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Exome sequencing as first-tier test for fetuses with severe central nervous system structural anomalies

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KEYWORDS: brain; central nervous system; chromosomal microarray; copy-number variant; exome sequencing; fetus; malformation; prenatal diagnosis

CONTRIBUTION

What are the novel findings of this work?
In this study of 114 cases that underwent termination of pregnancy following the detection of a major central nervous system anomaly, chromosomal microarray analysis (CMA) detected causative copy-number variants (CNVs) in 10% of fetuses. Among 86 CMA-negative cases, exome sequencing (ES) detected causative sequence variants in 44%. The ES bioinformatics pipeline also detected 13 of the causative and previously known non-causative CNVs.

What are the clinical implications of this work?
Our data suggest that ES could be considered as a first-tier clinical diagnostic test in the prenatal diagnosis of fetuses with major CNS anomalies, as it can detect both sequence variants and CNVs.

ABSTRACT

Objective *Prenatally detected central nervous system (CNS) anomalies present a diagnostic challenge. In this study, we compared the diagnostic yield of exome sequencing (ES) and chromosomal microarray analysis (CMA) in fetuses with a major CNS anomaly.*

Methods *This was a retrospective study of 114 cases referred for genetic evaluation following termination of pregnancy (TOP) due to a major CNS anomaly detected on prenatal ultrasound. All fetuses were first analyzed by CMA. All CMA-negative cases were offered ES. CMA-positive cases were reanalyzed using ES to assess its ability to detect copy-number variants (CNVs).*

Results *CMA identified a pathogenic or likely pathogenic (P/LP) CNV in 11/114 (10%) cases. Eighty-six CMA-negative cases were analyzed using ES, which detected P/LP sequence variants in 38/86 (44%). Among recurrent cases (i.e. cases with a previously affected pregnancy), the incidence of P/LP sequence variants was non-significantly higher compared with non-recurrent ones (12/19 (63%)* vs *26/67 (39%);* $P = 0.06$*). Among the 38 cases with an ES diagnosis, 20 (53%) were inherited and carried a significant risk of recurrence. Reanalysis of 10 CMA-positive cases by ES demonstrated that the bioinformatics pipeline used for sequence variant analysis also detected all P/LP CNVs, as well as three previously known non-causative CNVs.*

Conclusions *In our study, ES provided a high diagnostic yield (> 50%) in fetuses with severe CNS structural anomalies, which may have been partly due to the highly selected case series that included post-TOP cases from a specialist referral center. These data suggest that ES may be considered as a first-tier test for the prenatal diagnosis of major fetal CNS anomalies, detecting both P/LP sequence variants and CNVs. This is of particular importance given the time constraints of an ongoing pregnancy and the risk of recurrence in future pregnancies.*

INTRODUCTION

Approximately 2–5% of pregnancies present with a sonographically detected fetal anomaly that warrants further evaluation[1]. These anomalies range from an

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apparently isolated defect to multisystem malformations. A karyotype abnormality is found in 8–10% of cases with such an anomaly that undergo diagnostic testing, and a copy-number variant (CNV) is identified in an additional 6%, leaving the majority of cases without a specific genetic diagnosis[1–4]. This is because many anomalies have monogenic etiology (i.e. a single-gene disorder caused by a point mutation or small insertion/deletion)[1]. The detection of such genetic aberrations requires much higher resolution, which can be achieved only by next-generation sequencing technology, such as targeted multigene panels, exome sequencing (ES) or whole-genome sequencing. ES is sometimes applied as a second- or third-tier diagnostic test in selected cases of fetal anomaly. Recent work has shown that ES provides an additional yield of 5–57% over that of chromosomal microarray analysis (CMA) in the prenatal setting[5–9].

The central nervous system (CNS) is affected in 9% of fetal malformations but accounts for as much as one-third of all pregnancy terminations due to fetal anomaly. Moreover, CNS abnormalities are often diagnosed late in pregnancy[10]. When applying conventional methods (karyotyping followed by CMA or CMA alone) to the diagnosis of fetal CNS anomalies, the estimated diagnostic yield does not exceed 20%[11–13]. Thus, more than 80% of cases remain without a genetic diagnosis, probably owing to a large proportion of cases having monogenic etiology.

In this study, we compared the diagnostic yield of ES and CMA in fetuses with a major CNS anomaly leading to termination of pregnancy. In addition, we sought to determine the diagnostic yield for specific categories of CNS anomaly.

METHODS

This was a retrospective study of all cases referred to our institution for genetic evaluation following termination of pregnancy due to a major fetal CNS anomaly between 2014 and 2021. All patients underwent detailed neurosonographic examination in orthogonal planes, as described previously[14], at the Division of Obstetric Ultrasound at the Lis Maternity Hospital, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel, which is a tertiary referral center for fetal CNS anomalies. Patients then received comprehensive genetic counseling at the Prenatal Genetic Diagnosis Unit of the Genetics Institute. Following counseling, patients signed a standard clinical consent form for genetic testing.

Clinical data were retrieved retrospectively from electronic medical records at the Genetics Institute and prenatal ultrasound reports, as well as fetal brain magnetic resonance imaging (MRI) data, were also retrieved when available. All patient information was anonymized. Families with recurrent CNS anomalies were considered as a single case. Cases were further subcategorized according to clinical findings. Cases with multiple brain anomalies were categorized as 'complex', while cases with additional extracranial anomalies were categorized as 'multisystem'. Cases in which the anomaly affected only one brain developmental process were described separately according to the category, as determined by consensus: malformation of cortical development, midline anomaly (corpus callosal, septal, holoprosencephaly), brain cyst (subependymal, arachnoid), midbrain–hindbrain malformation and neural tube defect. These cases were defined as 'isolated severe CNS malformations'. Of note, mild isolated findings, such as mild ventriculomegaly, were not included in this study. Hydrocephalus secondary to aqueductal stenosis was considered a midbrain–hindbrain anomaly. Brain damage was not considered an isolated anomaly if the insult affected different regions of the brain simultaneously.

Genetic analysis

As routine practice, all cases of fetal anomaly subjected to termination of pregnancy underwent DNA extraction from amniotic fluid or fetal blood (obtained during feticide). Extraction from blood always produced sufficient DNA. Direct extraction following amniocentesis usually suffices for both CMA and ES, but, occasionally, cell culture is needed if both analyses are to be performed.

All cases were first analyzed by CMA, some prior to termination of pregnancy and some as part of the post-termination work-up. CMA was performed using a CytoScan 750K array (Affymetrix, Santa Clara, CA, USA). The platform is composed of 550 000 non-polymorphic CNV probes and more than 200 000 single-nucleotide polymorphism probes, with an average resolution of 100 kb. Analysis was performed using the Chromosome Analysis Suite (ChAS) software v 3.1 (Affymetrix). Detected CNVs were classified as either pathogenic (P), likely pathogenic (LP), benign, likely benign or as a variant of uncertain clinical significance (VOUS), according to the updated American College of Medical Genetics and Genomics (ACMG) and Clinical Genome Resource (ClinGen) guidelines[15]. A genomic map from the University of California, Santa Cruz Genome Browser[16] was used to map the locations of CNVs and gene content according to build hg19/GRCh37. The Database of Genomic Variants[17], as well as our internal database, provided catalogs of structural variations found in healthy controls.

All patients with a negative CMA result received further genetic counseling and were offered ES trio analysis. CMA-positive cases were reanalyzed using ES to assess its ability to detect CNVs. DNA samples from parents–fetus trios underwent ES. The first 15 cases were outsourced to external accredited clinical laboratories (Centogene or CeGat, Germany). Cases 1–5 have been reported previously[18]. The majority (71 initially evaluated cases and 10 reanalyzed cases (84%)) were sequenced and analyzed at the Tel Aviv Sourasky Genomics Center. Sequencing was performed on a NovaSeq 6000 sequencer (Illumina, San Diego, CA, USA) with 100-bp paired-end reads.

The entire bioinformatics pipeline from FASTQ files to the final report was performed using the Franklin genetic analysis platform (Genoox, Tel Aviv, Israel).

FASTQ files were aligned against the hg19/GRCh37 reference genome using BWA v 0.7.16[19]. Variant calling for single nucleotide variants (SNVs) and indels was performed using Genome Analysis Toolkit v 4.0.12.0[20] and FreeBayes v 1.1.0[21].

In addition to SNVs and small indels, the Franklin pipeline also detects CNVs using Genoox proprietary CNV-caller, which mainly utilizes coverage information and is expected to detect CNVs down to a resolution of two exons in the heterozygous state and a single exon in the homozygous state[22].

Sequence variant annotation, classification and prioritization, as well as CNV classification, were performed using Franklin according to ACMG guidelines[15,23]. The bioinformatics analysis was based solely on the prenatal phenotype. The minimal criteria for a sample to be included in the analysis were average coverage of 70× and at least 98% of variants with a quality score above 40. Family-case analysis was prepared considering different possible modes of inheritance. The variants were filtered, retaining the following: (1) rare variants with minor allele frequency < 5%; (2) protein-altering variants; (3) variants in coding regions or those up to 10 bp from splice junctions; (4) variants with a high or medium aggregated quality score. Variants were prioritized by Franklin based on their degree of pathogenicity and clinical information transcribed into Human Phenotype Ontology (HPO) terms[24].

Statistical analysis

Statistical analysis was performed using Social Science Statistics[25]; $P < 0.05$ was considered to indicate statistical significance.

RESULTS

The study included 114 consecutive cases following termination of pregnancy due to a major fetal CNS anomaly. MRI was performed in 25 cases at a median gestational age of 30 weeks. Postmortem confirmation of the imaging findings was obtained in 21 cases. All fetuses were first analyzed by CMA. Eleven (10%) cases had a P/LP CNV (Figure 1), most of which were deletions harboring haploinsufficient genes (Table 1). These included nine *de-novo* CNVs, one case of triploidy and a case with a terminal deletion of 3p26.1 and a terminal duplication of 8q24.3, compatible with an unbalanced reciprocal translocation. In the latter case, parental karyotyping revealed that the mother carried a balanced translocation 46,XX,t(3;8)(p26.1;q24.3), conferring a significant risk of recurrence in subsequent pregnancies.

Of the 103 CMA-negative cases, 13 patients declined further analysis by ES and, in four other cases, there was insufficient fetal DNA for further analysis (Figure 1). The remaining 86 cases underwent ES analysis. These included 82 fetus–parents trios, one solo fetal analysis, one fetus–mother duo and two quatros including both parents and two affected fetuses. P/LP (Class V/IV) sequence variants were detected in 38/86 (44%) cases (Table 2). In nine (10%) additional cases, a VOUS with potential clinical relevance was reported (Table S1). In the remaining 39 cases, no variant was reported (Table S2).

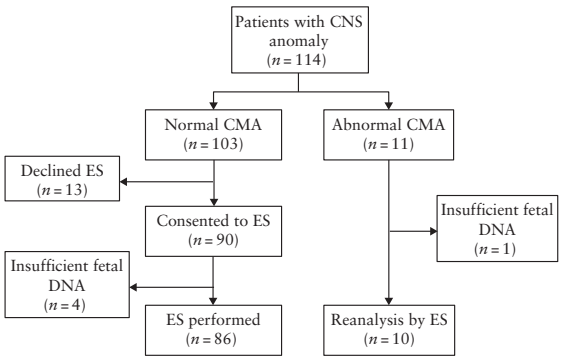

Figure 1 Flowchart summarizing genetic testing workflow in cases that underwent termination of pregnancy following detection of a fetal central nervous system (CNS) anomaly on ultrasound. CMA, chromosomal microarray analysis; ES, exome sequencing.

The incidence of P/LP sequence variants was non-significantly higher among the recurrent cases (i.e. cases with a previously affected pregnancy) compared with non-recurrent cases (12/19 (63%) *vs* 26/67 (39%), χ-square statistic = 3.56, $P = 0.06$). No difference in the incidence of P/LP sequence variants was noted between cases with an anomaly limited to the CNS and those with multisystem abnormalities (24/54 (44%) *vs* 14/32 (44%)). Of the cases with an ES diagnosis, 20/38 (53%) were inherited and carried a significant risk of recurrence (eight autosomal recessive (AR), five autosomal dominant (AD) and seven X-linked cases). The remaining 18 cases had a *de-novo* heterozygous variant in an AD or X-linked gene.

To evaluate the detection rate in different clinical categories, cases were subcategorized (Table 3). The highest diagnostic yield was observed in cases categorized as having multisystem (14/32 (44%)) and complex brain (14/24 (58%)) anomalies. Cases with specific isolated brain anomalies were too few to draw definitive conclusions. As a group, cases with isolated severe CNS malformation had the lowest diagnostic yield (7/21 (33%)), as did cases with brain damage (3/9 (33%)).

To assess whether the bioinformatics pipeline employed for SNV analysis (Franklin) can detect CNVs, we performed ES analysis in 10 of the 11 cases detected by CMA (Cases 9, 11, 15, 17, 18, 27, 47, 53, 63 and 71). In Case 54, there was insufficient DNA for ES analysis. Three additional cases had incidental, non-causative CNVs (Cases 16, 86 and 107). The CNV size ranged from 335 kb to 19 080 kb, and all CNVs were called correctly by the bioinformatics analysis pipeline. Triploidy of Case 15 was not detected directly by the pipeline. However, the homozygous/heterozygous ratio, a quality control metric, was 0.34, far below the normal range (0.45–0.80), suggesting such an abnormality.

In summary, 96 cases had undergone ES analysis (86 CMA-negative cases and 10 reanalyzed CMA-positive

Table 1 Pathogenic (P) and likely pathogenic (LP) copy-number variants detected by chromosomal microarray analysis (CMA) among 114 cases of fetal central nervous system anomaly

Case	*Clinical findings*	*Category*	*CMA result*	*Causative gene(s)*	*Genomic coordinates*	*Size (kb)*	*CMA classification*
9	Dysplastic CC, irregular ventricular wall, pontine hypoplasia	Complex brain	del14q22.3*	*OTX2*	arr14q22.3 (56 616 566–57 447 523) × 1	830	P
11	Dysplastic CC, signs of MCD	Complex brain	del16p13.3*	*CREBBP*	arr16p13.3 (3 650 502–4 005 644) × 1	335	P
15	Microcephaly, cerebellar hypoplasia, VSD, ARSA, echogenic kidneys, deviated stomach, absent gallbladder	Multisystem	Triploidy†	Multiple	arr(1–22X) × 3		P
17	Dysplastic CC, delayed sulcation, pontocerebellar hypoplasia	Complex brain	del5p15.33p14.3*	Multiple	arr5p15.33p14.3 (1–19 807 631) × 1	19 080	P
18	CC agenesis, fused thalami, abnormal midbrain	Complex brain	del5q14.3*	*MEF2C*	arr5q14.3 (87 265 522–88 674 041) × 1	1409	P
27	Vermian dysgenesis, abnormal anterior horns, signs of MCD, pelvic kidney	Multisystem	del12q24.33*	Multiple	arr12q24.33 (130 958 926–133 777 562) × 1	2818	LP
47	Macrocephaly, mild VM, dysplastic CC, signs of MCD, overgrowth	Multisystem	del8q22.1*	*PTDSS1*	arr8q22.1 (97 214 184–98 480 468) × 1	1266	P
53	Dysplastic CC, irregular ventricular walls, abnormal brainstem morphology, signs of MCD, annular pancreas	Multisystem	del3q13.31q21.2*	Multiple	arr3q13.31q21.2 (116 161 679–125 721 029) × 1	9559	P
54	Vermian hypoplasia	MBHB	del3q24q25.1‡	*ZIC1*	arr3q24q25.1 (143 357 046–149 188 029) × 1	5831	P
63	Asymmetric VM, dysplastic CC, signs of MCD	Complex brain	del17p13.3p13.2*	*PAFAH1B1 (LIS1)*	arr17p13.3p13.2 (838 750–4 034 456) × 1	3196	P
71	Dysplastic CC, signs of MCD, short long bones, IUGR, oligohydramnios	Multisystem	del3p26.1/dup8q24.3*§	Multiple	arr3p26.1 (61 891–5 443 206) × 1/ arr8q24.3 (144 794 838–1 462 957 710) × 3	5381/1501	P

*Deletion also detected by exome sequencing. †Triploidy suspected by exome sequencing due to low homozygosity/heterozygosity ratio. ‡Insufficient DNA for confirmation by exome sequencing. §Maternal balanced reciprocal translocation 46XX,t(3;8)(p26.2;q24.3). ARSA, aberrant right subclavian artery; CC, corpus callosum; IUGR, intrauterine growth restriction; MBHB, midbrain–hindbrain malformation; MCD, malformation of cortical development; VM, ventriculomegaly; VSD, ventricular septal defect.

Table 2 Pathogenic (P) and likely pathogenic (LP) variants detected by exome sequencing among 86 cases of fetal central nervous system anomaly with a negative result on chromosomal microarray analysis

Case	*Imaging findings*	*Clinical category*	*Gene*	*Variant(s)*	*Variant ACMG classification*	*Zygosity*	*Inheritance pattern*
1	VM, pachygyria, cerebellar hypoplasia, dysplastic CC	Complex brain	*TUBA1A*	NM_006009.4: c.1105G>A; p.(Ala369Thr)	P (PP5, PM1, PP2, PM2, PS2)	het (*de novo*)	AD
2	CC agenesis, signs of diffuse MCD	Complex brain	*ARX*	NM_139058.3: c.994C>T; p.(Arg332Cys)	LP (PM1, PM2, PM5, PP3, PP4)	hemi (mat)	XLR
3	Dysplastic CC, signs of MCD, asymmetric anterior horns, hypotelorism	Multisystem	*TUBB3*	NM_006086.4: c.728C>T; p.(Pro243Leu)	LP (PM1, PM2, PP3, PP2, PP4)	het (mat)	AD
5	Microcephaly, dysplastic CC, delayed sulcation, cerebellar hypoplasia	Complex brain	*VRK1*	NM_003384.3: c.1072C>T; p.(Arg358*)	P (PVS1, PM2, PP5)	hom	AR
28	Interhemispheric cyst, postaxial polydactyly	Multisystem	*GLI2*	NM_005270.5: c.2389del; p.(Thr797Profs*3)	P (PVS1, PS2, PM2)	het (*de novo*)	AD
35	HPE, dysplastic CC, fused diencephalon, Dandy–Walker malformation	Midline anomaly	*PEX2*	NM_000318.3: c.550del; p.(Cys184Valfs*8)	P (PVS1, PM2, PP5)	hom	AR
40	Myelomeningocele, Chiari Type-II malformation	NTD	*SCRIB*	NM_015356.5: c.1177C>T; p.(Gln393*)	LP (PVS1, PM2)	het (mat)	AD
42	Severe VM, abnormal BS morphology, signs of MCD	Complex brain	*GFAP*	NM_002055.5: c.1109T>C; p.(Leu370Pro)	LP (PM1, PM2, PP3, PP2)	het (*de novo*)	AD
46	Severe VM, dysplastic CC, Z-shaped hypoplastic BS, cerebellar hypoplasia, aqueductal stenosis, signs of MCD	Complex brain	*POMT1*	NM_007171.4: c.1045C>A; p.(Pro349Thr) NM_007171.4: c.2167dup; p.(Asp723Glyfs*8)	P (PVS1, PM2, PP3, PP4)	comp het	AR
49	Severe VM, dysplastic CC, aqueductal stenosis, Z-shaped BS, cerebellar hypoplasia, adducted thumb	Multisystem	*L1CAM*	NM_000425.5: c.1453C>T; p.(Arg485*)	P (PVS1, PM2, PP5)	hemi (mat)	XLR
50	Severe VM, dysplastic CC, aqueductal stenosis, Z-shaped BS, cerebellar hypoplasia, signs of MCD, ocular anomaly, retinal detachment	Multisystem	*POMGNT2*	NM_032806.6: c.1232_1233del; p.(Gln411Argfs*10)	P (PVS1, PM2, PP4)	hom	AR
55	Microcephaly, signs of MCD, hypotelorism	Multisystem	*CENPJ*	NM_018451.5: c.3243_3246del; p.(Ser1081Argfs*8)	P (PVS1, PM2, PP5)	hom	AR
61	Partial CC agenesis, thick septal leaves, double collecting system, SUA	Multisystem	*DCC*	NM_005215.4: c.2T>C; p.(Met1?)	LP (PVS1, PM2, PP4)	het (mat)	AD
62	VM, dysplastic CC, signs of MCD	MCD	*TUBB*	NM_001293212.2: c.1141C>T; p.(Leu381Phe)	LP (PM1, PM2, PP3, PP2, PP4)	het (mat)	AD
65	CC agenesis, interhemispheric cyst, large HC, signs of MCD, abnormal BS	Complex brain	*ERCC2*	NM_000400.4: c.2171T>C; p.(Met724Thr)	LP (PM2, PP3, PM1, PP4)	hom	AR
68	Brain tubers, cardiac rhabdomyomas	Multisystem	*TSC2*	NC_000016.9 (NM_000548.4): c.481+1G>A	P (PVS1, PM2, PS2)	het (*de novo*)	AD

Continued over.

Table 2 *Continued*

Case	*Imaging findings*	*Clinical category*	*Gene*	*Variant(s)*	*Variant ACMG classification*	*Zygosity*	*Inheritance pattern*
70	CC agenesis, interhemispheric cyst, signs of MCD, pontine hypoplasia	Complex brain	*COL4A1*	NC_000013.11 (NM_001845.5): c.388-1G>C	P (PVS1, PS2, PM2, PP4)	het (*de novo*)	AD
72	Dysplastic CC, signs of MCD, abnormal aortic valve, toe syndactyly	Multisystem	*FLNA*	NM_001110556.2: c.373G>A; p.(Asp125Asn)	LP (PM1, PM2, PM4, PP3, PP4)	hemi (mat)	XLD
73	IVH Grade IV	Brain damage	*COL4A2*	NM_001846.4: c.4151_4168del; p.(Ala1381_Gly1386del)	LP (PM2, PM4, PP4, PP1)	het (pat)	AD
76	Dysplastic CC, signs of MCD	Complex brain	*HUWE1*	NM_031407.7: c.12980G>A; p.(Arg4327Gln)	LP (PM2, PP1, PP2, PP3, PP4)	hemi (mat)	XLD
77	Dysplastic CC, abnormal frontal horns, Z-shaped BS, aqueductal stenosis, vermian dysplasia	Complex brain	*L1CAM*	NM_000425.5: c.3581C>T; p.(Ser1194Leu)	LP (PP5, PP4, PM2, PP3)	hemi (mat)	XLR
82	Small HC, dysplastic CC, vermian dysgenesis, PVPC, VSD, IUGR, SUA, double collecting system	Multisystem	*TAF1*	NM_004606.3: c.4010T>C; p.(lle1337Thr)	LP (PM2, PP1, PP4, PP5)	het (mat)	XLR
83	Atypical HPE	Midline anomaly	*TCF12*	NM_207037.2: c.207del; p.(Tyr70Metfs*17)	P (PVS1, PM2, PS2)	het (*de novo*)	AD
87	Dysplastic CC, abnormal basal ganglia, signs of MCD	Complex brain	*FOXG1*	NM_005249.5: c.686_687delinsAA; p.(Ile229Lys)	P (PM2, PM1, PM5, PS2)	het (*de novo*)	AD
88	Mild VM, signs of MCD (lissencephaly)	MCD	*PAFAH1B1*	NM_000430.4: c.368T>A; p.(Met123Lys)	LP (PS2, PM2, PP2, PP5)	het (*de novo*)	AD
89	Cephalocele, polycystic kidneys, postaxial polydactyly of hands and feet	Multisystem	*CC2D2A*	NM_001080522.2: c.1497del; p.(Glu500Lysfs*11) NC_000004.11 (NM_001080522.2): c.4179+1del	P (PVS1, PM2)	comp het	AR
90	Microcephaly, dysplastic CC, signs of MCD, prenasal edema	Multisystem	*CLTC*	NM_004859.4: c.4739A>G; p.(Asp1580Gly)	LP (PS2, PM2, PP3, PP2)	het (*de novo*)	AD
91	Severe VM, CC agenesis, small HC, cerebellar hypoplasia, signs of MCD	Complex brain	*ARID1B*	NM_017519.3: c.1637_1638del; p.(Pro546Argfs*93)	P (PVS1, PM2, PS2)	het (*de novo*)	AD
93	Large HC, VM, signs of MCD, cerebellar hypoplasia, molar tooth sign, dysplastic CC, hypertelorism, high forehead, postaxial polydactyly of hands, preaxial polydactyly of feet	Multisystem	*OFD1*	NC_000023.10 (NM_003611.2): c.2387+1G>C	P (PVS1, PM2, PP4)	hemi (mat)	XLR
94	Mild VM, Z-shaped BS, dysplastic CC, abnormal orbits, microphthalmia, irregular renal parenchyma	Multisystem	*KANSL1*	NM_001193466.1: c.2836A>G; p.(Arg946Gly)	LP (PS2, PM2, PP3, PP5)	het (*de novo*)	AD
97	Dysplastic CC, abnormal frontal horns, abnormal BS morphology, asymmetric brain, signs of MCD	Complex brain	*TUBA1A*	NM_006009.4: c.878A>G; p.(Asn293Ser)	LP (PM2, PP5, PS2)	het (*de novo*)	AD

Continued over.

Table 2 *Continued*

Case	*Imaging findings*	*Clinical category*	*Gene*	*Variant(s)*	*Variant ACMG classification*	*Zygosity*	*Inheritance pattern*
106	IVH Grade IV, microcephaly, right frontal horn disruption, disrupted CC	Brain damage	*COL4A1*	NM_001845.6: c.2086G>A; p.(Gly696Ser)	P (PM2, PP3, PP5, PS2)	het (*de novo*)	AD
109	Lissencephaly, PVPC	MCD	*CEP85L*	NM_001042475.3: c.232+2T>A	P (PVS1, PM2, PS2)	het (*de novo*)	AD
110	CC dysgenesis, macrocephaly, signs of MCD	Complex brain	*PIK3R2*	NM_005027.4: c.1117G>A; p.(Gly373Arg)	P (PS2, PM1, PM2, PP5)	het (*de novo*)	AD
111	Cephalocele, multicystic kidneys	Multisystem	*TMEM67*	NM_153704.6: c.1975C>T; p.(Arg659*)/c.1289-16_1289-12delCTTTT)	P (PVS1, PM2, PP5)/ LP (PS3, PM2, PM3)	comp het	AR
112	CC agenesis, ganglionic eminence lesions	Complex brain	*TUBB*	NM_178014.4: c.947T>C; p.(Val316Ala)	LP (PS2, PM1, PP4)	het (*de novo*)	AD
113	IVH Grade IV	Brain damage	*COL4A1*	NM_001845.6: c.1186C>T; p.(Arg396*)	P (PVS1, PM2, PS2)	het (*de novo*)	AD
114	PVPC	Subependymal cysts	*ATRX*	NM_000489.6: c.1186A>C; p.(Lys396Gln)	LP (PS2, PM2, PP3)	het (*de novo*)	X-linked

ACMG, American College of Medical Genetics and Genomics; AD, autosomal dominant; AR, autosomal recessive; BS, brainstem; comp, compound; CC, corpus callosum; HC, head circumference; hemi, hemizygous; het, heterozygous; hom, homozygous; HPE, holoprosencephaly; IUGR, intrauterine growth restriction; IVH, intraventricular hemorrhage; mat, maternal; MCD, malformation of cortical development; NTD, neural tube defect; pat, paternal; PVPC, periventricular pseudocysts; SUA, single umbilical artery; VM, ventriculomegaly; VSD, ventricular septal defect; XLD, X-linked dominant; XLR, X-linked recessive.

Table 3 Detection rate of pathogenic and likely pathogenic variants by chromosomal microarray analysis (CMA) and exome sequencing (ES) among 114 cases with fetal central nervous system abnormality, according to clinical category

Clinical category	*CMA*	*ES*
Multisystem	5/40 (13)	14/32 (44)
Complex brain	5/32 (16)	14/24 (58)
MCD	0/11 (0)	3/9 (33)
Brain damage	0/14 (0)	3/9 (33)
Subependymal/arachnoid cysts	0/5 (0)	1/4 (25)
Midline anomaly	0/4 (0)	2/3 (67)
MBHB	1/6 (17)	0/3 (0)
Neural tube defect	0/2 (0)	1/2 (50)
Total	11/114 (10)	38/86 (44)

Data are given as *n*/*N* (%). MBHB, midbrain–hindbrain malformation; MCD, malformation of cortical development.

cases). Thus, the combined detection rate of ES for both sequence variants and CNVs was 50%.

DISCUSSION

In the postnatal setting, ES has demonstrated an additional diagnostic yield for a variety of conditions of 25–45% beyond traditional methods. Multiple studies have also shown the efficacy of ES in the prenatal diagnosis of various fetal anomalies[5–7,12,26–32]. In a recent meta-analysis, the diagnostic yield for a variety of fetal anomalies, regardless of the affected organ, was 9–47%[33]. A higher rate was noted for fetuses showing multiple malformations[33]. Several studies have reported that, in cases with a CNS anomaly, the added diagnostic yield is 3–34%[6,7,12,18,30,34]. Finally, there are only a handful of studies focusing specifically on CNS anomalies, with a diagnostic yield ranging from 19% to 50%[35–38].

In our study, the added diagnostic yield of ES was 44%, with the highest yield observed in specific subcategories (44% in multisystem and 58% in complex brain anomalies). There was a non-significant trend towards a higher incidence of monogenic etiology in cases with recurring brain anomalies compared with first-time cases (63% *vs* 39%; $P = 0.06$). There was a significant risk of recurrence in 53% of cases that had an ES diagnosis. This information will be important for future reproductive choices. Several participating couples have already opted for preimplantation genetic testing (PGT).

In several cases with genetic etiology, we detected P/LP variants in AR genes that were in concordance with clinical manifestations. For example, Case 46 with *POMT1* variant and Case 50 with *POMGNT2* variant were suspected to have Walker–Warburg syndrome (OMIM 236670, 614830) (Table 2). However, in some cases, clinical manifestations did not correspond to the phenotype associated with the detected AR variants. For example, pathogenic variants in *VRK1* cause pontocerebellar hypoplasia Type 1A (*PCH1A*, OMIM 607596), characterized postnatally by hypoplasia of the ventral pons and cerebellum and neuronal loss and gliosis in the brainstem and basal ganglia. However, prenatally, Case 5 with a homozygous *VRK1* variant exhibited

a dysplastic corpus callosum (CC), delayed sulcation and cerebellar hypoplasia. This highlights the fact that prenatal manifestations may differ and be more severe when compared with those described postnatally. There is a need to expand the HPO terminology to include accurate prenatal manifestations. Such efforts are currently being undertaken by the Fetal Sequencing Consortium[39]. Moreover, this suggests that accurate diagnoses cannot rely on imaging alone, and that sequencing information provides additional important insights.

As expected, of the 22 cases with an AD mode of inheritance, 17 (77%) cases were *de novo*. Nonetheless, consideration should be given to the five inherited AD cases. In two maternally inherited cases, tubulinopathy was detected. In Case 3, a *TUBB3* (OMIM 614039) LP missense variant was detected in a fetus with cortical dysplasia and abnormal sulcation. In Case 62, a *TUBB* (OMIM 615771) LP missense variant was found in a fetus with CC hypoplasia, asymmetric hemispheres, ventriculomegaly, mega cisterna magna and colpocephaly. In Case 97, a *de-novo* LP missense variant in the *TUBA1A* gene (OMIM 611603) was found in a fetus with cortical dysplasia, abnormal cortical gyration and brainstem abnormality. Pathogenic variants in the tubulin isotypes are associated with various types of complex cortical dysplasia with other brain malformations. Perturbation of the tubulins may be associated with a spectrum of neurodevelopmental disorders with incomplete penetrance[40]. In Case 61, a maternally inherited *DCC* (OMIM 157600) loss-of-function variant was detected. On further examination, the otherwise unaffected mother was found to have mirror hand movements, a well-described phenomenon associated with this gene[41]. The fetus, however, was found to have partial agenesis of the CC, which is another known consequence of *DCC* mutations[42]. In Case 73, a paternally inherited *COL4A2* (OMIM 614483) deletion was associated with Grade IV intraventricular hemorrhage (IVH) in the index fetus and in the fetus in a previously terminated pregnancy. The carrier father had cerebral palsy (CP), most likely as a consequence of *in-utero* IVH. The father's niece, who also had CP, carried the same pathogenic variant. In Case 40, the fetus presented with Chiari Type-II malformation secondary to a neural tube defect. ES analysis revealed a maternally inherited stop codon in the *SCRIB* gene (OMIM *607733). Variants in *SCRIB* and other planar cell polarity genes have been associated with neural tube defects in animals[43] and humans[44,45]. The healthy mother had no clinical signs of a neural tube defect. These cases highlight the fact that even pathogenic variants may have variable expression and incomplete penetrance and stress the importance of obtaining a full medical history and examining other family members.

In addition to the 44% diagnostic yield of P/LP variants, nine (10%) other cases had a VOUS with potential clinical relevance (Table S1). Such VOUSs may prove over time to be benign or unrelated to the clinical scenario owing to data-sharing tools such as GeneMatcher[46] or the Franklin Users Community[47].

Our study has some limitations. First, our series consists of highly selected cases that were severe enough to warrant termination of pregnancy. Thus, our conclusions may not apply to all brain anomalies. Second, the study was carried out at a referral center for prenatal CNS anomalies and, as such, may have had selection bias due to severe and/or recurrent cases being more likely to have genetic etiology. Third, conclusions regarding specific clinical subgroups are limited owing to a relatively small number of cases in each subcategory.

In conclusion, we have shown that, in fetuses with major CNS anomalies, ES has an additional diagnostic yield of 44% over the 10% provided by CMA. Because ES can also detect many CNVs and given the tight timeframe in pregnancy, we suggest that ES should be considered as a first-tier test in the prenatal diagnosis of major CNS anomalies. Given the limited prenatal phenotype information, the additional prognostic information provided by ES is crucial for timely and evidence-based decision-making. Moreover, given that over half of the causative variants are inherited, their detection by ES provides valuable information regarding the risk of recurrence in subsequent pregnancies and facilitates counseling regarding reproductive choices, such as prenatal diagnosis or PGT. Further studies are needed to assess whether ES alone could be used for reliable CNV detection.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Table S1 Cases with variants of uncertain significance (VOUS) detected by exome sequencing (ES) among 86 cases of fetal CNS anomalies

Table S2 Cases with negative exome sequencing (ES) results among 86 cases of fetal CNS anomalies