Received: 3 March 2022 | Revised: 4 April 2022 | Accepted: 7 April 2022

DOI: 10.1002/jcla.24441

RESEARCH ARTICLE

WILEY

Chromosomal microarray in postnatal diagnosis of congenital anomalies and neurodevelopmental disorders in Serbian patients

Dijana Perovic | **Tatjana Damnjanovic** | **Biljana Jekic** | **Marija Dusanovic-Pjevic** | **Milka Grk** | **Ana Djuranovic** | **Milica Rasic** | **Ivana Novakovic** | **Nela Maksimovic**

Faculty of Medicine, Institute of Human Genetics, University of Belgrade, Belgrade, Serbia

Correspondence
Dijana Perovic, Faculty of Medicine, Institute of Human Genetics, University of Belgrade Faculty of Medicine, Visegradska 26a, 11000 Belgrade, Serbia.
Email: dijana.perovic@med.bg.ac.rs

Funding information
Ministarstvo Prosvete, Nauke i Tehnološkog Razvoja, Grant/Award Number: 175091

Abstract

Background: Array-based genomic analysis is a gold standard for the detection of copy number variations (CNVs) as an important source of benign as well as pathogenic variations in humans. The introduction of chromosomal microarray (CMA) has led to a significant leap in diagnostics of genetically caused congenital malformations and neurodevelopmental disorders, with an average diagnostic yield of 15%. Here, we present our experience from a single laboratory perspective in four years' postnatal clinical CMA application.

Methods: DNA samples of 430 patients with congenital anomalies and/or neurodevelopmental disorders were analyzed by comparative genome hybridization using oligonucleotide-based microarray platforms. Interpretation of detected CNVs was performed according to current guidelines. The detection rate (DR) of clinically significant findings (pathogenic/likely pathogenic CNVs) was calculated for the whole cohort and isolated or combined phenotypic categories.

Results: A total of 140 non-benign CNVs were detected in 113/430 patients (26.5%). In 70 patients at least one CNV was considered clinically significant thus reaching a diagnostic yield of 16.3%. The more complex the phenotype, including developmental delay/intellectual disability (DD/ID) as a prevailing feature, the higher the DR of clinically significant CNVs is obtained. Isolated congenital anomalies had the lowest, while the "dysmorphism plus" category had the highest diagnostic yield.

Conclusion: In our study, CMA proved to be a very useful method in the diagnosis of genetically caused congenital anomalies and neurodevelopmental disorders. DD/ID and dysmorphism stand out as important phenotypic features that significantly increase the diagnostic yield of the analysis.

KEYWORDS
chromosomal microarray, congenital anomalies, copy number variations, detection rate, neurodevelopmental disorders

1 | INTRODUCTION

Assessment of copy number variations (CNVs) on genomic level is recommended as a first-tier analysis for individuals with developmental delay (DD), intellectual disabilities (ID), autism spectrum disorder (ASD), and/or congenital anomalies.[1] Chromosomal microarray analysis (CMA) has been broadly implemented in clinical practice for the detection of those "middle size" genomic imbalances for over a decade. It encompasses several array-based genomic analyses including comparative genome hybridization (CGH) and single nucleotide polymorphism (SNP) microarrays. The diagnostic yield of CMA in the DD/ID category with or without conjoined morbidities varies among different studies but usually ranges between 10% and 20%, about 10% more than G-banded karyotype alone.[2-6]

CNVs are widespread in some regions of the genome. Most of them are benign phenotypic variations, but a small percent (e.g., less than 1% in ASD) are associated with various neurodevelopmental disorders.[7] Recurrent CNVs, usually flanked by segmental duplications and mediated by non-allelic homologous recombination events, are the cause of well-known microdeletion or microduplication syndromes.[8] However, losses or gains of genetic material could be based on replication error or DNA repair mechanisms, that could happen anywhere in the genome. Also, balanced genomic rearrangement in parents, like chromosomal translocations and inversions, predisposes to unbalanced aberrations in the offspring.[9-11]

Approximately 2% to 5% of children are born with major congenital malformations or express serious neurodevelopmental disorder during childhood.[12,13] DD and ID, included under a parent category of neurodevelopmental disorders, are considered complementary entities separated chronologically because very often DD in a child up to 5 years turns into ID at an older age. In broader conceptualization, epilepsy, autism or ASD, and other behavioral abnormalities along with some specific communication, learning, or motor disabilities, are included in neurodevelopmental disorders.[14] The exact prevalence of these disorders in our country is not known, but there is a striking combined prevalence of 17% among children 3 to 17 years in the United States,[15] making them the most prevalent chronic medical conditions in primary pediatric care. Etiologically, they represent a heterogeneous group of disorders, with genetic factors causing or contributing in at least a quarter to half of the cases.[16]

There are several algorithms for etiological investigations of DD/ID and related conditions. Screening for the most common or treatable disorders is usually recommended first, or the recommendations are based on the likelihood ratio models. Current guidelines include CMA and Fragile X testing as a first-line test,[16,17] although, it has been recommended that next-generation sequencing (NGS)-based methods should replace CMA as a first-line test in patients with neurodevelopmental disorders, based on significantly higher diagnostic yield.[18,19] However, a step-wise approach, which must be tailored to the specific clinical context and availability of local resources, is still a choice for most countries.

The Molecular Genetics Laboratory of the Institute of Human Genetics at the Faculty of Medicine in Belgrade started performing CMA analysis in 2015 for research and from 2017 is performing it for diagnostic purposes in postnatal settings. In this study, we have presented our first experience from four years' CMA application from a single laboratory perspective.

2 | MATERIALS AND METHODS

2.1 | Patients

The retrospective study included 430 patients (167 females, 263 males) referred to our Institute for array-CGH analysis during 2017–2020. All patients were examined by clinical genetic specialists or doctors of other specialties (pediatric neurologist, psychiatrist, cardiologist, etc.) who made detailed phenotypic reports based on the clinical features and additional tests including EEG, brain imaging, and metabolic analysis, when appropriate. Clinical data were collected based on the specialist's reports and questionnaire included in the referral list to our laboratory. All but three patients were of pediatric age, ranging from neonatal age to 18 years, and three adults were 26, 34, and 38 at the time of the analysis.

For most patients, array-CGH wasn't a first-line test. Classic karyotype and, in some cases, MLPA (Multiplex Ligation-dependent Probe Amplification) for most common microdeletion/microduplication syndromes were performed beforehand in other laboratories. Patients with autism or ASD, or suggestive clinical features, had negative Fragile-X testing. All patients, or their guardians, gave us informed consent. The study was approved by the Ethics Committee Faculty of Medicine, University of Belgrade (1322/VII-4).

2.2 | CNV detection by array comparative genome hybridization and interpretation

Patients' DNA was extracted from whole blood samples by the standard salting-out method. The array-CGH procedure was performed using Agilent microarray oligonucleotide platforms (SurePrint G3 Human CGH Microarray 8 × 60K—410 patients, and SurePrint G3 Human CGH +SNP Microarray 4 × 180K—20 patients) (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's protocol. Patients with autism, autistic spectrum disorder, or epilepsy, without congenital anomalies, were selected for microarray of higher resolution. The whole protocol of DNA digestion, labeling, and hybridization could be found on the manufacturer's website. Microarray slides were scanned with a DNA Microarray Scanner and data were obtained by Cytogenomic software (Agilent Technologies). Genomic positions were based on human genome reference sequence GRCh 37/hg19. Detected CNVs were not confirmed by the additional tests but samples with poorer quality control metrics (DLRSD score ≥0.3) were repeated, and CNVs smaller than 500 kb were reported as pathogenic or likely pathogenic only if they had a very good DLRSD ratio (≤0.2) and at least 10 probes affected.[20] The success rate of the analysis was over 99%.

All detected CNVs were analyzed and classified according to current guidelines.[21,22] The significance of detected variants has been evaluated by taking into account: type (gain/loss), size, gene content (especially dosage sensitivity), and inheritance pattern, all in the light of the patient's clinical phenotype. To achieve the best evaluation, a thorough review of both peer-reviewed literature and CNV databases for healthy and affected populations has been conducted: PubMed, Database of Genomic Variants (DGV), DECIPHER, ClinGen, and Online Mendelian Inheritance in Men (OMIM). Purely benign CNVs were not reported, and the detection rate (DR) has been calculated based on the other non-benign four categories (pathogenic, likely pathogenic, likely benign, and uncertain significance). We considered pathogenic and likely pathogenic CNVs as clinically significant (csCNV) and the DR of at least one such variant in one patient has been used to determine diagnostic yield in our study.

2.3 | Phenotypic categories and statistical analysis

With the respect to the heterogeneity of our sample, patients were divided into different single or combined phenotypic categories. According to their frequency, we considered six different clinical features: (1) DD/ID, (2) minor congenital anomalies (dysmorphism), (3) major congenital anomalies (including cardiovascular, urogenital, skeletal, or brain anomalies), (4) autism and ASD, (5) epilepsy, and (6) microcephaly. The detection rate of pathogenic/likely pathogenic CNVs has been calculated for each single or combined phenotypic category and compared with DR in the rest of the cohort. Statistical analysis has been performed by Pearson's chi-squared (χ^2) or Fisher's exact test using SPSS v.16.0 (SPSS Inc., Chicago, IL, USA).

3 | RESULTS

A total of 140 CNVs were detected in 113/430 patients (26.5%); 61 deletions (43.6%) and 79 (56.4%) duplications. Clinically significant CNVs were described in 70 patients, thus reaching a diagnostic yield of 16.3%. Variants of uncertain significance (VUS) have been detected in 29 cases (6.7%), and likely benign in 14 (3.2%). Thirty-three patients had rare or non-recurrent csCNVs and 37 patients had recurrent CNVs or syndromes with OMIM numbers. Their clinical and array-CGH findings are summarized in Tables 1 and 2, respectively (Supporting Information).

Patients with csCNVs had 92 different variations (48 deletions and 44 duplications): 50 patients had only one and 20 patients had two or more, but not necessarily all pathogenic/likely pathogenic. Fourteen patients had CNVs affecting two different chromosomes, mostly one duplication and one deletion, and 6 had a combination of discontinued gain and/or loss at the same chromosome. Overall, in 70 patients 39 deletions were considered causative (55.7%) in contrast to 31 duplications (44.3%). The duplications become predominant as classes change from pathogenic to likely benign, as expected. Thus, in the VUS and likely benign category there were 19 duplications and 10 deletions, and 11 duplications with 3 deletions, respectively. The largest number of clinically significant variants was found on chromosomes, 2, 22, and 15 (12, 10, and 9, respectively). In five patients supposed causal variant was on the X chromosome (3 females, 2 males). (Figure 1). The most common pathogenic CNVs were in regions 22q11.21 (4 deletions and 4 duplications) and 7q11.23 (3 deletions, 2 duplications).

CNV size ranged from 9 kb (detected on 4 × 180K slide) to 64 Mb. When distributed through different categories, presumed clinical significance also decreases with decreasing in size (Figure 2). There were some exceptions: 8 patients had csCNVs smaller than 500 kb. Three of them had recurrent pathogenic microdeletions (15q11.2 BP1-BP2, 16p11.2, and 17q21.31), one had *MECP2* duplication syndrome, three had intragenic deletion/duplications (one in *NRNX1* and two in *MYTL1* gene) and one had microduplication in 5p15.33 encompassing *MRLP36* and *NDUFS6* genes. The last four listed were classified as likely pathogenic. In the pathogenic/likely pathogenic category, the smallest CNV was 240 kb, and the largest that wasn't detected by conventional karyotype was 8300 kb or 64.28 Mb when array-CGH was the first-line test (median 2300 kb). In the VUS category, size ranged from 9 kb to 4,02 Mb (median 814 kb), and in the likely benign category 108 to 1708 kb (median 495 kb). In 23 patients gains or losses were larger than 5 Mb.

Figure 3 shows all diagnostic tests that were performed in other laboratories before patients were referred to our laboratory and the detection rates before and after aCGH.

One of the interesting findings in our cohort is that a relatively high number of patients with clinically significant results had more than one CNV detected: 20/70 (28.6%). Four of them had three or more CNVs including the same or two different chromosomes. Patient 2 (Table 1), a two-year-old boy with developmental delay and microcephaly, had complex genomic rearrangement including discontinued duplication-triplication-deletion spanning more than 8 Mb at 1q43-q44 region (arr[hg19]1q43 (240145375–240400485) × 4, 1q43 (240900722–242023977) × 4, 1q43 (242252160–242404158) × 1, 1q43-q44 (243508931–244464177) × 3, 1q44 (245000346–248262713) × 3–4, 1q44 (247074460–248684909) × 1). Patient 3 (Table 2), with Seathre-Chotzen phenotype suspected prenatally, and global DD postnatally also had complex rearrangement involving chromosome 7 with 3 deletions, two on *p* and one on *q* arm, and additional deletion on chromosome 5, although prenatal karyotype suspected unbalanced translocation between chromosomes 7 and 11 (arr[hg19]7p21.1–p15.3 (17975914–22797001) × 1; 7p12.1–p11.2 (52793551–54083685) × 1; 7q21.11 (78322150–81208583) × 1; 5p12–p11 (45519525–46100367) × 1). Parents' karyotypes, as well as array-CGH, were normal.

The sample was heterogeneous but DD/ID was the most consistent finding, confirmed in 373 patients (86.7%). For the rest 57 patients, 33 (7.7%) did not meet the criteria for DD or ID (referral diagnoses were mainly congenital anomalies, and/or epilepsy), and for 24 (5.6%) there were no accurate data or patients were in neonatal or early infant period when such diagnosis is not reliable.

TABLE 1 Array-CGH results and clinical phenotype of patients with rare or non-recurrent clinically significant CNVs

No case	Region	CNV type	Size (kb)	N	Age; gender	CNV class	Gene(s) of interest	Clinical phenotype
1	1p21.1–p13.2	del	7480	1	10 yr; F	LP	84 PK, 12 morbid	DD/ID, Epi, facial dysmorphia
2	1q43–q44	complex	8500	1	2 yr; M	P	*AKT3*, *NLRPN3*	DD, microcephaly, periodic fever
3	2p16.3	del	285	1	3 yr; M	LP dn	*NRNX1*, intragenic del	ASD, macrocrania
4	2p22.1	del	633	1	7 yr; M	LP	*SOS1*	IUGR, DD, plagiocephaly
5	2p22.2–p22.1	dupl	2730	1	4 yr; F	LP dn	24 PK, 4 morbid	CHD, ASD
6, 7	2p25.3	dupl	404	2	1 yr; M	LP	*MYT1L*, intragenic dupl	DD, microcephaly
			425		7 yr; M	LP pat		DD/ID, ASD
8	2q11.1–q11.2	del	1240	1	1 yr; F	LP mat	22 PK, 6 morbid	Premature birth, DD, craniosynostosis, microcephaly
9	2q13	dupl	1600	1	11 yr; F	LP	8 PK	Autism, moderate ID
10	2q23.3–q24.11	del	7250	1	4 yr; F	P	22 PK, 4 morbid	DD, CHD, microcephaly, facial dysmorphia
	14q24.1	del	221			LB		
11, 12	2q34	del	753	2	17 yr; F,	LP dn	*ERBB4* first two exons	Siblings with profound ID, behavioral disorder, hyperactivity
					10 yr;M			
13	3q21.1–q29	dupl	64280	1	newborn	P	362 PK, 85 morbid	IUGR, CHD, cleft palate, dysmorphic features
					F			
14	4q21.22–q21.23	del	2530	1	3 yr; F	LP	18 PK, 4 morbid	DD, mild facial dysmorphia
15	4q34.1–q34.3	del	5860	1	4 yr; M	P	*VEGFC*	Omphalocele, hydronephrosis, pterygium colli, lymphedema
16	5p15.33	dupl	320	1	5 yr; M	VUS mat		DD, ASD
		dupl	240			LP dn	*MRLP36*, *NDUFS6*	
17	6p25.3–p25.1	dupl	5370	1	17 yr; F	P	32 PK, 9 morbid	Mild ID, short stature, brachy- and clinodactyly,
	9p24.3–p24.1	del	4590				17 PK, 7 morbid (*SMARCA2*)	oligomenorrhoea, facial dysmorphia
18	6q14.3–q16.1	dupl	8300	1	1 yr; M	LP	32 PK, 7 morbid	Craniosynostosis (trigonocephaly), DD, facial
	15q13.1–q13.12	del	1570			VUS	7 PK,1 morbid	dysmorphia
19	6q25.1–q27	dupl	20,151	1	26 yr; F	P	87 PK, 21 morbid	Infertility, oligomenorrhoea, dysarthria, minor
	Xq25–q28	dupl	25,469				122 PK, 28 morbid	dysmorphisms
	Xq28	del	1975				48 PK, 18 morbid	
20	7p22.3–p22.1	del	6680	1	3 yr; M	P	70 PK, 17 morbid	DD, Epi, facial dysmorphia, hiatus hernia,
	8p23.3–p23.1	dupl	7530				40 PK, 3 morbid	intestinal perforation
21	7q35–q36.3	del	15,480	1	1 yr; M	P	101 PK, 19 morbid	IUGR, postnatal growth restriction,
	16q24.1–q24.3	dupl	3370			P	48 PK, 23 morbid	microcephaly, facial dysmorphia

TABLE 1 (Continued)

No case	Region	CNV type	Size (kb)	*N*	Age; gender	CNV class	Gene(s) of interest	Clinical phenotype
22	8p23.3–p23.2	del	2820	1	6 yr; M	LP	9PK, 2 morbid	ID, ASD
	8p23.2	dupl	1710				1PK	
23	8p23.3–p23.1	del	9040	1	newborn;	P	58PK, 4 morbid	Pierre-Robin sequence, CHD, hypotonia, dysmorphic features
	8q21.2–q24.3	dupl	59,440		F	P	277PK,67 morbid	
24	8p23.3–p22	dupl	17,018	1	19 yr; M	P	100 PK, 19 morbid	CHD, omphalocela, mild ID
	9p24.3–p24.2	del	4006				28PK, 6 morbid	
25	9q31.1–q31.3	del	5900	1	2 yr; M	P	32PK, 6 morbid	IUGR, neonatal convulsions, CHD, VUR, DD, facial dysmorphia
	12p12.1	dupl	1000			VUS	1 morbid: SHOX5	
26	10p15.3–p15.1	dupl	4960	1	2 yr; F	VUS	16PK, 3 morbid	Chronic juvenile arthritis—severe form, DD, facial dysmorphia, coloboma iris
	10q11.22–q11.23	del	5650			LP	44PK, 6 morbid	
	18p11.32–p11.31	del	4310			P	19PK, 3 morbid	
27	10q25.1-qter	dupl	24,440	1	3 mo; M	P	144PK, 31 morbid	DD, facial dysmorphia
28	15q13.1–q13.3	dupl	2660	1	11 yr; F	VUS pat	*FAN1*, *TRPM1*, *CHRNA7*	ID, Epi, hypothyreosis, diabetes insipidus, arthrogryposis, VUR, short stature (growth hormone), facial dysmorphia, brachydactyly
	16p13.11	dupl	1230			LP mat	*ABCC6*, *MYH11*, *NDE1* *LP mat*	
29	16p13.11–p.12.3	dupl	2300	1	8 yr; M	LP mat	*ABCC6*, *MYH11*, *NDE1*, *XYLT1*	Autism, Epi, myopia, partial syndactyly, widely spaced teeth
30	16q11.2–q22.2	dupl	24,560	1	6 mo; M	P	210 PK, 57 morbid	CHD, hypotonia, DD, facial dysmorphia
31	17q25.1–q25.3	dupl	7962	1	16 yr; F	P	217PK, 12 morbid	DD/ID, Epilepsia, facial dysmorphia
	17q25.3	del	755				16PK, 3 morbid	
32	5q32	dupl	574	1	6 yr; F	LB	8PK, 3 morbid	IUGR, ID, Epi, microcephaly, hand anomalies, hirsutism, facial dysmorphias
	Xp22.31	dupl	1560			LP	4PK, 1 morbid	
33	Xq21.23–q21.3	dupl	7280	1	14 yr; F	LP	12PK, 4 morbid	Severe ID, extremely obese, behavioral disorder, facial dysmorphia

Abbreviations: del, deletion; dupl, duplication; mo, month; F, female; M, male; P, pathogenic; LP, likely pathogenic; VUS, variants of unknown significance; LB, likely benign; dn, de novo; pat, paternal; mat, maternal; PK, protein coding (genes); DD, developmental delay; ID, intellectual disability; Epi, epilepsy; ASD, autism spectrum disorders; IUGR, intrauterine growth retardation; CHD, congenital heart disease; VUR, vesicoureteral reflux.

Dysmorphic features, mostly craniofacial dysmorphism, were the next most common characteristic, found in 232 (53.9%), followed by major congenital anomalies in 164 (38.1%) patients. Autism or ASD, epilepsy, and microcephaly had similar frequencies of 14.2%, 13.9%, and 13.7%, respectively.

To better evaluate phenotypic features and their contribution to the detection rate of clinically significant variants, we calculated DR for some isolated categories, when they were large enough, or "plus" categories including the main feature plus at least one other, and compared with DR in the remaining group. Most of the

TABLE 2 Array-CGH results and clinical phenotype of patients with recurrent CNVs and syndromes with OMIM number

No case	Region	CNV type	Size (kb)	N	Age; gender	CNV class	OMIM#	Clinical phenotype/syndrome
1	2q22.2–q22.3	del	2904	1	2 yr; F	P	235730	Mowat–Wilson sy
2	2q37.3	del	3600	1	2 yr; M	P	600430	Atresio oesophagei, TOF, Laryngomalatio, DD
3	5p12–p11	del	581	1	3 mo; M	P dn	101400	DD, Seathre-Chotzen syndrome
	7p21.1–p15.3	del	4800					
	7p12.1–p11.2	del	1300					
	7q21.11	del	2900					
4	7p22.1	del	712	1	1.5 yr; M	P	243310	Baraitzer Winter syndrome
5–7	7q11.23	del	1400	3	2 M,F	P	194050	Williams-Beuren syndrome
			1430					
8, 9	7q11.23	dupl	1150	2	M; F	P	609757	DD, mild facial dysmorphism
10, 11	15q11.2	del	395	2	9 yr; F	P	615656	ID, facial dysmorphia, seizures
			802		2 yr; F			DD, obesity, facial dysmorphia
12, 13	15q11.2–q13.1	del	4830	2	F	P	105830	Angelman syndrome
14	15q11.2–q13.1	dupl	9726	1	1.5 yr; M	P	608636	DD, hypotonia, hypospadia, facial dysmorphia
	15q13.2–q13.3	tripl	1500					
15	15q13.2–q13.3	del	1500	1	11 yr; F	LP pat	612001	DD/ID, ASD, facial dysmorphia
16	15q26.2–q26.3	del	7940	1	2 yr; F	P	612616	IUGR, CHD, VUR, facial dysmorphia (Dryer syndrome)
17	16p11.2	del	295	1	6 yr; M	P	613444	DD, hypotonia
18, 19	16p11.2	dupl	524	2	6/13 yr; F	P	614671	Epilepsy /Mild ID, dysphasia
20	16p11.2	dupl	856	1	13 yr; F	P	614671	DD/ID, strabismus, facial dysmorphia
	17q12	del	1300				614527	
21	17q21.31	del	442	1	11 yr; M	P	610443	Koolen de Vries syndrome
22	18p11.32–p11.21	del	14,570	1	6 mo; F	P	146390	DD, microcephaly, parieto-occipital meningocele, facial dysmorphia
23	18q21.33–q23	del	17,168	1	14 yr; M	P	601808	ID, facial dysmorphia
24	19p13.2–p13.12	dupl	2010	1	12 yr; F	P	613638	Microcephaly, short stature, CHD, borderline intelligence, facial dysmorphia
25–28	22q11.21	del	2250	4	M	P	188400	Di George/Velocardiofacial syndrome
			–2540					
29-32	22q11.21	dupl	2460	4	3 M, F	P mat	608363	Varies: from normal intelligence to mild ID, ASD, speech delay, Epilepsy, one case CHD
			–3200			(2)		
33. 34	22q13.3	del	241/1340	2	M, F	P	606232	Phelan- McDermid syndrome
35	Xp11.23–p11.22	dupl	5016	1	8 yr; F	P mat	300801	ID, facial and other minor dysmorphisms
36;37	Xq28	dupl	600	2	8/15 yr; M	P	300260	Severe DD/ID, macrocephaly, dysmorphic features (MECP2 dupl syndrome)
			351					

Abbreviations: del, deletion; dupl, duplication; trip, triplication; mo, month; F, female; M, male; P, pathogenic; LP, likely pathogenic; dn, de novo; pat, paternal; TOF, tracheoesophageal fistula; DD, developmental delay; ID, intellectual disability; ASD, autism spectrum disorders; CHD, congenital heart disease.

patients had complex phenotypes with more than one of the previously mentioned clinical features. The only isolated categories that we could single out were DD/ID without other special findings and isolated congenital anomalies. First, it was evident that in the group with isolated DD/ID (49 patients) there were only 2 patients with csCNVs. Comparing the DR of only 4.1% with DR in the

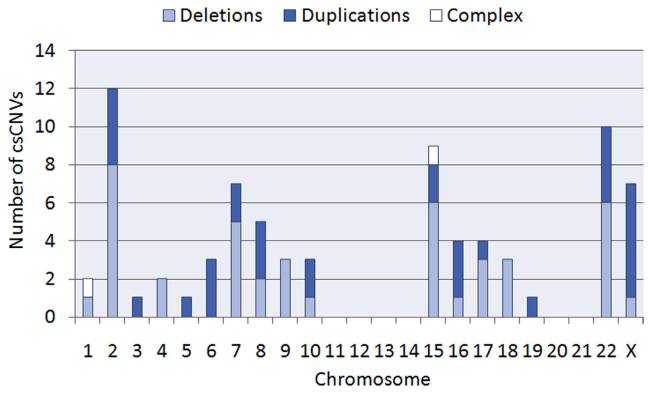

FIGURE 1 Chromosomal distribution of clinically significant CNVs in our cohort

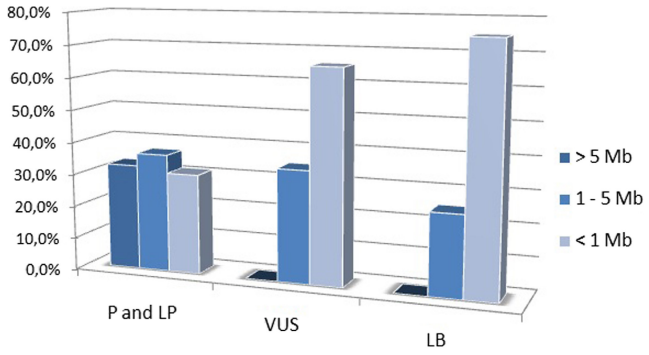

FIGURE 2 Distribution (percentage) of CNV types across different size categories. Abbreviations: P and LP, pathogenic and likely pathogenic; VUS, variants of unknown significance; LB, likely benign

remaining cohort—17.9%, we got a statistically significant difference ($p = 0.022$). Similarly, none of the 19 patients with isolated congenital anomalies (most often congenital heart disease and tracheoesophageal abnormalities) had csCNVs, as well as 17 patients with DD/ID and epilepsy, without other phenotypic features. Secondly, the more combined features have been present, the larger was csCNVs detection rate. We compared DR of the csCNVs in the group with one or two phenotypic features (33/288; 11.45%) and the group with three or more (37/142; 26.06%), and DR has been significantly higher in the latter group ($p = 0.0002$; OR 2.72; 95% CI 1.62–4.59).

In Figure 4, DRs of all "plus" phenotypic categories compared to DRs in the remaining cohort are represented. "DD/ID plus" category had significantly higher detection rate ($p = 0.002$) and that was even more emphasized in "dysmorphism plus" category ($p < 0.0001$ OR 4.02 95%CI: 2.02–8.20). For the rest of the categories, detection rates were similar.

4 | DISCUSSION

Congenital anomalies are usually evident soon after birth and are increasingly detected prenatally. One of the major concerns is that they will be accompanied by neurodevelopmental delay which is often the case in genetically caused malformations. Therefore, CMA is strongly suggested in neonates with structural malformations to achieve diagnosis as soon as possible and give appropriate genetic advice.[23]

In this study, we identified 140 non-benign copy number variations in 430 patients (26.5%) with congenital anomalies and/or neurodevelopmental disorders, but consider 70 of them to be causative or significantly contributing to the patient's phenotype, making the diagnostic yield of 16.3%. Our results are entirely in line with the literature data. Array-CGH was applied as a first-tier test, according to the current recommendations, in a small number of patients, mostly in the last year when the number of analyses performed on an annual basis increased. Therefore, this percentage largely reflects the diagnostic yield of the method applied to patients with unexplained DD/ID, congenital anomalies, and ASD when other tests (mainly karyotype and MLPA) did not give a clear genetic diagnosis.

Interpretation of rare or non-recurrent CNVs could be challenging. Patient 2 described in Results had complex rearrangement on 1q43–q44. Although pathogenic, based on the size and gene content of the region, it was difficult to interpret that finding in the light of the patients' phenotype. One of the duplicated segments contains *ZBTB18* and *AKT3* genes, associated with autosomal mental retardation 22 and microcephaly, respectively, in the case of reciprocal 1q43-q44 deletion (MIM612337). Duplication of *AKT3*, in contrast, leads to macrocephaly.[24] The reason for this contradiction probably lies in the fact that the aCGH cannot determine the precise localization and orientation of duplicated segments, and it is possible that the *AKT3*, in a complex rearrangement, had actually a loss of function, which would explain microcephaly in our patient. Another interesting fact, in this case, would be the duplication of the *NLRP3* gene whose "gain of function" mutations are described in CAPS (*Cryopirin-associated periodic syndromes*) and this boy had periodic febrile episodes that were diagnosed as PFAPA (*Periodic Fever, Aphthous Stomatitis, Pharyngitis, Adenitis*) syndrome by an immunologist. This aCGH finding led to the revision of the clinical diagnosis and consideration that duplication involving the *NLRP3* gene could explain the boy's immunological phenotype. Parents were not available for the analysis, but it is described that complex genomic rearrangements like this one are usually a consequence of "chromosomal catastrophes" involving replication mechanisms and happen *de novo*.[25]

The interpretation of CNVs could change over time. Also, the fact that some recurrent CNVs have incomplete penetrance poses a challenge for their interpretation and consequent genetic counseling. For example, patient 28, (Table 2) an 11-year-old girl with ID, epilepsy, endocrine disturbances, arthrogryposis, and dysmorphic features, had two recurrent duplications, one in region 15q13.1–q13.3 and the other in 16p13.11. She inherited the first duplication from the father and the second from the mother; both parents are reportedly healthy. In ClinGen dosage sensitivity curation, the 15q13.3 recurrent region (BP4-BP5; includes *CHRNA*) has "little evidence" (score 1), while the 16p13.11 region has "emerging

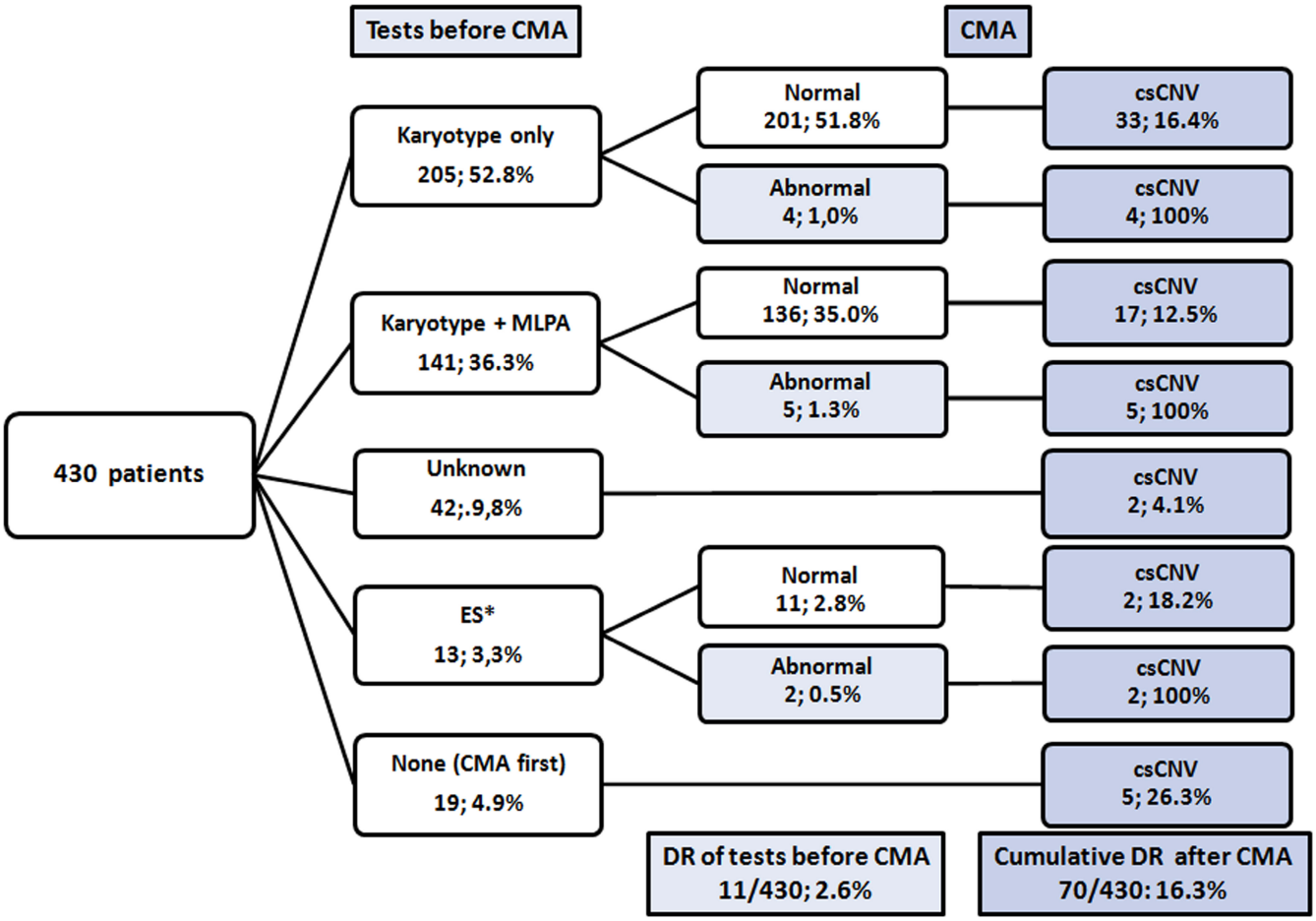

FIGURE 3 Results of diagnostic tests performed in other laboratories with detection rates before and after CMA. Abbreviations: CMA, chromosomal microarray; MLPA: Multiplex Ligation-dependent Probe Amplification for most common microdeletion/ microduplication syndromes, ES, exome sequencing, *± karyotype, MLPA with negative results, csCNV, clinically significant CNV

evidence" for triplosensitivity (score 2) (Clinical Genome Resource. https://search.clinicalgenome.org/kb/gene-dosage/ region/ISCA-37411, and https://search.clinicalgenome.org/kb/gene-dosage/region/ISCA-37415; accessed on January 10, 2022). We classified those CNVs as VUS, likely pathogenic, but remains unclear whether those two variants both inherited from one of the parents act together as a "two-hit" CNV model causing complex clinical phenotype in the patient, or the causative genetic variant is yet to be found, perhaps point mutation on exome/ genome sequencing.

Detection rate analysis based on single or isolated phenotypic categories in our cohort confirms previous findings. The more complex the phenotype, including developmental delay/intellectual disability as a prevailing feature, the higher the detection rate is obtained.[26-28] Similarly to Catusi et al.[28] patients were divided into "plus" categories (Figure 4) and DRs were compared between the examined category and the rest of the cohort. Again, it was clear that DD/ID and, notably, dysmorphism, stand out as important phenotypic features that significantly increase the diagnostic yield of the analysis.

Despite retrospective and multicentric sample collection, with variable quality of clinical reports, as the main limitations, one laboratory perspective in array-CGH performance and CNV interpretation could be also the strength of this study. Other limitations include the absence of clinical follow-up of patients, especially those that were newborns or infants at the time of referral and phenotyping, and a relatively high percentage of variants of unknown significance without other classification (6.7%). The main disadvantage was unknown inheritance in lots of cases (only in 8/29 VUS cases parents were tested) because the parents were not available for testing or we had varying knowledge of their phenotype. New ACMG and ClinGen guidelines for constitutional CNV interpretation and reporting[22] are helpful in the re-classification of those variants. This updated version includes a scoring system and recommendation of "uncoupling" the evidence-based classification of a variant from its potential implication for a particular individual. Understanding the clinical relevance of CNVs is a complex, continually evolving process, still prone to subjectivity.[29,30]

There have been several proposals that NGS technology-based genomic tests, like WES, should replace current ACMG guidelines for chromosomal microarray and Fragile- X as first-tier analyses in children with unexplained DD/ID and/or ASD. This is mostly supported by a significantly higher diagnostic yield of exome sequencing that

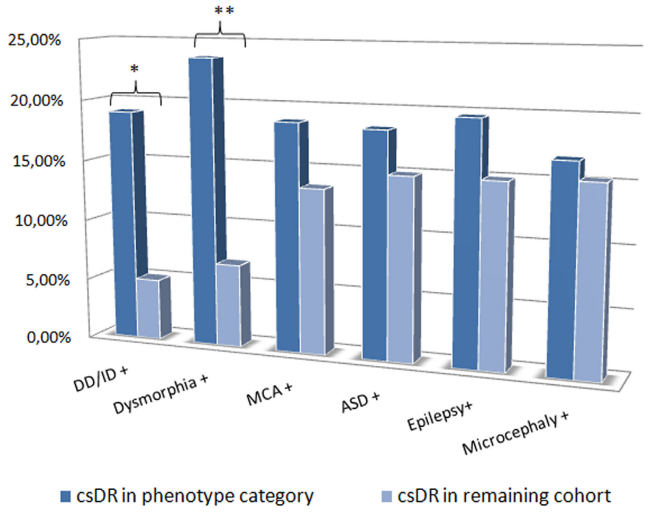

FIGURE 4 The detection rate of clinically significant CNVs according to "+" clinical categories of patients (single phenotypic category plus at least one other clinical sign) compared to DR in the remaining cohort. Abbreviations: DD, developmental delay; ID, intellectual disability, MCA, major congenital anomalies; ASD, autism spectrum disorders, csDR, clinically significant detection rate; * $p = 0.002$, ** $p < 0.0001$

outperforms the yield of chromosomal microarray.[18,19] In two siblings from our cohort, one CNV was missed on exome sequencing. Patients 11 and 12 (Table 1) are brother and sister born from healthy, non-consanguineous parents, who developed severe speech delay, intellectual disability, and behavioral problems. Among other genetic tests, WES was done in a laboratory elsewhere, and no causative variants were detected, noting that it was more than five years ago. By array-CGH, we detected 753 kb deletion of the 2q34 region. The deletion includes the first two exons of the *ERBB4* gene as well as proximal regulatory elements. Both siblings have the same variant, and none of the parents, suggesting that one of the parents could have gonadal mosaicism. *ERBB4* encodes tyrosine kinase receptors for neuregulin-1 that plays role in GABA-ergic circuit assembly and is essential for neurological development. Until recently, only one patient with similar deletion was described[31] and our variant was characterized as likely pathogenic. In 2021, Hyder et al.[32] described 9 more patients with similar deletion and phenotype of non-dysmorphic, often profound, DD and ID, sometimes with epilepsy and behavioral problems that fit completely to the phenotype of siblings from our cohort. The explanation for the fact that WES analysis did not detect this deletion is that at the time it was performed, read depth and NGS data processing were not appropriate for the detection of such CNVs. Although bioinformatics analysis of NGS data becomes better every year, detection of heterozygous CNVs from clinical WES data remains challenging due to biases in exome capture and variable sequence efficiency.

Currently, array-CGH is still the gold standard for detecting CNVs and probably it will be in the next five-year period. Furthermore, combining CMA and WES, although expensive, increases diagnostic yield, especially in recessive diseases, and accelerates novel gene discovery.[33,34]

ACKNOWLEDGMENTS

We would like to thank all patients included in the study. Our appreciation further goes to colleagues from University Children's Hospital "Tiršova", Mother and Child Health Care Institute of Serbia "Dr V. Čupić", Institute of Mental Health, and Obstetrics and Gynecology Clinic "Narodni Front" in Belgrade, for collaboration and valuable clinical reports.

CONFLICT OF INTEREST

The authors declared that they have no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Dijana Perovic https://orcid.org/0000-0003-1077-2373
Tatjana Damnjanovic https://orcid.org/0000-0003-0479-0620
Biljana Jekic https://orcid.org/0000-0001-6745-5403
Marija Dusanovic-Pjevic https://orcid.org/0000-0002-3564-2623
Milka Grk https://orcid.org/0000-0002-7534-9608
Ana Djuranovic https://orcid.org/0000-0002-8144-4433
Milica Rasic https://orcid.org/0000-0002-3269-7872
Ivana Novakovic https://orcid.org/0000-0003-0202-0673
Nela Maksimovic https://orcid.org/0000-0002-5815-573X

REFERENCES

1. Miller DT, Adam MP, Aradhya S, et al. Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. *Am J Hum Genet*. 2010;86:749-764.
2. Ahn JW, Bint S, Bergbaum A, Mann K, Hall RP, Ogilvie CM. Array CGH as a first line diagnostic test in place of karyotyping for postnatal referrals—results from four years' clinical application for over 8,700 patients. *Mol Cytogenet*. 2013;6:16.
3. Bartnik M, Wisniowiecka-Kowalnik B, Nowakowska B, et al. The usefulness of array comparative genomic hybridization in clinical diagnostics of intellectual disability in children. *Dev Period Med*. 2014;18:307-317.
4. Battaglia A, Doccini V, Bernardini L, et al. Confirmation of chromosomal microarray as a first-tier clinical diagnostic test for individuals with developmental delay, intellectual disability, autism spectrum disorders and dysmorphic features. *Eur J Paediatr Neurol*. 2013;17:589-599.
5. Lee JS, Hwang H, Kim SY, et al. Chromosomal microarray with clinical diagnostic utility in children with developmental delay or intellectual disability. *Ann Lab Med*. 2018;38:473-480.
6. Garcia-Acero M, Suarez-Obando F, Gomez-Gutierrez A. CGH analysis in Colombian patients: findings of 1374 arrays in a seven-year study. *Mol Cytogenet*. 2018;11:46.
7. Takumi T, Tamada K. CNV biology in neurodevelopmental disorders. *Curr Opin Neurobiol*. 2018;48:183-192.
8. Weise A, Mrasek K, Klein E, et al. Microdeletion and microduplication syndromes. *J Histochem Cytochem*. 2012;60:346-358.

9. Hastings PJ, Lupski JR, Rosenberg SM, Ira G. Mechanisms of change in gene copy number. *Nat Rev Genet*. 2009;10:551-564.
10. Roth DB, Wilson JH. Nonhomologous recombination in mammalian cells: role for short sequence homologies in the joining reaction. *Mol Cell Biol*. 1986;6:4295-4304.
11. Carvalho CMB, Pehlivan D, Ramocki MB, et al. Replicative mechanisms for CNV formation are error prone. *Nat Genet*. 2013;45:1319-1326.
12. Sheridan E, Wright J, Small N, et al. Risk factors for congenital anomaly in a multiethnic birth cohort: an analysis of the Born in Bradford study. *Lancet*. 2013;382:1350-1359.
13. Savatt JM, Myers SM. Genetic Testing in Neurodevelopmental Disorders. *Front Pediatr*. 2021;9: 526779.
14. Ismail FY, Shapiro BK. What are neurodevelopmental disorders? *Curr Opin Neurol*. 2019;32:611-616.
15. Zablotsky B, Black LI, Maenner MJ, et al. Prevalence and trends of developmental disabilities among children in the United States: 2009–2017. *Pediatrics*. 2019;144:2009–2017.
16. Moeschler JB, Shevell M, Moeschler JB, et al. Comprehensive evaluation of the child with intellectual disability or global developmental delays. *Pediatrics*. 2014;134:e903-e918.
17. Manning M, Hudgins L, Professional P, Guidelines C. Array-based technology and recommendations for utilization in medical genetics practice for detection of chromosomal abnormalities. *Genet Med*. 2010;12:742-745.
18. Srivastava S, Love-Nichols JA, Dies KA, et al. Meta-analysis and multidisciplinary consensus statement: exome sequencing is a first-tier clinical diagnostic test for individuals with neurodevelopmental disorders. *Genet Med*. 2019;21:2413-2421.
19. Martinez-Granero F, Blanco-Kelly F, Sanchez-Jimeno C, et al. Comparison of the diagnostic yield of aCGH and genome-wide sequencing across different neurodevelopmental disorders. *NPJ Genom Med*. 2021;6:25.
20. Baldan F, Passon N, Burra S, Demori E, Russo PD, Damante G. Quantitative PCR evaluation of deletions/duplications identified by array CGH. *Mol Cell Probes*. 2019;46:101421.
21. Kearney HM, Thorland EC, Brown KK, Quintero-Rivera F, South ST. Working group of the American College of Medical Genetics Laboratory Quality Assurance C. American College of Medical Genetics standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants. *Genet Med*. 2011;13:680-685.
22. Riggs ER, Andersen EF, Cherry AM, et al. Technical standards for the interpretation and reporting of constitutional copy-number variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen). *Genet Med*. 2020;22:245-257.
23. Resta N, Memo L. Chromosomal microarray (CMA) analysis in infants with congenital anomalies: when is it really helpful? *J Matern Fetal Neonatal Med*. 2012;25(Suppl 4):124-126.
24. Wang D, Zeesman S, Tarnopolsky MA, Nowaczyk MJ. Duplication of AKT3 as a cause of macrocephaly in duplication 1q43q44. *Am J Med Genet A*. 2013;161A:2016-2019.
25. Liu P, Erez A, Nagamani S, et al. Chromosome catastrophes involve replication mechanisms generating complex genomic rearrangements. *Cell*. 2011;146:889-903.
26. Shoukier M, Klein N, Auber B, et al. Array CGH in patients with developmental delay or intellectual disability: are there phenotypic clues to pathogenic copy number variants? *Clin Genet*. 2013;83:53-65.
27. Capkova Z, Capkova P, Srovnal J, et al. Differences in the importance of microcephaly, dysmorphism, and epilepsy in the detection of pathogenic CNVs in ID and ASD patients. *PeerJ*. 2019;7:e7979.
28. Catusi I, Recalcati MP, Bestetti I, et al. Testing single/combined clinical categories on 5110 Italian patients with developmental phenotypes to improve array-based detection rate. *Mol Genet Genomic Med*. 2020;8:e1056.
29. Tsuchiya KD, Shaffer LG, Aradhya S, et al. Variability in interpreting and reporting copy number changes detected by array-based technology in clinical laboratories. *Genet Med*. 2009;11:866-873.
30. Nowakowska B. Clinical interpretation of copy number variants in the human genome. *J Appl Genet*. 2017;58:449-457.
31. Kasnauskiene J, Ciuladaite Z, Preiksaitiene E, Utkus A, Peciulyte A, Kucinskas V. A new single gene deletion on 2q34: ERBB4 is associated with intellectual disability. *Am J Med Genet A*. 2013;161A:1487-1490.
32. Hyder Z, Van Paesschen W, Sabir A, et al. ERBB4 exonic deletions on chromosome 2q34 in patients with intellectual disability or epilepsy. *Eur J Hum Genet*. 2021.
33. Cheung SW, Bi W. Novel applications of array comparative genomic hybridization in molecular diagnostics. *Expert Rev Mol Diagn*. 2018;18:531-542.
34. Dharmadhikari AV, Ghosh R, Yuan BO, et al. Copy number variant and runs of homozygosity detection by microarrays enabled more precise molecular diagnoses in 11,020 clinical exome cases. *Genome Med*. 2019;11:30.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Perovic D, Damnjanovic T, Jekic B, et al. Chromosomal microarray in postnatal diagnosis of congenital anomalies and neurodevelopmental disorders in Serbian patients. *J Clin Lab Anal*. 2022;36:e24441. doi:10.1002/jcla.24441