Next-Generation Sequencing Gene Panels and “Solo” Clinical Exome Sequencing Applied in Structurally Abnormal Fetuses

Montse Pauta^a Berta Campos^b Maria Segura-Puimedon^b Gemma Arca^c
Alfons Nadal^d Albert Tubau^e Silvia Pina Perez^f Edda Marimon^g
Lourdes Martín^h Eva López-Quesadaⁱ Joan Sabrià^g Begoña Muñoz^j
Esperanza García^k Fernanda Paz Y. Miño^l Virginia Borobio^l Olga Gomez^l
Elisenda Eixarch^{l,m} Monica Lopez^h Montserrat Comas Rovira^f Antoni Borrell^{a,l}

^aInstitut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), BCNatal, Barcelona, Spain; ^bqGenomics, Esplugues de Llobregat, Catalonia, Spain; ^cDepartment of Neonatology, BCNatal, Hospital Clínic Barcelona, Barcelona, Spain; ^dDepartment of Pathology, Hospital Clínic Barcelona, Barcelona, Spain; ^eDepartment of Obstetrics and Gynecology, Son Llàtzer Hospital, Palma de Mallorca, Spain; ^fDepartment of Obstetrics and Gynecology, Parc Taulí Hospital, Sabadell, Catalonia, Spain; ^gDepartment of Obstetrics and Gynecology, Hospital Sant Joan de Déu, BCNatal, Esplugues de Llobregat, Catalonia, Spain; ^hDepartment of Obstetrics and Gynecology, Hospital Joan XXIII de Tarragona, Catalonia, Spain; ⁱDepartment of Obstetrics and Gynecology, Hospital Mútua de Terrassa, Terrassa, Spain; ^jDepartment of Obstetrics and Gynecology, Hospital Sant Joan de Reus, Reus, Spain; ^kDepartment of Obstetrics and Gynecology, Hospital Consorci Sanitari de Terrassa, Terrassa, Spain; ^lDepartment of Obstetrics and Gynecology, BCNatal, Hospital Clínic de Barcelona, Barcelona, Spain; ^mCentre for Biomedical Research on Rare Diseases (CIBER-ER), Madrid, Spain

Keywords

Congenital malformations · Fetal genetic analysis · Genetic disorder · Prenatal diagnosis · Single gene disorders · Exome sequencing

Abstract

Objective: The aim of the study was to assess the diagnostic yield of 2 different next-generation sequencing (NGS) approaches: gene panel and “solo” clinical exome sequencing (solo-CES), in fetuses with structural anomalies and normal chromosomal microarray analysis (CMA), in the absence of a known familial mutation. **Methodology:** Gene panels encompassing from 2 to 140 genes, were applied mainly in

persistent nuchal fold/fetal hydrops and in large hyper-echogenic kidneys. Solo-CES, which entails sequencing the fetus alone and only interpreting the Online Mendelian Inheritance in Man genes, was performed in multisystem or recurrent structural anomalies. **Results:** During the study period (2015–2020), 153 NGS studies were performed in 148 structurally abnormal fetuses with a normal CMA. The overall diagnostic yield accounted for 35% (53/153) of samples and 36% (53/148) of the fetuses. Diagnostic yield with the gene panels was 31% (15/49), similar to 37% (38/104) in solo-CES. **Conclusions:** A monogenic disease was established as the underlying cause in 35% of selected fetal structural anomalies by gene panels and solo-CES.

© 2021 The Author(s).
Published by S. Karger AG, Basel

Introduction

Structural fetal anomalies occur in approximately 2.5% of pregnancies [1] and most of them can be identified by ultrasound examination during pregnancy. When a fetal structural anomaly is detected by ultrasound, normally a chromosomal microarray analysis (CMA) is offered after amniocentesis, chorionic villi sampling, or fetal blood sampling because it provides up to a 6–10% incremental diagnostic yield [2, 3] above the 14% yield of a conventional karyotype [4].

The advent of high-throughput next-generation sequencing (NGS) allows sequencing the whole human genome in a single assay, while classic Sanger sequencing was limited to 1,000 nucleotides. To explore the frequency of monogenic disorders associated with fetal structural anomalies, whole-exome sequencing (WES) has been preferred over whole-genome sequencing because human exons account for only 2% of the whole genome and can explain 85% of monogenic disorders, and due to the cost and complexity of whole-genome sequencing analysis. The first prenatal diagnosis using WES was achieved in 2013 by the Baylor College group, when a mutation in the *NIPBL* gene associated with Cornelia de Lange syndrome was identified in a malformed fetus [5]. Applied to structurally abnormal fetuses with a normal CMA, a larger WES series of unselected fetal structural anomalies gave a 10–11% diagnostic yield [6, 7], lower than those observed in small series, including cases selected after genetic review that ranged from 10 to 32% [8, 9].

In 2015, our group initially applied NGS technology to diagnose monogenic disorders, using a gene panel if a clinically available phenotype-driven panel was available. Since 2016, when the observed fetal phenotype did not overlap with a previously described phenotype, we applied a simplified exome sequencing approach called “solo” clinical exome sequencing (solo-CES). The aim of the present study was to report our experience with the use of NGS gene panels and solo-CES in fetuses with selected structural anomalies and normal CMA.

Material and Methods

This is a prospective study conducted from January 2015 to October 2020 in which NGS studies were offered to pregnant women and partners presenting with selected structurally abnormal fetuses, normal CMA and no familial molecular diagnosis. This cohort includes consecutive cases.

Fetal Phenotyping

Our center is a tertiary referral center with an average of 400 cases of fetal abnormalities per year. Ultrasound examinations were performed by expert Fetal Medicine specialists in the participating centers, while extended echocardiogram, neurosonography, or magnetic resonance imaging contributed to the diagnostic approach when appropriate. Pregnant women with any fetal anomaly were offered an invasive procedure and quantitative fluorescent polymerase chain reaction (QF-PCR) and CMA was performed on fetal samples. In terminated or stillborn fetuses, post-mortem studies were also recommended. In continuing pregnancies, postnatal follow-up was assessed.

DNA Extraction and NGS

Fetal DNA was extracted from chorionic villi, amniotic fluid, or fetal blood, although in some samples, cell culturing was required as a preliminary step. Remaining fetal DNA from CMA was sent to the NGS laboratory together with peripheral blood of both biological parents, with the exception of pregnancies conceived by gamete donation. Genomic DNA was obtained from fetal samples and peripheral blood lymphocytes of both parents to be used for subsequent segregation studies, if required. DNA quality was determined by measuring optical density with a DeNovix DS-11. Human exome was enriched using the MedExomeSeqCap EZ assay (Roche-Nimblegen, Pleasanton, CA, USA) and massively sequenced using NextSeq 500 (Illumina, San Diego, CA, USA) equipment. An average 30× coverage depth was achieved along the target regions (exons and ± 75 intronic nucleotides flanking the exon-intron boundaries of all genes).

Gene Panel Analysis

If a clinically available phenotype-driven panel was available, a gene panel was used. “Gene panel” was defined as the NGS test that analyzes multiple genes at once, ranging from 2 to 140 in our study, associated with a specific fetal phenotype. Gene panels offer a middle ground between sequencing just a single gene, and sequencing every gene in the genome. Six gene panels were applied in more than one case: (a) RASopathies-hydrops panel in unexplained fetal hydrops/persistent nuchal fold, defined as an increased nuchal translucency persisting in the second trimester as an increased nuchal fold (>5 mm at 15–17 weeks and >6 mm at 18–22 weeks)/cystic hygroma; (b) Congenital Anomalies of the Kidney and Urinary Tract (CAKUT) panel [10] in large hyperechogenic kidneys; (c) Osteogenesis imperfecta panel in suspicion of osteogenesis imperfecta (OI); (d) Tuberous sclerosis panel in single or multiple cardiac rhabdomyomas; (e) Craniosynostosis panel in fetal craniosynostosis; and (f) Lissencephaly panel in fetal lissencephaly. Furthermore, 5 gene panels were used only once: Cornelia de Lange panel, visceral myopathy panel, Cardiomyopathy panel, Fanconi panel [11], and CHARGE panel. The number of genes studied in each of the 11 gene panels are shown in Table 1, and the genes and the name of the laboratory are listed in online supplementary Table 1 (see www.karger.com/doi/10.1159/000519701 for all online suppl. material). In skeletal dysplasias other than OI, exome sequencing was preferred over a gene panel analysis.

Solo-Clinical Exome Sequencing

During the period 2016–2020, when the observed fetal phenotype did not overlap with a previously described phenotype, we applied a simplified exome sequencing approach called solo-CES, given that

Table 1. Diagnostic yield achieved by NGS gene panels and solo-CES

NGS gene panel (31%: 15/49)			
Gene panel type	Included genes (n)	Definitive diagnoses (N/N)	Diagnostic yield (%)
RASopathies-hydrops panel	130	8/23	35
CAKUT panel	140	4/7	57
Osteogenesis imperfecta panel	22	1/4	25
Tuberous sclerosis panel	2	1/4	25
Craniosynostosis panel*	11	1/4	25
Lissencephaly panel**	24	0/2	0
Cornelia de Lange panel	5	0/1	0
Visceral myopathy panel	12	0/1	0
Cardiomyopathy panel†	119	0/1	0
Fanconi panel§	22	0/1	0
CHARGE panel	2	0/1	0
Solo-CES (37%: 38/104)			
Indication		Definitive diagnoses (N/N)	Diagnostic yield (%)
Multisystem anomalies		28/69	41
Recurrent anomalies		3/12	25
Skeletal dysplasia		5/10	50
Complex CNS anomalies		2/8	25
Increased nuchal translucency		0/4	0
Complex cardiac defects		0/1	0

CAKUT, congenital anomalies of the kidney and urinary tract; CNS, central nervous system; NGS, next-generation sequencing; solo-CES, solo-clinical exome sequencing. * In one of the 3 fetuses with a negative panel result, solo-CES was subsequently performed as complex CNS anomaly. ** Both fetuses had a negative panel result and underwent a subsequent solo-CES, 1 as a complex CNS anomaly and the second as a recurrent anomaly. † This fetus had a negative panel result and solo-CES was subsequently performed as a complex CNS anomaly. § After a negative panel result, solo-CES was subsequently performed as a multisystem anomaly.

the fetus alone was sequenced and only the Clinical Genomics Database and Online Mendelian Inheritance in Man (OMIM) genes were interpreted [12]. Solo-CES was offered in consecutive cases presenting with: (a) Multisystem anomalies (at least 2 major anomalies from different anatomical systems); (b) recurrent anomalies (a similar major anomaly in consecutive pregnancies); and (c) severe skeletal dysplasias with at least bilateral upper and/or lower limbs micromelia below -3 SD, not resembling OI. Solo-CES was occasionally performed in 3 further anomaly types: (a) complex central nervous system anomalies after fetal brain specialists suspected a monogenic defect; (b) complex cardiac defects after fetal heart specialists suspected a monogenic defect; and (c) increased nuchal translucency above the 99th percentile as a self-funded personal choice.

During the final 26 months of the study (July 2018–October 2020), pregnancies affected with multisystem or recurrent structural fetal anomalies from 7 further participating centers were also recruited and studied by means of solo-CES. Informed consent was obtained independently from each parent, including an option of opting out of being informed of secondary and incidental findings. At the pretest genetic counseling session, prospective parents were informed about the proposed genetic test and advantages and limitations were discussed [13].

Data Filtering and Classification of Variants

After sequencing, raw FASTQ data were obtained and processed via the following steps: (1) Variants were called using the Genome Analysis Toolkit to find sites with differences between the sample sequence data and the reference genome. After alignment of reads against the human reference genome sequence, using standard procedures to identify single nucleotide variants and small insertions and deletions. Variants were annotated using ANNOVAR software and splicing variants were considered up to ± 10 bp from the exon-intron boundary. (2) Variants were filtered according to quality parameters. (3) Only rare variants in coding sequence or exon-intron boundaries with a frequency under 0.5% in the Genome Aggregation Database (GnomAD), or variants considered pathogenic in public databases were collected and evaluated for their potential relationship to Mendelian diseases, and were analyzed manually via extensive bibliographic research. (4) Quality of variants was then confirmed. (5) Potential variants were assessed in relation to the index case phenotype and classified following the American College of Medical Genetics and Genomics (ACMG) guidelines [14]. Population (GnomAD) and disease (ClinVar, or Human Gene Mutation Database) databases were used as a starting point to assess the variant's frequency in the gen-

Table 2. Positive results obtained by NGS gene panels and solo-CES: ultrasound findings, gene involved, genomic variant, variant type according ACMG, zygosity, and associated monogenic syndrome with the inheritance pattern for each case

NGS study	N	Ultrasound findings	Gene	Genomic variants (nucleotide)	ACMG variant type	Zygosity	Associated conditions (inheritance pattern)
Gene panel	15/49	<i>Rasopathies-hydrups panel</i>					
		Fetal hydrups; large cystic hygroma	FOXS2	NM_005251:c.461_463delACA*	L.Pat.	Het.	Lymphedema-distichiasis syndrome (AD and de novo)
		Persistent hygroma	KRAS	NM_033360:c.65A > G	Pat.	Het.	Noonan syndrome (AD and de novo)
		Fetal hydrups; cystic hygroma; single umbilical artery; bilateral talipes	RIT1	NM_006912:c.268A > G	Pat.	Het.	Noonan syndrome (AD and de novo)
		Cystic hygroma	RIT1	NM_006912.5:c.245T > G	Pat.	Het.	Noonan syndrome (AD and de novo)
		Cystic hygroma	RIT1	NM_006912.6:c.245T > G	Pat.	Het.	Noonan syndrome (AD and de novo)
		Fetal hydrups	GATA1	NM_002049:c.1019delG	Pat.	Het.	GATA1-related X-linked cytopenia (LX, inherited from mother)
		Fetal hydrups	GUSB	NM_000181:c.1084G > C	Pat.	Het.	Mucopolysaccharidosis VII (AR)
		Fetal hydrups	PIEZO1	NM_001142864:c.1490delG* (i) NM_001142864:c.5289C > G*	Pat./Pat.	Comp. Het.	Hereditary lymphedema III (AR)
<i>CAKUT panel</i>							
		Bilateral renal hypoplasia; abnormal upper limbs; oligohydramnios	NEK8	NM_178170.3:c.325_327delTTC	L.Pat.	Hom.	Renal-hepatic-pancreatic dysplasia 2 (AR)
		Bilateral renal hyperplasia; oligohydramnios	NPHP3	NM_153240.5:c.434_437delAAAG	Pat.	Het. UPD	Renal-hepatic-pancreatic dysplasia 2 (AR)
		Bilateral renal hyperplasia; fetal hydrups; polydactyly	TTC8	NM_144596.3:c.69delC (i) c.799-?_909+ *	Pat./Pat.	Comp. Het.	Bardet-biedl syndrome (AR)
		Bilateral renal hyperplasia; bladder not seen; oligohydramnios	PKHD1	NM_138694.3:c.842G > A* (i) c.1529delG	L. Pat/Pat.	Comp. Het.	Polycystic kidney disease (AR)
<i>Osteogenesis imperfecta panel</i>							
		Prognathism; asymmetric fetal growth restriction; micromelia	COL1A2	NM_000089:c.2350-2A > G*	Pat.	Het.	Osteogenesis imperfecta (AD, de novo)
<i>Tuberous sclerosis panel</i>							
		Cardiac rhabdomyoma	TSC2	NM_000548.3:c.2251C > T	Pat.	Het.	Tuberous sclerosis (AD, de novo)
<i>Craniostosis panel</i>							
		Craniostosis	FGFR2	NM_000141.4:c.1024T > G	L.Pat.	Het.	Pfeiffer syndrome (AD, de novo)

Table 2 (continued)

NGS study	N	Ultrasound findings	Gene	Genomic variants (nucleotide)	ACMG variant type	Zygosity	Associated conditions (inheritance pattern)
Solo-clinical exome sequencing	38/104	<i>Multisystem anomalies</i>					
		Ventriculomegaly; tetralogy of fallot; hypospadias; bilateral talipes	ASCC1	NM_001198798: c.157dupG	Pat.	Hom.	Spinal muscular atrophy with congenital bone fractures 2 (AR)
		VSD; nuchal edema; feet and hands malposition	ASCC1	NM_001198799: c.397_398delAT	Pat.	Hom.	Spinal muscular atrophy with congenital bone fractures 2 (AR)
		Situs inversus; agenesis of the inferior vena cava; VSD; duodenal atresia; Polysplenia	PKD1L1	NM_138295: c.2276A > T*	VUS	Hom.	Visceral heterotaxy 8 (AR)
		Tetra-amelia; bilateral cleft lip; macroglossia; retrognathia	RSP02	NM_178565: c.277T > A*	L.Pat	Hom.	Tetra-amelia syndrome (AR)
		Macrocephaly with dilatation of the III-IV ventricle; agenesis of corpus callosum; Dandy-Walker malformation	KIF7	NM_198525: c.2917C > T	Pat.	Hom.	Joubert syndrome 12 (AR)
		Agenesis of corpus callosum; multicystic kidney dysplasia	KIF7	NM_198525: c.2896_2897del	Pat.	Hom.	Acrocallosal syndrome (AR)
		Ventriculomegaly; subependymal cysts; cerebellar dysplasia; large dysplastic kidneys	SUCLG1	NM_003849: c.626C > A	L.Pat.	Hom.	Mitochondrial depletion syndrome 9 (AR)
		Mild ventriculomegaly; mandibular retrognathia; hypoplastic aortic arch; right hand polydactyly; micropenis	CPLANE1	NM_023073: c.4394_4397dupTAGG (:) NM_023073: c.3290-2A > G	Pat./Pat.	Comp. Het.	Joubert syndrome with orofaciocigital defect (AR)
		Dandy-Walker malformation; hypoplastic mitral valve; hypertrophy of left ventricle; short femur; polydactyly	EVC	NM_153717: c.721A > T (:) NM_153717: c.2041G > T*	Pat./Pat.	Comp. Het.	Ellis-van Creveld syndrome (AR)
		Agenesis of corpus callosum; Dandy-Walker malformation; micromelia; fracture of tibia	PCNT	NM_006031: c.2282G > A* (:) NM_006031: c.4675A > T*	VUS/VUS	Comp. Het.	Microcephalic osteodysplastic primordial dwarfism type II (AR)
		Micromelia; feet malposition; extended knees	COL27A1	NM_032888: c.2548G > A (:) NM_032888: c.3249+1G > T*	L.Pat./Pat.	Comp. Het.	Steel syndrome (AR)
		Communicant hydrocephalus; hypotelorism; partial agenesis of corpus callosum; short femur; fetal growth restriction	DHCR7	NM_001360: c.964-1G > C (:) NM_001360: c.452G > A	Pat./Pat.	Comp. Het.	Smith-Lemli-Opitz syndrome (AR)
		Generalized edema; arthrogryposis; short femur; feet malposition	DHCR7	NM_001360: c.1228G > A (:) NM_001360: c.452G > A	Pat./Pat.	Comp. Het.	Smith-Lemli-Opitz syndrome (AR)
		Hydrocephalus; agenesis of corpus callosum; retrognathia; lumbar hyperlordosis	EFTUD2	NM_001142605: c.2287_2314del*	Pat.	Het.	Mandibulofacial dysostosis with microcephaly (AD and de novo)
		Micrognathia; esophageal atresia; aberrant subclavian artery	EFTUD2	NM_001142605: c.146_168del*	Pat.	Het.	Mandibulofacial dysostosis, guion-Almeida type (AD and de novo)
		Megacystis; anhydramnios; complete syndactyly of all fingers and toes	FGFR2	NM_022970: c.758C > G	Pat.	Het.	Apert syndrome (AD and de novo)

Table 2 (continued)

NGS study	N	Ultrasound findings	Gene	Genomic variants (nucleotide)	ACMG variant type	Zygosity	Associated conditions (inheritance pattern)
Microcephaly; polymicrogyria; hypoplasia of the cerebellum; bilateral talipes; aberrant subclavian artery		Microcephaly; polymicrogyria; hypoplasia of the cerebellum; bilateral talipes; aberrant subclavian artery	<i>ASXL1</i>	NM_015338: c.1582G > T	Pat.	Het.	Bohring-Opitz syndrome (AD and de novo)
		VSD; coarctation of aorta; Horseshoe kidney	<i>KMT2D</i>	NM_003482: c.13450C > T	Pat.	Het.	Kabuki syndrome (AD and de novo)
		Hypoplastic aortic arch; VSD; unilateral renal dysplasia	<i>KMT2D</i>	NM_003482: c.13450C > T	Pat.	Het.	Kabuki syndrome (AD and de novo)
		Ventriculomegaly; partial agenesis of corpus callosum; hypospadias	<i>TCF4</i>	NM_001083962: c.1733G > T	Pat.	Het.	Pitt-Hopkins syndrome (AD and de novo)
		Agenesis of corpus callosum; low-set ears; VSD; Ventricular hypertrophy; short femur; generalized edema; polyhydramnios	<i>BRAF</i>	NM_004333: c.1785T > G	Pat.	Het.	Cardiofaciocutaneous syndrome (AD and de novo)
		Left enlarged kidney; double collecting system; fetal growth restriction	<i>ANKRD11</i>	NM_001256183: c.4420G > T	Pat.	Het.	KBG syndrome (AD and de novo)
		Ventriculomegaly; severe fetal growth restriction	<i>SLC25A24</i>	NM_013386: c.992C > G*	L.Pat.	Het.	Fontaine syndrome (AD and de novo)
		Dysgenesis of corpus callosum; absent splenium (NT >99 percentile); VSD	<i>KANSL1</i>	NM_001193466: c.2785_2786delAG	Pat.	Het.	Koolen-de vries syndrome (AD and de novo)
		Holoprosencephaly; proboscis; hemivertebrae; malposition of the hand	<i>GLI2</i>	NM_005270: c.3323G > T	VUS	Het.	Holoprosencephaly 9 (AD) (inherited from a parent affected with a microform)
		Lobar holoprosencephaly; partial agenesis of corpus callosum; bilateral cleft lip and palate; micropenis	<i>SIX3</i>	NM_005413: c.110G > A*	VUS	Het.	Holoprosencephaly 2 (AD) (inherited from a parent affected with a microform)
		Phocomelia and cardiac defect	<i>TBX5</i>	NM_181486: c.663+1G > A	Pat.	Het.	Holt-Oram syndrome (AD) (inherited from a father with thumb agenesis)
		Bilateral severe ventriculomegaly; abnormal facial profile	<i>KDM5C</i>	NM_001146702: c.3923C > T*	L.Pat	Het.	Intellectual disability syndromic; claes-Jensen type (X-linked)
		<i>Recurrent anomalies</i>					
		Fetal hydrops	<i>SEC23B</i>	NM_001172745: c.716A > G	L.Pat.	Hom.	Congenital dyserythropoietic anemia type II (AR)
		Lissencephaly	<i>ASPM</i>	NM_018136.4: c.7551T > G	L.Pat.	Het.	Microcephaly with simplified gyral pattern (AR)
		Arthrogryposis multiplex	<i>DOK7</i>	NM_001164673: c.230C > T (:) NM_173660: c.532+4A > G*	L.Pat./L.Pat.	Comp. Het.	Fetal akinesia deformation sequence (AR)
		<i>Skeletal displasias</i>					
		Skeletal dysplasia	<i>COL2A1</i>	NM_001844.4: c.3319G > A	Pat.	Het.	Spondyloepiphyseal dysplasia congenita (AD, de novo)

Table 2 (continued)

NGS study	N	Ultrasound findings	Gene	Genomic variants (nucleotide)	ACMG variant type	Zygosity	Associated conditions (inheritance pattern)
		Micromelia; vertebral fusion; distal arthrogyriposis	CANT1	NM_138793: c.1037C > T	L.Pat.	Hom.	Desbuquois dysplasia 1 (AR)
		Micromelia	HSPG2	NM_001291860: c.6823G > A (i) NM_001291860: c.3581G > T*	L.Pat/L. Pat	Comp. Het.	Schwartz-Jampel syndrome (AR)
		Micromelia; unilateral bended femur	DYNC2H1	NM_001080463: c.6128G > A + NM_001080463: c.8343A > G* (i) NM_001080463.2: c.12460C > T	VUS/VUS	Comp. Het.	Short-rib thoracic dysplasia 3 (AR)
		Micromelia; bilateral bended femurs	RMRP	NR_003051.3: n.28G > A (i) NR_0030513: n.71A > G	L.Pat./L.Pat.	Comp. Het.	Anauxetic dysplasia (AR)
		<i>CNS complex anomalies</i>					
		Ventriculomegaly; septo-optic dysplasia; cerebellar dysgenesis	CHD7	NM_017780: c.5833C > T	Pat.	Het.	CHARGE syndrome (AD, de novo)
		Microcephaly; lissencephaly; dysgenesis of corpus callosum	FOXG1	NM_005249: c.167_176del	L.Pat.	Het.	Congenital Rett syndrome (AD, de novo)

VSD, ventricular septal defect; Pat., pathogenic, L.Pat., likely pathogenic; VUS, variant of uncertain significance; Het., heterozygous; Hom., homozygous; Comp. Het., compound heterozygous; CAKUT, congenital anomalies of the kidney and urinary tract; CNS, central nervous system; solo-CES, solo-clinical exome sequencing; ACMG, American College of Medical Genetics and Genomics. *Not previously reported.

eral population and whether it was already described as pathogenic in disease databases. (6) Furthermore, the Varsome [15, 16] algorithm was applied taking into account the type of variant (non-sense, frameshift, exon deletion, and intronic) and computational *in-silico* predictive programs.

Reports inform about pathogenic, likely pathogenic, and extremely rare variants of uncertain significance (VUS) identified in genes that are related to the index case phenotype or overlapping phenotypes. Secondary findings were reported following the ACMG guidelines for the communication of pathogenic and likely pathogenic variants in actionable genes [17].

Sanger sequencing in the index case and parents was performed to confirm the inheritance pattern of the most promising candidates. Negative results were reviewed to search for possible causative genes and ensure correct coverage in the NGS experiment. A multidisciplinary Clinical Review Committee composed of 6 members: a Maternofetal Medicine specialist and Prenatal Geneticist (A.B.), a Pediatric Geneticist (G.A.), a Perinatal Pathologist (A.N.), a Molecular Geneticist (B.C.), a Genetic Counselor (M.S.), and the Project Coordinator (M.P.), reviewed all the candidate variants and the negative results. The committee could request reviewing the coverage of specific genes in negative results, or expanding the parental segregation analysis or the study of previous siblings or other members of the family.

Ethical Approval

This study was approved by the Institutional Review Board of the Hospital Clínic de Barcelona (HCB/2018/0077), and written informed consent was obtained from the parents.

Results

During the 6-year study period (January 2015–October 2020), 149 selected structurally abnormal fetuses with a normal CMA in the absence of a known familial mutation were recruited. All women consented to NGS studies, although one missed the blood draw appointment, resulting in 148 studied fetuses. Mean maternal age was 34 years, range (18–47 years) and the mean gestational age at ultrasound diagnosis was 20 + 2 weeks (range 10–34 weeks). There were 5 fetuses in which both gene panel and solo-CES were consecutively performed, leading to an overall of 153 NGS studies. In fetuses from our center, 49 gene panels and 69 solo-CES were performed, together with 35 solo-CES in fetuses referred from the other participating centers. The number of cases studied yearly increased from 5 cases (2015), 10 cases (2016), 11 cases (2017), 38 cases (2018), 46 cases (2019), to 43 (2020). In 5 fetuses, after a negative result with the NGS gene panel, the study was extended to solo-CES.

Overall, NGS studies revealed the cause of the anomalies observed in 53 of the 153 fetuses studied, resulting in a 35% incremental diagnostic yield over CMA (Table 1). According to the ACMG classification, there were 40

Table 3. Perinatal outcome of the 148 pregnancies studied, according to whether a definitive diagnosis was provided by NGS studies

	Stillbirth	Termination of pregnancy	Neonatal survival	Neonatal death	Total
Negative result	7	60	23	5	95
Definitive diagnosis provided	4	43	4	2	53
Total	11	103	27	7	148

pathogenic, 22 likely pathogenic, and 7 VUS reassigned by the multidisciplinary Clinical Review Committee as likely causative, after parental segregation studies (online suppl. Table 2 [23–28]). Hence, medical examination of the corresponding parent was indicated when an inherited VUS of a gene with an autosomal dominant inheritance, related to the observed fetal phenotype had an incomplete penetrance or variable expression. Among the 53 monogenic disorders diagnosed, 26 (49%) had an autosomal dominant inheritance (23 caused by a de novo variant), 25 (49%) had an autosomal recessive inheritance (14 compound heterozygous and 11 homozygous variants), and 2 cases (3.7%) had an X-linked recessive inheritance (Table 2).

NGS gene panels were applied in 49 cases, with a 31% (15/49) diagnostic yield (Table 1). The gene panel most used ($n = 23$) was the RASopathies-hydrops gene panel, which in the last years of the study included 130 genes associated with RASopathies and fetal hydrops, and resulted in a 35% (8/23) diagnostic yield (Table 2). The CAKUT panel was applied in 7 cases of large hyperechogenic kidneys with a 57% (4/7) diagnostic yield. The OI panel had a 25% (1/4) diagnostic yield (Table 1). Reporting times ranged from 2 to 4 weeks.

Solo-CES demonstrated a 37% (38/104) diagnostic yield. The observed test performance for fetuses with multisystem major anomalies was 41% (28/69), 25% (3/12) for recurrent major anomalies, and 50% (5/10) for severe skeletal dysplasias (Table 2). The mean depth of coverage of the targeted coding region was at least 50×. Typically, the turnaround time in negative cases was 3 weeks, while an extra 3 weeks were needed for Sanger sequencing in positive cases.

Repeat testing by solo-CES after a negative gene panel result yielded a diagnosis in 2 of the 5 cases. In the case with a negative result after a lissencephaly panel, a variant was detected in the *ASPM* gene, not included in the panel (microcephaly with simplified gyral pattern), and in the case of the cardiomyopathy panel, a pathogenic variant in the *KMT2D* gene (Kabuki syndrome) was detected with solo-CES. A single (1%) secondary finding was found by

solo-CES in the *MYBPC3* gene, not related to the fetal phenotype (rachischisis and malformed vertebrae and ribs), but causing familial hypertrophic cardiomyopathy. This variant was inherited from the mother who was counseled about this finding and referred to the cardiologist. Regarding incidental findings, a “de novo” pathogenic variant was found in the *MED13L* gene, associated with intellectual disability, in a case of multisystem anomalies. This gene was included in the filtering of the variants in the CES since it has been described in association with intellectual disability [18].

Regarding pregnancy outcomes, 103 (69%) of the 148 pregnancies studied were terminated upon parental request, and a definitive diagnosis was reached in 43 (42% diagnostic yield) of these cases, although only 5 (12%) diagnoses were reported before termination (Table 3). Among the 45 continuing pregnancies, a definitive diagnosis was reached in 10 (22% diagnostic yield) of the cases. In 4 of these, the fetus was delivered stillborn, and among the 6 liveborn fetuses there were 2 neonatal demises. Among the 35 fetuses without a definitive diagnosis from continuing pregnancies, 7 were delivered stillborn, and 28 were born alive, although 5 infants died in the neonatal period and another infant died at 7 months (Table 3).

Discussion

Our study demonstrates an overall diagnostic yield of 36% (53/148) among fetuses with selected structural anomalies. When the NGS studies managed to provide a causative diagnosis, an accurate prognostic profile for the proband, together with the recurrence risk, were established. The observed diagnostic yield in NGS gene panels applied to those fetal conditions with an available phenotype-driven panel was 31% (15/49), with the most effective panels identified as the CAKUT and RASopathies-hydrops panels. Our approach for exome sequencing using solo-CES, which entails sequencing the fetus alone and interpreting only the OMIM genes, provided a 37%

(38/104) diagnostic yield, and was proven particularly efficient in multisystem anomalies, skeletal dysplasias, and recurrent anomalies.

NGS-based diagnostic panels, initially used in pediatrics, have been subsequently applied to prenatal cases when the observed fetal phenotype matched the conditions covered by these gene panels. Gene panels analyze multiple genes at once, ranging from 2 to 140 in our study, selected because they had been described as associated with a specific fetal phenotype. The most characteristic panel is that containing genes for Noonan syndrome, applied in persistent nuchal edema after an increased first trimester nuchal translucency. However, in 2 large prenatal series in which a RASopathies panel was applied, a lower yield (2.9–9.4%) than that observed in our study (35%) was achieved [19, 20]. This discrepancy may be explained by different inclusion criteria, as we requested not just an isolated increased nuchal translucency (as in the 2 previous studies), but also an increased nuchal fold persisting in the second trimester. The prenatal use of a gene panel for skeletal dysplasias [21] and for CAKUT panel in hyperechogenic large kidneys [10] has also been reported to achieve a remarkable yield. However, in our center, we preferred to use solo-CES for skeletal dysplasias, other than suspected OI, given that the gene coverage of the panel available was found to be incomplete during the first cases of the study. A recent study sought to compare WES with a simulated application of commercial gene panels' diagnostic yield in children susceptible of having a genetically heterogeneous condition, concluding that, had a panel been selected instead, 23% of WES-diagnosed children would not have been diagnosed [22].

Prenatally, WES can be particularly useful in cases where a clinically available phenotype-driven panel cannot provide an answer, as variants in genes that were not on the specific prenatal panel for the phenotype being tested can be detected by WES [8]. The 2 largest WES studies, carried out in Britain and the United States, were reported in the same issue of the *Lancet* in February 2019. In the British PAGE study, among 610 fetuses with structural anomalies analyzed by trio-WES, a diagnostic genetic variant was identified in 8.5% of the fetuses, which recently increased to 11% in the final analysis (Kilby M. D, personal communication). The American study included DNA samples from 234 eligible trios. Diagnostic genetic variants were identified in 10% of the families, while variants with bioinformatic signatures, possibly indicative of pathogenicity, but with insufficient evidence to be considered diagnostic, were found in another 20% of the remaining fetuses [6]. In these 2 large series, the

diagnostic yield declined in comparison with previous WES studies and also with our study, due to different inclusion criteria, given that fetal structural anomalies of any kind were included in both studies.

The vast majority of prenatal NGS studies use a trio-WES approach, which allows screening 3 exomes simultaneously, from the fetus and the parents. Our group has introduced a simplified exome sequencing method, based on a decreased number of exomes sequenced and genes interpreted. Instead of performing 3 exomes ("trio" analysis), the fetus and both parents, we sequenced the fetus alone ("solo" analysis). Instead of interpreting all the 23,000 genes of the human genome described, we interpreted the 4,000 genes described in association with an abnormal phenotype by the OMIM database, known as the "clinical exome" or "medical exome." In positive cases, Sanger sequencing was subsequently applied for variant confirmation and parental segregation analysis. As expected, the solo-CES cost is less than one third of a trio-WES, and this difference is crucial for any public health system.

The main drawback of the solo-CES approach, as compared with trio analysis, is that a second step is required in positive cases to elucidate whether the candidate variants are inherited from one of the parents or if they are "de novo" findings. We took advantage of the recommended Sanger sequencing confirmation of positive NGS results to simultaneously test the parents. In our study, the turnaround time typically was 6 weeks in positive cases, exceeding by 3 weeks the time for negative cases. This is not an optimal turnaround time if the results would affect pregnancy management. However, in our setting, solo-CES was restricted to pregnancies with severe fetal anomalies, where, often, the decision to terminate the pregnancy had already been made based solely on the ultrasound findings. Otherwise, in our country termination for severe anomalies is allowed until the end of pregnancy. A strong point in favor of sequencing the fetus alone is that we encounter fewer incidental findings in the parental genomes.

Our study is not without limitations. The main limitation is the inability to extrapolate the diagnostic yield for the specific fetal anomalies studied herein to other fetal anomalies not included in this study, as highlighted in the discussion. Proper comparisons between groups are hampered by the resulting small sample size after stratification according to NGS methods. A fair comparison with trio analysis, which would audit whether any pathogenic variants in our cohort were missed, using the solo-CES method, is not possible in the present series, and this should then be considered a limitation and a suggestion for a fur-

ther study. Another limitation, which is intrinsic to the new technique, is the fact that a large proportion of candidate variants carry an uncertain significance because there is no information about them. Nowadays, exome sequence interpretation is not as straightforward for clinicians as karyotype or CMA. A multidisciplinary team is needed to interpret previously undescribed variants.

The main strength of our study is the clinical applicability of the proposed solo-CES approach, given that results were delivered to the women and could be used clinically for reassessing fetal prognosis and for improving neonatal management in ongoing pregnancies. In addition, this low-cost exome sequencing method is available for less than one-third of the current trio-WES cost, including the cost of parental segregation analysis. Resources applied to health care are limited and a reduced exome sequencing cost may facilitate its introduction in many countries, although a cost-effectiveness analysis is beyond the scope of this study. In contrast, single gene studies appear to be inefficient and gene panels may be useful when the provider regularly updates its gene content.

Acknowledgments

The authors thank Esther Titos, Gloria Poblador, and M. Dolores Jiménez from the Molecular Core Laboratory of the Medical Diagnostic Center, Hospital Clínic Barcelona; Laia Rodríguez-Revenge from the Genetic and Molecular Department for her help with the fetal DNA samples; and Sandra Oms for her assistance in parental blood draw. The authors would also like to thank Emma Triviño from CatLab, Neus Baena from Parc Taulí Genetic Laboratory, Loreto Martorell from Hospital Sant Joan de Déu, Jordi Rosell from Son Espases Genetic and Molecular Department, and Alberto Plaja from Vall d'Hebron Genetic Laboratory for their help in handling fetal DNA samples. Also, Iosifina Stergiotou for her suggestions regarding the English review of the manuscript.

References

- 1 Dolk H, Loane M, Garne E. The prevalence of congenital anomalies in Europe. *Adv Exp Med Biol*. 2010;686:349.
- 2 Wapner RJ, Martin CL, Levy B, Ballif BC, Eng CM, Zachary JM, et al. Chromosomal microarray versus karyotyping for prenatal diagnosis. *N Engl J Med*. 2012;367(23):2175–84.
- 3 Callaway JLA, Shaffer LG, Chitty LS, Rosenfeld JA, Crolla JA. The clinical utility of microarray technologies applied to prenatal cytogenetics in the presence of a normal conventional karyotype: a review of the literature. *Prenat Diagn*. 2013;33(12):1119.
- 4 Nicolaides KH, Snijders RJM, Campbell S, Gosden CM, Berry C. Ultrasonographically detectable markers of fetal chromosomal abnormalities. *Lancet*. 1992;340(8821):704.
- 5 Yang Y, Muzny DM, Reid JG, Bainbridge MN, Willis A, Ward PA, et al. Clinical whole-exome sequencing for the diagnosis of Mendelian disorders. *N Engl J Med*. 2013;369(16):1502.

Statement of Ethics

This study was approved by Institutional Review Board of Hospital Clínic de Barcelona (HCB/2018/0077), and written informed consent was obtained from parents.

Conflict of Interest

The authors report no conflict of interest.

Funding Sources

This study was partly supported by a grant from Fondo de Investigaciones Sanitarias (Grant #PI17/01153), Instituto de Salud Carlos III, and Fondo Europeo de Desarrollo Regional (FEDER), Ministerio de Economía y Competitividad, Spain. The preliminary results of this study were presented in the top abstracts' session at the 23rd International Conference on Prenatal Diagnosis and Therapy in Singapore (2019).

Author Contributions

A.T.; S.P.; E.M.; L.M.; E.L.; J.S.; B.M.; E.G.; F.P.; V.B.; O.G.; E.E.; M.L.; and M.C. gave their substantial contributions to the acquisition of data for the work. G.A. and A.N. gave their substantial contribution to the interpretation of the found variants and phenotype. B.C. and M.S. carried out the interpretation of exome variants and participated in the committee. They contributed to the manuscript. M.P. gave substantial contributions to the design of the work, analysis, and interpretation of data for the work and drafting the work under the supervision of A.B. who revised it critically for important intellectual content and approved the final version to be published, aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

Data Availability Statement

All data generated during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

- 6 Petrovski S, Aggarwal V, Giordano JL, Stosic M, Wou K, Bier L, et al. Whole-exome sequencing in the evaluation of fetal structural anomalies: a prospective cohort study. *Lancet*. 2019;393(10173):758–67.
- 7 Lord J, McMullan DJ, Eberhardt RY, Rinck G, Hamilton SJ, Quinlan-Jones E, et al. Prenatal exome sequencing analysis in fetal structural anomalies detected by ultrasonography (PAGE): a cohort study. *Lancet*. 2019;393(10173):747–57.
- 8 Vora NL, Powell B, Brandt A, Strande N, Hardisty E, Gilmore K, et al. Prenatal exome sequencing for fetuses with ultrasound abnormalities and challenges. *Genet Med*. 2017;19(11):1207.
- 9 Normand EA, Braxton A, Nassef S, Ward PA, Vetrini F, He W, et al. Clinical exome sequencing for fetuses with ultrasound abnormalities and a suspected Mendelian disorder. *Genome Med*. 2018;10(1):74.
- 10 Bullich G, Domingo-Gallego A, Vargas I, Ruiz P, Lorente-Grandoso L, Furlano M, et al. A kidney-disease gene panel allows a comprehensive genetic diagnosis of cystic and glomerular inherited kidney diseases. *Kidney Int*. 2018;94(2):363.
- 11 Bogliolo M, Pujol R, Aza-Carmona M, Muñoz-Subirana N, Rodríguez-Santiago B, Casado JA, et al. Optimised molecular genetic diagnostics of Fanconi anaemia by whole exome sequencing and functional studies. *J Med Genet*. 2020;57(4):258.
- 12 Amberger J, Bocchini CA, Scott AF, Hamosh A. McKusick's Online Mendelian Inheritance in Man (OMIM®). *Nucleic Acids Res*. 2009;37(Database issue):D793.
- 13 Jelin AC, Vora N. Whole exome sequencing: applications in prenatal genetics. *Obstet Gynecol Clin North Am*. 2018;45(1):69.
- 14 Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American college of medical genetics and genomics and the association for molecular pathology. *Genet Med*. 2015;17(5):405.
- 15 Kopanos C, Tsiolkas V, Kouris A, Chapple CE, Aguilera MA, Meyer R, et al. VarSome: the human genomic variant search engine. *Bioinformatics*. 2019;35(11):1978.
- 16 Kopanos C, Tsiolkas V, Kouris A, Chapple C, Aguilera MA, Meyer R, et al. VarSome: the human genomic variant search engine. *Bioinformatics*. 2019;35(11):1978.
- 17 Kalia SS, Adelman K, Bale SJ, Chung WK, Eng C, Evans JP, et al. Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2016 update (ACMG SF v2.0): a policy statement of the American college of medical genetics and genomics. *Genet Med*. 2017;19(2):249.
- 18 Adegbola A, Musante L, Callewaert B, Maciel P, Hu H, Isidor B, et al. Redefining the MED13L syndrome. *Eur J Hum Genet*. 2015;23(10):1308.
- 19 Stuurman KE, Joosten M, Van Der Burgt I, Elting M, Yntema HG, Meijers-Heijboer H, et al. Prenatal ultrasound findings of rasopathies in a cohort of 424 fetuses: update on genetic testing in the NGS era. *J Med Genet*. 2019;56(10):654.
- 20 Sinajon P, Chitayat D, Roifman M, Wasim S, Carmona S, Ryan G, et al. Microarray and RASopathy-disorder testing in fetuses with increased nuchal translucency. *Ultrasound Obstet Gynecol*. 2020;55(3):383.
- 21 Zhou X, Chandler N, Deng L, Zhou J, Yuan M, Sun L. Prenatal diagnosis of skeletal dysplasias using a targeted skeletal gene panel. *Prenat Diagn*. 2018;38(9):692.
- 22 Dillon OJ, Lunke S, Stark Z, Yeung A, Thorne N, Gaff C, et al. Exome sequencing has higher diagnostic yield compared to simulated disease-specific panels in children with suspected monogenic disorders. *Eur J Hum Genet*. 2018;26(5):644.
- 23 Vetrini F, D'Alessandro LCA, Akdemir ZC, Braxton A, Azamian MS, Eldomery MK, et al. Bi-allelic mutations in PKD1L1 are associated with laterality defects in humans. *Am J Hum Genet*. 2016.
- 24 Le Fevre A, Baptista J, Ellard S, Overton T, Oliver A, Gradhand E, et al. Compound heterozygous Pkd1l1 variants in a family with two fetuses affected by heterotaxy and complex Chd. *Eur J Med Genet*. 2020;63(2):103657.
- 25 Rauch A, Thiel CT, Schindler D, Wick U, Crow YJ, Ekici AB, et al. Mutations in the pericentrin (PCNT) gene cause primordial dwarfism. *Science*. 2008;319(80):816.
- 26 Rodríguez-García ME, Cotrina-Vinagre FJ, Cruz-Rojo J, Garzón-Lorenzo L, Carnicero-Rodríguez P, Pozo D, et al. A rare male patient with Fontaine progeroid syndrome caused by p.R217H de novo mutation in SLC25A24. *Am J Med Genet Part A*. 2018.
- 27 Writzl K, Maver A, Kovačič L, Martínez-Valero P, Contreras L, Satrustegui J, et al. De novo mutations in SLC25A24 cause a disorder characterized by early aging, bone dysplasia, characteristic face, and early demise. *Am J Hum Genet*. 2017;101(5):844.
- 28 Zhang W, Taylor SP, Ennis HA, Forlenza KN, Duran I, Li B, et al. Expanding the genetic architecture and phenotypic spectrum in the skeletal ciliopathies. *Hum Mutat*. 2018;39(1):152.