly								+		+	2/10			2/12	
Pes planus				+					+		2/10			2/12	
Hirsutism/hypertrichosis		+	+								2/10			2/12	
Umbilical hernia			+						+		2/10			2/12	

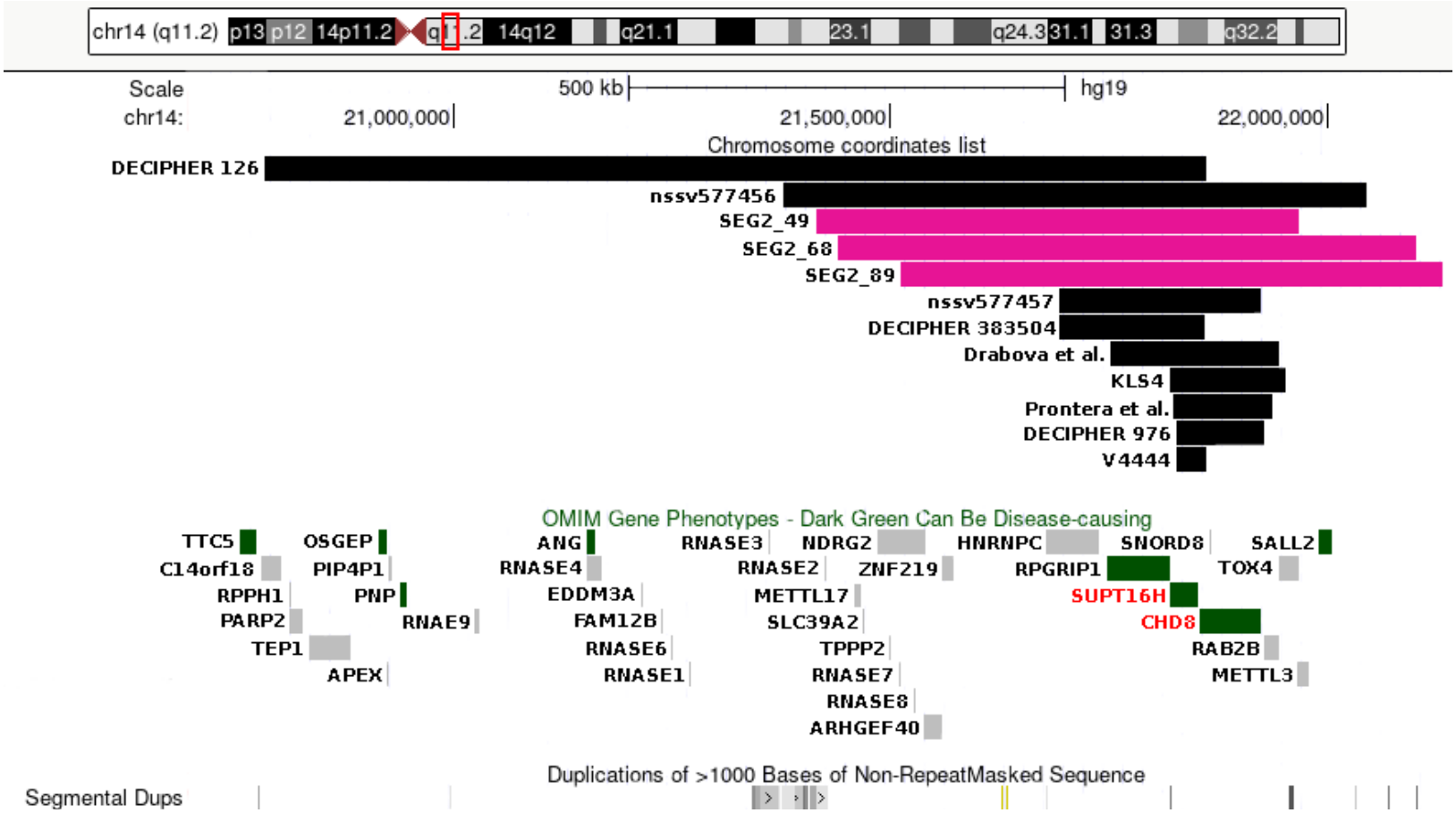

Figure 5. Microdeletions of chromosome 14q11.2

Pink bars denote the presented patients, black bars represent patients from the literature/databases.

In alignment with current knowledge, ID/DD and ASD were the most common features reported (83% and 50%, respectively). Most frequent associated features were abnormal pinna morphology, muscular hypotonia, brachydactyly, high palate (noted in four patients each), and micrognathia, syndactyly, abnormal repetitive mannerisms, prominent Cupid's bow and irregular dentition (three patients each). Including only the 10 individuals with detailed phenotypes, 40% were reported to have macrocephaly. Notably, neither of our patients had increased HC; on the contrary, SEG2_49 had microcephaly (with generalized growth delay). This is presumably due to other, as of yet unknown genetic factors counteracting the effect of *CHD8*. Taken together with the other two children with normal HC, these results highlight the fact that macrocephaly is not an obligatory symptom in SUPT16H-CHD8 microdeletions. Our patients enable further phenotypic expansion: growth delay/short stature, muscular hypertonia/spasticity, ventriculomegaly and hirsutism/hypertrichosis, deep-set eyes and STPC are all novel associated features noted in 2/3 patients in the current study (Lengyel et al., 2022).

Given that to date only ~10 patients have been reported with <1 Mb microdeletions in this region, and that this number does not rise significantly if larger (1-5 Mb) microdeletions are considered, it is quite surprising that three carriers were identified in our small cohort (two were of Arabic descent, while the third was Central European).

5.5. CMA results in patients with phenotypic features reminiscent of Silver-Russell syndrome

Silver–Russell syndrome (SRS) is an imprinting disorder that causes pre- and postnatal growth failure. Other common features include significant feeding difficulties (often requiring tube feeding), hypoglycaemia, body asymmetry, scoliosis, and NDDs. SRS patients are eligible for growth hormone replacement therapy, which can improve height, body composition, and motor development; but can lead to premature adrenarche, precocious central puberty and insulin resistance (Wakeling et al., 2017). SRS is primarily a clinical diagnosis: the Netchine-Harbinson Clinical Scoring System [NH-CSS, (Azzi et al., 2015)] is the current gold-standard score used to obtain a clinical diagnosis of SRS. The NH-CSS includes six criteria, four of which are objective:

- small for gestational age (*birth weight and/or birth length z-score ≤ -2 for gestational age*)
- postnatal growth delay (*height z-score at 24 ± 1 months ≤−2 or height z-score ≤−2 below mid-parental target height*)
- relative macrocephaly at birth (*HC z-score at birth ≥1.5 above birth weight and/or length z-score*)
- body asymmetry [*leg length discrepancy of ≥0.5 cm or arm asymmetry or leg length discrepancy <0.5 cm with at least two other asymmetrical body parts (one non-face)*].

The other two criteria are more subjective, but the scoring system includes clear clinical definitions:

- protruding forehead (*forehead projecting beyond the facial plane on a side view between ages 1-3 years*)
- feeding difficulties and/or low BMI *(BMI z-score ≤−2 at 24 months or current use of a feeding tube or cyproheptadine for appetite stimulation*)

The clinical diagnosis of SRS can be made if a patient reaches four points on the scoring system, and molecular diagnostic testing should be considered above three points. The NH-CSS is very sensitive (98%) and has a high negative predictive value (89%) (Wakeling et al., 2017). Relative macrocepahly and protruding forehead are the most reliable differentiating features from non-syndromic SGA.

Molecular testing enables confirmation of the clinical diagnosis in approximately 60% of cases and defines the subtype (Wakeling et al., 2017). The classical form of SRS is caused by variations at the telomeric end of the short arm of chromosome 11, i.e. hypomethylation of the H19/IGF2 intergenic differentially methylated region (IG-DMR) that results in reduced paternal *IGF2* expression and increased maternal *H19* expression, or various CNVs affecting 11p15.5 region (Wakeling et al., 2017). Another relatively common (affecting less than 10% of SRS patients) molecular subtype is the maternal UPD of chromosome 7 [upd(7)mat]. In these cases the SRS phenotype is thought to result from the altered expression of an imprinted growth-regulatory gene. Candidate genes currently include *GRB10* (7p12.1) and *MEST* (7q32) (Wakeling et al.,

2017). Methylation anomalies and copy number changes involving chromosomes 11 and 7 can be tested simultaneously with MS-MLPA.

Less common molecular defects that have been identified in patients with a clinical SRS diagnosis, or should be considered in the differential diagnosis include upd(20)mat, upd(16)mat, pathogenic SNVs in *CDKN1C* or *IGF2* genes, Temple syndrome, and several pathogenic CNVs described in the literature (Baba et al., 2022; Lengyel et al., 2021a; Wakeling et al., 2017).

5.5.1. Temple syndrome

Temple syndrome (OMIM #616222) is a rare imprinting disorder caused by dysregulation of chromosome region 14q32 harbouring paternally expressed *DLK1* and *RTL1*, and maternally expressed *MEG3*, *RTL1as*, *MEG8*, a sno- and a microRNA gene cluster. The imprinting control regions include two DMRs that regulate parent-of-origin specific expression, both of which are methylated on the paternal allele. A third DMR was identified later, and is methylated on the maternal allele (Gillessen-Kaesbach et al., 2018). Temple syndrome is most commonly caused by upd(14)mat, but is occasionally due to paternal deletions or rare imprinting defects. Upd(14)mat can be associated with robertsonian translocations invloving chromosome 14. Interestingly, Temple syndrome is often characterized as both an SRS-like and a PWS-like syndrome. Features overlapping SRS are SGA, relative macrocephaly and hypoglycaemia, features overlapping PWS are small hands and feet, obesity and polyphagia, while features shared by all tree syndromes include short stature, growth failure in early childhood, feeding difficulties in infancy, muscular hypotonia, DD and scoliosis. Temple syndrome patients can face additional health problems such as precocious puberty (irrespective of growth hormone supplementation), early type 2 diabetes mellitus or maturity-onset diabetes of the young (MODY), and hypercholesterinaemia. Cognitive deficit is usually mild or can manifest in learning difficulties only (Brightman et al., 2018; Gillessen-Kaesbach et al., 2018; Ioannides et al., 2014; Kagami et al., 2017).

In the discussed patient cohort, one boy was diagnosed with Temple syndrome. Patient SEG2_62 (DECIPHER ID: 517835, Tables 6 and 7) presented with birth weight at the 3rd percentile for gestational age, postanatal somatic and psychomotor DD, relative macrocephaly, high and prominent forehead, generalized muscular hypotonia

and myopathic features on electromyography. Brain MRI revealed a partial empty sella and an abnormally thin pituitary gland stalk. Additional symptoms included delayed bone age, micropenis and unilateral retractile testis. He had episodes of hypoglycaemia and was diagnosed with growth hormone deficiency. His NH-CSS was 4/6 points. Due to his complex phenotype CMA testing was performed initially, which identified a deletion of chromosome X (6537108_8167604 Mb; GRCh37) encompassing the STS gene (OMIM* 300747). After a dermatology consult revealed generalized dry skin, discrete white scaling on the extensor surfaces and ichtyiotic lesions on the ears and the scalp, the boy was diagnosed with a mild form of X-linked ichthyosis (OMIM #308100). An additional finding on the SNP array was a large LOH on chromosome 14 (20052038_106871264 Mb; GRCh37). Follow-up MLPA proved the presence of maternal UPD 14. The child's primary diagnosis is thus Temple syndrome.

5.5.2. Pathogenic CNVs

Two further patients with SRS-like phenotypes received a definitive diagnosis owing to CMA: one child (SEG2_37, reported in Lengyel et al., 2021a) who was included in the original study cohort, and an additional patient who was tested in the year 2021 after the original study had been closed (reported in Baba et al., 2022).

SEG2_37 (DECIPHER ID: 414204) is a young boy who presented with global DD, significant growth delay, relative macrocephaly, a protruding forehead, triangular face, joint hypermobility, and feeding difficulties. Brain MRI revealed mild ventriculomegaly and focal hyperintensities in the frontal lobe. His NH-CSS was 4/6 points (Table 7, for more details see Lengyel et al., 2021a). His phenotype prompted testing for SRS, but MS-MLPA excluded molecular abnormalities of 11p15, and UPD affecting chromosomes 7 and 14. CMA subsequently identified a 10 Mb large deletion of the long arm of chromosome 16: arr[GRCh37]16q22.2q23.3(72155844_82148404)x1 (Tables 6 and 14). Partial 16q deletions have surprising phenotypic overlap despite variability in breakpoint and size. Commonly reported features are SGA, feeding difficulties, failure to thrive, short stature, NDDs, large anterior fontanel and/or delayed closure of the cranial sutures, frontal bossing, low-set ears with morphological abnormalities, narrow thorax, wide intermamillary distance, and minor anomalies of the hands and feet (Lengyel et al., 2021a). In the previous case study this child's phenotype

was compared to 18 patients from international databases who had deletions with at least 50% overlap (see Table 1 in Lengyel et al., 2021a). The most common feature was ID/DD, followed by growth abnormalities and minor anomalies of the forehead. Additional symptoms shared by multiple individuals were muscular hypotonia, abnormalities of the pinna, wide anterior fontanel and/or delayed closure of the fontanel, high anterior hairline, microcephaly, and CHD (Lengyel et al., 2021a). Two patients had cataracts; both with deletions encompassing *MAF* gene, one of the 16 disease-associated genes within SEG2_37's deleted 16q region. *MAF* (*177075) is involved in the differentiation of T-cells and chondrocytes and in the development of the eye. Pathogenic variation is associated with AD disorders cataract 21 (#601202) and Aymé-Gripp syndrome (#601088). Main features of the latter syndrome include congenital cataracts, sensorineural hearing loss, NDDs, short stature, brachycephaly and facial minor anomalies (Amudhavalli et al., 2018; Niceta et al., 2015). Aymé-Gripp syndrome and previously reported 16q microdeletion cases have overlapping phenotypes, highlighting *MAF* as an inmportant candidate gene (Lengyel et al., 2021a). SEG2_37, and indeed various 16q microdeletions also show significant overlap with SRS. The presented patient's genetic and phenotypic data corroborates the differential diagnostic importance of 16q22.2q23.3 deletions.

Patient 2021.1 (Baba et al., 2022) was born after a pregnancy complicated by IUGR, urgent C-section was performed due to abnormal flowmetry at 30 weeks of gestation. Apgar scores at 1 and 5 minutes were 8 and 9, respectively. Birth weight, height and HC were reduced [990 g (z-score -1.79), 37 cm (z-score -1.39), 27 cm (z-score -0.23)]. The child had complicated perinatal adaptation and newborn period (he required intensive care due to sepsis). Hearing, vision and psychomotor development were normal. At the age of 1 and a half years height was 80 cm (z-score -1.45), weight was 7.1 kg (z-score -3.87) and HC was 44 cm (z-score -4.06). The patient exhibited a triangular face and a protruding forehead. He developed feeding difficulties requiring a nasogastric tube for two years. By applying the NH-CSS, a score of 3/6 was obtained (he had relative macrocephaly at birth – HC z-score is 1.56 higher than birth weight z-score – but later relative microcephaly developed, which is uncharacteristic of SRS). Molecular testing for the SRS typical alterations by MS-MLPA was negative. CMA revealed a 77 Kb deletion affecting the *PLAG1* and the *CHCHD7* genes:

arr[GRCh37]8q12.1(57079399_57155945)x1; *de-novo* occurrence was proven by quantitative PCR of the parental DNA samples (Baba et al., 2022).

Relevant data regarding the two discussed patients are summarized in Table 14.

Table 14. Patients with SRS-like phenotypes and their causative CNVs

SGA: small for gestational age, IUGR: intrauterine growth retardation, NH-CSS: Netchine-Harbinson Clinical Scoring System, JH: joint hypermobility, CALM: café au lait macule

	SEG2_37	**Patient 2021.1**
CNV	arr[GRCh37]16q22.2q23.3(72155844_82148404)x1	arr[GRCh37]8q12.1(57079399_57155945)x1
Size	9.992 Mb	77 Kb
Candidate gene(s)	*WWOX, MAF*	*PLAG1*
SGA	no	no (*but IUGR*)
Relative macrocephaly at birth	yes	yes (*later microcephaly*)
Postnatal growth delay	yes	no
Feeding difficulties	yes	yes
Protruding forehead	yes	yes
Body asymmetry	no	no
NH-CSS	**4/6**	**3/6**
DD/ID	yes	no
Other	triangular face, JH, CALMs, delayed eruption of teeth, ear abnormality	triangular face, complicated perinatal adaptation

PLAG1 (*603026) has been implicated in SRS (coined SRS4, #618907) recently through both pathogenic SNVs (Abi Habib et al., 2018; Inoue et al., 2020; Meyer et al., 2021) and CNVs (Brereton et al., 2021; Fernández-Fructuoso et al., 2021). *PLAG1* is widely expressed in fetal development and regulates several growth factors, including *IGF2* (Karim et al., 2011). As mentioned above, the 11p15 form of SRS is related to *IGF2*, which is important in the growth and proliferation of cells (Brereton et al., 2021).

PLAG1 haploinsufficiency decreases *IGF2* expression (Fernández-Fructuoso et al., 2021). Fernandez-Fructuoso et al. reported a patient with a 2.1 Mb large deletion of chromosome 8q12.1 who achieved 4/6 points on the NH-CSS (Fernández-Fructuoso et al., 2021). The same year, Brereton at al. reported another pair of brothers with large (2.9 Mb) maternally inherited 8q12.1 deletions. Both had an NH-CSS score of 4/6 as well, additional features included triangular face and clinodactyly. The younger brother had a more severe phenotype including DD. He was shown to have a complex chromosomal rearrangement (CCR) associated with two additional deletions of chromosomes 22q11.2 and 8p23.3p23.1, which can likely explain his further developmental problems. Their mother was subsequently shown to have a balanced CCR (Brereton et al., 2021). DECIPHER database lists five deletion cases (filtered <5 Mb), neither patient has any additional variants. Four of the entries include phenotypic data, albeit scarce; two patients (IDs 350153 and 340694) have growth abnormalities (short stature and IUGR, respectively). There are too few patients for statistically significant genotype-phenotype correlation, but in contrast to the epimutation forms of the disease *PLAG1* variants seem not to be associated with body asymmetry (Brereton et al., 2021).

In comparison to the currently reported cases carrying *PLAG1* deletions, the presented patient 2021.1 exhibited the smallest deletion (77 Kb vs. the smallest entry in DECIPHER is 230 Kb). This finding unequivocally confirms that *PLAG1* is the SRS causing gene in 8q12.1 (Baba et al., 2022).

5.6. Variants of unknown significance

To this day, classification of CNVs is often confounded by lack of sufficient evidence, and is dynamic as the accumulation of data, case reports, functional analyses, etc. can lead to reclassification of VUS. Improved genetic counselling and optimal patient care necessitates further studies to resolve the ambiguity of the not insignificant number of uncertain results. Below a selection of VUS from our patient cohort are discussed.

5.6.1. Patients with a single potentially pathogenic VUS

Patient SEG2_71 (DECIPHER ID: 517871) is a girl with a complex phenotype including global DD, IUGR, short stature, craniofacial and skeletal minor anomalies,

vertebral anomalies, generalized hypotonia, incoordination, speech articulation difficulties, bilateral sensorineural hearing impairment, refraction error, CHD, gallbladder cysts, ectopic kidney and bilateral inguinal hernia (Table 10). After normal karyotyping CMA revealed a 2.6 Kb large deletion (Table 9) encompassing *RAC3* gene (OMIM *602050), encoding a GTPase of the RAS protein RHO subfamily (de Curtis, 2019). DECIPHER does not list any functionally similar CNVs, and only one sequence variant within *RAC3* (ID 287569). This patient had severe feeding difficulties in infancy and failure to thrive, GERD, visual impairment, inguinal hernia, central hypotonia, agenesis of the corpus callosum, severe GDD and minor anomalies. A handful of patients with heterozygous pathogenic *RAC3* variants have recently been reported in the scientific literature (Costain et al., 2019; Hiraide et al., 2019; White et al., 2018). Main phenotypic features of the associated NDD [neurodevelopmental disorder with structural brain anomalies and dysmorphic facies, (#618577)] include moderate to severe DD/ID, abnormal muscle tone, brain imaging abnormalities (corpus callosum anomalies, ventriculomegaly, polymicrogyria and heterotopia were reported in more than one patient), non-specific craniofacial minor anomalies, feeding difficulties, and less frequently, seizures, urogenital, respiratory and endocrinological abnormalities (Hiraide et al., 2019; Scala et al., 2021). Current evidence suggests that this novel disorder arises due to constitutive activation of the Rho GTPase due to gain-of-function gene defects. Animal studies corroborate this, as the full deletion of the gene does not impede fertility, viability or brain organization of knockdown mice. The mice do show behavioral abnormalities, however (de Curtis, 2019). Overall, the proposed disease mechanism does not strongly implicate the presented patient's VUS deletion as potentially disease-causing. Nonetheless, considering that both *RAC3* and the highly homologous *RAC1*, and several other members of the RAC signalling pathways are associated with NDDs (Scala et al., 2021), that the RAC GTPases are important regulators of neuronal morphogenesis and synaptic plasticity, and that *RAC3* is highly expressed in the developing nervous system, we would like to draw attention to the presented patient's deletion as a possible disease modifier and/or NDD risk factor.

Patient SEG2_81 (DECIPHER ID: 517874) has a 154 Kb large chromosome 20 duplication (arr[GRCh37]20p11.21(24554628_24708699)x3) encompassing *SYNDIG1* (OMIM*614311) gene (Table 9). WES came back negative. The 5-year-old boy

presented with moderate global developmental delay. His speech delay is particularly severe as he has no spoken words; receptive speech is also impaired, albeit to a lesser degree than expressive (Table 10). He was initially diagnosed with ASD, but this diagnosis was later revised due to the complete failure of autism-specific developmental therapies - which led to persistent resistance from the parents - and the overall longitudinal clinical picture. He is currently under the care of an expert neuropsychologist; extensive medical evaluations have unfortunately been repeatedly delayed, partly due to non-compliance. Preliminary neuropsychiatric opinion states that the patient has a complex NDD affecting the quality of many neurodevelopmental domains, including social interactions, behavior, and processing functions. A previous brain MRI (performed in 2017) was normal except for mild atrophy of the posterior third of the truncus corporis callosi. He has no other major or minor congenital anomalies. Patient SEG2_81's 20p11.21 duplication was inherited from the father (Table 3). The encompassed gene, *SYNDIG1*, is a brain-specific transmembrane protein proven to be a regulator of excitatory synapse development in rat brain (Díaz, 2012). Further study has shown that *SYNDIG1*-deficient excitatory synapses have impaired structure and function, thus suggesting an important role in normal synapse development (Chenaux et al., 2016). No comparable duplications of *SYNDIG1* have been reported in the literature or online databases. In the presented case, paternal inheritance further complicates classification. Nevertheless, the currently suspected pathogenesis of patient SEG2_81's phenotype implicates faulty synaptic development (Lengyel et al., 2022).

5.6.2. Patients with more than one VUS – second-hit theory

Patient SEG2_57 (DECIPHER ID: 517870) is a young boy with severe DD/ID, behavioral stereotypies and multiple congenital anomalies (bilateral talipes equinovarus, bilateral complete syndactyly of the fingers and the toes, nail dystrophy, macrocephaly and postnatal overgrowth, craniofacial minor anomalies, spasticity, mild congenital heart disease, and strabismus; Figure 6 and Table 10). The etiology of the child's phenotype is complicated by two factors: there are contradictory reports regarding the possibility of perinatal hypoxia; and four days after a syndactyly correcting operation, he presented sudden loss of vision, multiple symptomatic focal epileptic attacks and diffuse hypoxic-ischaemic encephalopathy with cytotoxic cerebral oedema. This

happened unexpectedly, and no cause has been identified to date (genetic variants causing malignant hyperthermia were ruled out from a blood sample, but were not tested from muscle biopsy). There were no further seizures from this point onward, and treatment (carbamazepine) was discontinued after one year.

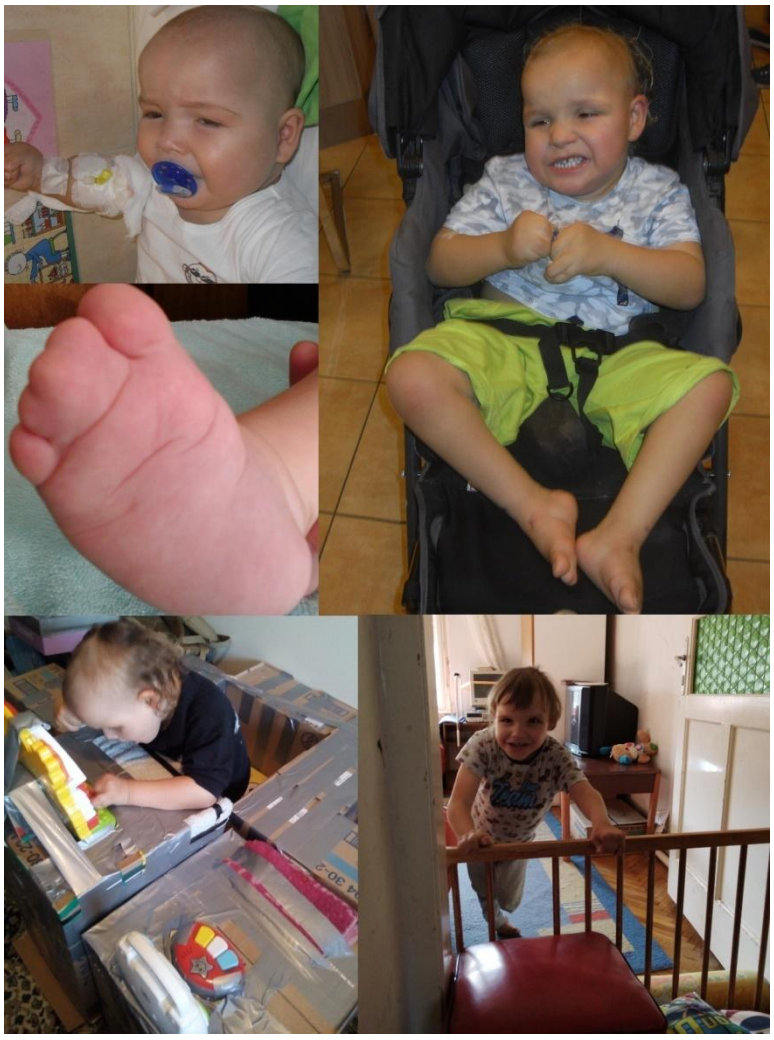

Figure 6. Phenotype of patient SEG2_57

Close-up of one of the child's feet (syndactyly and pes equinovarus). Top right: age 3 years. At this age he was unable to sit without assistance. He learned to sit and hold himself up while leaning on something at age 5 years (bottom left), and to pull himself to a standing position at age 7 years (bottom right). Identifiable photographs are published with the written consent of the parents.

Pediatric neurologists retrospectively identified very mild cerebral atrophy when re-evaluating the patient's pre-surgery brain MRI. At the 6 month follow-up, MRI revealed

severe cortical necrosis as sequelae to the encephalopathy, as well as diffuse mild cerebral atrophy. The boy has consistently slightly elevated serum lactate levels. His karyotype was determined to be 46,XY; pathogenic *GLI3* (OMIM *1654240) variants causing Greig cephalopolysyndactyly (OMIM #175700) were ruled out at an early stage of genetic investigations; WES was preformed later and identified no pathogenic variants or VUS that might explain the phenotype. Mitochondrial genome analysis performed on peripheral blood was negative. CMA analysis identified three VUS (Table 9), one of which encompasses an NDD-associated disease-causing gene. His 384 Kb large chromosome 4 duplication (arr[GRCh37]4q24(102058416_102443207)x3) contains *PPP3CA* (OMIM *114105) gene. The encoded calcineurin A protein (the catalytic subunit of calcineurin, which is responsible for calmodulin-calcineurin interactions) has an important role in synaptic vesicle recycling through regulation of response to calcium levels (Myers et al., 2017); and is associated with epilepsy and other NDDs. Loss-of-function pathogenic variants cause autosomal dominant developmental and epileptic encepalopathy 91 (OMIM#617711). This disorder is characterized by early-onset epilepsy (severity of which is variable and may be correlated with the specific variant), moderate/severe DD/ID, developmental regression, autistic behavior, generalized muscular hypotonia or spasticity, talipes equinovarus, feeding difficulties, cortical vision loss, cerebral atrophy and delayed myelination (Panneerselvam et al., 2021; Rydzanicz et al., 2019; Yang et al., 2020). Gain-of-function pathogenic variants lead to a syndrome characterized by arthrogryposis, cleft palate, craniosynostosis and ID (OMIM #618265). Postnatal growth delay, plagio- or trigonocephaly, vesicoureteral reflux, gracile bones, brachydactyly, generalized seizures, behavioral stereotypies may also be present (Mizuguchi et al., 2018). The other two VUS of this patient are a 504 Kb 20p11.23 duplication (arr[GRCh37]20p11.23(19240620_19745197)x3), encompassing *SLC24A3* gene (OMIM *609839), important for calcium homeostasis (Kraev et al., 2001); and an 81 Kb Xq25 deletion (arr[GRCh37]Xq25(124088718_124169834)x0), containing part of *TENM1* gene (OMIM *300588), relevant in synaptic organization (Mosca et al., 2012). The phenotype of the presented SEG2_57 patient greatly overlaps with the disorders associated with *PPP3CA* (severe DD/ID, talipes equinovarus, cerebral atrophy, vision loss, and arthrogryposis, abnormal cranial morphology, behavioral stereotypies). The

duplication of this gene plausibly contributed to the patient's complex disorder, genotype-phenotype correlation is however greatly confounded by the presence of multiple uncertain environmental factors. The syndromic origins of the child's seizures and vision loss are questionable, but they might be attributable to decompensation of an existing genetic disorder (Lengyel et al., 2022). The complete syndactyly affecting all four extremities remains unexplained, which seems counter-intuitive at first glance, however, approximately 60% of syndactyly cases are sporadic, and hereditary cases are often isolated and multifactorial (Jordan et al., 2012).

Patient SEG2_77 (DECIPHER ID: 411577) is a boy with speech articulation disorder, ASD, ADHD, learning difficulties (due to behavioral problems, not cognitive disability), sensorineural hearing impairment, tracheal and anal stenosis, scoliosis and minor anomalies (Table 10). He carries two VUS, an Xq28 deletion encompassing genes associated with colorblindness, and a 16p11.2 deletion (Table 9 and Figure 3C). The affected 16p region contains one OMIM gene, *TP53TG3* (*617482), which is unlikely to be haploinsufficient according to the DECIPHER score (%HI=94.39), and is not associated with any known disease or phenotype. A nearly identical deletion is a DGV Gold Standard Variant (gssvL43686), with an overall frequency of 1.35%. I have found two similar deletions in patients from international databases (excluding those that also encompass one or both of the pathogenic recurrent 16p11.2 regions). ClinVar database records a VUS (nssv581526), which is nearly identical to SEG2_77's deletion with microcephaly and global DD listed in the phenotypic information. DECIPHER contains one deletion classified as likely pathogenic (ID 328130; chr16:30361048_33660219, GRCh37). The reported features include delayed speech and language development, global brain atrophy, global DD, ID, gait disturbance, tremor and seizures. This patient has no other CNV; however, the gene content in their 16p11.2 deletion is much higher compared to SEG2_77's, and includes multiple genes linked to NDDs and/or neurological disorders [e.g. *FUS* (OMIM *137070) and *STX1B* (OMIM *601485)]. Although these two patients from the databases are not directly comparable to SEG2_77, they each presented with various NDDs, and speech development was affected in all three children. This could suggest that the disturbance of more proximal 16p11.2 regions might compromise normal neurodevelopment, especially in regards to speech and language, possibly due to altered chromatin

interactions (Lengyel et al., 2020). Analysis with SVInterpreter strengthens this supposition – SEG2_77's deletion disrupts several chromatin loops and clustered interactions. Nearby genes *SETD1A* (*611052) and *KAT8* (*609912) are both associated with NDDs. The former encodes a component of the histone methyltransferase complex, and is associated with epilepsy, early-onset, with or without developmental delay (#618832) and neurodevelopmental disorder with speech impairment and dysmorphic facies (#619056), both inherited in an AD manner. The latter gene encodes a lysine acetyltransferase and is important in genome-wide epigenetic regulation. The associated AD disorder, Li-Ghorgani-Weisz-Hubshman syndrome (#618974) is likewise a recently characterized NDD.

The phenotypes of these patients could be, at least partially, attributed to their co-occuring CNVs, in line with the second hit model (Girirajan et al., 2010; Redaelli et al., 2019, Lengyel et al., 2020, 2022). As mentioned previously, Girirajan et al. observed an eight-fold increased risk of DD in children carrying two large CNVs (Girirajan et al., 2012). It is also worth mentioning, that common genetic variants have been shown to contribute to the risk and variability of severe NDDs in a genome-wide association study (Niemi et al., 2018).

5.7. Limitations and ongoing research objectives

The main limitations of this study are the small sample size, the different CMA platforms utilized (not all patients were tested using SNP arrays, therefore LOHs suggestive of syndromes caused by UPDs could have been missed), and the lack of information regarding inheritance in the majority of patients.

An important next step is the search for causative sequence variants for those patients who obtained uncertain or negative CMA results. This has begun, but is still in its early phase (so far ~30% of patients with VUS and 25% of patients with negative CMA have completed additional genetic testing; one of the VUS carriers and seven of the negative patients subsequently received a definitive diagnosis).

Further research objectives include functional analyses for the potentially pathogenic VUS, and international collaborations in the hopes of identifying therapeutic targets.

6. Conclusions

In this study I have systematically analyzed the CMA results of NDD patients presenting at our Unit's Genetic Counselling out-patient clinic between 2011 and 2020. Through my efforts I have made the following observations and have come to the subsequent conclusions.

1) The scientific community recommends reevaluation of VUS every 2-3 years. I have conducted reevaluation of the CNVs identified in the presented patients according to the most recent ACMG guidelines (Riggs et al., 2020), through rigorous literature and database review and by applying the recently published SVInterpreter online tool (Fino et al., 2021). My work has enabled the reclassification of VUS in eleven patients, five of whom can now be considered carriers of clinically significant variants. Despite these efforts, 24 CNVs stil remain clinically uncertain. Overall 13/78 patients (16.67%) received uncertain CMA results, highlighting the continued need for data sharing.

2) The diagnostic yield in the presented patient cohort was determined to be 37.18% (Lengyel et al., 2022). At first glance this seems high compared to the 15-20% given in the scientific literature. However, these estimates were based on early studies conducted on very large cohorts with broad selection criteria, but with lower resolution array platforms than are available today. In the current study, the number of patients was comparatively small while the selection criteria were rather strict, necessitated by availability constraints and funding considerations. The diagnostic yield of this study is comparable to publications with similar cohorts and higher resolution platforms.

3) Given the above availability and funding difficulties it would seem prudent to delineate phenotypic clues that could help indicate the laborious and costly CMA. Although the small sample size limits statistical analyses, the following clinical features increased the probability of identifying a disease-causing CNV in the presented patients: postnatal growth delay, brain imaging abnormalities, global DD, macrocephaly and pectus excavatum (Lengyel et al., 2022). The first three corroborate literature data. Interestingly, in this cohort macrocephaly - and not microcephaly – emerges as an important phenotypic clue. This is most likely related to the small sample size and possible sampling bias; i.e. patients with obvious microcephaly are often sent to clinical genetics consultations, and are therefore "overrepresented" in the entire cohort,

similarly to ID. This result corroborates the need for CMA in children who have macrocephaly but lack symptoms of a specific overgrowth syndrome or skeletal dysplasia (Kamien et al., 2018).

4) Despite the small sample size, the presented cohort enables discussion of recurrent CNVs on the short arm of chromosome 16 and microdeletions of the long arm of chromosome 14 as well.

a. Chromosome 16p has a high percentage of SDs, predisposing it to NAHR, and therefore recurrent rearrangements. Three notable recurrent CNV regions on 16p are the ~500 Kb large NDD predisposition region on 16p12.2, the distal (BP2-BP3; ~220 Kb, encompassing *SH2B1*) and the proximal (BP4-BP5; ~600 Kb) 16p11.2 regions. A reciprocal duplication to the 16p12.2 recurrent microdeletion has been identified in patient SEG2_32. The duplication has conflicting classifications in the scientific literature and online databases. I argue for its consideration as an NDD risk factor similar to the reciprocal deletion, and present findings regarding SEG2_32 as further corroboration. One patient, SEG2_53 has been identified as a carrier of the distal 16p11.2 deletion, and she presents the expected phenotype. Patient SEG2_45 carries a deletion directly distal to the BP2-BP3 region, further implicating the region as pathogenic, as he lacks the typical phenotype. Significantly, this patient shows that the upstream region does not convey similar phenotypic consequences through position effects. Four of the presented patients have been revealed as carriers of BP4-BP5 16p alterations. Their phenotypes consolidate literature data. Patients SEG2_26 and SEG2_27 inherited their duplication from their mother, who was only mildly effected, if at all. Furthermore, the brothers presented with varying severity, showcasing the interfamilial variable expressivity linked to recurrent rearrangements (Lengyel et al., 2020).

b. Three patients were shown to be carriers of overlapping microdeletions of 14q11.2 encompassing *CHD8* and *SUPT16H* genes. These children allowed delineation of several new phenotypic features associated with the deletion, including growth delay/short stature, muscular hypertonia/spasticity and ventriculomegaly. Importantly, macrocephaly should no longer be considered a cardinal feature of the microdeletion (Lengyel et al., 2022).

5) The analyzed patients also allowed for ascertainment of a phenotypic subgroup, namely patients with characteristics resembling Silver-Russell syndrome. SRS has long been associated with great molecular heterogeneity, which is further corroborated in this study. Temple syndrome, a rare imprinting disorder most commonly caused by maternal UPD of chromosome 14, is one of the most important differential diagnoses of SRS, and is often routinely included in diagnostics. Patient SEG2_62, who achieved 4/6 points on the NH-CSS, has been diagnosed with Temple syndrome. This was possible due to the use of a hybrid array platform, highlighting the usefulness of SNP arrays. The CMA also identified a chromosome X deletion, therefore this child has a dual diagnosis of Temple snydrome and X-linked ichthyosis. Two further patients with SRS-like phenotypes carried pathogenic CNVs. Deletions of the long arm of chromosome 16 show great phenotypic overlap with each other, and SRS as well. The 16q22.2q23.3 deletion in patient SEG2_37 is a novel SRS-like disorder, and *MAF* emerges as an important candidate gene (Lengyel et al., 2021a). Finally, in recent years variants of *PLAG1* gene have been emerging as SRS-disease-causing. The pathogenicity of the gene and 8q12.2 deletions is confirmed through the presented patient 2021.1 (Baba et al., 2022).

6) As mentioned above, a relatively high proportion of VUS have been identified in the presented cohort, classification of which is confounded by lack of scientific knowledge. VUS represent a great difficulty in everyday genetic counselling. To facilitate future reclassification endeavours, I present detailed phenotypic data, and current literature and database information regarding the VUS identified in our patients. Of these, I discuss in detail two novel variants containing genes possibly relevant for the associated phenotypes: SNVs in *PPP3CA* gene have been associated with two complex disorders, both of which show partial overlap with patient SEG2_57's phenotype; and *SYNDIG1*, a gene implicated in faulty synaptic development, duplicated in patient SEG2_81 with a complex NDD (Lengyel et al., 2022). The general applicability of the two-hit model is addressed (Lengyel et al., 2020).

7. Summary

Neurodevelopmental disorders are genetically heterogeneous pediatric conditions. The first tier diagnostic method for uncovering copy number variations (CNVs), one of the most common genetic etiologies in affected individuals, is chromosomal microarray (CMA). This study reports clinical and genetic data of the first, relatively large Hungarian cohort (n=78) of pediatric patients whose genetic testing included CMA.

The diagnostic yield of CMA in this cohort of patients with NDDs and/or congenital anomalies was 37.18%. The high diagnostic rate is partly due to rigorous patient selection criteria necessitated by availability and funding considerations. Several phenotypic features that increased the likelihood of finding a diagnosis in the patient group were identified: postnatal growth delay, brain imaging abnormalities, global developmental delay, macrocephaly, and pectus excavatum.

A subgroup of patients with chromosome 16p rearrangements is discussed. The presented carriers of the distal and proximal recurrent CNVs corroborate literature data. Neighboring breakpoints identified in two patients support the pathogenicity of the distal 16p11.2 microdeletion and *SH2B1* gene. Patients with the rare, non-recurrent 14q11.2 microdeletion allowed delineation of new clinical features. Patients with phenotypes reminiscent of Silver-Russell syndrome corroborate the underlying molecular heterogeneity. Deletion of 16q22.2q23.3 emerges as an SRS-like disorder, and the *PLAG1* locus on chromosome 8q12.2 is confirmed as SRS-disease-causing.

Classification, and therefore genetic counseling of VUS is confounded by the lack of scientific knowledge. To facilitate interpretation efforts, detailed phenotypic information corresponding to all VUS identified in our cohort is provided. Two novel rare variants containing genes (*PPP3CA* and *SYNDIG1*, respectively) possibly relevant for the associated clinical phenotypes are discussed. A proximal 16p11.2 VUS that speaks to the importance of 3D chromatin interactions is presented, and the applicability of the generalized two-hit model is discussed.

8. Összefoglalás

Az idegrendszer fejlődési zavarai (neurodevelopmental disorders; NDD) összetett, heterogén genetikai eredetű gyermekkori kórképek. Az egyik fő kóroki csoportot a kópiaszám eltérések (copy number variations, CNV) képezik; azonosításuk standard diagnosztikai módszere a kromoszómák microarray (CMA) vizsgálata. Kutatásom fő célja egy CMA-val vizsgált NDD betegcsoport (n=78) klinikai és genetikai adatainak szisztematikus elemzése volt. A kohort CMA vizsgálatának nemzetközi vonatkozásban is viszonylag magas diagnosztikus rátája (37.18%) többek közt a szigorú beválogatási feltételeknek tudható be, ami összefüggésben van a vizsgálat hazai elérhetőségével, finanszírozási nehézségével is. A betegek tünettanának statisztikai elemzésével több olyan fenotípus jegyet (születés utáni növekedési elmaradás, agyi képalkotó eljárással azonosított rendellenesség, globális fejlődési elmaradás, macrocephalia, pectus excavatum) állapítottam meg, melyek jelenléte növelte a diagnózis felállításának a valószínűségét. Dolgozatomban részletesen kifejtem a 16-os kromoszóma rövid karjának rekurrens rendellenességeivel összefüggő tünettant. Kiemelendő, hogy két betegben azonosított szomszédos töréspontok alátámasztják a disztális 16p11.2-es régió és a benne található *SH2B1* gén patogenitását. A ritka, egyedi töréspontokkal rendelkező 14q11.2-es deléciót hordozó betegek lehetővé tették a kapcsolódó fenotípus pontosítását. Silver-Russell-szindrómára jellegzetes tünettant mutató gyermekek CMA eredményei megerősítik a szindróma molekuláris heterogenitását: a leírt 16q22.2q23.3 deléció új, “Silver-Russell-like” kórképnek tekinthető, a 8q12.2 deléció genotípus-fenotípus összefüggéseinek vizsgálata bebizonyítja az érintett *PLAG1* gén betegségokozó szerepét Silver-Russell-szindrómában. A bizonytalan klinikai jelentőségű eltérések (variants of unknown significance, VUS) klasszifikációja, és egyúttal genetikai tanácsadása a családok felé, igen nehezített a hiányos evidenciák miatt. Munkámban részletesen bemutatom a betegcsoportban azonosított VUS-okhoz kapcsolódó fenotípusos és tudományos információkat. Kiemelek két ritka variánst, amelyek az érintett gének (*PPP3CA* és *SYNDIG1)* alapján potenciálisan kórokiak. A bemutatott VUS-okon keresztül kirajzolódik a 3D kromatin kölcsönhatások fontossága és a kettős-ütés modell általános alkalmazhatósága.

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10. Author's publications

10.1. Publications related to the dissertation

- **Lengyel A**, Pinti É, Pikó H, Jávorszky E, David D, Tihanyi M, Gönczi É, Kiss E, Tóth Z, Tory K, Fekete G, Haltrich I. (2020) Clinical and genetic findings in Hungarian pediatric patients carrying chromosome 16p copy number variants and a review of the literature. Eur J Med Genet, 63(10): 104027. https://doi.org/10.1016/j.ejmg.2020.104027
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- **Lengyel A**, Pinti É, Eggermann T, Fekete G, Haltrich I. (2021) Deletion of 16q22.2q23.3 in a Boy with a Phenotype Reminiscent of Silver-Russell Syndrome. Mol Syndromol, 12(5): 300-304. http://doi.org/10.1159/000515941
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- Baba N, **Lengyel A**, Pinti E, Yapici E, Schreyer I, Liehr T, Fekete G, Eggermann T. (2022) Microdeletions in 1q21 and 8q12.1 depict two additional molecular subgroups of Silver-Russell syndrome like phenotypes. Mol Cytogenet, 15(1): 19. https://doi.org/10.1186/s13039-022-00596-z
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- **Lengyel A**, Pinti E, Pikó H, Árvai K, Abonyi T, Némethi Z, Fekete G, Haltrich I. (2022) Clinical evaluation of rare copy number variations identified by chromosomal microarray in a Hungarian neurodevelopmental disorder patient cohort. Mol Cytogenet, 15(47). https://doi.org/10.1186/s13039-022-00623-z
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10.2. Other publications

- **Lengyel A**, Pinti É, Bertalan R, Bajnóczky K, Tóth Zs, Kiss E, Fekete Gy, Haltrich I. (2018) Barangolás egy ritka betegség világában: a cri du chat szindróma. Gyermekgyógyászat, 69(2): 113-118.
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11. Acknowledgments

First and foremost, I would like thank my supervisor, Dr. Haltrich Irén, who has been tirelessly mentoring me for nearly a decade. Likewise, Dr. Fekete György, our Professor at the Genetics Division, has been helping me through my clinical practice from the very beginning. Both are true role models, who lead by example, and have shaped me as a professional and as a person.

I would also like to thank my constant colleague, co-author, and dear friend, Dr. Pinti Éva, for always lending me valuable professional insight, as well as a sympathetic ear.

I am grateful for the help, advice and kindness I have received from all my other colleagues at the Genetics Division: Abonyi Tünde, Gönczi Józsefné, Gudlin Gabriella, Kiss Eszter, Dr. Kovács Árpád Ferenc, Dr. Németh Krisztina, Némethi Zaránd, Staub Krisztina, Tóth Zsuzsa and Varga Xénia.

I would also like to acknowledge all my co-authors for their guidance and for their exemplary work both in the clinical and the diagnostic setting. I must highlight Dr. Tory Kálmán and Dr. Jávorszky Eszter, who have been trusted collaborators for many years. Dr. Karcagi Veronika, Dr. Pikó Henriett, Dr. Ujfalusi Anikó and Dr. Árvai Kristóf have greatly facilitated my research, and have bettered the lives of our shared patients. I have been honored by the counsel of the following international collaborators: Dr. Dezső Dávid, Dr. Thomas Eggermann, Dr. Oskar Haas, Dr. Karin Nebral and Dr. Naomi Baba.

My PhD studies would not have been possible without the support of Professor Szabó Attila, the current Director of the Pediatric Center, and his predecessors, Professors Kovács Gábor and Szabó András.

I would be remiss not to acknowledge my colleagues at the Pediatric Center's Tűzoltó street Unit for their devoted and selfless work in caring for our patients.

I would like to express my warmest gratitude to all the patients presented in this thesis, and their families.

Finally, words cannot express my gratitude to my family and friends for their life-long support, patience and love.