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Research Paper

Advancing Neuropediatric Rare Disease Diagnosis Through Clinical Genome Sequencing

Fabio Sirchia, MD [a,b,1], Silvia Kalantari, MD [b,1], Diana Carli, MD, PhD [c], Mariia Zadorozhna, PhD [a,d], Francesco Bassanese, MD [e], Erin Thorpe Venti, MS, CGC [f], Ryan J. Taft, PhD [f], Akanchha Kesari, PhD [g], Lorena Sorasio, MD [h], Vincenzo Antona, MD [i], Andrea Guala, MD [j,k], Agnese Feresin, MD [l], Anna Basile, MSc [a], Francesco Licciardi, MD [m], Jessica Garau, PhD [d], Paolo Gasparini, MD [l,n], Enrico Grosso, MD [o], Alessandro Mussa, MD, PhD [p,q], Giovanni Battista Ferrero, MD, PhD [r,s], Alfredo Brusco, PhD [o,t], Elisa Giorgio, PhD [a,d,*]

[a] Department of Molecular Medicine, University of Pavia, Pavia, Italy
[b] Medical Genetics Unit, IRCCS San Matteo Foundation, Pavia, Italy
[c] Department of Medical Sciences, University of Turin, Turin, Italy
[d] Neurogenetics Research Centre, IRCCS Mondino Foundation, Pavia, Italy
[e] Neonatal and Pediatric Unit, P.O Oltrepò - ASST Pavia, Pavia, Italy
[f] Genetic Alliance, Damascus, Maryland
[g] Illumina Inc, San Diego, California
[h] Pediatrics Unit, Santa Croce e Carle Hospital, Cuneo, Italy
[i] Department of Health Promotion, Mother and Child Care, Internal Medicine and Medical Specialties "G. D'Alessandro", University of Palermo, Palermo, Italy
[j] A.B.C. Associazione Bambini Cri du Chat Scientific Committee, Firenze, Italy
[k] Department of Pediatrics, Castelli Hospital, Verbania, Italy
[l] Department of Medicine, Surgery and Health Sciences, University of Trieste, Trieste, Italy
[m] Immuno-Reumatologia, Pediatria Specialistica Universitaria, Ospedale Infantile Regina Margherita, Torino, Italy
[n] Institute for Maternal and Child Health, IRCCS "Burlo Garofolo", Trieste, Italy
[o] Unit of Medical Genetics, Città della Salute e della Scienza University Hospital, Turin, Italy
[p] Department of Public Health and Pediatric Sciences, University of Turin, Turin, Italy
[q] Pediatric Genetics Unit, Regina Margherita Children Hospital, Turin, Italy
[r] Department of Clinical and Biological Sciences, University of Torino, Orbassano, Italy
[s] Medical Genetics Unit and Thalassemia Center, San Luigi University Hospital, Orbassano, Italy
[t] Department of Neurosciences Rita Levi-Montalcini, University of Turin, Turin, Italy

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ABSTRACT

Background: Many patients with rare genetic diseases remain undiagnosed or receive a molecular diagnosis only after years. In this study, we want to evaluate the usefulness of clinical genome sequencing (cGS) in a cohort of complex neuropediatric patients with undiagnosed rare genetic diseases.
Methods: Between 2018 and 2022, our Medical Genetics Units in Torino, Trieste and Pavia partnered with the iHope program, a philanthropic initiative by Illumina Inc., with the aim of offering family-based cGS within the Italian National Health Service (Servizio Sanitario Nazionale) diagnostic process. A multidisciplinary team of pediatricians, clinical geneticists, and molecular biologists selected 64 cases. Inclusion criteria consisted of suspicion of an ultra-rare monogenic disease and at least one negative result from a first-tier genetic test.
Results: A definitive molecular diagnosis was achieved in 57.8% of the patients. All patients and families underwent clinical re-evaluation to assess the diagnostic relevance of the laboratory findings, which led us to reclassify 10 variants of unknown significance as responsible for the probands' phenotypes.

* Communications should be addressed to: Prof. Giorgio; Department of Molecular Medicine; University of Pavia; Via Forlanini 14; 27100 Pavia, Italy.
E-mail address: elisa.giorgio@unipv.it (E. Giorgio).
[1] These authors contributed equally to the paper.

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Diagnoses impacted patients' management, enabling palliative care referrals, avoiding unnecessary invasive tests, and guiding follow-up treatments.
Conclusions: Our study confirms that the use of cGS in a rare disease setting increased the diagnostic yield even in complex cases where other methods had previously failed. We speculate that introducing cGS as first-tier test within the Italian Servizio Sanitario Nazionale might offer both diagnostic and economic advantages.

Introduction

Rare genetic diseases (RGDs) impact an estimated 3.5-8% of the global population, accounting for approximately 273-624 million individuals affected worldwide.[1,2] These conditions are a significant cause of hospitalization and mortality among infants in neonatal intensive care units.[3] Of note, about 90% of RDGs affecting children have major neurological effects. Patients with RGDs frequently undergo a prolonged and complex "diagnostic odyssey"—a process that involves multiple clinical evaluations, imaging studies, and laboratory tests. This often delays or complicates appropriate clinical management. Reducing the length of this odyssey could yield substantial clinical, psychosocial, and economic benefits, both for patients and national health services.[4-6]

When a RGD is suspected, a stepwise genetic testing approach is commonly followed. This typically starts with a conventional karyotype or chromosomal microarray and progresses through targeted next-generation sequencing (NGS) gene panels, clinical exome sequencing (CES), or complete exome sequencing (ES). However, this approach can be costly and time consuming, and it leads to a molecular diagnosis in less than half of all cases across various patient cohorts.[7-11]

Due to advancements in sequencing technology and decreasing costs, genome sequencing (GS) has recently become a viable option in clinical settings, offering several distinct advantages. Unlike ES, which covers only 1-2% of the genome, GS spans approximately 90% of the genome,[12] enabling the identification of additional variant types including variants in noncoding regions. GS also eliminates the need of an exon-capturing step before sequencing, thereby reducing the "wet laboratory" phase and providing a more uniform coverage of the genome.[13,14] In addition, GS is highly effective in detecting copy number variants (CNVs), with a sensitivity comparable to, if not greater than, chromosomal microarray analysis.[7,12,15-17] Furthermore, GS can assess mitochondrial DNA, balanced chromosomal rearrangements, and uniparental disomies (UPDs), reducing the need for separate analyses like mitochondrial DNA testing, conventional karyotyping, and SNP-arrays.[18] In clinical settings, GS has achieved high detection rates for clinically relevant variants, ranging from 11% to ~70% across various patient subgroups.[7,11,19-24]

Despite its advantages, the clinical adoption of GS faces many challenges, including substantial data storage requirements, and increased analytic demands due to the huge number of variants identified per genome.[12] These challenges can be mitigated by focusing *in silico* bioinformatics analyses on the coding regions and exon-intron junctions of known disease-related genes (clinical genome sequencing [cGS]).

The Italian National Health Service (Servizio Sanitario Nazionale [SSN]) provides universal health care access to Italian citizens and is funded through taxation. However, this system faces increasing pressure from a rapidly aging population, the growing prevalence of chronic diseases, rising health care demand, and the aftermath of COVID-19 pandemic. These challenges have highlighted the urgent need for resource optimization.[25] Given these challenges, it is of paramount importance that diagnostic genetic testing offered through the Italian SSN is cost-effective.

We believe that diagnostic cGS should be implemented in routine clinical care due to its high diagnostic rate and increasing cost-effectiveness.[26] In this study, we present the experience of three Italian centers that, in collaboration with the iHope Program, provided family-based cGS to complex pediatric patients with undiagnosed RGDs as part of the Italian SSN diagnostic process.[27]

Materials and Methods

Patients

cGS tests were provided to 64 patients and their families followed in pediatric subspecialty clinics at Città della Salute e della Scienza University Hospital in Turin, the Institute for Maternal and Child Health, IRCCS "Burlo Garofolo" in Trieste, and Policlinico San Matteo Fondazione IRCCS in Pavia over a 4-year period (January 2018 to May 2022). Patients were selected by a multidisciplinary team of pediatricians, clinical geneticists, and molecular biologists. Patients were eligible to participate if the following criteria were met: (1) signs and symptoms consistent with a RGD without an etiological diagnosis; (2) negative first-tier genetic tests, including routine array-Comparative genomic hybridization (array-CGH; 60-K Agilent) or specific gene-disease panels; (3) available DNA from the trio (patient, mother, and father); (4) no health insurance or private funding to cover cGS costs (Fig 1). Patients and families were offered the option of learning about secondary findings as defined by the American College of Medical Genetics and Genomics (ACMG) at the time of testing.[28] Informed written consent was obtained for each participant. The study was approved by the Ethical Committee of Azienda Ospedaliera Universitaria Città della Salute e della Scienza di Torino (Torino, Italy) (AM IRB Torino 256/2022 approved 17/06/2022 prot 68301). Testing was provided at no-cost to the families through the iHope program.

To assess cost-effectiveness of cGS as first-tier test, total costs of investigations were calculated based on the Italian Health System tabs (https://www.istituto-besta.it/documents/447318/0/Tariffario+esami+laboratorio+2021_18.03.2022.pdf/c3c95a95-e33a-e28f-eb99-4b62d36c96c0 for costs in the 2018-2022 period; https://www.trovanorme.salute.gov.it/norme/renderNormsanPdf?anno=2024&codLeg=104476&parte=1%20&serie=null for actual costs). Total costs were considered, and minimum, maximum and average costs were calculated.

Clinical genome sequencing (cGS)

Clinical GS of trios was performed by the Illumina Clinical Services Laboratory in San Diego, California, as previously described.[27] In brief, cGS was performed on DNA extracted from whole blood. A polymerase chain reaction (PCR)-free library preparation protocol was utilized, and samples were sequenced with paired-end 150 base pair reads to an average of ≥30-fold

coverage. Variants' calling and annotation were performed exploiting the most updated version of NorthStar tool and Nirvana annotator. Variants were prioritized via the laboratory's proprietary pipeline (v8.10.12; see supplementary materials for further details) and reported according to build 37.1 of the Human Reference Genome (http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/human/). Prioritization of variants included interrogation of single nucleotide variants (SNVs), small insertions and deletions (indels), and CNVs for all samples. B-allele frequency analysis was employed to detect mosaic CNVs and UPDs, as previously described.[16] Clinically relevant SNVs and indels were confirmed by Sanger sequencing; structural variants and UPDs were confirmed by SNP array, quantitative real-time-PCR (qRT-PCR), and karyotype experiments. Mitochondrial SNVs at an allele fraction greater than or equal to 3% were interpreted. From November 2019 forward, short tandem repeat expansions were assessed for a defined set of genes.[27]

Evaluation of the phase of the two CAPN3 variants (cis or trans) by TA-cloning

A 2,506 bp region containing both *CAPN3* variants was amplified using KAPA2G Fast HotStart DNA Polymerase (Sigma-Aldrich, Shanghai, China) from the patient's DNA (primers: Fw 5′-TGTGTTAGTCCTGGGGTCTTCC-3'; Rv 5′-TGGGTCACTGGGTATTGAGG-3′). The PCR product was cloned into a TA-plasmid (pGEM-T Easy, Promega) and transformed into Escherichia coli JM109 cells (Promega). White colonies (n = 30) were cultured in Lysogeny broth medium with 100 mg/mL ampicillin, extracted (ZymoPURE II Plasmid MidiPrep Kit, Zymo Research) and screened by Sanger sequencing. Phasing was determined by analyzing whether variants occurred in the same or distinct clones.

Outcomes and analyses

Diagnostic yield was assessed as the number of patients with positive test results out of the total number of patients undergoing the test. We consider a positive test an analysis identifying a likely pathogenic or pathogenic variant(s) in a disease-associated gene(s) consistent with the clinical presentation and expected disease inheritance pattern. The cohort was assessed overall, and in two groups distinguished by prior genetic testing. Cohort 1 includes patients who did not have ES before cGS testing and cohort 2 includes patients with prior ES.

Results

Cohort overview

We recruited 64 unrelated cases (34 males and 30 females) from pediatric subspecialty clinics in Turin, Trieste, and Pavia over a 4-year period (Figs 1 and 2B). The mean proband age at the time of enrollment was 6 years and 8 months (range: one month to 17 years). All probands had a previously negative chromosomal microarray test. Additional negative genetic testing pursued before cGS included karyotype analysis (n = 20), NGS targeted gene sequencing panels (n = 7), single-gene sequencing (n = 5), *FMR1* triplet repeat expansion testing (n = 4), and methylation-specific multiplex ligation-dependent probe amplification analysis (n = 3) (Table 1). For 53 families, no further genetic testing was performed (cohort 1; Table 2). Clinical exome sequencing (cES) was conducted for four patients, while exome sequencing (ES) had previously been performed for seven patients, all of whom had received negative diagnostic results (11/64; cohort 2; Table 3). The total costs of prior diagnostic testing in our cohort were calculated using both the Italian National Health System reimbursement tariffs applicable during the 2018–2022 period and the current tariffs (Supplementary Table 1). Using the 2018-2022 tariffs, the mean overall cost was €2,866.17 ± 2,074.90, whereas recalculation with current tariffs yielded a lower mean cost of €1,705.78 ± 967.06. As expected, costs were substantially higher in cohort 2, with a mean of €6,798.68 ± 1,804.46, compared with €2,049.98 ± 1,126.28 in cohort 1 (2018-2022 costs; Supplementary Table 1).

Trio cGS was performed for all cases except one (patient 21), where duo cGS analysis was conducted due to confirmed non-paternity. Most patients (70.3%; 45/64) presented with syndromic developmental delay. Nine patients were neonates with dysmorphisms, neurological symptoms, and malformations; four cases exhibited a neuromuscular phenotype; two had syndromic overgrowth conditions; three patients presented with skeletal anomalies; and one patient exhibited an epileptic encephalopathy phenotype (Fig 2A). Clinical GS results were consistently delivered within 3 months after the request. For urgent clinical cases, such as oncological follow-up or rapidly deteriorating conditions, results were expedited and available within 2 weeks.

A positive test was reached in 37 cases, resulting in an overall diagnostic yield of 57.8% (37/64) (Fig 2C). The two most frequent patterns of inheritance in our cohort are autosomal dominant (19/37 patients; 51.4%) and autosomal recessive (9/37; 24.3%) (Fig 2D). All autosomal dominant variants, except for one, occurred *de novo* (Tables 2 and 3).

In case 41 (Tables 1 and 3), cGS revealed two heterozygous variants in the *CAPN3*, namely a maternally inherited silent variant affecting the last nucleotide of exon 11 (c.1524 G > A; NM_000070.2) and a *de novo* splicing variant affecting the acceptor splice site of exon 11 (c.1194-2A > G; NM_000070.2) (Table 3). The TA-cloning analysis allowed us to define the phase of the two variants and to confirm the diagnosis: the variant c.1194-2A > G was found to have arisen *de novo* on the paternal allele of the *CAPN3* gene and was confirmed to be *in trans* with the variant of maternal origin (c.1524 G > A).

A total of 22 variants identified in 16 cases, initially classified as variants of unknown significance (VUS) by the Illumina laboratory, were reclassified based on subsequent analyses as either likely pathogenic (n = 10) or likely benign (n = 12) (Table 4). Reclassification was achieved by reverse phenotyping and/or family segregation studies of the variants. In 2/16 cases (cases 27 and 57; Tables 2 and 4), *in vitro* functional studies performed on patients' RNA or placenta samples allowed to support the pathogenicity of the identified variants and to further corroborate cGS results.[29,30] Reclassification of VUS to likely pathogenic provided a definitive diagnosis in 3/16 cases, while enabling a double diagnosis in 2/16 cases (Table 4). Conversely, reclassification to likely benign excluded a genetic diagnosis in 5/16 patients (Table 4).

In one case (patient 8), cGS identified maternal UPD of chromosome 1, which was not diagnostic *per se*, but it raised suspicion of an autosomal recessive condition caused by an unidentified variant in a gene located on chromosome 1. The maternal UPD was reported to the family as variant of uncertain significance (Fig 2C).

The diagnostic rate was higher in cohort 1 (Table 2), which included 53 patients who had not undergone prior cES or ES testing, reaching 62.3% (33/53) (Fig 2E). Among the 11 patients who had previously undergone cES or ES with negative results (cohort 2, Table 3), cGS provided a definitive diagnosis in four cases, resulting in a diagnostic rate of 36.4% (4/11) (Fig 2F). Of these four positive diagnoses, three (patients 9, 30, and 41) could theoretically have been achieved through cES or ES but were missed. Here, the lack of diagnosis was primarily related to challenges in variant interpretation, especially for synonymous and splice-altering variant, and

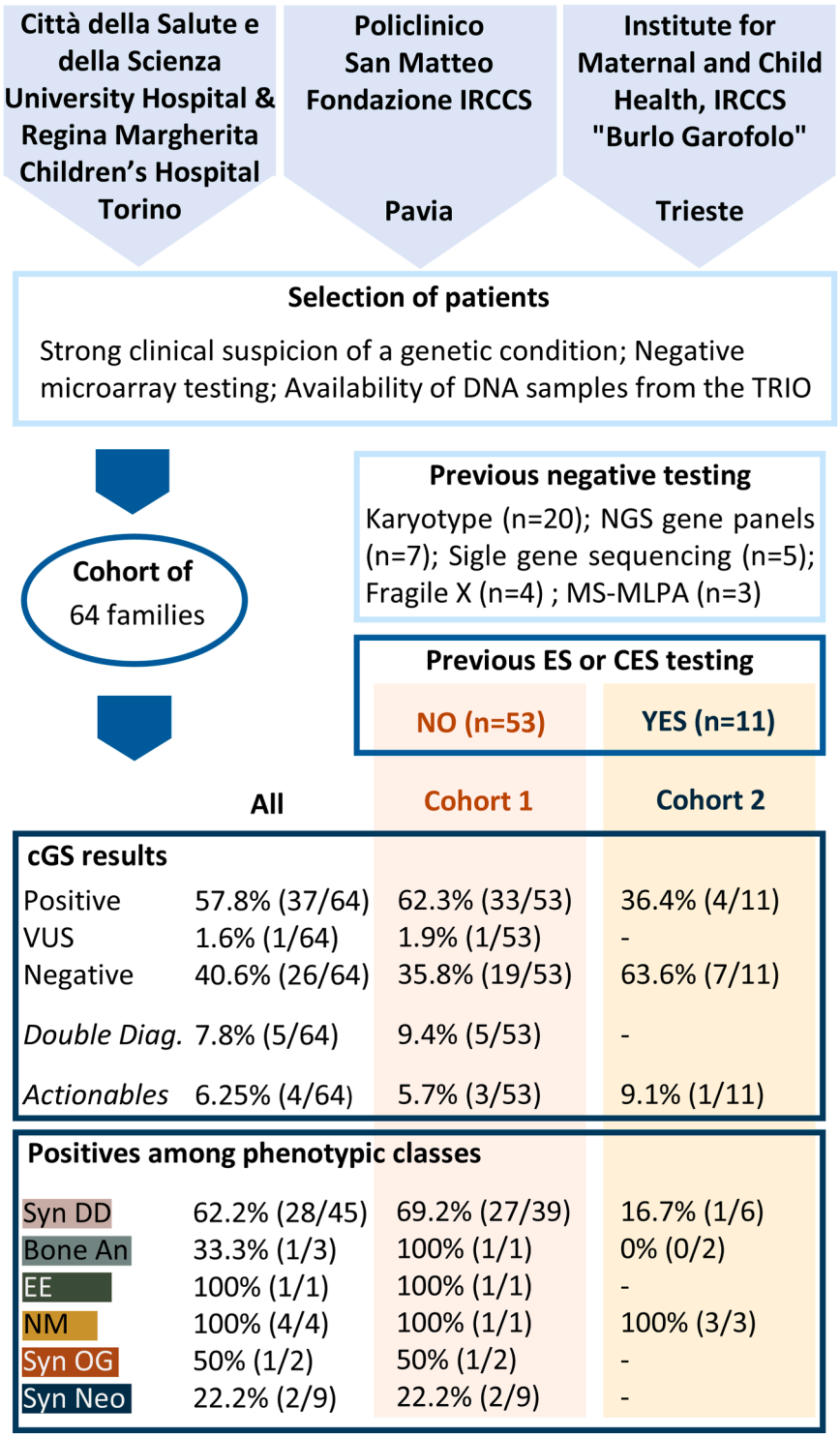

FIGURE 1. Flowchart of patient selection and cGS results. Patient selection process and summary of the clinical genome sequencing (cGS) findings is reported. Bone An = bone abnormalities; CES = clinical exome sequencing; cGS = clinical Genome Sequencing; Double Diag = double genetic diagnosis; EE = epileptic encephalopathy; ES = exome sequencing; MS-MLPA = methylation-specific multiplex ligation-dependent probe amplification; NGS = next-generation sequencing; NM = neuromuscular disorder; Syn DD = syndromic developmental delay; Syn Neo = syndromic presentation in a neonate without developmental delay; Syn OG = syndromic overgrowth; VUS = variant of uncertain significance. The color version of this figure is available in the online edition.

incomplete segregation analyses, highlighting nontechnical limitations of exome-based diagnostics. In contrast, the pathogenic variant in the small nuclear RNA gene *RNU4ATAC* identified in patient 63 was detectable exclusively through cGS.

A double genetic diagnosis was reached in five patients (7.8%) (Tables 2 and 4; Fig 2D), while in six cases, a second diagnosis was excluded by reclassification of a VUS variant to likely benign based on clinical evaluation and family segregation studies (Table 4).

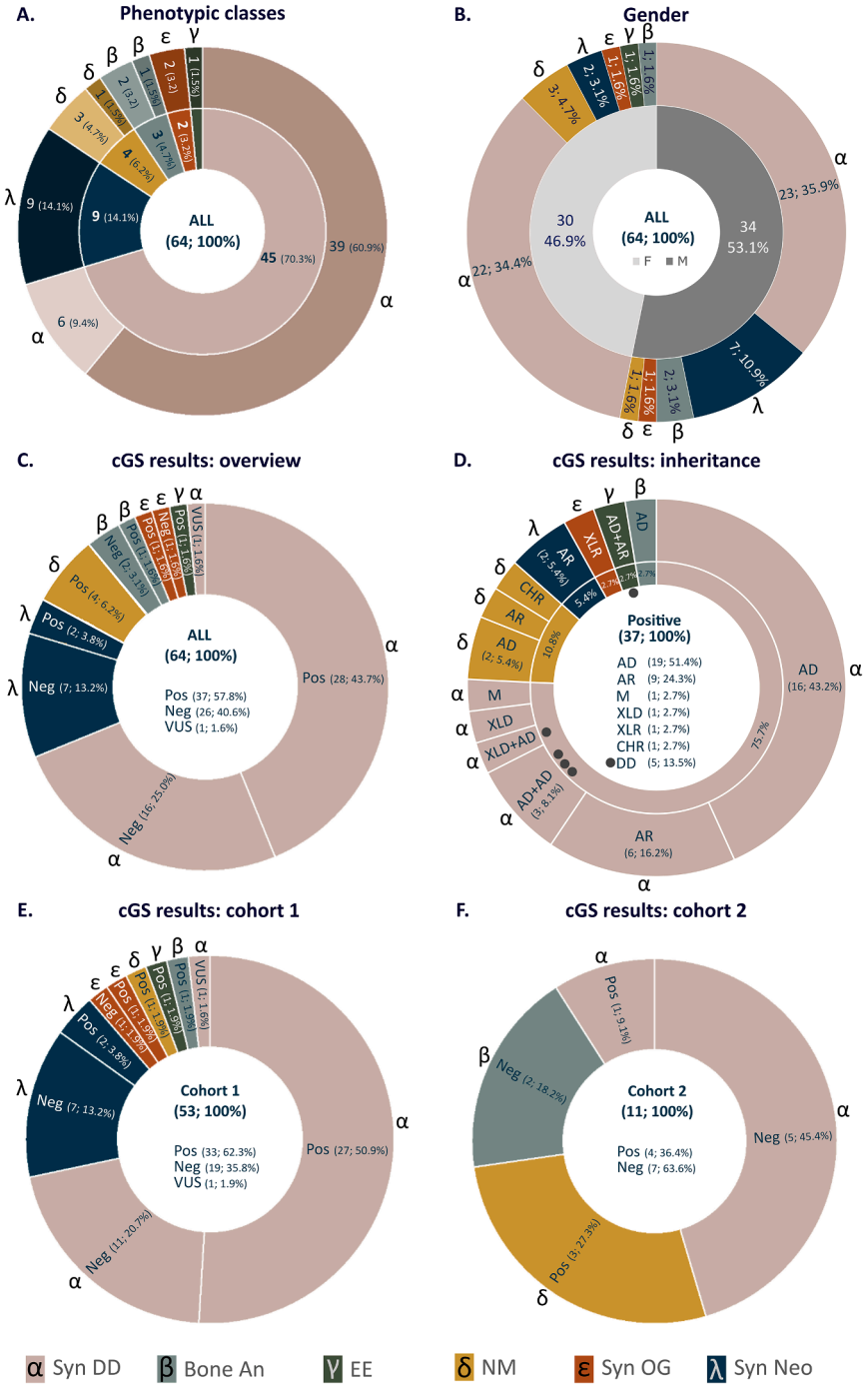

FIGURE 2. Summaries and statistics. Total numbers and corresponding percentages are reported for each category. Phenotypic classes are coded by both color and Greek letters. Syn DD, syndromic developmental delay (α); Bone An, bone abnormalities (β); EE, epileptic encephalopathy (γ); NM, neuromuscular disorder (δ); Syn OG, syndromic overgrowth (ε); Syn Neo, syndromic presentation in a neonate without developmental delay (λ). (A) The patient cohort (n = 64, 100%) is subdivided into the six phenotypic classes (inner circle, α–λ), with each segment representing a specific category. The outer circle further divides patients within each phenotypic class into two subgroups: cohort 1 (darker shades) and cohort 2 (lighter shades). (B) Gender distribution of the total cohort is shown. The inner ring represents the cohort subdivided by gender: female (F, light gray) and male (M, dark gray). The outer ring further categorizes patients within each gender group by phenotypic class (α–λ). (C) Overview of clinical genome sequencing (cGS) results across all phenotypic classes (α–λ). Results are classified as positive (Pos), negative (Neg), or variant of unknown significance (VUS). (D) Patients with a positive genetic diagnosis are represented by phenotypic class (α–λ) and inheritance pattern (outer circle). Dots indicate patients with double genetic diagnoses. AD, autosomal dominant; AR, autosomal recessive; M, mitochondrial; XLD, X-linked dominant; XLR, X-linked recessive; CHR: chromosomal anomaly; DD: double genetic diagnosis. (E and F) Results for cohort 1 (n = 53) and cohort 2 (n = 11) are reported across phenotypic classes (α–λ) and categorized as positive (Pos), negative (Neg), or variant of unknown significance (VUS), respectively.

TABLE 1.
Overview of Patient Cohort Selected for Clinical GS Analysis

Patient	Age	Years/ Months	Gender	Phenotype	Macrocategory	Previous Analysis	Family	Cohort
1	6.8	6 years, 10 months	F	Severe hypotonia, DD, muscular hypotrophy, epilepsy, hypoplasia of the corpus callosum and Arnold-Chiari malformation type 1, dysmorphic features	Syndromic developmental delay	Array-CGH	Trio	1
2	14.1	14 years, 2 months	M	Intellectual disability, cryptorchidism, strabismus, epilepsy, mild hypotonia, mild ataxia, dysmorphic features	Syndromic developmental delay	Array-CGH both on blood and skin samples	Trio	1
3	4.6	4 years, 7 months	M	Mild hypotonia, frequent respiratory infections, severe DD, microcephaly, dysmorphic features	Syndromic developmental delay	Array-CGH, NGS panel for Kabuki Syndrome, NGS panel intellectual disability	Trio	1
4	9.2	9 years, 2 months	F	Severe DD, severe ID, generalized epilepsy, microcephaly, dysmorphic features	Syndromic developmental delay	Array-CGH	Trio	1
5	3.1	3 years, 1 months	M	Global DD, hypoplasia of the corpus callosum, dysmorphic features	Syndromic developmental delay	Array-CGH	Trio	1
6	7.3	7 years, 4 months	M	Severe pre and postnatal microcephaly, bilateral cryptorhidism, ID, dysmorphic features	Syndromic developmental delay	Array-CGH, ES both on blood and skin samples	Trio	2
7	2.4	2 years, 5 months	F	ID, DD, dysmorphic features, alteration of ocular motility, nystagmus, clinodactyly of the IV and V toes	Syndromic developmental delay	Karyotype, array-CGH	Trio	1
8	8.0	8 yo	M	DD, severe speech delay, mild ID, congenital scoliosis, tethered cord, pectus carinatum, leg-lenght asymmetry, bilateral cryptorchidism, dysmorphic features, mild muscular dystrophy	Neuromuscular disorder	Array-CGH, *FMR1*	Trio	1
9	9.8	9 years, 10 months	F	Severe progressive Meuromuscular disorder, high CPK levels	Neuromuscular disorder	Array-CGH, cES	Trio	2
10	0.6	7 months	M	Normal development during the first 3 months of file, then progressive severe hypotonia, epilepsy, renal tubulopathy, corneal opacity, sensorineural deafness, bell-shaped thorax, high concentrations of spinal fluid proteins	Syndromic developmental delay	Array-CGH	Trio	1
11	14.1	14 years, 1 months	M	Severe DD and ID, epilepsy, Hirschprung disease, scoliosis, pectus carinatum, camptodactyly, nail hypoplasia, thin corpus callosum, frontotemporal atrophy, dysmorphic features	Syndromic developmental delay	Array-CGH	Trio	1
12	3.8	3 years, 9 months	M	Severe progressive microcephaly, ID, DD, lack of speech, dysmorphic features	Syndromic developmental delay	SNP-Array, *UBE3A* single gene sequencing	Trio	1
13	3.6	3 years, 8 months	M	Hypotonia, speech delay, mild ID, hearing loss, Leigh-like cerebral anomalies, hepatic steatosism metabolic acidosis, dysmorphic features	Syndromic developmental delay	Array-CGH	Trio	1
14	11.5	14 years, 11.5 months	M	Bilateral cryptorchidism, DD, mild/moderate ID, dysmorphic features	Syndromic developmental delay	Karyotype, SNP-Array, *FMR1*	Trio	1
15	6.3	6 years, 4 months	F	ID, choanal atresia, nasopharyngeal lipoma, bilateral sensorinueral hearing loss, bilateral ear fistulas, finger pads, elongated fingers, aplasia/hypoplasia of nipples, dysmorphic features	Syndromic developmental delay	Karyotype, SNP-Array, *CHD7* single gene sequencing	Trio	1
16	2.7	2 years, 9 months	M	DD, sensoneural hearing loss, delayed bone age, dysmorphic features	Syndromic developmental delay	Karyotype, SNP-Array	Trio	1
17	3.2	3 years, 3 months	M	Hypotonia, DD, blue sclerae, proptosis, midface hypoplasia, ear anomalies, atrial septal defect, rhizomelia, delayed bone age, hypertrichosis, hepatosplenomagaly	Syndromic developmental delay	SNP-Array	Trio	1
18	5.8	5 years, 10 months	M	Bilateral sensorineural deafness, neutropenia, postnatal overgrowth	Syndromic overgrowth	Array-CGH, *PTPN11*, *WNT5A*, *DVL1*, *GPC3*, *GPC4;* NGS panel for RASopathies	Trio	1
19	4.4	4 years, 5 months	M	DD, language delay, severe dysphagia, microcephaly, dysmorphic features	Syndromic developmental delay	SNP-Array	Trio	1
20	0.4	4 months	M	Severe hypotonia, blindness, ophtalmoplegia, MRI abnormalities, dysmorphic features	Syndromic presentation in a neonate	Array-CGH; Congenital Myasthenic syndromes gene panel; *DMPK* CTG triplet repeats.	Trio	1
21	2.5	2 years, 6 months	M	Microcephaly, facial asymmetry, arachnodactyly, clenched in fists, hyporeactive to external stimuli, vocal cord paresis, MRI anomalies, dysmorphic features	Syndromic developmental delay	Karyotype, array-CGH	Duo	1
22	9.9	9 years, 11 months	F	Severe DD and regression, sensory processing disorder, very high pain threshold, stereotypic movements, prognathism, epicanthus, acute lymphoblastic leukemia	Syndromic developmental delay	SNP-Array	Trio	1

(*continued on next page*)

TABLE 1 (*continued*)

Patient	Age	Years/ Months	Gender	Phenotype	Macrocategory	Previous Analysis	Family	Cohort
23	11.4	11 years, 5 months	F	Distal renal tubular acidosis, conductive hearing loss, microcephaly, ID, severe astigmatism, toes brachysyndactyly, heart anomalies (interatrial aneurysm, midbasal hypokinesia of the interventricular septum and hypertrabeculation of apical and mid-left ventricle segments)	Syndromic developmental delay	Array-CGH, ES	Trio	2
24	10.4	10 years, 5 months	F	Bilateral cleft palate, bilateral congenital varus metatarsal, global DD and severe ID, microcephaly, small corpus callosum, dysmorphic features	Syndromic developmental delay	Karyotype, array-CGH	Trio	1
25	9.6	9 years, 7 months	F	DD, ID, ADHD, brachydactyly, dysmorphic features	Syndromic developmental delay	Karyotype, SNP-Array	Trio	1
26	5.9	5 years, 11 months	F	ID, DD, growth delay, short stature, hyposthenia, microcephaly, thin corpus callosum, diffuse cortical atrophy, kyphosis, dysmorphic features	Syndromic developmental delay	Karyotype, array-CGH	Trio	1
27	0.3	3 months	M	Hypotonia, severe respiratory distress, dysmorphic features	Syndromic presentation in a neonate	Karyotype, array-CGH, *SMN1* gene sequencing	Trio	1
28	2.8	2 years, 9 months	F	Severe global DD, hypotonia, chronic neutropenia, growth delay, pectus excavatum, joint hypermobility, patent foramen ovale, MRI anomalies	Syndromic developmental delay	Array-CGH	Trio	1
29	14.1	14 years, 1 months	M	Severe ID, DD, autism, lymphedema, cryptorchidism, hyperlaxity, dysmorphic features and bilateral club feet	Syndromic developmental delay	Karyotype, Array-CGH, MS-MLPA in 15q11.2-q13	Trio	1
30	2.5	2 years, 6 months	F	Motor delay, Meuromuscular disorder and normal cognitive development	Neuromuscular disorder	Array-CGH, cES	Trio	2
31	8.3	8 years, 3 months	F	Severe ID, diaphragmatic hernia, dysmorphic features	Syndromic developmental delay	Karyotype, array-CGH	Trio	1
32	2.2	2 years, 3 months	F	Fetal US: IUGR, Dandy-Walker malformation and bilateral talipes. At birth: bilateral talipes, scoliosis and soft palate cleft. Severe global DD (no walking, no language at 26 months), chronic respiratory failure, patent ductus arteriosus, dysmorphic features	Syndromic developmental delay	Karyotype, array-CGH	Trio	1
33	13.0	12 years, 12 months	M	Growth delay, congenital hip dysplasia, severe congenital bilateral glaucoma, delayed bone age, small ears, short stature, joint laxity	Bones abnormalities	Array-CGH, ES	Trio	2
34	0.2	3 months	M	Limited opening of the mouth, poor sucking and swallowing, cervical syringomielia, transiet apneas, transient hypotonia/hypertonia, dysmorphic features	Syndromic presentation in a neonate	Array-CGH	Trio	1
35	0.1	1 month	F	Congenital bilateral cataract, hypoacusia, dysmorphic features	Syndromic presentation in a neonate	Prenatal array-CGH	Trio	1
36	14.3	14 years, 3 months	M	Short stature, global DD, growth delay, sacral dimple at birth, tetralogy of Fallot, renal asymmetry, hypernasality, brachydactyly with digit clubbing, dysmorphic features	Syndromic developmental delay	Array-CGH	Trio	1
37	0.9	11 months	M	MRI anomalies (ventriculomegaly, bilateral cystic periventricular leukomalacia with some anteromedian pseudocysts), hepatosplenomegaly, hypocacemia, immunodeficiency (chronic neutropenia, lymphopenia), hemophagocytosis, hypotonia, regression with loss of psychomotor functions, ligamentous hyperlaxity, dysmorphic features	Syndromic presentation in a neonate	SNP-Array	Trio	1
38	0.1	1 month	M	Severe hypotonia, microcephaly, distal bilateral arthrogryposis and bilateral talipes calcaneovalgus, respiratory failure, MRI abnormalities (bilateral microphthalmia, optic disc hypoplasia, widespread pachygyria and frontal lobe lissencephaly, cerebellar hemispheres and vermis hypoplasia, thinning of corpus callosum, and enlargement of subarachnoid spaces, ventricles, and basal cisterns), dysmorphic features	Syndromic presentation in a neonate	SNP-Array	Trio	1
39	1.0	1 yo	F	Progressive microcephaly, MRI anomalies (thin and unmyelinated corpus callosum and enlarged ventricles), axial hypotonia, global DD, reduced motor activity, positional plagiocephaly, one café-au-lait spot, increased lactic acid and pyruvic acid	Syndromic developmental delay	Array-CGH	Trio	1
40	13.0	13 yo	M	Tetralogy of Fallot, bilateral cryptorchidism, growth delay, global DD and ID, occasional episodes of epistaxis and gingivorrhagia with prolonged PT, mild neutropenia and mild thrombocytopenia	Syndromic developmental delay	Array-CGH, Karyotype, *PTPN11*, *RAF1*, *SOS1*, *BRAF*, *KRAS*, *SHOC2* gene panel analysis	Trio	1
41	11.3	11 years, 4 months	F	Severe Meuromuscular disorder, elevated CPK levels, mild bilateral ptosis and retraction of Achillean tendons	Neuromuscular disorder	Array-CGH, cES	Trio	2

TABLE 1 (*continued*)

Patient	Age	Years/ Months	Gender	Phenotype	Macrocategory	Previous Analysis	Family	Cohort
42	6.7	6 years, 8 months	M	IUGR, microcephaly, DD, mild ID, ventricular and renal asymmetry, delayed bone age, cryptorchidism, dysmorphic features (high frontal hairline, short palpebral fissure, full lips, toe syndactyly, V finger clinodactily, mild strabismus)	Syndromic developmental delay	Array-CGH, MS-MLPA chromosome 11p15.5	Trio	1
43	0.1	1 months	F	Hypotonia, MRI anomalies (bilateral white matter hyperintensity and hypogyration), heart anomalies (patent foramen ovale, hepatic vein drainage directly into the right atrium), failed otoacoustic emission test for the left ear, dysmorphic features (depressed nasal bridge, bulbous nose, crumpled ears with small lobes)	Syndromic presentation in a neonate	Array-CGH	Trio	1
44	6.0	5 years, 11 months	M	Global DD, cleft lip and palate, bilateral inguinal hernia, heart anomalies (intervetricular muscular defect, ventricular hypertrophy), pulmonary hypertension, umbilical hernia, macroglassia, gastroesophageal reflux, recurrent lung infections	Syndromic developmental delay	Array-CGH, MS-MLPA chromosome 11p15.5, molecular analysis for Kabuki syndrome	Trio	1
45	7.6	7 years, 7 months	F	Global DD with ID, microcephaly, severe congenital hip dysplasia, recurrent otitis, dysmorphic features, severe short stature	Syndromic developmental delay	Array-CGH, NGS panel for Kabuki syndrome	Trio	1
46	13.7	13 years, 8 months	M	DD, mild ID, bilateral talipes equinovarus and congenital muscular torticollis	Syndromic developmental delay	Array-CGH, FMR1	Trio	1
47	0.3	4 months	M	Microcephaly, MRI anomalies (dysgenesis of the telencephalic commissural plate, mild dysmorphisms of the lateral ventricles), severe respiratory distress	Syndromic presentation in a neonate	Array-CGH	Trio	1
48	0.1	1 month	M	Abnormal EEG with burst suppression, hypoplasia of the corpus callosum, apnea, poor spontaneous mobility and suction	Syndromic presentation in a neonate	NIPT for trisomy 13, 18 and 21; Array-CGH	Trio	1
49	7.4	7 years, 5 months	F	Severe ID, epileptic encephalopathy, MRI anomalies (dysmorphic left hippocampus, slight posterior thinning of the corpus callosum), hyperlaxity, coarse facies with dysmorphic features, nystagmus	Syndromic developmental delay	Karyotype, Array-CGH, ES	Trio	2
50	13.6	13 years, 7 months	M	Perimembranous ventricular septal defect, bilateral V finger camptodactyly, foot first finger distal phalanx agenesis, tenosynovitis of both wrists, finger joint contractures, decreased range of motion in multiple joints, bilateral coxa	Bones abnormalities	Del22q11 FISH, Array-CGH, ES	Trio	2
51	0.1	1 month	F	Bowed and short femurs with recurrent bilateral fractures, severe demineralization of long bones, Wormian bones, cortical thinning and rarefaction of humeral bones and scapulae, possible gray sclerae	Bones abnormalities	Array-CGH, *FGFR3* single gene sequencing	Trio	1
52	15.0	15 years, 1 month	M	Heart anomalies (pulmonary atresia, VSD, overriding aorta), hypospadia, right cryptorchidism, short stature, ID, astigmatism and myopia, fine motricity abnormalities, dysmorphic features, feet and hands brachydactyly	Syndromic developmental delay	Karyotype, 22q11 FISH, Array-CGH	Trio	1
53	5.3	5 years, 3 months	F	DD, severe astigmatism, heart anomalies (pulmonary and tricuspid atresia with intact septum), bilateral club foot	Syndromic developmental delay	Array-CGH, *PMP22, MPZ, MFN2, GDAP1, GJB1* single gene analysis	Trio	1
54	5.9	5 years, 10 months	F	DD, ID, bilateral congenital clubfoot, hip dysplasia, mild joint laxity, polydactyly with tapered fingers and bilateral clinodactyly of the V finger of the hands, mild anorectal malformation, dysmorphic features	Syndromic developmental delay	Array-CGH	Trio	1
55	13.1	13 years, 2 months	F	Slowly progressive ataxia with cerebellar leukoencephalopathy, mild cognitive impairment, Ewing's Sarcoma/PTNET of the scalp	Syndromic developmental delay	Karyotype with DEB, Array-CGH, *POLG* single gene sequencing	Trio	1
56	0.2	2 months	F	Epileptic encephalopathy with status epilepticus, lactic acidosis, axial hypertonia and T2-hyperintensity in the putamen with pseudo-cystic lesions	Epileptic encephalopathy	Array-CGH	Trio	1
57	0.1	1 month	M	Prenatal US: overgrowth, polyhydramnios. At birth macrosomia, supernumerary nipples, square coarse facies, macroglossia, left preauricular pit, left iris and retinal coloboma, heart anomalies (subcentimetric Gerbode ventricular septal defect, a slightly hypoplastic left ventricle, and a tortuous ductus arteriosus), bilateral renal cysts, splenomegaly	Syndromic overgrowth	Prenatal karyotype, array-CGH	Trio	1
58	14.3	14 years, 4 months	F	DD and ID, failure to thrive, short stature, dysmorphic features, alveolar rhabdomyosarcoma of the upper lip, Ewing/PNET sarcoma of the chest, cortical desmoid tumor of the femur	Syndromic developmental delay	Karyotype with DEB, array-CGH	Trio	1
59	15.7	15 years, 8 months	M	Mild cognitive impairment, growth hormone deficiency, dysmorphic features, nonspecific MRI anomalies, congenital right club foot, patent foramen ovale, hyperopia	Syndromic developmental delay	Array-CGH, *KMT2D*, *KDM6A*, *RAP1A* and *RAP1B* gene panel, cES	Trio	2
60	15.7	15 years, 9 months	F	Midfacial cleft, microphthalmia, progressive drug-resistant epileptic encephalopathy and intestinal malrotation	Syndromic developmental delay	Karyotype, array-CGH	Trio	1

(*continued on next page*)

TABLE 1 (*continued*)

Patient	Age	Years/ Months	Gender	Phenotype	Macrocategory	Previous Analysis	Family	Cohort
61	6.8	6 years, 10 months	F	Spastic-dystonic tetraparesis, DD, sensorineural hearing loss, microcephaly, white matter abnormalities, delayed growth, dysmorphic features	Syndromic developmental delay	Array-CGH, mitochondrial DNA analysis, trio ES.	Trio	2
62	6.9	6 years, 11 months	M	Born from a monochorionic biamniotic pregnancy, with selective abortion of the other twin due to congenital malformations (IUGR, horseshoe kidney, anhydramnios, vertebral and cardiac anomalies). DD, naso-frontal cutaneous fistula, facial dysmorphisms hearing impairment	Syndromic developmental delay	Prenatal karyotype, array-CGH	Trio	1
63	3.9	3 years, 11 months	F	Growth delay, microcephaly, ID, MRI anomalies (optic nerve coloboma, symmetrical lyssencephaly, insular heterotopy, corpus callosum hypoplasia, "molar tooth" sign), dysmorphic features	Syndromic developmental delay	Array-CGH, NGS panel for Joubert syndrome, ES	Trio	2
64	16.8	16 years, 9 months	M	DD, ASD, scoliosis, teeth agenesis, short stature, pectus carinatum, cryptorchidism, dysmorphic features	Syndromic developmental delay	Array-CGH, *FMR1*	Trio	1

Abbreviations:
cES = Clinical exome sequencing
CGH = Comparative genomic hybridization
CPK = Creatine kinase
DD = Double genetic diagnosis
DEB = Diepoxybutane
ES = Exome sequencing
GS = Genome sequencing
MRI = Magnetic resonance imaging
MS-MLPA = Methylation-specific multiplex ligation-dependent probe amplification
NGS = Next-generation sequencing
PNET = Primitive neuroectodermal tumour
VSD = Ventricular septal defect
This table summarizes patient demographics, phenotypes, prior negative genetic testing, and DNA availability for the 64 families included. Patients are divided into two cohorts: cohort 1 (no prior exome testing; see Table 2) and cohort 2 (prior exome testing with negative results; see Table 3).

All families consented to the analysis of ACMG secondary finding,[28] and secondary and incidental findings were identified in four families. These included paternally inherited pathogenic variants in *F2* (patient 3), in *APC* (patient 1) or in *CDKN2A* (patient 43) genes, and a pathogenic variant in *RYR1* gene identified in a parent (mother of patient 56). These findings enabled cascade testing for other family members.

The most common class of variants identified by cGS in our cohort was SNVs (37/42). Moreover, a combination of intragenic deletions/duplications and SNVs was identified in two patients (patients 20 and 56), an indel variant was identified in one patient (patient 24), a maternal UPD was identified in one patient (patient 5), a mosaic trisomy of chromosome 9 was diagnostic for one patient (patient 8), a mitochondrial DNA pathogenic variant was identified in one patient (patient 13), and a pathogenic variant affecting a small nuclear RNA (snRNA) was identified in one patient (patient 63). As expected, no CNVs were identified through cGS, consistent with the prior negative chromosomal microarray testing.

Among the 26 cases with a negative result (40.6%; 26/64), patient 19 was subsequently enrolled in a research project focused on discovering novel disease-causing genes. This effort led to the identification of a pathogenic variant in the *SPEN* gene.[31]

In all positive cases, the definitive genetic diagnosis significantly improved clinical management and family counseling by enabling precise estimation of recurrence risk. Specifically, for patients 1, 14, and 57, the diagnosis prompted the initiation of oncological follow-up.

Instructive clinical cases

Case 1

Patient 8 (Tables 1 and 2) is an 8-year-old boy, delivered by cesarean section at 36 weeks of gestation (wg) due to podalic presentation and premature rupture of membranes. Prenatal screening was unremarkable, and family history was noncontributory. At birth, his weight was 2295 g (25th centile) and his length was 46 cm (25th-50th centile). The Apgar scores were 8 and 9 at one and 5 minutes, respectively. Bilateral cryptorchidism and a unilateral inguinal hernia were noted at birth. A severe scoliosis and tethered cord syndrome were diagnosed in the first months of life, and dorsal X-rays revealed 13 ribs and 13 thoracic vertebrae.

Regarding psychomotor development, the patient exhibited neonatal hypotonia, walked at 12 months, and had severe language delay, using only "mum" and "dad" at 9 months, and no additional words until the age of 4 years. He received special education support from 3 years of age and had a full-scale IQ of 64 at 8 years. Audiometric screening was normal, and the patient had moderate hyperopia.

He was evaluated for the first time by a clinical geneticist at 8 years of age (Supplemental Figure 1). On examination, his height was 121 cm (10th centile), weight was 20 kg (3rd centile), and cranial circumference was 51 cm (25th centile). The patient displayed right parieto-occipital plagiocephaly, mild facial asymmetry, thin lips, a right preauricular pit, and a simplified right ear compared to the left. Mild ptosis, frail nails on the hands and feet, pectus carinatum, lower limb dysmetria (right leg 72 cm, left leg 69 cm), bilateral flat feet, fetal pads on toes, a mild bilateral sandal gap, bilateral toes camptodactyly, and mild joint hyperlaxity were also noted.

Array-CGH and *FMR1* gene testing had been previously performed with negative results. Trio cGS revealed a mosaic trisomy of chromosome 9, confirmed by traditional karyotyping on peripheral blood (93/100 metaphases with a 46, XY karyotype and 7/100 with 47, XY+9) (Supplemental Figure 1). The trisomy 9 mosaicism was considered responsible for the proband's phenotype,[32] and no chromosomal anomalies were found in the parental blood samples by karyotypes.

TABLE 2.
Clinical GS Findings in Cohort 1 (No Prior Exome Testing)

Patient	Macrocategory	Variants Identified That Overlap With the Reported Phenotype	Variants Reported at the Diagnosis	Variants Reported After 2022	cGS Result	Diagnosis (Disease, OMIM Number, Inheritance)	Variant Type	Incidental Findings	Phenotype Associated With Incidental Finding	Double Diagnosis
1	Syndromic developmental delay	ASXL1 (NM_015338.5): c.2789 G > A p.(Trp930*) [het-DN] - P	RCV003985236		Positive	Bohring-Opitz Syndrome (OMIM #605039), AD	SNV	-	-	No
2	Syndromic developmental delay	PACS1 (NM_018026.3): c.607 C > T p.(Arg203Trp) [het-DN] - P PRKCG (NM_002739.3): c.529+2T > G p.? [het-DN] - LP	RCV000032781 -	-	Positive	Schuurs-Hoeijmakers syndrome (OMIM #615009), AD Spinocerebellar ataxia 14 (OMIM #605361), AD	SNV	-	-	Yes
3	Syndromic developmental delay	DYRK1A (NM_001396.3): c.787 C > T p.(Arg263*) [het-DN] - P	-	RCV003126661	Positive	Mental retardation, autosomal dominant 7 (OMIM #614104), AD	SNV	F2 [het-pat &prob]	Thrombophilia 1 due to thrombin defect (OMIM #188050), AD	No
4	Syndromic developmental delay	ASXL3 (NM_030632.1): c.3349 C > T p.(Arg1117*) [het-DN] - P	-	RCV000263069	Positive	Bainbridge-Ropers syndrome (OMIM #615485, AD)	SNV	-	-	No
5	Syndromic developmental delay	Maternal UPD chromosome 1	-	-	VUS	Maternal UPD chromosome 1	UPD	-	-	No
7	Syndromic developmental delay	DDX3X (NM_001356.3): c.1292 T > G p.(Leu431Arg) [het-DN] - LP COL2A1 (NM_01356.3): c.2491 G > T p.(Gly831*) [het-DN] - P	- RCV003985110	RCV003985694	Positive	Intellectual developmental disorder, X-linked (OMIM #300958), XL Stickler syndrome (OMIM #108300), AD	SNV	-	-	Yes
8	Neuromuscular disorder	Mosaic trisomy chromosome 9	-	-	Positive	Mosaic trisomy 9	Aneuploidy	-	-	No
10	Syndromic developmental delay	RRM2B (NM_015713.4): c.472 G > A p.(Ala158Thr) [het-pat] - LP RRM2B (NM_015713.4): c.581 A > G p.(Glu194Gly) [het-mat] - P	- -	RCV003985157 -	Positive	Mitochondrial DNA depletion syndrome (OMIM #612075), AR	SNV	-	-	No
11	Syndromic developmental delay	PIGO (NM_032634.3): c.2854+1G > A p.? [het-pat] - LP PIGO (NM_032634.3): c.79 G > A p.(Gly27Ser) [het-mat] - VUS	- -	RCV001270714 RCV001270715	Positive	Hyperphosphatasia with mental retardation syndrome 2 (OMIM #614749), AR	SNV	-	-	No
12	Syndromic developmental delay	HIVEP2 (NM_006734.3): c.5764del p.(Asp1922Thrfs*6) [het-DN] - P CSNK2A1 (NM_001895.3): c.997 C > T p.(Arg333*) [het-DN] - LP	- -	RCV003985171 RCV001270882	Positive	Mental retardation, autosomal dominant 43 (OMIM #616977), AD Okur-Chung neurodevelopmental syndrome (OMIM #617062), AD	SNV	-	-	Yes
13	Syndromic developmental delay	MT-TK m.8357 T > C; n.63 T > C - LP	-		Positive	Leigh syndrome (OMIM #256000), M	mDNA	-	-	Yes- > No
14	Syndromic developmental delay	NFIX (NM_001271043.1): c.167 T > A p.(Met56Lys) [het-DN] - LP	-	RCV001263215	POSITIVE	Malan syndrome (OMIM #614753), AD	SNV	-	-	No
15	Syndromic developmental delay	KMT2D (NM_003482.3): c.10595 T > C p.(Ile3532Thr) [het-DN] - LP	-	RCV001880218	Positive	Kabuki syndrome 1 (OMIM #147920), AD	SNV	-	-	Yes- > No
16	Syndromic developmental delay	ANKRD11 (NM_013275.5): c.5558dup p.(Asp1854Argfs*96) [het-mat] - LP	-	RCV003985168	Positive	KBG syndrome (OMIM #148050), AD	SNV	-	-	No
17	Syndromic developmental delay	–			Negative	-	-	-	-	No
18	Syndromic overgrowth	–			Negative	-	-	-	-	No
19		–			Negative	-	-	-	-	No

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TABLE 2 (*continued*)

Patient	Macrocategory	Variants Identified That Overlap With the Reported Phenotype	Variants Reported at the Diagnosis	Variants Reported After 2022	cGS Result	Diagnosis (Disease, OMIM Number, Inheritance)	Variant Type	Incidental Findings	Phenotype Associated With Incidental Finding	Double Diagnosis
	Syndromic developmental delay									
20	Syndromic presentation in a neonate	RYR1 (NM_000540.2): c.11314 C > T p.(Arg3772Trp) [het-pat] - LP RYR1 (NM_000540.2): 4.4 Kb loss, chr 19:g.38988140-38992568del [het-mat] - LP	- -	RCV002289771 -	Positive	Central Core Disease (OMIM #117000), AR	SNV + intra del	-	-	No
21	Syndromic developmental delay	TUBB3 (NM_006086.3): c.785 G > A p.(Arg262His) [het-ukn] - P CREBBP (NM_004380.2): c.2417 T > C p.(Met806Thr) [het-ukn] - VUS	RCV000203607 -	RCV001270737	Positive	Fibrosis of extraocular muscles, congenital, 3A (OMIM #600638), AD Rubistein Taybi Syndrome (OMIM #180849), AD	SNV	-	-	Yes
22	Syndromic developmental delay	SHANK3 (NM_033517.1): c.3679dup p.(Ala1227Gly*56) [het-DN] - P	-	-	Positive	Phelan-McDermid syndrome (OMIM #606232), AD	SNV	-	-	No
24	Syndromic developmental delay	SATB2 (NM_015265.3): c.1979_1981del p.(Ile660del) [het-DN] - P	-	RCV003985220	POSITIVE	Glass syndrome (OMIM #612313), AD	Indel and SNV	-	-	Yes- > No
25	Syndromic developmental delay	PDE4D (NM_001104631.1): c.2051A > G p.(Glu684Gly) [het-DN] - LP	-	RCV001270704	POSITIVE	Acrodysostosis type 2 (OMIM #614613), AD	-	-	-	No
26	Syndromic developmental delay	–			Negative	-	SNV	-	-	No
27	Syndromic presentation in a neonate	LRPPRC (NM_133259.4): c.2056A > G p.(Ile686Val) [het-pat] - LP LRPPRC (NM_133259.4): c.1921-7A > G [het-mat] - LP	- -	RCV001270836 RCV001270837	Positive	Mitochondrial complex IV deficiency, nuclear type 5 (OMIM #220111), AR	-	-	-	No
28	Syndromic developmental delay	SETBP1 (NM_015559.2): c.2561 C > A p.(Ser854Tyr) [het-DN] - LP	-	RCV001270830	Positive	Schinzel-Giedion midface retraction syndrome (OMIM #269150), AD	SNV	-	-	No
29	Syndromic developmental delay	–			Negative	-	SNV	-	-	No
31	Syndromic developmental delay	–			Negative	-	SNV	-	-	No
32	Syndromic developmental delay	PIEZO2 (NM_022068.3): c.8045 G > T p.(Gly2682Val) [het-DN] - LP	-	RCV001728097	Positive	Marden-Walker syndrome (OMIM #248700), AD	-	-	-	Yes- > No
34	Syndromic presentation in a neonate	–			Negative	-	-	-	-	No
35	Syndromic presentation in a neonate	–			Negative	-	-	-	-	No
36	Syndromic developmental delay	SRCAP (NM_006662.2): c.7330 C > T p.(Arg2444*) [het-DN] - P	RCV000023895		Positive	Floating-Harbor syndrome (OMIM #136140), AD	-	-	-	No
37	Syndromic presentation in a neonate	–			Negative	-	SNV	-	-	No

38	Syndromic presentation in a neonate	–			Negative	-	-	-	-	No
39	Syndromic developmental delay	PDHA1 (NM_000284.3): c.1142_1145dup p.(Trp383Serfs*6) [het-DN] - P	RCV000011627		Positive	Pyruvate dehydrogenase E1-alpha deficiency (OMIM #312170), XLD	SNV	-	-	No
40	Syndromic developmental delay	PACS1 (NM_018026.3): c.607 C > T p.(Arg203Trp) [het-DN] - P	RCV000032781		Positive	Schuurs-Hoeijmakers syndrome (OMIM #615009), AD	SNV	-	-	No
42	Syndromic developmental delay	NSD2 (NM_001042424.3): c.2523del p.(Ser842Alafs*17) [het-DN] - P	-	-	Positive	Rauch-Steindl syndrome (OMIM #619695), AD	SNV	-	-	Yes- > No
43	Syndromic presentation in a neonate	–			Negative	-	SNV	CDKN2A [het-pat and prob]	CDKN2A-related cancer susceptibility (OMIM #155755, #155601, #606719), AD	No
44	Syndromic developmental delay	SMPD1 (NM_000543.4): c.769_777del p.(Arg257_Leu259del) [het-pat] - LP SMPD1 (NM_000543.4): c.340 G > A; p.Val114Met [het-mat] - LP	–	-RCV000801532	Positive	Niemann-Pick type A (OMIIM #257200) and B (OMIIM #607616), AR	-	-	-	No
45	Syndromic developmental delay	–			Negative	-	SNV	-	-	No
46	Syndromic developmental delay	–			Negative	-	SNV	-	-	No
47	Syndromic presentation in a neonate	–			Negative	-	-	-	-	No
48	Syndromic presentation in a neonate	–			Negative	-	-	-	-	No
51	Bones abnormalities	COL1A1 (NM_000088.3): c.3531+1G > A p.? [het-DN] - P	RCV000689406		Positive	Osteogenesis imperfecta type 1 (OMIM # 166200), AD	-	-	-	No
52	Syndromic developmental delay	–			Negative	-	SNV	-	-	No
53	Syndromic developmental delay	SLC12A6 (NM_133647.1): c.619 C > T p.(Arg207Cys) [het-pat] - LP SLC12A6 (NM_133647.1): c.3076 C > T p.(Arg1026*) [het-mat] - LP	RCV000005657 RCV002260549		Positive	Agenesis of the corpus callosum with peripheral neuropathy (OMIM #218000), AR	-	-	-	No
54	Syndromic developmental delay	–			Negative	-	SNV	-	-	Yes- > No
55	Syndromic developmental delay	PPP1R21 (NM_001135629.2): c.292_294del p.(Ser98del) [het-mat] - VUS PPP1R21 (NM_001135629.2): c.1728 G > T; p.Glu576Asp [het-pat] - VUS	- -	RCV002990616 RCV002260565	Positive	Neurodevelopmental disorder with hypotonia, facial dysmorphism, and brain abnormalities (OMIM #619383), AR	SNV	-	-	No
56	Epileptic encephalopathy	NARS2 (NM_024678.5): c.1385 T > C p.(Ile462Thr) [het-mat] - VUS NARS2 (NM_024678.5): Dup of exons 7-11 in NARS2 - seq[GRCh37]dup(11)(q14.1) -chr11:g.78165386_78231070dup [het-pat] - VUS	- - - -	RCV001795572 - - -	Positive	Combined oxidative phosphorylation deficiency 24 (OMIM #616239), AR Developmental and epileptic encephalopathy 99 (OMIM #619606), AD	SNV + intra dup	RYR1 [het-mat]	Malignant hyperthermia susceptibility (OMIM #145600), AD	Yes

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TABLE 2 (*continued*)

Patient	Macrocategory	Variants Identified That Overlap With the Reported Phenotype	Variants Reported at the Diagnosis	Variants Reported After 2022	cGS Result	Diagnosis (Disease, OMIM Number, Inheritance)	Variant Type	Incidental Findings	Phenotype Associated With Incidental Finding	Double Diagnosis
		ATP1A3 (NM_152296.4): c.1231delC; p.Leu411CysfsTer47 [het-DN] - LP								
57	Syndromic overgrowth	GPC3 (NM_004484.3): c.1645 A > G p.(Ile549Val), [hem-mat] - LP	-	RCV002260575	Positive	Simpson-Golabi-Behmel syndrome (OMIM #312870), XLR	SNV	-	-	No
58	Syndromic developmental delay	CTCF (NM_006565.3): c.1699 C > T p.(Arg567Trp) [het-DN] - P	RCV000074335		Positive	Intellectual developmental disorder, autosomal dominant 21 (OMIM #615502), AD	SNV	-	-	No
60	Syndromic developmental delay	-			Negative	-	-	-	-	No
62	Syndromic developmental delay	-			Negative	-	-	-	-	No
64	Syndromic developmental delay	CTCF (NM_006565.3): c.1622 A > T p.(His541Leu) [het-DN] - LP	-	RCV002467371	Positive	Intellectual developmental disorder, autosomal dominant 21 (OMIM #615502), AD	SNV			No

Abbreviations:
AD = Autosomal dominant; AR = Autosomal recessive; DN = De novo; GS = Genome sequencing; Hem = Hemizygous; Het = Heterozygous; Hom = Homozygous; Indel = Insertion and deletion; Intra Del = Intragenic deletion; Intra dup = Intragenic duplication; LP = Likely pathogenic; M = Mitochondrial; Mat = Maternal; mDNA = Mitochondrial DNA; P = Pathogenic; Pat = Paternal; Prob = Proband; SNV = Single nucleotide variant; ukn = Unknown (nonpaternity, not possible to establish whether the variants were de novo or not); UPD = Uniparental disomy; VUS = Variant of uncertain significance; XLD = X-linked dominant; XLR = X-linked recessive; Yes - > No = Double diagnosis excluded due to reclassification of a VUS as likely benign.
Results for each patient include patient ID, patient's phenotype as phenotypic macrocategory, the identified causative variant/s, the ClinVar ID When available at the time of diagnosis or after 2022, cGS outcome, clinical diagnosis and the nature of the identified variant. Additional details include the presence of actionable pathogenic/likely pathogenic variants and any cases of double genetic diagnosis.

TABLE 3.
Clinical GS Findings in Cohort 2 (Prior Negative Exome Testing)

Patient	Macrocategory	Variants Overlapping With Reported Phenotype	Variants Reported at the Diagnosis	Variants Reported After 2022	cGS Result	Diagnosis (Disease, OMIM Number, Inheritance)	Variant Type	Incidental Findings	Phenotype Associated With Incidental Finding	Double Diagnosis
6	Syndromic developmental delay	–	-	-	Negative	-	-	-	-	No
9	Neuromuscular disorder	*COL6A3* (NM_004369.3): c.6156 G > A p.? [het-DN] - LP	-	RCV003985176	Positive	Ulrich Myopathy 1 (OMIM #254090), AD	SNV	-	-	No
23	Syndromic developmental delay	–	-	-	Negative	-	-	-	-	No
30	Neuromuscular disorder	*LMNA* (NM_170707.3): c.745 C > T p.(Arg249Trp) [het-DN] - P	RCV000015621		Positive	Muscular dystrophy, congenital (OMIM #613205), AD	-	-	-	No
33	Bones abnormality	–	-	-	Negative	-	SNV	-	-	No
41	Neuromuscular disorder	*CAPN3* (NM_000070.2): c.1524 G > A p.? [het-mat] - LP *CAPN3* (NM_000070.2): c.1194-2A > G p.? [het-DN] - P	RCV001196491 -	RCV001249767	Positive	Muscular dystrophy, limb-girdle, autosomal recessive 1 (OMIM #253600), AR	SNV	*APC* [het -pat]	Familial adenomatous polyposis 1 (OMIM # 175100), AD	No
49	Syndromic developmental delay	–	-	-	Negative	-	-	-	-	No
50	Bones abnormality	–	-	-	Negative	-	-	-	-	No
59	Syndromic developmental delay	-	-	-	Negative	-	-	-	-	No
61	Syndromic developmental delay	-	-	-	Negative	-	-	-	-	No
63	Syndromic developmental delay	*RNU4ATAC* (NR_023343.1): n.16 G > C [het-pat] - LP *RNU4ATAC* (NR_023343.1): n.55 G > A [het-mat] - P	- -	- -	Positive	RNU4ATAC-related spliceosomopathies (OMIM #601428), AR	-			No

Abbreviations:
AD = Autosomal dominant; AR = Autosomal recessive; DN = De novo; GS = Genome sequencing; Hem = Hemizygous; Het = Heterozygous; Het = Heterozygous; Hom = Homozygous; LP = Likely pathogenic; Mat = Maternal; P = Pathogenic; Pat = Paternal; SNV = Single nucleotide variant.
For each patient, this table includes patient ID, patient's phenotype as phenotypic macrocategory, the identified causative variant/s, the ClinVar ID when available at the time of diagnosis or after 2022, cGS outcome, clinical diagnosis and the nature the identified variant, Along with the presence of actionable pathogenic/likely pathogenic variants and double genetic diagnoses.

Case 2

Patient 56 (Tables 1 and 2) is a 2-month-old infant born after spontaneous birth at 40 weeks of gestation. Choroid plexus cysts were detected at 20 weeks of gestation but were not present in subsequent prenatal ultrasounds. At birth, her weight was 2670 g (25th centile), length was 49 cm (10th-25th centile), and cranial circumference was 34.5 cm (50th-75th centile). The Apgar scores were 7 and 9 at one and 5 minutes, respectively. Family history was unremarkable.

On the first day of life, the infant displayed axial hypertonia, hyperexcitability, mild extremity tremors, and impaired suction. Electroencephalography revealed sharp waves but no epileptiform activity. On the second day of life, the patient developed a fever (38°C), and broad-spectrum antibiotics were initiated. Cerebrospinal fluid examination was normal, but an *Escherichia coli* infection was detected, together with elevated lactic acid levels (111 mg/dL, normal range: 5.7-22 mg/dL). Neonatal metabolic screening was unremarkable. Heart ultrasound demonstrated a patent *foramen ovale* and *ductus arteriosus*, while abdominal ultrasound and ophthalmologic examination were normal. Brain magnetic resonance imaging (MRI) at 3 weeks showed mild thinning of the *corpus callosum*. A muscle biopsy was requested to evaluate for a mitochondrial disorder.

TABLE 4.
Reclassification of Variants of Unknown Significance (VUS)

Patient	Macrocategory	VUS	Reclassification	Notes	Final Report
2	Syndromic developmental delay	*PRKCG* (NM_002739.3): c.529+2T > G p.? [het-DN]	Likely pathogenic	ACMG criteria applied: PVS1 strong, PM2 supporting and PS2 strong.	Positive, DD
13	Syndromic developmental delay	MT-TK m.8357 T > C; n.63 T > C	Likely pathogenic	Re-evaluated due to coherence with clinical presentation and familiar segregation.	Positive, DD
		BRWD3 (NM_153252.4): c.1118 A > G p.Asn373Ser - [hem-mat]	Likely benign	Hemizygous variant identified in an unaffected maternal uncle.	excluded
15	Syndromic developmental delay	*KMT2D* (NM_003482.3): c.10595 T > C p.(Ile3532Thr) [het-DN]	Likely pathogenic	ACMG criteria applied: PM2 supporting, PP5 supporting, PP4 supporting and PS2 strong.	Positive, DD
		SMCHD1 (NM_015295.2): c.4823 T > G p.(Ile1608Ser) [het-pat]	Likely benign	Variant inherited from a healthy father (clinical re-evaluation of the father following the NGS results).	excluded
18	Syndromic overgrowth	*COL11A1* (NM_080629.2): c.1604 C > G p.(Ala535Gly) [het-pat]	Likely benign	Variant inherited from a healthy father (clinical re-evaluation of the father following the NGS results).	Negative
24	Syndromic developmental delay	*DPM1* (NM_003859.1): c.779 C > T p.(Thr260Ile) [hom-mat/pat]	Likely benign	Variant pathogenicity ruled out based on patient's normal transferrin analysis.	Positive, DD excluded
27	Syndromic presentation in a neonate	*LRPPRC* (NM_133259.4): c.2056A > G p.(Ile686Val) [het-pat]	Likely pathogenic	ACMG PM3 criterion added.	Positive
		LRPPRC (NM_133259.4): c.1921-7A > G [het-mat]	Likely pathogenic	Validation of the impact of the intronic variant on splicing by RNA analysis (ACMG PS3 criterion applied).	
32	Syndromic developmental delay	*PIEZO2* (NM_022068.3): c.8045 G > T p.(Gly2682Val) [het-DN]	Likely pathogenic	ACMG criteria applied: PP3 moderate, PM2 supporting, BP1 supporting and PS2 strong.	Positive, DD
		FLNA (NM_001110556.1): c.695 C > T p.(Ala232Val) [het-mat]	Likely benign	Variant inherited from a healthy mother (clinical re-evaluation of the mother following the NGS results).	excluded
34	Syndromic presentation in a neonate	*PIGA* (NM_002641.3): c.1418 G > C p.(Arg473Thr) [hem-mat]	Likely benign	ACMG criteria applied: PM2 supporting, BP4 strong, BP3 supporting.	Negative
38	Syndromic presentation in a neonate	*DYNC1H1* (NM_001376.4): c.7421 C > T p.(Ala2474Val) [het-mat]	Likely benign	Variant inherited from a healthy mother (clinical re-evaluation of the mother following the NGS results).	Negative
42	Syndromic developmental delay	*BRWD3* (NM_153252.4): c.4384 A > G p.(Ser1462Gly) - [hem-mat]	Likely benign	ACMG criteria applied: BS2 strong, BP4 moderate, BP1 supporting.	Positive, DD excluded
44	Syndromic developmental delay	*CIC* (NM_015125.4): c.1927G > A p.(Gly643Arg) [het-pat]	Likely benign	ACMG criteria applied: BS1 strong, BS2 strong, BP4 strong, BP1 supporting, BP6 supporting.	Positive, DD excluded
45	Syndromic developmental delay	*PDE4D* (NM_001104631.1): c.1774 A > C p.(Lys592Gln) [het-pat]	Likely benign	Clinical re-evaluation of the patient and the healthy father, including cranial and hands X-rays and Hormonal evaluation, were not coherent with the molecular finding.	Negative
54	Syndromic developmental delay	*OFD1* (NM_003611.2): c.2224 G > A p.(Ala742Thr) [het-mat]	Likely benign	Variant reclassified based on lack of coherence with clinical presentation and re-evaluation of the healthy mother.	Negative
		OTX2 (NM_172337.2): c.397 C > T p.(Pro133Ser) [het-mat]	Likely benign	Variant reclassified based on lack of coherence with clinical presentation and re-evaluation of the healthy mother.	
56	Epileptic encephalopathy	*NARS2* (NM_024678.5): c.1385 T > C p.(Ile462Thr) [het-mat]	Likely pathogenic	ACMG PM3 criterion added.	Positive, DD
		NARS2 (NM_024678.5): Dup of exons 7-11 in *NARS2* seq[GRCh37]dup(11) (q14.1) - chr11:g.78165386_78231070dup [het-pat]	Likely pathogenic	Intragenic duplication causing a out-of-frame insertion of 475 nucleotides. ACMG PVS1 criterion added.	
57	Syndromic overgrowth	*GPC3* (NM_004484.3): c.1645 A > G p.(Ile549Val), [hem-mat]	Likely pathogenic	Functional experimental validation (ACMG PS3 criterion) based on immunohistochemical staining of GPC3 on placenta (see Fiandrino et al, 2022).	Positive
64	Syndromic developmental delay	*CTCF* (NM_006565.3): c.1622 A > T p.(His541Leu) [het-DN]	Likely pathogenic	ACMG criteria applied: PP3 supporting, PM2 moderate, PP2 supporting, PS2 strong.	Positive

Abbreviations:
DD = Double genetic diagnosis; DN = De novo; Hem = Hemizygous; Het = Heterozygous; Het = Heterozygous; Hom = Homozygous; Mat = Maternal; Pat = Paternal.
For each patient, this table includes patient ID, patient's phenotype as phenotypic macrocategory, the identified VUS, the reclassification outcome with a concise explanation of the rationale behind it, and the resulting diagnostic report.

The patient was seen by the clinical geneticist at one month of age and showed no significant dysmorphism on examination. Array-CGH had previously been performed and yielded negative results. Trio cGS was requested. The patient's clinical condition deteriorated rapidly, leading to severe epileptic encephalopathy and respiratory failure at 3 months of age. The cGS results were available one week after her demise. The analysis identified a heterozygous maternally inherited variant [c.1385 T > C; p.Ile462Thr] in the *NARS2* gene (NM_024678.5) as well as a heterozygous paternally inherited a 66-kb duplication encompassing exons 7 to 11 of the same gene [seq[GRCh37]dup(11) (q14.1) - chr11:g.78165386_78231070dup] (Supplemental Figure 1), with one breakpoint located in intron 6 and another in intron 11. These variants were determined to be causative of combined oxidative

phosphorylation deficiency 24 (OMIM #616239), with autosomal recessive inheritance. In addition, cGS revealed a *de novo* likely pathogenic variant in the *ATP1A3* gene [c.1231delC; p.(Leu411-CysfsTer47)] (Supplemental Figure 1), which is associated with a spectrum of neurological disorders with autosomal dominant inheritance. Due to the presence of compound heterozygous variants in *NARS2*, it was difficult to fully interpret the contribution of the *ATP1A3* variant to the patient's severe clinical course.

Finally, cGS detected an incidental finding in the *RYR1* gene in the mother, who was asymptomatic. The variant [NM_000540.3: c.1589 G > A; p.(Arg530His)] is associated with susceptibility to malignant hyperthermia type 1 (OMIM #145600), an autosomal dominant condition. Cascade testing was initiated for the maternal side of the family.

In this case, the cGS results could not inform clinical management because of the patient's early demise. Had the results been available earlier, they might have supported a more timely reconsideration or discontinuation of invasive interventions. Nevertheless, the findings remained clinically meaningful, informing reproductive decision-making in subsequent pregnancies and enabling comprehensive genetic counseling for the family, particularly in the context of an incidental finding that required segregation analysis in the extended family.

Case 3

Patient 63 (Tables 1 and 3; Supplemental Figure 1) is a 4-year-old girl born at 37 + 2 weeks of gestation via spontaneous birth. Severe fetal growth restriction was identified during prenatal ultrasonography starting at 20 weeks of gestation. At birth, her weight, length, and head circumference were markedly below average: 1573 g (<< 3rd centile), 38 cm (<< 3rd centile), and 27.7 cm (<< 3rd centile), respectively. The Apgar scores were 8 at one minute and 8 at 5 minutes. The family history was unremarkable, with a healthy older sister. After birth, she was admitted to the neonatal intensive care unit due to fetal growth restriction and respiratory distress. Metabolic and infectious screenings were normal. Karyotyping and array-CGH were normal.

Initial clinical investigations revealed bilateral optic nerve coloboma on ophthalmologic examination. Electroencephalography and auditory evoked potentials were unremarkable. Brain MRI findings included microcephaly, hypoplasia of the corpus callosum, lateral ventricle enlargement, molar tooth sign, and bilateral frontal lissencephaly. Echocardiography identified a small interatrial septal defect, while abdominal ultrasound was normal.

During her first 2 years, the patient experienced recurrent infections and exhibited significant psychomotor delay. She achieved independent sitting at 30 months but remained unable to walk independently by the age of nearly 4 years, requiring a walker for mobility. Language skills were limited to a few simple words during the last examination. At 3 years of age, vertebral MRI revealed a cleft from C3 to C5. At 3 years and 10 months, she was evaluated by a clinical geneticist. At that time, her growth parameters included a height of 86.5 cm (−3.2 S.D.), weight of 11.5 kg (−2.7 S.D.), and head circumference of 40.5 cm (−6.1 S.D.). Notable dysmorphic features included microcephaly, proptosis, protruding ears with a slim upper portion, bilateral fifth finger clinodactyly, bilateral hockey-stick palmar creases, and systemic hypertonia (Supplemental Figure 1). A NGS panel including genes associated with Joubert syndrome and congenital disorders of glycosylation was requested, but no pathogenic variants were identified. Subsequently, clinical GS revealed compound heterozygous variants in the *RNU4ATAC* gene (NR_023343.1): the paternally inherited c.16 G > C (classified as pathogenic) and the maternally inherited c.55 G > A (classified as likely pathogenic) (Supplemental Figure 1). These findings led to the diagnosis of *RNU4ATAC*-opathy with autosomal recessive inheritance.[33]

Discussion

GS is widely regarded as the most comprehensive single test for RGDs. It extends beyond coding regions and can detect SNVs, CNVs, and more complex genomic rearrangements.[34] Despite its advantages, GS has yet to become a routine diagnostic tool in many countries, including Italy, due to its high costs, the substantial volume of data requiring analysis and storage, and challenges associated with data interpretation.

Our study shows the potentiality of cGS in a selected cohort of pediatric syndromic cases, achieving an overall diagnostic yield of 57.8%. Notably, the diagnostic rate was higher among patients who had not undergone prior cES or ES analyses (cohort 1; 62.3%) compared to those with negative results from a previous exome testing (cohort 2; 36.4%). This finding is consistent with prior studies, which have shown that the increase in the diagnostic rate for cGS following a nondiagnostic ES is modest.[11] Notably, in cohort 2, the absence of a molecular diagnosis after prior cES was mainly due to limitations in variant prioritization and interpretation, particularly for synonymous and splicing variants, rather than to insufficient genomic coverage. Within the three most represented phenotypic categories in our cohort, we observed a diagnostic yield of 100% for cases with a neuromuscular phenotype (4/4), 22.2% for neonates with a syndromic presentation (2/9), and 62.2% for patients presenting with syndromic developmental delay (28/45).

Compared to ES, cGS is more comprehensive and offers substantial advantages. Notably, it can identify pathogenic variants in noncoding genomic regions, as demonstrated in patient 63, where a pathogenic variant in a small nuclear RNA (snRNA) was identified. Furthermore, cGS is more reliable for detecting intragenic deletions or duplications compared to ES, as exemplified by cases 20 and 56. Of note, cGS enables the identification of a wide range of variant types in a single assay as in case 56. Here a double genetic diagnosis was established revealing the co-occurrence of a missense variant and an intragenic duplication in the recessive gene *NARS2* and a dominant *de novo* variant in the *ATP1A3* gene. The compound heterozygosity would likely be missed by ES, at least in the past, resulting in an incomplete diagnosis for the patient and failure to identify the *NARS2* carrier status in the parents. This could significantly challenge the genetic counseling and the recurrence risk assessment within the family. cGS is also efficient in identifying mosaic aneuploidies, such as the mosaic trisomy 9 observed in patient 8, which are often missed by conventional microarray. Furthermore, it can detect mitochondrial DNA anomalies not visible by ES, as in patient 13. Moreover, cGS allows the identification of secondary and incidental findings and their reporting in accordance with ACMG guidelines,[28] positively impacting counseling and prevention as demonstrated in five families within our cohort.

Re-evaluation of cGS results is also critical to reach a definitive diagnosis in patients affected by RGDs. In our cohort, 22 variants initially reported as VUS in the diagnostic report have been reclassified as likely pathogenic (10 variants, 8 cases) or likely benign (12 variants, 11 cases), following clinical assessment and family segregation analyses. This underscores the importance of interpreting NGS results in a clinical context to ensure the most accurate diagnostic outcomes.

In our cohort, all patients had undergone chromosomal microarray testing, a standard procedure within the Italian SSN. As a result, no CNVs were identified through cGS, despite its high accuracy for CNV detection.[16,23] This highlights the potential of cGS to serve as a first-tier diagnostic test in various clinical contexts.[9,24,35] Its implementation as a primary test could reduce the

diagnostic odyssey faced by patients with RGDs, streamline the diagnostic process, and improve clinical management by facilitating faster diagnoses and recurrence risk assessment.

The decreasing cost of GS is making its routine clinical implementation increasingly feasible. Notably, the mean cost associated with patients who remain without a molecular diagnosis has been estimated to be at least €35,000-60,000 over the course of prolonged diagnostic evaluations. In this context, the systematic adoption of GS has the potential to improve cost-effectiveness by shortening diagnostic trajectories and increasing diagnostic yield. Consistent with this, recent studies evaluating the cost-effectiveness of GS in pediatric populations have demonstrated that its use as a first-tier test shortens diagnostic odysseys and reduces overall health care costs, particularly in critical care settings,[20,36-40] including studies conducted within the Italian health care system.[38-40] In our study, the mean cost of prior genetic testing alone reached €6,798 per patient in cohort 2 (range €5,335-9,481), indicating that the cumulative cost of sequential, non-diagnostic testing is already comparable to that of GS, thereby supporting GS as a cost-effective earlier-tier diagnostic approach. Task forces should assess its cost-effectiveness and develop guidelines for its integration into the Italian SSN, as has been done in other European countries.[9,18,24,26,41,42] These evaluations should also take into account the recent availability of rapid GS, which was not considered in the present study as it was not available during the 2018-2022 study period, but which now represents a valuable diagnostic tool for critically ill pediatric patients, particularly in neonatal intensive care settings.

Despite all the advantages offered, cGS has several limitations. As a clinical test, interpretation and reporting is often restricted to well-characterized genomic regions, limiting its utility for identifying novel gene-disease associations or variants in deep intronic regions. However, raw GS data can be reanalyzed in a research setting when a diagnostic test is inconclusive, as illustrated by case 19 (Table 1).[31] In addition, cGS cannot reliably interpret variants affecting three-dimensional genome structure or epigenetic features such as histone modifications and DNA methylation. Traditional short-read sequencing also struggles with long trinucleotide repeat expansions,[43] and its lower coverage depth compared to ES may reduce the sensitivity for detecting mosaic variants or those in difficult-to-sequence regions.[11] This study has several limitations that should be acknowledged. The cohort was highly selected and enriched for patients with complex, predominantly syndromic presentations. While this intentional design reflects a specialist multidisciplinary referral setting and likely contributed to the higher diagnostic yield observed compared to previous reports,[24] it may limit the generalizability of our findings to unselected rare disease populations. Nevertheless, this strategy facilitates a cost-effective diagnostic strategy by prioritizing comprehensive clinical characterization over repeated or exploratory testing. Prior diagnostic testing was also heterogeneous, reflecting differences in local diagnostic workflows within the Italian National Health Service during the 2018-2022 period. Although this variability complicates direct comparisons with standardized diagnostic strategies, it mirrors real-world clinical practice and was mitigated by the application of a uniform, multidisciplinary phenotyping process at study entry. Regarding mitochondrial variant detection, the $\geq$3% heteroplasmy threshold used in this study reflects a validated analytical choice adopted during the study period. Although a 5% cutoff is commonly used as a conservative standard to minimize false positives, our pipeline reproducibly detected mitochondrial variants down to ~3% heteroplasmy, improving analytical sensitivity, with all variants interpreted in the appropriate clinical context and confirmed using orthogonal methods. Overall, our findings should be interpreted as a proof of concept demonstrating the diagnostic utility of cGS in a specialist referral setting, an approach that is particularly relevant for optimizing resource allocation within national health care systems.

Conclusions

In summary, our experience with cGS in the Italian health care context has been highly positive, yielding high diagnostic rates for patients who had previously undergone extensive diagnostic evaluations. This project was supported by funding from the iHope program.[27] We hope that cGS will be further implemented in the Italian SNN, enhancing diagnostic outcomes and cost-effectiveness by consolidating multiple tests into a single comprehensive approach for known genetic conditions.

Author contributions

E.G.: Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review and editing. F.S.: Conceptualization, Supervision, Writing - review and editing. D.C.: Conceptualization. S.K.: Data curation, Writing - original draft. M.Z.: Data curation, Writing - original draft. F.B.: Data curation, Writing - original draft. A.B.a.: Data curation. E.T.V.: Formal analysis. R.J.T.: Formal analysis. A.K.: Formal analysis. A.B.r.: Funding acquisition, Supervision, Writing - review and editing. L.S.: Investigation, V.A.: Investigation. A.G.: Investigation. A.F.: Investigation. F.L.: Investigation. J.G.: Investigation. G.B.F.: Writing - review and editing. A.M.: Writing - review and editing. P.G.: Writing - review and editing.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplemental material.

Declaration of competing interest

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Supplementary data

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