Copy number variation analysis in 189 Romanian patients with global developmental delay/intellectual disability

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Abstract

Background: Developmental delay and intellectual disability represent a common pathology in general population, involving about 3% of the pediatric age population, the genetic etiology being often involved. The aim of this study was to determine the clinically relevant copy number variants in patients diagnosed with global developmental delay/intellectual disability in our population, using the chromosomal microarray analysis.

Methods: We analyzed 189 patients diagnosed with global developmental delay/intellectual disability, presented in Clinical Emergency Hospital for Children, Cluj-Napoca. The patients were completely clinically investigated, including dysmorphic and internal malformations evaluation, psychiatric, neuropsychological and metabolic evaluation, standard karyotyping. Genomic analysis was done using chromosomal microarray analysis.

Results: Pathogenic findings (including uniparental disomy) and variants of unknown significance were detected in 53 of 189 patients (28.04%). Pathogenic copy number variants and uniparental disomy were observed in 35 of 189 patients (18.51%). Two patients presented uniparental disomy for chromosome 15, one with clinical phenotype of Prader-Willi syndrome and the other with clinical phenotype with Angelman syndrome. Within the category of pathogenic findings, the recurrent copy number variants were seen in 21 of 35 patients (60%).

Conclusions: The increased percentage of pathogenic structural variants observed in patients with global developmental delay/intellectual disability analyzed by chromosomal microarray technique supports its use in patients with a non-specific phenotype such as these neurodevelopmental disorders. The high percentage of recurrent pathogenic variants between these findings is a finding that support their initial evaluation when a genetic testing algorithm could be a useful option.

Keywords: Global developmental delay, Intellectual disability, Chromosomal microarray analysis, Copy number variants, Etiology

Background

Developmental delay and intellectual disability represent a common pathology, affecting 1–3% of children, the etiology being represented by genetic factors in more than a half of these patients [1–3]. Global developmental delay (GDD) is a diagnosis reserved for a child under five years, being defined as a significant delay, under two standard deviations (SD), in two or
more developmental domains (gross or fine motor abilities, speech/language, cognition, social/personal and activities of daily living) [4]. Intellectual disability (ID) is a diagnosis established beginning with the age of five years, when the following three criteria are met simultaneously: defective intellectual function (usually measured by intellectual coefficient), defective adaptive function (conceptual, social, or practical skills) and onset of these deficits during the developmental period [5]. Not all the patients with GDD diagnosis will fulfill the criteria for ID diagnosis after the age of five years.

With advanced genomic technologies, as chromosomal microarray analysis (CMA) and exome/genome sequencing, the genetic etiology in GDD/ID is now identified in more than 50% of these patients [3, 5].

The G-bands karyotype identified numerical or structural chromosomal abnormalities in approximately 5% of GDD/ID patients (in some studies, up to 15% of cases) [5–8], 21 trisomy being the most frequently seen (in about 70% of these patients) [6, 8]. Recurrent microdeletions/microduplications (mainly involving 22q11.2, 7q11.23, 17p11.2, 15q11–13, 16p11.2, 1q21.1 and other regions) are observed in about 5% of cases, usually being identified by Fluorescent In Situ Hybridization (FISH), Multiplex Ligation-dependent Probe Amplification (MLPA) or quantitative Polymerase Chain Reaction (qPCR) techniques [8, 9]. A first-tier test in genetic investigations in GDD/ID is now represented by CMA, due to an important diagnostic yield, of 15–25% in patients with GDD/ID [10–13], preferred over G-bands karyotype, FISH, MLPA or qPCR techniques, due to a higher sensitivity and better genomic resolution for copy number variants (CNVs) detection [10].

Pathogenic single nucleotide variants (SNVs) or indels variants, in monogenic or oligogenic disorders, are identified by exome/genome sequencing in 15–30% of GDD/ID patients, tests usually performed after a negative CMA analysis [10, 12–15]. The other unexplained causes in GDD/ID patients could be related to environmental teratogens (including the fetal alcohol exposure, valproate exposure or infections), perinatal factors (prematurity, asphyxia, or other neonatal complications) or postnatal causes (as CNS infections, traumatisms, toxic, psychosocial environment).

The aim of this study was to determine the clinically relevant CNVs in Romanian children diagnosed with GDD/ID, using Single Nucleotide Polymorphism (SNP) array technology.

Methods
We analyzed 189 patients diagnosed with GDD/ID, presented in Clinical Emergency Hospital for Children Cluj-Napoca, between January 1st 2015 and July 1st 2017. The age of the patients was between 1 and 18 years. The inclusion criteria was the diagnosis of GDD or ID. An exclusion criteria was the presence of 21 trisomy confirmed by karyotype. GDD/ID diagnosis was based on the intelligence quotient evaluated by Wechsler Intelligence Scale for Children test (WISC-IV) and development quotient (for children younger than 6 years), evaluated by Portage test and A Developmental NEuroPSYchological Assessment test (NEPSY). The patients were completely clinically investigated, including dysmorphological evaluation, internal malformations evaluation, psychiatric and neuropsychological examinations, metabolic evaluation, standard karyotyping. Brain imaging and electroencephalogram (EEG) were indicated by the neurologist. Other investigations was performed depending on clinical indication of each patient.

The research was approved by Ethics Committee of Clinical Emergency Hospital for Children, Cluj-Napoca. Written informed consent was obtained from the parents of all the participants in the study.

High density SNP array analysis
The deoxyribonucleic acid (DNA) was purified by Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), using 3 ml peripheral blood, sample collected for each patient. Then, a SNP array analysis was done using Infinium OmniExpress-24 BeadChip array kit (Illumina, San Diego, CA, USA) and the platform iScan System (Illumina, San Diego, CA, USA). The SNP array kit allowed the analysis of about 700,000 markers. For bioinformatic analysis it was use the Genome Studio software version 2.0 (Illumina, San Diego, CA, USA). The interpretation of each CNV was done using the recommendations of American College of Medical Genetics [16, 17].

Results
The study group included 189 patients, 91 girls (48.14%) and 98 boys (51.85%), with an age between three and 18 years (Table 1). The average age was 11.17 years and 28 of 189 patients (14.81%) were five years old and under the age of five years (with the GDD diagnosis), the others 161 patients (85.19%) were older (with ID diagnosis). Pathogenic findings (including pathogenic CNVs and uniparental disomy - UPD) and variants of unknown significance (VOUS) were detected in 53 of 189 patients (28.04%). Pathogenic CNVs and UPD were observed in 35 of 189 patients (18.51%). Clinical characteristics are described in Table 1.

The pathogenic CNVs detected in our patients are described in Table 2. Two patients presented UPD for
chromosome 15, one with clinical phenotype of Prader-Willi syndrome and the other with clinical phenotype of Angelman syndrome (patients 76 and 78). Among pathogenic CNVs, 22 patients (66.7%) presented deletions and 11 patients (33.3%) had duplications.

Recurrent pathogenic CNVs were observed in 21 of 35 patients (60%) with pathogenic findings, thus: 15q11.2-q31.1 deletion (two patients), 4p16 deletion (two patients), 22q11.21 deletion (two patients), 22q11.21 duplication (one patient), 16p11.2 proximal deletion (two patients), 16p11.2 proximal duplication (one patient), 18p11 duplications (two patients), 18p11 deletion (one patient), 7p11.23 deletion (one patient), 5q35 deletion (one patient), 1q21 deletion (two patients), 1p36 deletion (one patient), 17p11.2 duplication (one patient), 17q21.31 deletion (one patient), Xp22.31 deletion (one patient) (Table 2). The clinical phenotype was suggestive for the etiological diagnosis in four of 189 patients (2.11%) and confirmed by SNP array analysis, thus: Wolf-Hirschhorn syndrome (4p16 deletion), Williams syndrome (7p11.23 deletion), Sotos syndrome (5q35 deletion) and Prader-Willi syndrome (15q11.2-q31.1 deletion). For most patients, the clinical phenotype was not suggestive for a particular etiology.

The patients observed with VOUS in our study group are described in Table 3.

Table 1  Clinical characteristics in patients with GDD/ID

| Clinical characteristics | n (%) |
|-------------------------|-------|
| Gender                  | 189 patients |
| Male                    | 98 (51%) |
| Female                  | 91 (48%) |
| Age                     | 189 patients |
| < or = 5 years          | 28 (14%) |
| > 5 years               | 161 (85%) |
| CNVs                    | 189 patients |
| Pathogenic              | 33 (17.4%) |
| Uniparental disomy      | 2 (1%) |
| VOUS                    | 18 (9.5%) |

Clinical features in patients with pathogenic CNVs

| Clinical features | n (%) |
|-------------------|-------|
| Dysmorphic features | 27 (81%) |
| Short stature     | 2 (6%) |
| Obesity           | 6 (18%) |
| GDD/ID            | 33 (100%) |
| Microcephaly      | 1 (3%) |
| Epilepsy          | 4 (12%) |
| Autism spectrum disorders | 1 (3%) |
| Hypotonia         | 1 (3%) |
| Language delay    | 3 (9%) |
| Associated internal malformation | 7 (21%) |

Discussions

In this study group of Romanian patients with GDD/ID we identified pathogenic CNVs, UPD or VOUS in 28% of patients. Pathogenic CNVs and UPD were seen in 18.5% of patients. The recurrent pathogenic CNVs were seen in 60% of patients with pathogenic findings (CNVs or UPD).

A similar percentage of pathogenic findings analyzing patients with GDD/ID was also seen in other studies [18–25], supporting the important diagnosis yield given by this analysis, indicated as first-tier test in GDD/ID [10]. A genomic approach for the patients with an unspecific phenotype such as isolated or syndromic GDD/ID is useful, in our research, the clinical etiological diagnosis was indicated in only 2% of cases, similar with other study [9]. Recurrent CNVs were identified in 60% of pathologic findings, the same percentage being observed by other study [26], these CNVs having in some cases a potential recognizable phenotype, even if quite variable in some patients, compared to the classical clinical picture. This could be an argument to continue giving an importance to the phenotype evaluation, which could bring a diagnosis in some patients, that can be confirmed more easily and less expensive by MLPA technique. The same recurrent CNVs seen in our study, described above, were also noted by other studies [26, 27]. Chromosome 18 was often involved in pathogenic CNVs, four patients presenting large deletion/duplication: 18q21.2-q23 duplication, 18p11.32-p11.21 duplication and 18p11.32-p11.21 deletion.

Some patients presented some very rare and particular CNVs, which will be described below. The patient 3, a 12-year-old boy with isolated GDD/ID, presented as a particularity a pathogenic 22q11.1-q11.21 duplication of 1.5 Mb (cat eye syndrome) associated to a pathogenic Xq27.1-q27.3 duplication of 7.4Mb duplication, the last one including more OMIM genes, SOX3 being a known morbid OMIM gene, coding for a transcription factor implicated in neurodevelopment, which is associated with X-linked intellectual disability and panhypopituitarism or growth hormone deficiency. These features were described for other patients in literature, our patient presenting isolated GDD/ID without endocrine or other features [28–31]. The patient 5, a 18-year-old girl with GDD/ID and dysmorphic signs, presented 29.4 Mb duplication of 1q41-1q44 region, which included 43 morbid OMIM genes (including ZBTB18), a similar CNV being described in other patients, most of them also presenting short stature or associated internal malformations [32–35], features not observed in our patient.

In patient 6, a 11-year-old boy with GDD/ID, epilepsy, autism spectrum disorder (ASD) and obesity was detected a 16p13.2-16p13.13 duplication (3.8 Mb),
Table 2  Pathogenic CNVs observed in our GDD/ID patients

| Patient | CNV (del/dup) | Chr       | Start (hg19) | Stop (hg19) | Size (Kb) | Known Genetic Syndrome | Patient phenotype |
|---------|---------------|-----------|--------------|-------------|-----------|------------------------|-------------------|
| 1       | Del           | 17q12     | 34,856,055   | 36,248,918  | 1392      | 17q12 deletion syndrome | GDD/ID, dysmorphic features, ataxia |
| 3       | Dup           | Xq27.1-q27.3 | 139,283,418 | 146,699,586 | 7416      | SOX3 deletion           | GDD/ID            |
| 5       | Dup           | 22q11.1-q1.21 | 17,397,498  | 18,984,519  | 1587      | Cat Eye syndrome        |                  |
| 6       | Dup           | 16p13.13-p13.2 | 8,226,775   | 12,071,213  | 3844      | 16p13.2 deletion syndrome | GDD/ID, ASD, epilepsy, obesity |
| 45      | Del           | 14q32.2   | 99,448,000   | 100,800,103 | 1352      | 14q32 deletion syndrome | GDD/ID, short stature, dysmorphic features |
| 55      | Del           | 5q35.2-5q35.3  | 175,346,223 | 177,484,097 | 2137      | Sotos syndrome          | Sotos syndrome, GDD/ID, dysmorphic features, language delay, obesity, CNS and renal malformation |
| 59      | Del           | 1q21.2–21.2 | 146,501,348  | 147,911,246 | 1409      | 1q21.1 deletion syndrome | GDD/ID, dysmorphic features |
| 61      | Dup           | 16p11.2   | 28,615,243   | 29,028,905  | 413       | 16p11.2 duplication syndrome | GDD/ID, dysmorphic features |
| 62      | Dup           | 18p11.32–11.21 | 112,535     | 14,791,236  | 18,678    | 18p Deletion syndrome   | GDD/ID, dysmorphic features |
| 66      | Dup           | 17p11.2   | 16,777,177   | 20,239,827  | 3462      | Potocki-Lupski syndrome | GDD/ID, dysmorphic features, obesity |
| 67      | Del           | 22q11.21  | 18,886,915   | 21,462,353  | 2575      | DiGeorge syndrome       | GDD/ID, obesity, dysmorphic features |
| 71      | Del           | 6q15q21   | 91,305,608   | 111,699,368 | 20,393    | 6q syndrome deletion    | GDD/ID, dysmorphic features |
| 90      | Del           | 4p16.1-p16.3 | 71,566      | 8,357,645   | 8268      | 4p deletion syndrome    | Wolf-Hirschhorn syndrome |
| 91      | Del           | 16p11.2   | 29,595,483   | 30,187,676  | 592       | 16p11.2 deletion syndrome | GDD/ID, language delay, dysmorphic syndrome, obesity |
| 106     | Del           | 9p24.3-p13.1 | 46,587      | 39,179,289  | 39,132    | 9p deletion syndrome    | GDD/ID, dysmorphic syndrome |
| 109     | Dup           | 16p24.3   | 89,542,695   | 89,656,251  | 113       | 16q24.3 deletion syndrome | GDD/ID, dysmorphic features |
| 117     | Del           | 18p11.32–11.31 | 13,034     | 4,390,081   | 4377      | 18p Deletion syndrome   | GDD/ID, dysmorphic features |
| 118     | Del           | 15q11.2-q31.1 | 23,656,946  | 28,535,266  | 4878      | Prader-Willi syndrome   | GDD, hypotonia     |
| 130     | Del           | 15q11.2-q31.1 | 23,656,946  | 28,535,266  | 4878      | Prader-Willi Syndrome   | GDD, hypotonia     |
| 136     | Del           | 1p36.33-1p36.32 | 82,154      | 8,321,782   | 3739      | 1p36 deletion syndrome  | GDD/ID, dysmorphic syndrome |
| 149     | Dup           | 16p11.2   | 29,595,483   | 30,187,676  | 620       | 16p11.2 duplication syndrome | GDD/ID, short stature, deafness |
| 150     | Del           | 7q11.23   | 73,110,603   | 73,702,525  | 592       | Williams syndrome       | GDD/ID, dysmorphic syndrome |
| 151     | Del           | Xp22.31   | 6,456,940    | 8,135,053   | 1678      | Xp22.3 microdeletion syn‑ drome | GDD/ID, dysmorphic syndrome |
| 153     | Del           | 7p15.3p21.1 | 18,814,931  | 23,539,546  | 4726      | Partial monosomy 7p     | GDD/ID, dysmorphic syndrome |
| 154     | Del           | 17q21.31  | 44,163,925   | 44,177,103  | 13        | 17q21.31 deletion syndrome (KANSL1 – exon 3) | GDD/ID, dysmorphic features, cardiac and genitourinary malformation |
| 156     | Dup           | 16p12.2-p11.2 | 21,610,804  | 30,198,151  | 8587      | 16p11.2–p12.2 deletion syndrome | GDD/ID, dysmorphic features |
| 157     | Dup           | 18p11.21–11.32 | 13,034     | 15,375,878  | 15,362    | 18p Deletion syndrome   | GDD/ID, epilepsy |
| 160     | Del           | 16p11.2   | 28,595,483   | 28,995,097  | 401       | 16p11.2 deletion syndrome | GDD/ID, dysmorphic features |
| 161     | Del           | 22q11.21  | 18,889,490   | 21,797,812  | 2908      | DiGeorge syndrome       | GDD/ID, dysmorphic features, cardiac and renal malformation |
| 165     | Del           | 1q21.1    | 145,394,955  | 145,755,813 | 360       | 1q21.1 deletion syndrome | GDD/ID, epilepsy, dysmorphic features, forearm agenesis |
| 166     | Del           | 4q22.2-4q24 | 94,543,233  | 107,486,817 | 12,943    | 4q deletion syndrome    | GDD/ID, dysmorphic syndrome, language delay |
| 184     | Del           | 4p16.2–16.3 | 48,283      | 5,405,805   | 5357      | 4p deletion syndrome    | GDD/ID, epilepsy, cardiac malformation, dysmorphic features |
| 189     | Dup           | 18q21.2–23 | 48,866,388  | 77,888,708  | 29,022    | 18q21q24 duplication    | GDD/ID, microcephaly, epilepsy, dysmorphic features |

CNV copy number variant, del deletion, dup duplication, chr chromosome, kb kilobase, GDD global developmental delay, ID intellectual disability, ASD autism spectrum disorder
including GRIN2A gene - known to be associated with epilepsy and GDD/ID - and also 16p13.2 region - known to be associated with 16p13.2 duplication syndrome - USP7 gene usually involving ASD and GDD/ID - these features were also described in our patients [27]. The pathogenic CNVs described in patient 45 – a 3-year-old girl with GDD/ID, short stature and dysmorphic features - is a 14q32.2 deletion (1.3 Mb), which included genes involved in ID, as YY1 gene, responsible of Gabriela de Vries syndrome [36], overlapping CNVs were described in Decipher patients (260,834, 291,402), with similar phenotypes as our patient, the cases with this CNV are very rare. 6q15-q21 deletion of 20.3 Mb seen in patient 71 is another rare CNV already noted in association with GDD/ID [37–39], including an important number of OMIM genes involved in neurodevelopment. In patient 153, presenting with GDD/ID and dysmorphic features, was observed the 7p15.3-p21.1 deletion (4.7 Mb), also described in association with ID [40], for this patient it is interesting that TWIST1 gene, associated with Saetre-Chotzen syndrome, is also included in this deletion, being responsible for dysmorphic features presented in our patient. The deletion in 4q22.2-q24 region in patient 166, who presents GDD/ID, dysmorphic features and language delay is also a very rare CNVs, it was described in patients with similar features [41, 42].

**Conclusion**

The pathogenic findings, as pathogenic CNVs or UPD, were observed in 18.5% patients, thus supporting the use of chromosomal microarray technique in patients with a non-specific phenotype such as GDD/ID. Recurrent CNVs were observed in 60% patients of those with pathogenic findings, as: 15q11.2-q31.1 deletion, 4p16 deletion, 22q11.21 deletion, 22q11.2 duplication, 16p11.2 deletion, 16p11.2 duplication, 18p11 duplications, 18p11 deletion, 7p11.23 deletion, 5q35 deletion, 1q21 deletion, 1p36 deletion, 17p11.2 duplication, 17q21.31 deletion, Xp22.31 deletion.

**Abbreviations**

GDD: Global developmental delay; SD: Standard deviation; ID: Intellectual disability; CMA: Chromosomal microarray analysis; FISH: Fluorescent in situ

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**Table 3** VOUS observed in our GDD/ID patients

| Patient | CNV/UPD | Chromosome | Start | Stop  | Size (Kb) | Major genes involved | Patient phenotype                  |
|---------|---------|------------|-------|-------|-----------|----------------------|-------------------------------------|
| 3       | Dup     | 3q26.1     | 161,577,780 | 166,471,417 | 4893       | BCHE, SI             | GDD/ID                              |
| 4       | Dup     | 4q28.2-4q28.3 | 130,609,436 | 138,430,265 | 7820       | PCDH10, PABPC4L      |                                     |
| 5       | Del     | 11q25      | 133,531,291 | 134,868,407 | 1337       | JAM3, ACAD8, NCAPD3  | GDD/ID, dysmorphic features         |
| 6        | Dup     | 17q21.33   | 48,263,589  | 48,607,252  | 344        | COL1A1, XYLTY2       | GDD/ID, autism spectrum disorder    |
| 62      | Dup     | 21p11.1    | 34,097,891  | 34,853,011  | 755        | IFNAR2, PARK20       | GDD/ID, dysmorphic features         |
| 65      | Dup     | 15q12      | 26,874,395  | 26,888,344  | 14         | GABR3                | GDD/ID                              |
| 68      | Del     | 10q21      | 68,107,483  | 68,150,124  | 42         | CTNNA3               | GDD/ID, dysmorphic features         |
| 84      | Del     | 12p12.1    | 23,836,212  | 23,840,513  | 4.3        | SOX5                 | GDD/ID, dysmorphic features, hypotonia |
| 85      | Del     | 1q34       | 237,584,925 | 237,597,163 | 12         | RYR2                 | GDD/ID, dysmorphic features, deafness, mitral insufficiency |
| 110     | Dup     | 16q22.1    | 70,513,384  | 70,519,783  | 6          | COG4                | GDD/ID                              |
| 123     | Del     | 19p13.11   | 33,882,222  | 33,893,008  | 10         | PEPD                 | GDD/ID, dysmorphic features, spastic paraplegia |
| 163     | Dup     | 3q27.1     | 184,010,230 | 184,038,969 | 28         | PARK18               | GDD/ID, autism spectrum disorder, language delay |
| 164     | Del     | 6p25.1     | 5,256,116   | 5,391,419   | 135        | FARS2, LYMR4         | GDD/ID, obesity, hypospadias, language delay |
| 173     | Dup     | 22q11.21   | 18,877,787  | 19,008,108  | 130        | DGCGR5, DGCGR6, DGCGR9, PRODH | GDD/ID, ataxia                       |
| 178     | Dup     | 22q11.21   | 18,895,227  | 19,008,108  | 112        | DGCGR5, DGCGR6, DGCGR9, PRODH | GDD/ID, dysmorphic features          |
| 183     | Del     | 10q22.3    | 79,313,729  | 79,331,919  | 18         | KCNMA1               | GDD/ID, West syndrome, ataxia       |
| 185     | Del     | 18q21.1    | 43,655,010  | 43,743,081  | 88         | ATP5A1               | GDD/ID, microcephaly, dysmorphic features, short stature |
| 186     | Del     | Xp11.4     | 38,230,704  | 38,246,882  | 16         | OTC                  | GDD/ID, obesity, cryptorchidism     |
| 188     | Del     | Xp11.4     | 38,235,792  | 38,256,737  | 20         | OTC                  | GDD/ID, dysmorphic features         |

CNV: copy number variant, UPD: uniparental disomy, kb: kilobase
hybridization; MLPA: Multiplex ligation-dependent probe amplification; qPCR: Quantitative polymerase chain reaction; CNVs: Copy number variants; SNVs: Single nucleotide variants; Indels: Insertion and/or deletion of nucleotides into genomic DNA (deoxyribonucleic acid), less than 1 kb in length; CNS: Central nervous system; SNP array: Single nucleotide polymorphism array; WISC-IV: Wechsler Intelligence Scale for Children; NEPSY: Developmental NEuroPSYcho-logical Assessment; EEG: Electroencephalogram; DNA: Deoxyribonucleic acid; UPD: Uniparental disomy; YOUS: Variant of unknown significance; Del: Deletion; Dup: Duplication; Chr: Chromosome; Kb: Kilobase; ASD: Autism spectrum disorder; Mb: Megabase.

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Authors’ contributions
DM (conceptualization, methodology, validation, investigation, manuscript writing, manuscript supervising), SO (methodology, validation, investigation, manuscript writing), SB (methodology, investigation), DS (methodology, investigation), MM (methodology, investigation), MP (methodology, validation, investigation), CZ (methodology, investigation), CAE (methodology, validation, investigation, manuscript supervising). All authors read and approved the final manuscript.

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Availability of data and materials
Relevant data generated or analyzed during this study are included in this published article. The other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All methods and genetic testing were carried out in accordance with the ethical standards on human experimentation, of the hospital committee and with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments. The research was approved by Ethic Committee of Clinical Emergency Hospital for Children, Cluj-Napoca. Written informed consent was obtained from the legal guardians of all the participants in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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