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

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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
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. 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.

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. 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. 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.

Approximately 2% to 5% of children are born with major congenital malformations or express serious neurodevelopmental disorder during childhood. 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. 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, 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.

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. 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. 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.
All detected CNVs were analyzed and classified according to current guidelines. 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 (χ²) 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 x 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 2p15.33 encompassing MRLP36 and NDUF56 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 (247044640–248684909) × 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.
| 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 morbidity | 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 morbidity | CHD, ASD                                               |
| 6, 7    | 2p25.3             | dupl        | 404       | 2 | 1 yr; M     | LP         | MYT1L, intragenic del | DD, microcephaly                                       |
| 8       | 2q11.1–q11.2       | del         | 1240      | 1 | 1 yr; F     | LP mat    | 22 PK, 6 morbidity | 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 morbidity | DD, CHD, microcephaly, facial dysmorphism              |
| 14q24.1 |                   |             |           |   |             |            |                     |                                                        |
| 11, 12  | 2q34               | del         | 753       | 2 | 17 yr; F, 10 yr; M | LP dn     | ERBB4 first two exons | Siblings with profound ID, behavioral disorder, hyperactivity |
| 13      | 3q21.1–q29         | dupl        | 64280     | 1 | newborn     | P          | 362 PK, 85 morbidity | IUGR, CHD, cleft palate, dysmorphic features           |
| 14      | 4q21.22–q21.23     | del         | 2530      | 1 | 3 yr; F     | LP         | 18 PK, 4 morbidity | DD, mild facial dysmorphism                           |
| 15      | 4q34.1–q34.3       | del         | 5860      | 1 | 4 yr; F     | 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 morbidity | Mild ID, short stature, brachy- and clinodactyly, oligomenorrhoea, facial dysmorphia |
| 9p24.3–p24.1 | del     | 4590       |           |   |             |            |                     |                                                        |
| 18      | 6q14.3–q16.1       | dupl        | 8300      | 1 | 1 yr; M     | LP         | 32 PK, 7 morbidity | Craniosynostosis (trigonocephaly), DD, facial dysmorphia |
| 15q13.1–q13.12 | del   | 1570       |           |   |             | VUS        | 7 PK, 1 morbidity | dysmorphia                                            |
| 19      | 6q25.1–q27         | dupl        | 20,151    | 1 | 26 yr; F    | P          | 87 PK, 21 morbidity | Infertility, oligomenorrhoea, dysarthria, minor dysmorphisms |
| Xq25–q28 | dupl          | 25,469     |           |   |             |             | 122 PK, 28 morbidity |                                                        |
| Xq28    | del                | 1975       |           |   |             |             | 48 PK, 18 morbidity |                                                        |
| 20      | 7p22.3–p22.1       | del         | 6680      | 1 | 3 yr; M     | P          | 70 PK, 17 morbidity | DD, Epi, facial dysmorphism, hiatus hernia, intestinal perforation |
| 8p23.3–p23.1 | dupl     | 7530       |           |   |             |            | 40 PK, 3 morbidity |                                                        |
| 21      | 7q35–q36.3         | del         | 15,480    | 1 | 1 yr; M     | P          | 101 PK, 19 morbidity | IUGR, postnatal growth restriction, microcephaly, facial dysmorphia |
| 16q24.1–q24.3 | dupl     | 3370       |           |   |             | P          | 48 PK, 23 morbidity |                                                        |
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
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

| 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 syndrome                                         |
| 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                                       |
| 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 dysmorphism, seizures                               |
|         |                 |          | 802       | 2   | 2 yr; F     |           |           | DD, obesity, facial dysmorphism                                 |
| 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 dysmophia                   |
|         | 15q13.2–q13.3   | trip     | 1500      |     |             |           |           |                                                               |
| 15      | 15q13.2–q13.3   | del      | 1500      | 1   | 11 yr; F    | LP pat    | 612001    | DD/ID, ASD, facial dysmophia                                   |
| 16      | 15q26.2–q26.3   | del      | 7940      | 1   | 2 yr; F     | P         | 612616    | IUGR, CHD, VUR, facial dysmophia (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, dysphasis                                   |
| 20      | 16p11.2         | dupl     | 856       | 1   | 13 yr; F    | P         | 614671    | DD/ID, strabismus, facial dysmophia                            |
| 21      | 17q12           | del      | 1300      |     |             |           | 614527    |                                                               |
| 22      | 17q21.31        | del      | 442       | 1   | 11 yr; M    | P         | 610443    | Koolen de Vries syndrome                                       |
| 23      | 18q21.33–q23    | del      | 17,168    | 1   | 14 yr; M    | P         | 601808    | ID, facial dysmophia                                           |
| 24      | 19p13.2–p13.12  | dupl     | 2010      | 1   | 12 yr; F    | P         | 613638    | Microcephaly, short stature, CHD, borderline intelligence, facial dysmophia |
| 25–28   | 22q11.21        | del      | 2250      | 4   | M           | P         | 188400    | Di George/Velocardiofacial syndrome                            |
| 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 |
|         |                 |          |           |     |             |           |           |                                                               |
| 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) |

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.
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 (Cryopyrin-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...
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.\textsuperscript{26-28} Similarly to Catusi et al.,\textsuperscript{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\textsuperscript{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.\textsuperscript{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
One patient with similar deletion was described, and our variant is essential for neurological development. Until recently, only tors for neuregulin-1 that plays role in GABA-ergic circuit assembly have gonadal mosaicism. And none of the parents, suggesting that one of the parents could have proximal regulatory elements. Both siblings have the same variant, ERBB4 deletion includes the first two exons of the gene as well as proximal regulatory elements. By array-CGH, we detected 753 kb deletion of the 2q34 region. The variants were detected, noting that it was more than five years ago. Tests, WES was done in a laboratory elsewhere, and no causative 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, and our variant was characterized as likely pathogenic. In 2021, Hyder et al. 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

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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.

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**SUPPORTING INFORMATION**

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

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