Exome Sequencing in 200 Intellectual Disability/Autistic Patients: New Candidates and Atypical Presentations

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Abstract: Intellectual disability (ID) and autism spectrum disorder (ASD) belong to neurodevelopmental disorders and occur in ~1% of the general population. Due to disease heterogeneity, identifying the etiology of ID and ASD remains challenging. Exome sequencing (ES) offers the opportunity to rapidly identify variants associated with these two entities that often co-exist. Here, we performed ES in a cohort of 200 patients: 84 with isolated ID and 116 with ID and ASD. We identified 41 pathogenic variants with a detection rate of 22% (43/200): 39% in ID patients (33/84) and 9% in ID/ASD patients (10/116). Most of the causative genes are genes responsible for well-established genetic syndromes that have not been recognized for atypical phenotypic presentations. Two genes emerged as new candidates: CACNA2D1 and GPR14. In conclusion, this study reinforces the importance of ES in the diagnosis of ID/ASD and underlines that “reverse phenotyping” is fundamental to enlarge the phenotypic spectra associated with specific genes.

Keywords: exome sequencing; intellectual disability; autism spectrum disorder

1. Introduction

Intellectual disability (ID) is characterised by significant limitations in intellectual functioning (reasoning, learning, problem-solving) and adaptive behaviour (conceptual, social, and practical skills) that originate before the age of 18 [1]. Affecting 1–3% of the world’s population, ID represents an important socio-economic problem in healthcare [2,3]. ID is characterized by limitations in cognitive functions that manifest as an intelligence quotient (IQ) below 70. ID may be “isolated” or “syndromic” when patients have peculiar facies, specific physical signs and/or an abnormal growth pattern [4].

Autism spectrum disorder (ASD) is characterized by deficient social interactions, poor or absent communication, repetitive behaviours, and apparently limited interests [5]. ASD generally becomes apparent after the first year of life and it has been reported in an increasing number (2.2–2.7%) of children, with boys four times more likely to be affected.
than girls [6,7]. From a clinical point of view, the autism spectrum disorder is subdivided into “syndromic” when a co-occurrence between autism (ASD) and dysmorphic features, also other somatic or neurobehavioral abnormalities could be observed [8].

ID and ASD often co-exist and identifying the etiology of the two conditions remains challenging due to disease heterogeneity. Accurate clinical, as well as molecular diagnoses are essential for a deeper understanding of the pathogenesis of these conditions and for devising tailored treatments [4]. Until the advent of the first next generation sequencing (NGS) platforms, a large fraction of cases remained not diagnosed, with many families undergoing a “diagnostic odyssey” [9]. The introduction of exome or genome sequencing (ES/GS) has significantly improved diagnostic rates in individuals with suspected ID/ASD genetic disorders refractory to conventional diagnostic testing [10].

In the present study, ES was generated for a total of 200 individuals (84 ID and 116 ID/ASD patients). Pathogenic or likely pathogenic (P/LP) variants were found in 43 individuals (22%), with 45 variants of uncertain significance in an additional 20% (40/200). Our data strongly support the value of large-scale sequencing, especially ES within proband-parent trios, as an effective first-choice diagnostic tool.

2. Materials and Methods

2.1. Selection of Patients and DNA Samples’ Preparation

Genetic counselling was carried out to evaluate each patient’s personal and familial history. Parents provided and signed a written informed consent at the Medical Genetics department of the University of Siena, Italy, for exome sequencing analysis, clinical data usage, and the use of DNA samples from the tested individuals for both research and diagnosis purposes. We analysed a total of 200 patients affected by ID and ID/ASD (84 with ID and 116 with ID and ASD) collected from January 2019 until the end of March 2021.

Genomic DNA from the parents was isolated from EDTA peripheral blood samples using MagCore HF16 (Diatech Lab Line, Jesi, Ancona, Italy) according to the manufacturer’s instructions.

2.2. Exome Sequencing

Sample preparation was performed following the Illumina DNA Prep with Enrichment manufacturer protocol. A bead-based transposome complex is used to perform tagmentation, a process that fragments the genomic DNA and then tags it with adapter sequences in one step. After saturation with input DNA, the bead-based transposome complex fragments a set number of DNA molecules. This fragmentation provides flexibility to use a wide DNA input range to generate normalized libraries with a consistent tight fragment size distribution. Then a limited-cycle PCR adds adapter sequences to the ends of a DNA fragment. A subsequent target enrichment workflow is then applied. Following pooling, the double-stranded DNA libraries are denatured and biotinylated. Illumina Exome Panel v1.2 (CEX) probes are hybridized to the denatured library fragments. Then Streptavidin Magnetic Beads (SMB) capture the targeted library fragments within the regions of interest. Then the indexed libraries are eluted from beads and further amplified before sequencing. The exome sequencing analysis was performed on the Illumina NovaSeq6000 System (Illumina San Diego, CA, USA) according to the NovaSeq6000 System Guide. Reads were mapped against the hg19 reference genome using the Burrow-Wheeler aligner BWA [11]. Variant calling was obtained using an in-house pipeline which takes advantage of the GATK Best Practices workflow [12].

Prioritization of the variants was obtained excluding polymorphisms (minor allele frequency, MAF <0.01), synonymous variants, variants classified as benign or likely benign. Frameshift, stopgain, and splice site variants were prioritized as pathogenic. Missense variants were predicted to be damaging by CADD-Phred prediction tools. The potential impact of variants on splicing was evaluated using Alamut® Visual software—version
2.11–0 (Interactive Biosoftware, Rouen, France), which employs five different algorithms: SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, and HumanSplicingFinder. The following public databases were used for the interpretation of the variants: ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/, accessed on 9 June 2021), LOVD (https://databases.lovd.nl/shared/genes, accessed on 9 June 2021), the Human Genome Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php3, accessed on 9 June 2021).

3. Results
3.1. Clinical Characteristics of Patients

We enrolled 200 families (574 individuals total) with at least one proband with an unexplained diagnosis of ASD/ID-related phenotype. In particular, 181 families had one affected proband and 4 families had two affected probands. ES was sequenced to an average depth of 100×, respectively, with ~94% of bases covered ≥ 20×. The study population had a mean age of 15 years and was 64% male (128/200). We divided into four groups on the basis of the age (0–10; 11–18; 19–30; 31–49) and gender (Figure 1). All individuals displayed ID, 58% (116/200) associated with ASD. The totality of the patients had been subjected to genetic testing prior to enrolment in this study. Our ID and ID/ASD cohort was characterized by the presence of additional associated clinical findings for 90% of the patients (179/200). These included epilepsy (n = 41/200, 20%), hypotonia (n = 10/200, 5%), and MRI abnormalities observed in 13 patients. Craniofacial dysmorphisms (n = 106/200, 53%) were found in 53% of the cases. The clinical descriptions of the 200 patients are summarized in Table S1.

![Figure 1. Demographic analysis of the cohort, filtered by age and gender.](image-url)
3.2. P/LP Variants Identified by ES

ES was performed in 200 probands with ASD and/or ID and 41 different pathogenic/likely pathogenic (P/LP) variants were identified with a detection rate of 22% (Table S2). Clinical features of patients with pathogenic variants in disease genes were described in Table 1.

Variants were classified based on frequency, mutation category, literature, and databases such as ClinVar. Affected individuals were categorized based on the number of parents that were sequenced along with the proband(s): proband-parent trios (184); duos with one parent (6); and proband-only singletons (10). Most P/LP variants were missense variants (15/200; 8%), while 5% (9/200) were nonsense, 7% (13/200) frameshift and 2% (4/200) splicing variants. Most (62%) P/LP variations occurred de novo, while 29% of individuals inherited P/LP variants as heterozygotes or homozygotes. Four out of 200 (2%) participants who harboured a P/LP result were sequenced with one or no biological parent and thus have unknown inheritance.

Mutations in the following genes were found in patients suffering from ID: ADNP, AP4M1, ATP1A3, BSCL2, CACNA1A, CSNK2B, CTU2, DEPDC5, DYRK1A, EFTUD2, HK1, IQSEC2, KANSL1, KIF1A, KMT2A, MBOAT7, MED13L, MMACHC, POGZ, PTPN11, RHOBTB2, SHANK3, SPTBN2, TBCE, TUBA1A, WDR45, WFS1. In patients presenting autism and ID we found pathogenic variants in: BCOR, DDX3X, FGFR3, KCNQ3, SHANK3, SYNGAP1, TREX1, UPF3B, WFS1 (Table S2).

Two de novo P/LP variants were found in the new candidate genes: CACNA2D1 and GPR14 (Table S3), and the corresponding clinical pictures were reported in Table 2.

3.3. Uncertain Variants Identified by ES

We further reported 45 uncertain variants including in this number the variants that are currently considered to have an uncertain significance in the databases and other missense variants that have not been previously described in the scientific literature (Table S4). The effect on the encoded mutated proteins has been predicted using CADD (combined depletion annotation depletion). In our study the majority of uncertain and of P/LP variants fall in genes that play a role in the axon guidance and in the neurodevelopment processes (https://reactome.org/PathwayBrowser/#/, accessed on 6 July 2021).
Table 1. Clinical features of patients with pathogenic variants in disease genes.

| Patient | Gene | Variant (HGVS) | Protein (HGVS) | Gender | Age (Years) | ID/ASD | Craniofacial Dysmorphisms | Additional Clinical Signs | OMIM Phenotype (n°) |
|---------|------|----------------|---------------|--------|-------------|--------|--------------------------|-------------------------|---------------------|
| #1      | ADNP | c.2314dup      | p.(Thr772Asnfs *16) | Male   | 7           | ID     | Yes (Plagiocephaly, prominent forehead, sparse eyebrows, broad nasal bridge, small ears, uplifted earlobes, conical teeth) | Bilateral clinodactyly of the 5th toe, supernumerary nipple, epilepsy, heteroaggressive behaviour | Helsmoortel-van der Aa syndrome (#615873) |
| #2      | AP4M1| c.1257_1282del c.1317G > A p.(Val421Alafs *98) p.(Trp439 *) | Female | 18      | ID          | No     | Yes (microcephaly, triangular face, widow’s peak, thick eyebrows, large nose, flat philtrum, thin lips, high vaulted and narrow palate) | Short stature, clinodactyly of the fifth finger, laryngomalacia, splenium thinning, epilepsy, hearing loss, flat foot, stereotypies, epilepsy, speech defect | Spastic paraplegia 50, autosomal recessive (#612936) |
| #3      | ATP1A3| c.2116G > A | p.(Gly706Arg) | Female | 22          | ID     | No | Epilepsy, ataxia, limited speech | | Alternating hemiplegia of childhood 2 (#614820)/CAPOS syndrome (#601338)/Dystonia-12 (#128235) |
| #4      | BCOR | c.776C > T | p.(Ser259Leu) | Male   | 14          | ID and ASD | Yes (low hairline, periorbital swelling, big mouth, full lips) | Stereotypies | Microphthalmia, syndromic, 2 (#300166) |
| #5      | BSCL2| c.856C > T | p.(Arg286 *) | Female | 22          | ID     | Yes (elongated faces, prominent ears, high nasal bridge, open mouth and protruded tongue) | Dystonia, myoclonia, drug-resistant epilepsy, language delay, psychomotor delay, bruxism, hypertrichosis | Dystonia, myoclonia, hypertrichosis, language delay, drug-resistant epilepsy, psychomotor delay | Encephalopathy, progressive, with or without lipodystrophy (#61924) |
| #6      | BSCL2| c.856C > T | p.(Arg286 *) | Female | 16          | ID     | Yes (faces with coarse features, synophia, bulbous nose, large ears, large mouth) | | Developmental and epileptic encephalopathy 42 (#617106); Episodic ataxia, type 2 (#108500); Migraine, familial hemiplegic, 1 (#141500); Migraine, familial hemiplegic, 1, with progressive cerebellar ataxia (#141500); Spinocerebellar ataxia 6 (#183086) |
| #7      | CACNA1A| c.4055G > A | p.(Arg1352Gln) | Male   | 14          | ID     | Yes (Craniosynostosis, long filter, long eyelashes, thin upper lip) | Stereotypies, alternating hemiplegia, language delay | |
| Patient | Gene | Variant (HGVS) | Protein (HGVS) | Gender | Age (Years) | ID/ASD | Craniofacial Dysmorphisms | Additional Clinical Signs | OMIM Phenotype (n°) |
|---------|------|---------------|---------------|--------|-------------|--------|--------------------------|--------------------------|-----------------------|
| #8      | CSNK2B | c.170del   | p.(Glu57Glyfs *15) | Female | 20          | ID     | Yes (Open mouth, protruding tongue, narrow palate, dental anomalies) | Epilepsy, hypotonia, developmental delay, absent language | Poirier-Bienvenu neurodevelopmental syndrome (#618732) |
| #9      | CTU2  | c.881C > A  | p.(Ser294 *)   | Female | 22          | ID     | Yes (broad eyebrows, low anterior and posterior hairline, forehead hypertelorism, prominent ears, eye asymmetry, large nose with wide nostrils, large mouth, dental anomalies) | Epilepsy, hypotonia, lower limb hypertonia, recurrent infections, thinning corpus callosum, growth retardation, pectus excavatum, clinodactyly of the 5th fingers. | Microcephaly, facial dysmorphism, renal agenesis, and ambiguous genitalia syndrome (#617057) |
| #10     | DEPDC5 | c.1453C > T | p.(Arg485 *)  | Male   | 11          | ID     | Yes (low hairline, flattened nasal bridge, bulbous nasal tip, anteverted nostrils, full lips, small and widely spaced teeth, anteverted ears) | Epilepsy, language delay | |
| #11     | DDX3X | c.976C > T  | p.(Arg326Cys) | Female | 12          | ID and ASD | Yes (Microcephaly, long face, smooth and long philtrum, strabismus, up slanting palpebral fissures) | Developmental delay, stereotypic hand movements, hypotonia, bruxism, sialorrhea, corpus callosum hypoplasia | Intellectual developmental disorder, X-linked, syndrome; Snijders Blok type (#300958) |
| #12     | DYRK1A | c.1669C > T | p.(Gln557 *)  | Male   | 16          | ID     | Yes (Microcephaly, narrow forehead, frontal bossing, depressed nasal bridge, short philtrum, prognathism) | Hypotonia, epilepsy, stereotypies, absent language | Mental retardation, autosomal dominant 7 (#614104) |
| #13     | EFTUD2 | c.702 + 1G > A | NA           | Female | 22          | ID     | Yes (prominent columnella, sloping forehead, high and scattered eyebrows, deeply set eyes, nose with anteverted nostrils, narrow palate, prominent incisors, prominent ears) | Hypertrichosis, small hands and feet, joint hyperextensivity | Mandibulofacial dysostosis, Guion-Almeida type (#603892) |
| #14     | FGFR3  | c.749C > G  | p.(Pro250Arg) | Female | 4           | ID and ASD | Yes (craniosynostosis, flat forehead, down slanting palpebral fissures, high nasal bridge) | Language delay, hyperchromic and hypochromic spots | Muenke syndrome (#602849) CATSHL syndrome (#610474) |
| Patient | Gene | Variant (HGVS) | Protein (HGVS) | Gender | Age (Years) | ID/ASD | Craniofacial Dysmorphisms | Additional Clinical Signs | OMIM Phenotype (n°) |
|---------|------|----------------|---------------|--------|-------------|--------|---------------------------|-------------------------|-----------------------|
| #15     | HK1  | c.1367C > T    | p.(Thr456Met) | Male   | 16          | ID     | Yes (proptosis, long eyelashes, synophria, narrow palate, dental anomalies, small mouth, and full lips) | Bilateral cryptorchidism, psychomotor delay, poor vision, ventricular system dilation, immature hippocampal structures, spastic paraparesis, epilepsy, hypothyria, absent language | Neurodevelopmental disorder with visual defects and brain anomalies (#618547) |
| #16     | IQSEC2 | c.3780del | p.(Gln1261Serfs*136) | Female | 22          | ID     | No | Strabismus, cerebral atrophy, sialorrhea, motor problems, bruxism | Language delay, epilepsy, aggressiveness | Mental retardation, X-linked 1/78 (#309530) |
| #17     | KANSL1| c.985_986del | p.(Leu329Glu fs*22) | Female | 6           | ID     | Yes (cleft palate, deeply set eyes, high nasal bridge, bulbous nasal tip) | Yes (triangular facies, high nasal bridge, bulbous nasal tip, open mouth, micrognathia, narrow and downturned eyelids) | Language delay, epilepsy, aggressiveness | Koolen-De Vries syndrome (#610443) |
| #18     | KCNQ3 | c.688C > T    | p.(Arg230Cys) | Male   | 39          | ID and ASD | Stereotypies, aggressiveness, bladder anomalies | Spastic paraparesis, behaviour disorder, slight enlargement of the interfolial spaces of the cerebellar hemispheres, hypertone Language delay, cerebellar and worm atrophy, psychomotor delay, brain abnormalities, hypertrichosis, bilateral clinodactyly of the second/third/fourth/fifth toes | Seizures, benign neonatal, 2 (#121201) |
| #19     | KIF1A | c.37C > T     | p.(Arg13Cys)  | Female | 16          | ID     | No | | Spastic paraplegia 30, autosomal dominant (#610357) |
| #20     | KIF1A | c.914C > T    | p.(Pro305Leu) | Female | 10          | ID     | Yes (sparse eyebrows, small nose, thin upper lip, dental anomalies, chubby cheeks) | | Spastic paraplegia 30, autosomal dominant (#610357) |
| #21     | KIF1A | c.914C > T    | p.(Pro305Leu) | Male   | 49          | ID     | No | Ataxia, spastic paraparesis, angiomata, nystagmus, epilepsy | Hypertrichosis of the legs, arms and lumbar region, large hands, short fifth ray of the foot | Spastic paraplegia 30, autosomal dominant (#610357) |
| #22     | KMT2A | c.5256delA   | p.(Ala1753Profs*70) | Male   | 16          | ID     | Yes (thick eyebrows, narrow and short eyelids, depressed helix, nose with bulbous tip, thick lips) | | Wiedemann-Steiner syndrome (#605130) |
| Patient | Gene | Variant (HGVS) | Protein (HGVS) | Gender | Age (Years) | ID/ASD | Craniofacial Dysmorphisms | Additional Clinical Signs | OMIM Phenotype (n°) |
|---------|------|----------------|---------------|--------|-------------|--------|--------------------------|--------------------------|------------------------|
| #23     | MBOAT7 | c.477C > G | p.(Tyr159 *) | Female | 2           | ID     | No                       | Motor stereotypies, epilepsy, thinning of the corpus callosum, ventricular enlargement, Syndactyly of the second and third toes, small feet, hypertrophy of the limbs and truncal obesity, strabismus | Mental retardation, autosomal recessive 57 (#617188) |
| #24     | MED13L | c.72 + 1G > T | NA           | Female | 49          | ID     | Yes (sparse eyebrows, hypertelorism, narrow eyelids, gingival hypertrophy) | Mental retardation and distinctive facial features with or without cardiac defects (#616789) | Methylmalonic aciduria and homocystinuria, cbLC type (#277400) |
| #25     | MMACHC | c.440G > C | p.(Gly147Ala) | Male   | 12          | ID     | Yes (synophria, horizontal eyebrows, wide nasal tip, anteverted nostrils, long filter, dental anomalies) | Spastic paraparesis, language delay, polyneuropathy, Hyperactivity, blepharophimosis, brachydactyly, nail hypoplasia, kidney abnormalities, language delay | Methylmalonic aciduria and homocystinuria, cbLC type (#277400) |
| #26     | POGZ  | c.1180_1181del | p.(Met394Valfs *9) | Female | 6           | ID     | Yes (microcephaly, deeply set eyes, nose with bulbous tip, anteverted nostrils, full lips) | White-Sutton syndrome (#616364) |
| #27     | POGZ  | c.1180_1181del | p.(Met394Valfs *9) | Male   | 12          | ID     | Yes (narrow bitemporal diameter, narrow and upward eyelid rims, deep philtrum, proptosis, exaggerated Cupid’s bow, buccal rim pointing downwards, uplifted ear lobe) | Hypotonia, obesity | White-Sutton syndrome (#616364) |
| #28     | POGZ  | c.1180_1181del | p.(Met394Valfs *9) | Female | 44          | ID     | No                       | Microcephaly, brachydactyly, nail hypoplasia | White-Sutton syndrome (#616364) |
| #29     | PTPN11 | c.1471C > A | p.(Pro491Thr) | Male   | 2           | ID     | Yes (high forehead, low-set ears with large, downward-pointing auricles, down-slanting eyelids, broad nasal tip, long and thick filter, exaggerated Cupid’s bow) | Café au lait spots in the thoracic and lumbar region, nail hypoplasia of the fifth toe, neurodevelopmental delay, cryptorchidism | Noonan syndrome 1 (#163950) |
Table 1. Cont.

| Patient | Gene     | Variant (HGVS) | Protein (HGVS) | Gender | Age (Years) | ID/ASD | Craniofacial Dysmorphisms | Additional Clinical Signs                                                                                          | OMIM Phenotype (n°)                        |
|---------|----------|----------------|----------------|--------|-------------|--------|----------------------------|------------------------------------------------------------------------------------------------------------------|--------------------------------------------|
| #30     | RHOBTB2  | c.1382G > A    | p.(Arg461His)  | Male   | 18          | ID     | Yes (microcephaly, thick eyebrows, deeply set eyes, square chin) | epilepsy, thinning corpus callosum, language delay, cold extremities, cerebellar atrophy, muscle hypertrophy  | Developmental and epileptic encephalopathy 64 (#618004) |
| #31     | SHANK3   | c.2717_2718dup | p.(Ser907Alafs *3) | Male   | 45          | ID and ASD | Yes (alopecia in the fronto-temporal region, depressed ocular region, thin upper lip) | Absent language, obsessive behaviour, tapered fingers with widening of the intermediate interphalangeal joints, nail dystrophy, clinodactyly of the third toe, stereotypies, mouth chewing automatisms epilepsy, psychomotor delay Language delay, psychomotor delay, manual stereotypies, increased tolerance to pain Epilepsy, neurodevelopmental regression, stereotypies, syndactyly Strabismus, hyperchromic spot in the thoracic area, cerebellar atrophy, language delay, ataxia Stereotypies, self-harm, cold extremities, sphincter control not acquired, language regression Scoliosis, sialorrhea, optic atrophy, psychomotor delay epilepsy, spastic paraparesis, absent language. | Phelan-McDermid syndrome (#608232) |
| #32     | SHANK3   | c.2313 + 1G > A | NA             | Male   | 21          | ID and ASD | No                                                                 | Phelan-McDermid syndrome (#606232) |
| #33     | SHANK3   | c.3250_3253del | p.(Leu1084Cysfs *9) | Male   | 18          | ID     | No                                                                 | Phelan-McDermid syndrome (#606232) |
| #34     | SPG7     | c.233T > A     | p.(Leu78 *)    | Female | 10          | ID     | Yes (high forehead, spaced teeth, pursed lips attitude)            | Spastic paraplegia 7, autosomal recessive (#607259)                                                     |
| #35     | SPTBN2   | c.1310G > A    | p.(Arg437Gln)  | Male   | 17          | ID     | Yes (sloping forehead, prognathism)                               | Spino cerebellar ataxia 5 (#600224)                                                               |
| #36     | SYNGAP1  | c.2337−1G > A  | NA             | female | 26          | ID and ASD | No                                                                 | Mental retardation, autosomal dominant 5 (#612621)                                                   |
| #37     | TBCE     | c.464T > A     | p.(Ile155Asn)  | Male   | 21          | ID     | Yes (bullaous nasal tip, full lips, spaced teeth)                 | Encephalopathy, progressive, with atrophy and optic atrophy (#617207)                               |
| Patient | Gene | Variant (HGVS) | Protein (HGVS) | Gender | Age (Years) | ID/ASD | Craniofacial Dysmorphisms | Additional Clinical Signs | OMIM Phenotype (n°) |
|---------|------|----------------|----------------|--------|-------------|--------|----------------------------|--------------------------|------------------------|
| #38     | TREX1| c.558_573del   | p.(Phe186Leufs *24) | Male   | 10          | ID and ASD | No                         | Gastroesophageal reflux, sphincter control not acquired, stereotyped behaviour | Aicardi-Goutieres syndrome 1 dominant and recessive (#225750) |
| #39     | TUBA1A| c.352G > A     | p.(Val118Met)     | Male   | 9           | ID     | Yes (advanced hairline, long and thick eyebrows, anteverted nostrils, long filter, thin upper lip) | Yes (advanced hairline, long and thick eyebrows, anteverted nostrils, long filter, thin upper lip) | Lissencephaly 3 (#611603) |
| #40     | URF3B| c.1288C > T    | p.(Arg430 *)      | Male   | 7           | ID     | Yes (Wide forehead, arched eyebrows, deeply set eyes, pointed chin) | Yes (Wide forehead, arched eyebrows, deeply set eyes, pointed chin) | Mental retardation, X-linked syndromic 14 (#300676) |
| #41     | WDR45| c.66del        | p.(Cys23Alafs *15) | Female | 20          | ID     | Yes (thick eyebrows, prominent upper arch, hyperemic gums, high palate) | Yes (thick eyebrows, prominent upper arch, hyperemic gums, high palate) | Neurodegeneration with brain iron accumulation 5 (#800894) |
| #42     | WFS1 | c.124C > T     | p.(Arg42 *)       | Male   | 6           | ID and ASD | No                         | Yes (microcephaly, synophria, long eyelashes, bulbous nasal tip) | Wolfram-like syndrome, autosomal dominant (#614296) |
| #43     | WFS1 | c.1230_1233del | p.(Leu412Serfs *29)  | Female | 14          | ID     | No                         | Yes (microcephaly, synophria, long eyelashes, bulbous nasal tip) | Wolfram-like syndrome, autosomal dominant (#614296) |

* means change in a stop codon.
Table 2. Clinical features of patients with pathogenic variants in the new candidate genes.

| Patient | Gene | Variant (HGVS) | Protein (HGVS) | Gender | Age (Years) | ID/ASD | Craniofacial Dysmorphisms | Additional Clinical Signs |
|---------|------|----------------|----------------|--------|-------------|--------|---------------------------|--------------------------|
| #44     | CACNA2D1 | c.659–2_659-1insT | NA | Female | 14 | ID | Yes (deeply set eyes, squared facies, bulbous nasal tip, full lips, horizontal eyebrows, enlarged nasal bridge, mouth with downward corners, antverted nostrils) | Language delay, epilepsy |
| #45     | GPR14 (before UTS2R) | c.844C > T | p.(Gln282 *) | Male | 6 | ID and ASD | Yes (long and large nose, broad nasal bridge, antverted nostrils, deep philtrum) | Frequent infections |

* means change in a stop codon.
4. Discussion

This study emphasizes the clinical diagnostic relevance of ES in patients with ID and/or autism with additional clinical features. In particular, in a cohort of 200 patients, we reached a diagnostic yield of 22% (43/200) with a higher rate in ID patients (33/84; 39%) with respect to ID and ASD patients (10/116; 9%). The diagnostic yield is lower with respect to other studies that employ ES in neurodevelopmental disorder (30–43%) [13]. ES has technological limitations, including the inability to detect noncoding variants, copy number variants (CNVs), epigenetic changes, and trinucleotide repeat expansion [14]. In our cohort, 30% of cases have not been screened for CNVs and this could have underestimated the presence of other pathogenic genetic alterations, in particular in patients with ASD. Until recently, whole genome chromosomal microarray was recommended as a first-tier clinical genetic test for detecting disease-causing CNVs in individuals with ASD [15–17].

Most of the previous diagnoses failed for the atypical phenotypic presentation of well-established genetic syndromes. The DDX3X mutated patient (#11) shows Rett-like spectrum features with typical hand-washing stereotypes and was initially screened for mutations in MECP2, FOXG1 and CDKL5 genes [18–21]. Differently, a hundred patients are reported in literature mutated in DDX3X with various clinical features including hypotonia, movement disorder, behavioural problems, corpus callosum hypoplasia and epilepsy [22,23]. Another atypical clinical picture was manifested by a patient bearing the mutation c.688C > T (p.(Arg230Cys)) in KCNQ3, who did not suffer from any status epilepticus but showing ID, autism, stereotypies, aggressiveness, bladder anomalies; he also presented craniofacial dysmorphisms (patient #18). KCNQ3 pathogenic alterations are generally linked to the occurrence of seizures but recently patients with no EEG abnormalities have been described [24]. KIF1A gene was found altered in three unrelated cases with different phenotypic presentations (patients #19, #20, #21). KIF1A mutations cause NESCAV syndrome (NESCAVS), a neurodegenerative disorder characterized by global developmental delay, progressive spasticity, ID, speech delay, learning disabilities and/or behavioural abnormalities [25]. The mutation c.37C > T (p.(Arg13Cys)) in KIF1A was found in patient #19 with spastic paraparesis, behaviour disorder, slight enlargement of the interfolial spaces of the cerebellar hemispheres, hypertone and no craniofacial dysmorphisms. In particular the same mutation c.914C > T (p.(Pro305Leu)) was shown in two different patients (#20, #21). One of these, presented language delay, cerebellar and vermis atrophy, psychomotor delay, brain abnormalities, hypertrichosis, bilateral clinodactyly, and facial dysmorphisms (patient #20). The other showed ataxia, spastic paraparesis, angioma, nystagmus, and seizures (patient #21). Mutations in POGZ are associated with the White-Sutton syndrome, which is a neurodevelopmental disorder characterized by delayed psychomotor development and a characteristic constellation of dysmorphic facial features [26]. Additional features may include hypotonia, sensorineural hearing impairment, visual defects, joint laxity, and gastrointestinal difficulties [27]. The pathogenic variant c.1180_1181del (p.(Met394Valfs*9)) was carried by two siblings and their mother (#26, #27, #28). The sister exhibited craniofacial dysmorphisms and ID; she also showed hyperactivity, blepharophimosis, brachydactyly, nail hypoplasia, kidney abnormalities and language delay as additional clinical signs (patient #26). The brother was affected by ID, hypotonia, obesity and had some craniofacial dysmorphisms (patient #27). Their mother instead displayed ID, microcephaly, brachydactyly, and nail hypoplasia (patient #28). Another example, SHANK3 was found altered in three unrelated patients (#31, #32, #33). Mutations in SHANK3 cause Phelan-McDermid syndrome, a developmental disorder with variable features including neonatal hypotonia, global developmental delay, absent to severely delayed speech, autistic behaviour, and minor dysmorphic features [28–30]. One of the three mutated patients did not show neither autism nor dysmorphic features and was initially classified as a “non-syndromic” ID case.

The following new candidate genes for ID/ASD have emerged: CACNA2D1 and GPR14. They all show de novo truncating variants (patients #44–#45). CACNA2D1 encodes the alpha-2/delta subunit of skeletal muscle and brain voltage-dependent calcium
channels [31]. A genomic aberration affecting the CACNA2D1 gene has been previously characterized in patients with epilepsy and ID, pinpointing the gene as an interesting candidate gene for these clinical features [32]. Mice-bearing point mutations in the CACNA2D1 gene have an abnormal central nervous system synaptic transmission [33]. GPR14 gene, encoding the orphan G protein-coupled receptor 14 for Urotensin II, is widely expressed in the brain and spinal cord [34]. We found 45 variants of unknown significance (VUS) in 40 patients. These variants are 22/84 (26%) in ID patients and 18/116 (16%) in ID/ASD patients. These variants are mostly missense 42/45 (93%) and CADD ≥ 25 in 20/42 (48%) of cases. With increased knowledge over time, exome reanalysis may change the clinical interpretation of a VUS. Thus, it is important to list all the VUS, analyse them periodically and write a report in case of changes to provide a timely response for patients and families. An accurate molecular diagnosis allows for precise genetic counselling and has the potential to change clinical management.

5. Conclusion

ES was able to avoid a sort of “diagnostic odyssey” for a significant fraction of families consisting in the step-by-step application of the traditional genetic methods. ES revealed atypical phenotypic presentations and new candidate genes for ID/ASD. Further studies are needed to better characterize the contribution of new candidates and to show how their haploinsufficiency can determine ID/ASD.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/brainsci11070936/s1, Table S1: Clinical features of the 200 ID and ID/ASD patients; Table S2: P/LP variants identified in disease genes; Table S3: P/LP variants identified in candidate disease genes; Table S4: Uncertain variants identified by ES

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Data Availability Statement: NGS data has been deposited in publicly accessible repositories. The data can be found here: http://nigdb.cineca.it/.

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