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ARTICLE

Implementation of fetal clinical exome sequencing: Comparing prospective and retrospective cohorts

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ABSTRACT

Purpose: We compared the diagnostic yield of fetal clinical exome sequencing (fCES) in prospective and retrospective cohorts of pregnancies presenting with anomalies detected using ultrasound. We evaluated factors that led to a higher diagnostic efficiency, such as phenotypic category, clinical characterization, and variant analysis strategy.
Methods: fCES was performed for 303 fetuses (183 ongoing and 120 ended pregnancies, in which chromosomal abnormalities had been excluded) using a trio/duo-based approach and a multistep variant analysis strategy.
Results: fCES identified the underlying genetic cause in 13% (24/183) of prospective and 29% (35/120) of retrospective cases. In both cohorts, recessive heterozygous compound genotypes were not rare, and trio and simplex variant analysis strategies were complementary to achieve the highest possible diagnostic rate. Limited prenatal phenotypic information led to interpretation challenges. In 2 prospective cases, in-depth analysis allowed expansion of the spectrum of prenatal presentations for genetic syndromes associated with the *SLC17A5* and *CHAMP1* genes.
Conclusion: fCES is diagnostically efficient in fetuses presenting with cerebral, skeletal, urinary, or multiple anomalies. The comparison between the 2 cohorts highlights the importance of providing detailed phenotypic information for better interpretation and prenatal reporting of genetic variants.

Introduction

Ultrasound detection of fetal abnormalities, which occurs in approximately 2% to 4% of pregnancies,[1,2] is an essential part of routine obstetrical care. When fetal structural anomalies are suspected, invasive procedures (ie, amniocentesis, chorionic villus sampling [CVS]) are offered for prenatal genetic diagnosis. Historically, karyotype testing was the first-line method to investigate chromosomal anomalies, providing a diagnosis in approximately 30% of fetuses with abnormal ultrasound findings.[3] With the introduction of chromosomal microarray analysis (CMA), the diagnostic rate of chromosomal anomalies increased by 4% to 7%.[3-5] However, the search for a genetic diagnosis in the remaining cases was costly and time-consuming before the introduction of next-generation sequencing (NGS). The encouraging results of exome sequencing (ES) in pediatric patients[6-10] raised interest for its application in a prenatal setting. Since 2014, several studies comprising small and selected cohorts of aborted fetuses reported high diagnostic yields (in the range of 50%-80%).[11-15] Recently, 2 large and

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unselected prospective cohorts were reported with a lower diagnostic rate (8%-10%).[16,17] As the use of ES increases in prenatal care, medical professionals (eg, referring physicians, laboratory geneticists, genetic counselors) are faced with complex technical and ethical challenges that must be addressed collectively.[18]

Herein we report fetal clinical exome sequencing (fCES) in a series of 303 pregnancies displaying abnormal ultrasound findings (with no selection of the type of fetal anomalies) and normal quantitative fluorescent–polymerase chain reaction and CMA results, including 183 pregnancies for which the diagnosis was obtained during the pregnancy and hence contributed to the clinical decision process. Importantly, the retrospective and prospective analyses were carried out by the same center and in the same time frame, allowing direct comparison of the diagnostic yields between the 2 cohorts. In particular, we discuss the effect of the availability of detailed phenotypic information for the establishment of genetic diagnoses through fetal NGS-based analyses. In addition, our series expanded the range of fetal phenotypes investigated by genome-wide methods because it included fetuses presenting with anomalies detected using ultrasound anomalies that have rarely been explored by NGS (ie, intrauterine growth restriction, amniotic fluid abnormalities) and uncovered novel associations between known genotypes and fetal phenotypes. Finally, our study aimed to share data analysis workflow strategies and interpretation challenges.

Materials and Methods

Study design and participants

Patients were recruited from 14 centers (Supplemental Table 1) between October 2016 and June 2020, and their enrollment was conducted in parallel for both cohorts. However, because of a long turnaround time (TAT) at the beginning of the study, the cases recruited initially mostly consisted of interrupted pregnancies. The study design is summarized in Figure 1A. For the retrospective study cohort, couples who had experienced a pregnancy characterized by fetal anomalies for which the fetal sample was available were recruited. For the prospective study cohort, on detection of fetal structural anomalies during a routine ultrasound examination, parents who opted for invasive testing were offered participation. Inclusion and exclusion criteria were the same for both cohorts. Inclusion criteria were increased nuchal translucency (NT) (>99th percentile and/or ≥3.5 mm at 11-14 weeks ultrasound scan), amniotic fluid anomalies, or any major structural fetal malformation(s). Exclusion criteria were fetuses presenting isolated soft markers (Supplemental Table 2), known monogenic disease within the family, identification of an etiology (chromosomal abnormality, infection) explaining the fetal phenotype, and simplex cases for which no parental DNA was available (ie, only trio/duo analysis was performed). Families were informed about the technical aspects and the limitations of the study by a clinical geneticist or a trained gynecologist, and written informed consent was obtained. The parents could opt in or out of the return of incidental findings. This study was approved by the ethical committee of the Hôpital Erasme, Brussels, Belgium, under the reference P2016/236.

Clinical data collection

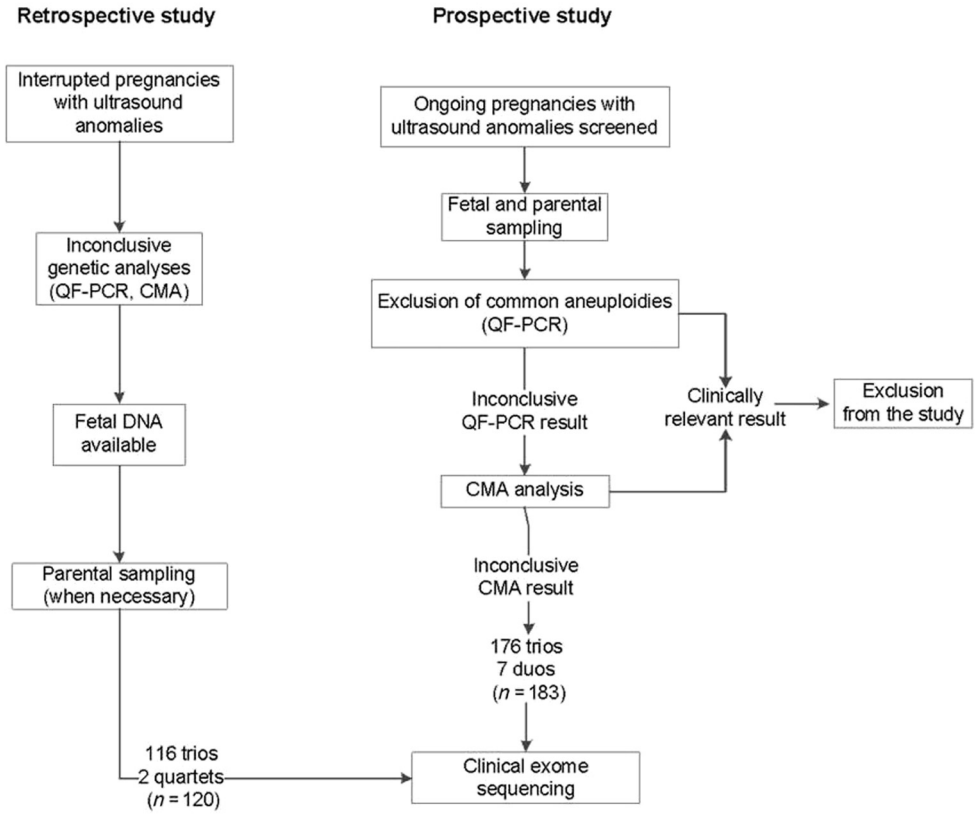
In the retrospective study, information about fetal phenotypes was collected from imaging data and postmortem examinations when available and used for genotype–phenotype correlation during fCES analysis. In the prospective study, fCES interpretation was based on imaging data available at the time of the analysis. Retrospectively, clinical data were reviewed in all cases, and phenotypes were annotated using Human Phenotype Ontology (HPO) (https://hpo.jax.org/app/) terms,[19] allowing classification in several phenotypic categories (Supplemental Table 3).

fCES

Library preparation and sequencing

In the prospective study, fetal DNA was obtained from CVS, amniotic fluid, or fetal blood that remained unused after routine investigations. When the amount of DNA from these samples was insufficient, DNA was extracted from cultured samples. In the retrospective study, cultured fibroblasts or fetal biopsies were used for DNA extraction if no other type of sample was available. Parental blood samples were also collected for DNA extraction. In all prenatal samples, maternal contamination and presence of the most common aneuploidies were excluded by quantitative fluorescent–polymerase chain reaction (Elucigene QST*R Plus v2, Elucigene Diagnostics). CMA on a CytoSure Constitutional v3 8×60k array (Oxford Gene Technology) was performed to exclude copy number variations (CNVs). Library preparation was performed using KAPA HyperPrep/HyperPlus Library Preparation Kit (Roche NimbleGen Inc). An in-house SeqCap EZ Choice XL Probes (Roche NimbleGen Inc) targeting the coding exons of genes associated with Mendelian disorders was used (4 designs) (Supplemental Table 4). Libraries were sequenced on Illumina HiSeq 1500/NovaSeq 6000 (Illumina Inc). Bioinformatics pipeline was run at Brussels Interuniversity Genomics High Throughput core (BRIGHTcore) (http://www.brightcore.be/). The mean coverage of fetal samples was between 250× and 300×, and the parental samples were sequenced at 150×. Reads were aligned to the reference genome (GRCh37/hg19) using the Burrows-Wheeler Aligner (version 0.7.10), and variant calling was performed using Genome Analysis Toolkit (version 3.3).

A



B

	Type of analysis	In silico gene panel	Inheritance patterns investigated	Main advantages	Main disadvantages
Analysis step 1	Trio/duo	Congenital anomalies gene panel (Supplemental Table 5)	de novo, homozygous and compound heterozygous, X-linked (male fetuses)	•Timesaving •Limited complexity of the analysis (VUS, incidental findings etc.)	Diagnoses missed in: •genes not included in the gene panel •AD genes with incomplete penetrance •AR genes which fit with fetal phenotype (only one class IV/V variant identified)
Analysis step 2	Trio/duo	NA (all fCES gene content)	de novo, homozygous and compound heterozygous, X-linked (male fetuses)	•Diagnostic gain in genes not previously associated to in utero phenotypes •Subsequent curation of in silico gene panels (step 1/3) based on the positive diagnoses	•Time consuming •Increase the complexity of the analysis (VUS, incidental findings etc.) Diagnoses missed in: •AD genes with incomplete penetrance •AR genes which fit with fetal phenotype (only one class IV/V variant identified)
Analysis step 3*	Simplex	Ad hoc gene panel/s (ie, specific genes involved in the fetal phenotype)	de novo, homozygous and compound heterozygous, X-linked, autosomal dominant, autosomal recessive with only one hit	Diagnostic gain in: •AD genes with incomplete penetrance •AR genes which fit with fetal phenotype (only one class IV/V variant identified)	•Time consuming •Increase the complexity of the analysis (VUS, incidental findings etc.)

Figure 1 A. Schematic representation of the study flow. B. Summary of the multistep strategy used. *Optional—performed when fetal anomalies were specific for 1 system or were highly suggestive of defined genetic disorders. AD, autosomal dominant; AR, autosomal recessive; CMA, chromosomal microarray analysis; fCES, fetal clinical exome sequencing; NA, not applicable; QF-PCR, quantitative fluorescent–polymerase chain reaction; VUS, variant of unknown significance.

Variant filtering

Variant filtering and interpretation were carried out through Highlander (https://sites.uclouvain.be/highlander/) according to allele frequency and effect on protein and inheritance pattern (de novo, homozygous and compound heterozygous, X-linked [XL] inheritance in the case of male fetuses). fCES data were analyzed by a laboratory scientist in collaboration with prenatally involved clinical geneticists using the collected clinical information about fetal phenotypes (as described in the clinical data collection section). In particular, variant filtering included several steps (Figure 1B). First, a comprehensive in silico panel of genes (up to 1273 genes) involved in congenital anomalies (developed from Pangalos et al[13] and Fetal anomalies panel from Genomics England PanelApp [https://panelapp.genomicsengland.co.uk/]) (Supplemental Table 5) was analyzed using a trio/duo-based approach, exploring the different inheritance patterns. Second, trio/duo analysis of the entire clinical exome (up to 4867 genes) (Supplemental Table 4) was accomplished for all inheritance modes. If the results of the previous analyses were negative and the fetal anomalies were specific for 1 system or were highly suggestive of defined genetic disorders, further in silico gene panels were investigated exclusively in the proband (simplex analysis) using Genomics England PanelApp, GeneReviews (https://www.ncbi.nlm.nih.gov/books/NBK1116/), HPO, or the most recent literature. In selected cases (ie, only 1 likely pathogenic/pathogenic variant detected in genes responsible for recessive disorders), single exon CNVs were detected using Copy Number Variation Detection In NGS gene panels[20] and exonic CNV data were visualized using in-house software. This exonic CNV pipeline was run retrospectively.

Variant interpretation

Variant interpretation and classification followed international guidelines (pathogenic: class V; likely pathogenic: class IV; variant of unknown significance (VUS): class III; likely benign: class II; benign: class I).[21] For class V, we used stringent criteria because the analysis had to be performed with phenotypic information that was limited to the prenatal stages (ie, fetal ultrasound and, if available, nuclear magnetic resonance imaging) and, for the majority of prospective cases, absent/limited postnatal phenotypic characterization; therefore, only the variants already reported in the literature as pathogenic were classified as class V. Nonetheless, highly suspicious novel variants (eg, loss-of-function variant in a dosage-sensitive gene) within genes related to the fetal phenotype were classified as likely pathogenic (class IV) and reported. In addition, if further information was needed for correct interpretation of the data or if a potential discrepancy between the genetic findings and the fetal phenotype was noted, candidate variants were discussed in a multidisciplinary team.

Our ethical review board–approved informed consent form did not mention the possibility of opting in/out of reporting secondary variant information. Therefore, the American College of Medical Genetics and Genomics recommendations for reporting of secondary findings[22] were not followed. In particular, fetal incidental findings were reported only if highly penetrant pathogenic/likely pathogenic variants were detected in genes known to cause moderate or severe childhood-onset disorders. Pathogenic or likely pathogenic variants in genes known to cause medically actionable dominant conditions (inherited cancer syndromes, cardiovascular, and others) were reported exclusively in the parental reports if they had consented to the return of these results.

Variant validation

Variants were confirmed by Sanger sequencing if they arose as a de novo event in the index case or if the variant call-specific metrics (eg, read depth, allele balance, strand bias) and/or their genomic context (eg, presence of repeats, pseudogenic regions) were considered suboptimal.

Statistical analysis

The number of diagnostic variants in the 2 cohorts was compared using a χ^2 test performed using GraphPad Prism v.7.0 (GraphPad Software).

Results

A total of 300 couples were recruited, and 303 fetal samples (120 terminated and 183 ongoing pregnancies) were processed for fCES (Supplemental Tables 6 and 7). Fetal DNA was obtained from amniocentesis (68.9%, 209/303), CVS (14.1%, 43/303), fetal blood (6.6%, 20/303), or tissue samples (10.2%, 31/303). Male to female ratios were 0.94 (89/94) and 1.2 (66/54) in the prospective and retrospective cohorts, respectively. fCES was mostly performed in trio (176 prospective and 116 retrospective cases), followed by duo (7 prospective cases) or quartet (4 retrospective cases belonging to 2 couples). In the prospective group pregnancy, outcomes were available for 105 of 183 fetuses (57%). Of these, the parents opted for termination in 45 pregnancies (25%), 6 ended in miscarriages (3%), 2 ended in neonatal deaths (1%), and 52 were livebirths (28%). In contrast, in the retrospective cohort, the pregnancy outcome was known in most cases (109/120, 91%). Ninety of them were terminated (82.5%), 16 ended in miscarriages (15%), and 3 ended in neonatal death (2.5%).

Among the prospective cases, warning signs were variable. Some phenotypic categories represented a small sample size (<10 cases), such as anomalies of the endocrine system (0.5%, 1/183), digestive tract (1.6%, 3/183), genital system (1.6%, 3/183), spine (1.6%, 3/183), face (2.2%, 4/183), fluid regulation (2.2%, 4/183), musculature (2.7%, 5/183), and amniotic fluid volume (3.3%, 6/183) (Figure 2B). Fetuses presenting with abnormalities in growth (5.5%, 10/183), urinary tract (6%, 11/183), cardiovascular system

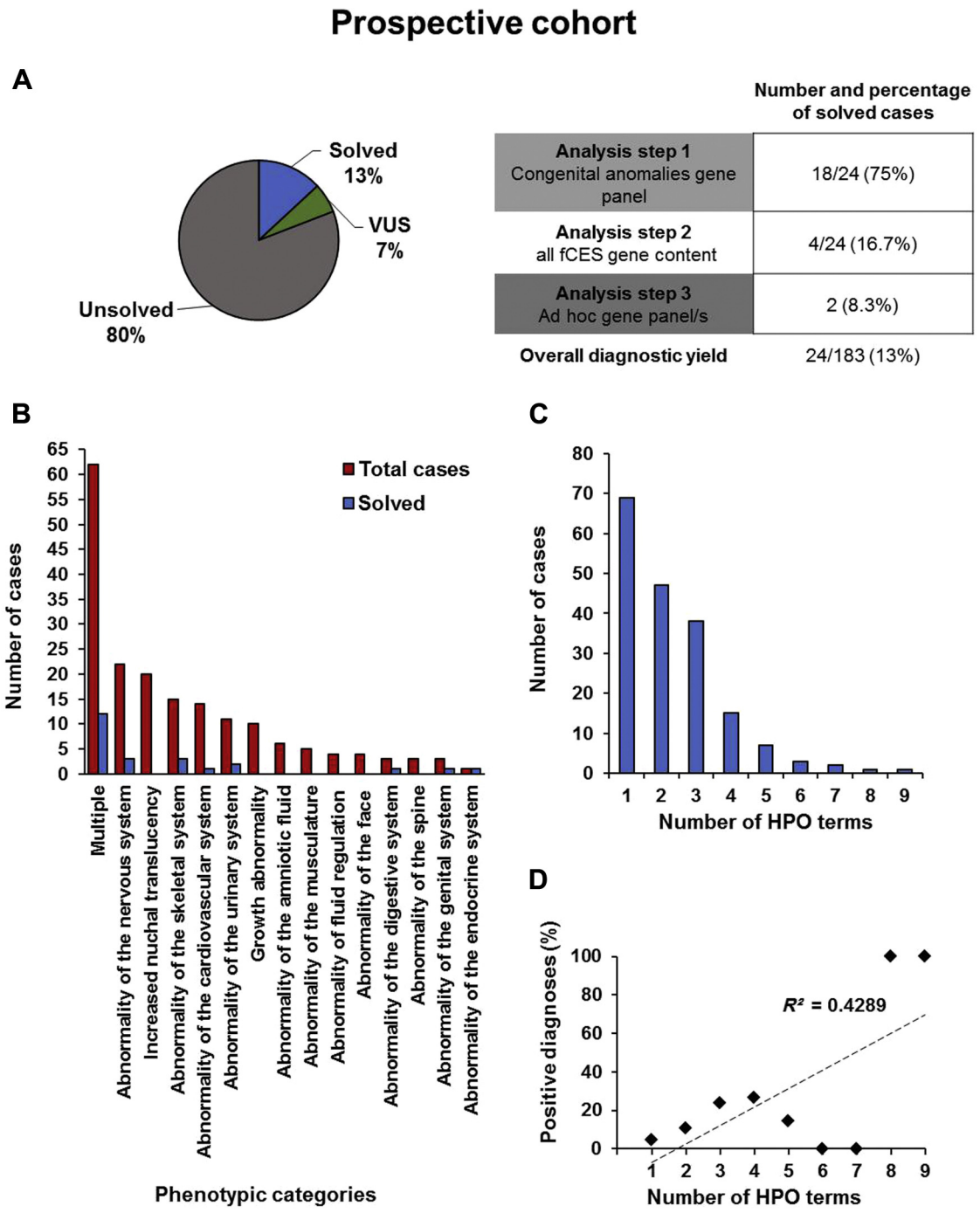


Figure 2 A. Overall fCES results in the prospective cohort and diagnostic yield of each analysis step. B. Proportion of solved cases for each phenotypic category. C. Distribution of HPO terms. D. Correlation between the positive diagnoses and the number of HPO terms. fCES, fetal clinical exome sequencing; HPO, Human Phenotype Ontology; VUS, variant of unknown significance.

(7.7%, 14/183), skeleton (8.2%, 15/183), nervous system (12%, 22/183), multiple organs (33.9%, 62/183), and NT (10.9%, 20/183) represented larger cohorts (Figure 2B). In contrast, most of the retrospective cases displayed multiple (65.8%, 79/120) and cerebral abnormalities (11.7%, 14/120), followed by fetuses with spinal (6.7%, 8/120), urinary (5.8%, 7/120), fluid regulation (3.3%, 4/120), facial (3.3%, 4/120), skeletal (1.7%, 2/120), cardiovascular (0.8%, 1/120), and digestive (0.8%, 1/120) defects (Figure 3B).

In the prospective cohort, our multistep variant analysis process provided a genetic diagnosis in 24 of 183 cases (13%) (Figure 2A, Table 1). None of the 7 cases for which a duo-based analysis was performed could be solved. Most diagnostic variants (18/24, 75%) were found through analysis of the congenital anomalies gene panel (Figures 1B and 2A). Four additional cases (4/24, 16.7%) were solved through analysis of the whole clinical exome (Figures 1B and 2A). A specific in silico gene panel in simplex analysis

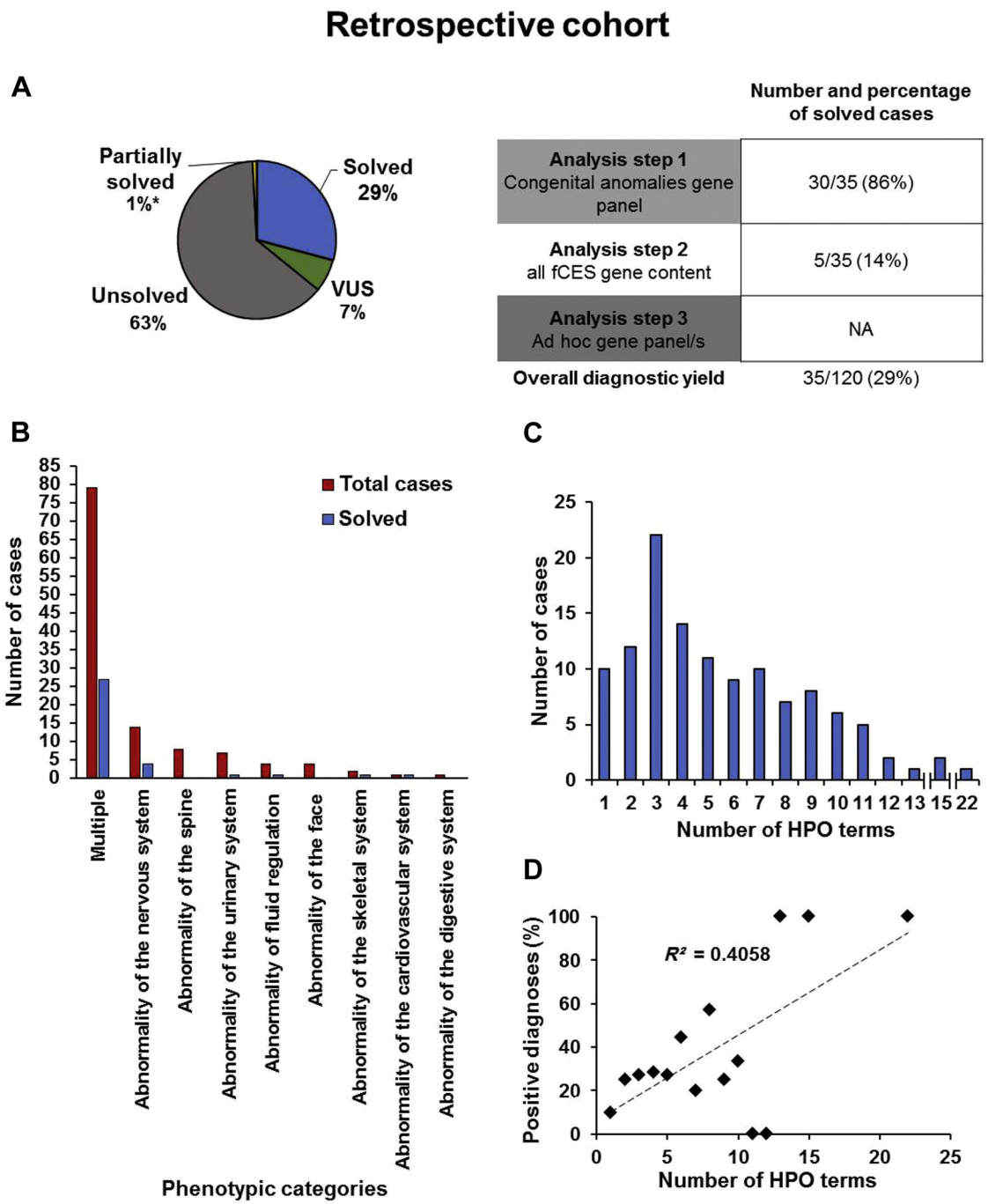


Figure 3 A. Overall fCES results in the retrospective cohort and diagnostic rate of each analysis step. B. Proportion of solved cases for each phenotypic category. C. Distribution of HPO terms. D. Correlation between the positive diagnoses and the number of HPO terms. *Only one pathogenic variant in an autosomal recessive gene (case R56) (Supplemental Table 7). fCES, fetal clinical exome sequencing; HPO, Human Phenotype Ontology; NA, not applicable; VUS, variant of unknown significance.

(ie, only filtering on the proband variants) solved 2 more cases (2/24, 8.3%) (Figure 2A). Among the positive cases, only 1 presented a relevant family history (ie, affected fetus from previous pregnancy). When only consanguineous couples ($n = 12$) were taken into account, the diagnostic yield increased to 42% (5/12). In the whole prospective cohort, autosomal dominant (AD) disorders accounted for 46% ($n = 11$) of cases and all were caused by a de novo variant (genes: *PIK3CA*, *ZIC2*, *TSC2*, *NRAS*, *COL1A1*, *CHAMP1*, *ARID1B*, *TP63*, *RASA1*) (Table 1). Autosomal recessive (AR) disorders were diagnosed in 11 cases (46%), including 6 (54.5%) with compound heterozygous variants and 5 (45.5%) with homozygous variants (genes: *MKS1*, *PKHD1*, *PKD1*, *SLC7A9*, *TPO*, *RBM8A*, *SLC17A5*, *COQ9*, *BBS7*) (Table 1). In this last subgroup, consanguinity was noted in 4 of 5 cases, and a founder pathogenic variant was

Table 1 Overview of the diagnoses identified in the prospective cohort

Case Number	Phenotypic Category	Prenatal Findings	Post-mortem Exams/ Postnatal Finings	Overall HP Terms	Consanguinity	Recurrence of the Disorder	Transcript	Gene	Variant(s) and Protein Effect(s)	Variant Classification Phenotype's Contribution	Inheritance	fCES Interpretation Strategy that Identified the Pathogenic Variant(s)	Disorder	OMIM	Additional Findings	Classification Phenotype's Contribution	Inheritance	Pregnancy Outcome
P3	Multiple	Occipital encephalocele, omphalocele, polycystic kidney dysplasia	Postaxial foot polydactyly, bilateral postaxial polydactyly, bile duct proliferation	HP:0002085; HP:0001539; HP:0000113; HP:0001830; HP:0006136; HP:0001408	N	N	NM_017777.4	*MKS1*	c.1408-34_ 1408-6del	V Full	Recessive homozygote	*In silico* gene panel - Congenital anomalies (design 2)	Meckel syndrome 1	249000	N	-	-	TOP at 12w
P4	Abnormality of the urinary system	Polycystic kidney dysplasia	NA	HP:0000113	N	N	NM_138694.4	*PKHD1*	c.5321G>A p.(Cys1774Tyr) c.8312T>C p.(Val2771Ala)	IV, V Full	Compound heterozygote	*In silico* gene panel - Congenital anomalies (design 2)	Polycystic kidney disease 4, with or without hepatic disease	263200	N	-	-	Newborn
P6[a]	Multiple	Large for gestational age, macrocephaly, polyhydramnios, pulmonary hypoplasia	Diaphragmatic eventration, hypertelorism, abnormality of the hairline, intestinal duplication (small bowel, distal part)	HP:0001520; HP:0000256; HP:0009110; HP:0001561; HP:0002089; HP:0000316; HP:0009553; HP:0100668	N	N	NM_006218.4	*PIK3CA*	c.1030G>A p.(Val344Met)	V Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	PIK3CA-Related Overgrowth Spectrum	615108/ 612918/ 602501	N	-	-	TOP at 34w
P7	Multiple	Fetal cystic hygroma (4.25mm), omphalocele, congenital diaphragmatic hernia, pulmonary hypoplasia	NA	HP:0010878; HP:0001539; HP:0000776; HP:0002089	N	N	NM_000202.8	*IDS*	c.1072C>A p.(Pro358Thr)	IV Partial	X-linked	*In silico* gene panel - Congenital anomalies (design 2)	Mucopolysaccharidosis II	309900	N	-	-	TOP at 26w
P26	Abnormality of the urinary system	Oligohydramnios, polycystic kidney dysplasia, hyperechogenic kidneys	NA	HP:0001562; HP:0000113; HP:0004719	Y	N	NM_ 001009944.3	*PKD1*	c.3820G>A p.(Val1274Met)	IV Full	Recessive homozygote	Clinical Exome	Polycystic kidney disease 1[c]	173900	N	-	-	Fetal demise
P32[b]	Abnormality of the nervous system	Cerebellar hypoplasia, absent septum pellucidum, holoprosencephaly (Middle interhemispheric variant)	Agenesis of corpus callosum, focal polymicrogyria, abnormality of the falx cerebri (hypoplasia), periventricular heterotopia, muscular hypotonia, depressed nasal bridge, short nose, anteverted nares	HP:0001321; HP:0001331; HP:0001360; HP:0001274; HP:0032471; HP:0010653; HP:0007165; HP:0001252; HP:0005280; HP:0003196; HP:0000463	Y	N	NM_007129.5	*ZIC2*	c.1109G>A p.(Cys370Tyr)	IV Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	Holoprosencephaly 5	609637	N	-	-	Newborn
P41	Abnormality of the digestive system	Antenatal hyperechoic colon	Cystinuria, ornithinuria, hyperlysinuria, argininuria	HP:0003131; HP:0003532; HP:0003297; HP:0003268	N	N	NM_001243036.2	*SLC7A9*	c.1A>G p.(Met1?) c.313G>A p.(Gly105Arg)	IV, V Full	Compound heterozygote	Clinical Exome	Cystinuria	220100	N	-	-	Newborn

(continued)

Table 1 Continued

Case Number	Phenotypic Category	Prenatal Findings	Post-mortem Exams/ Postnatal Finings	Overall HP Terms	Consanguinity	Recurrence of the Disorder	Transcript	Gene	Variant(s) and Protein Effect(s)	Variant Classification Phenotype's Contribution	Inheritance	fCES Interpretation Strategy that Identified the Pathogenic Variant(s)	Disorder	OMIM	Additional Findings	Classification Phenotype's Contribution	Inheritance	Pregnancy Outcome
P83	Multiple	Cardiac rhabdomyoma, astrocytoma, cortical tubers	NA	HP:0009729; HP:0009592; HP:0009717	N	N	NM_000548.5	*TSC2*	c.1001T>G p.(Val334Gly)	IV Full	Probably de novo (duo analysis)	*In silico* gene panel - Congenital anomalies (design 2)	Tuberous sclerosis-2	613254	N	-	-	TOP
P84	Multiple	Increased nuchal translucency, generalized edema, talipes equinovarus	NA	HP:0010880; HP:0007430; HP:0001762	N	N	NM_002524.5	*NRAS*	c.34G>A p.(Gly12Ser)	V Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	Noonan syndrome 6	613224	N	-	-	Fetal demise in utero at 15w
P108	Abnormality of the skeletal system	Femoral bowing (bilateral)	NA	HP:0002980	N	N	NM_000088.4	*COL1A1*	c.1876G>A p.(Gly626Ser)	V Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	Osteogenesis imperfecta, type III/IV	259420/ 166220	N	-	-	TOP at 17w
P128	Abnormality of the endocrine system	Goiter, hypothyroidism	Hearing impairment	HP:0000853; HP:0000821; HP:0000365	N	N	NM_000547.5	*TPO*	c.209C>T p.(Pro70Leu) c.1184_1187 dupGCCG p.(Ala397Profs *76)	IV, V Full	Compound heterozygote	Clinical Exome	Thyroid dyshormonogenesis 2A	274500	N	-	-	Newborn
P129	Abnormality of the skeletal system	Bilateral radial aplasia, aplasia/ hypoplasia of the humerus, radial club hand	NA	HP:0004977; HP:0006507; HP:0004059	N	N	NM_005105.5	*RBM8A*	c.67+32G>C Microdeletion 1q21.1	IV Full	Compound heterozygote	Genomics England PanelApp: Radial dysplasia	Thrombocytopenia-absent radius syndrome	274000	N	-	-	Newborn
P131	Multiple	Increased nuchal translucency (4mm), congenital diaphragmatic hernia	NA	HP:0010880; HP:0000776	N	N	NM_001164144.3	*CHAMP1*	c.2134A>T p.(Lys712*)	IV Full	De novo	Clinical Exome	Mental retardation, autosomal dominant 40	616579	N	-	-	TOP
P132	Multiple	Agenesis of corpus callosum, abnormality of the helix	NA	HP:0001274; HP:0011039	N	N	NM_001363725.2	*ARID1B*	c.2918delT p.(Met973ArgfsTer11)	IV Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	Coffin-Siris syndrome 1	135900	N	-	-	Newborn
P136	Multiple	Hydrops fetalis, hyperechogenic kidneys, hepatomegaly	Peritoneal effusion, pleural effusion, short lower limbs, hypertelorism, depressed nasal ridge, labial hypertrophy, hypoplasia of first ribs, delayed calcaneal ossification	HP:0001789; HP:0004719; HP:0002240; HP:0030995; HP:0002202; HP:0006385; HP:0000316; HP:0000457; HP:0000065; HP:0006657; HP:0008142	N	N	NM_012434.5	*SLC17A5*	c.308G>A p.(Trp103*) dup ex 8-9	IV, V Full	Compound heterozygote	Genomics England PanelApp: Fetal hydrops/ CoNVaDING	Salla disease	604369	N	-	-	TOP at 25w

(continued)

Table 1 Continued

Case Number	Phenotypic Category	Prenatal Findings	Post-mortem Exams/ Postnatal Finings	Overall HP Terms	Consanguinity	Recurrence of the Disorder	Transcript	Gene	Variant(s) and Protein Effect(s)	Variant Classification Phenotype's Contribution	Inheritance	fCES Interpretation Strategy that Identified the Pathogenic Variant(s)	Disorder	OMIM	Additional Findings	Classification Phenotype's Contribution	Inheritance	Pregnancy Outcome
P146	Multiple	Oligohydramnios, severe intrauterine growth retardation, cerebellar hypoplasia, hypoplasia of the corpus callosum, cardiomegaly, echogenic fetal bowel, hyperechogenic kidneys, fetal ascites, abnormality of neuronal migration	NA	HP:0001562; HP:0008846; HP:0001321; HP:0001320; HP:0001640; HP:0010943; HP:0004719; HP:0001791; HP:0002269	Y	N	NM_020312.4	*COQ9*	c.197_198delAG p.(Gln66ArgfsTer6)	IV Full	Recessive homozygote	*In silico* gene panel - Congenital anomalies (design 3)	Coenzyme Q10 deficiency, primary, 5	614654	*IL7R* (NM_ 002185.5): c.83-1G>A (secondary finding)	IV Unknown	Recessive homozygote	Newborn death after 1h of life
P158	Multiple	Increased nuchal translucency (7mm), pleural effusion, hydrops fetalis, toe syndactyly	NA	HP:0010880; HP:0002202; HP:0001789; HP:0001770	N	N	NM_003722.5	*TP63*	c.728G>A p.(Arg243Gln)	V Partial	De novo	*In silico* gene panel - Congenital anomalies (design 3)	Ectrodactyly, ectodermal dysplasia, and cleft lip	604292	*LZTR1* (NM_ 006767.4): c.594-3C>T c.988A>G p.(Ser330Gly)	III Unknown	Compound heterozygote	Not available
P167	Multiple	Mild fetal ventriculomegaly, cardiomegaly, abnormality of the cerebral vasculature, abnormality of neck blood vessel	NA	HP:0010952; HP:0001640; HP:0100659; HP:3000037	N	N	NM_002890.3	*RASA1*	c.261_262delAG p.(Gly89ArgfsTer22)	V Full	De novo	*In silico* gene panel - Congenital anomalies (design 3)	Capillary malformation-arteriovenous malformation 1	608354	N	-	-	TOP at 23w
P170	Abnormality of the genital system	Urogenital sinus anomaly, fetal ascites	Generalized edema, broad neck, low-set ears, hand polydactyly, foot polydactyly	HP:0100779; HP:0001791; HP:0007430; HP:0000475; HP:0000369; HP:0001161; HP:0001829	N	N	NM_176824.3	*BBS7*	c.1119delA p.(Lys373AsnfsTer9) c.712A>G p.(Arg238Gly)	IV Full	Compound heterozygote	*In silico* gene panel - Congenital anomalies (design 3)	Bardet-Biedl syndrome 7	615984	N	-	-	Newborn

Details are provided only when the patients agreed with personal data publication (19/24).

[a]Case already published in PMID: 31568861.

[b]Case already published in PMID: 32695376.

[c]Usually AD but prenatal cases having AR inheritance have been described (PMID: 31079206, 22034641, 23624871).

present in the remaining case. One XL disorder was found (1/24, 4.2%) (gene: *IDS*) (Table 1), and a digenic diagnosis was proposed in 1 case (1/24, 4.2%). Interestingly, 2 cases involved detection of a single nucleotide change along with a CNV (genes: *RBM8A* and *SLC17A5*) (Table 1). The highest diagnostic yields in the prospective cohort (among subgroups with ≥10 cases) were obtained for skeletal (20%, n = 3/15), multiple (19%, n = 12/62), urinary (18%, n = 2/11), and cerebral anomalies (14%, n = 3/22) (Figure 2B). Three cases from our prospective series warranted further description (see Supplemental Case Reports for details). The discovery of a novel fetal phenotype caused by a novel variant within a known gene is illustrated by case P131 for which a de novo truncating variant of the *CHAMP1* gene (Supplemental Figure 2, Supplemental Case Reports), associated with AD mental retardation (OMIM 616579), was detected in a fetus presenting with increased NT and congenital diaphragmatic hernia. Case P136, presenting with hydrops fetalis, hyperechogenic kidneys, and hepatomegaly, was solved by the detection of 1 pathogenic variant in the *SLC17A5* gene (Salla disease, OMIM 604369)—by a simplex analysis of an in silico panel hydrops fetalis—along with a *SLC17A5* microduplication detected after the CNV analysis through Copy Number Variation Detection In NGS gene panels (Supplemental Figure 1, Supplemental Case Reports), showing the need for multiple analysis strategies. In numerous cases, medical teams are faced with variant interpretation challenges, as in case P178 presenting with spina bifida, lemon sign, and mild fetal ventriculomegaly for which compound heterozygous VUS were detected in the *SCRIB* gene (Supplemental Figure 3, Supplemental Case Reports), a candidate gene for neural tube defects.

Most fetuses in the prospective cohort were classified using 1 to 3 HPO terms (Figure 2C), with a median number of 2 terms. Although the correlation between frequency of a positive diagnosis and the number of HPO terms was not statistically significant, the trend of the plot may suggest that fCES diagnostic yield could be partially determined by an accurate phenotypic description (Figure 2D).

In the retrospective cohort, a diagnosis was reached in 35 of 120 cases (29%) through multistep analysis (Figure 3A, Table 2). The diagnostic rate was thus significantly higher in the retrospective than in the prospective cohort (N = 303; $X^2_{(2)}$ = 11.9; P < .001). Most variants (30/35, 86%) were found using the gene panel for congenital anomalies (Figures 1B and 3A), and the remaining cases required analysis of all the fCES genes (5/35, 14%) (Figure 3A). Among the solved cases, 5 were characterized by a positive family history (5/35, 14%). When only consanguineous couples were considered (n = 15), the diagnostic rate increased to 40% (6/15). AD disorders were diagnosed in 49% (17/35) with mostly de novo variants (15/35, 43%) (genes: *ACTA1*, *MYH3*, *HRAS*, *PTPN11*, *DVL1*, *FLNB*, *RIT1, BRAF, JAG1, KMT2D, COL1A1, GREB1L*) (Table 2). Parental mosaicism was identified in 2 of the cases affected by AD syndromes (genes: *COL1A1, GREB1L*) (Table 2). Similar to AD disorders, AR diseases were diagnosed in 17 cases (17/35, 49%) with compound heterozygous (9/35, 26%) and homozygous variants (8/35, 23%) (genes: *NEB*, *ASCC1*, *ASPM*, *GBE1*, *B3GALNT2, ISPD, CEP290, PIEZO1, TUBGCP6, TNNT3, DDX11, ALG3, ETFA*) (Table 2). In 5 of 8 fetuses (63%) presenting with homozygous variants, the parents were consanguineous, whereas in most other cases the variants were hotspot variants. Moreover, an XL disorder was diagnosed (1/35, 3%) (gene: *IDS*) (Table 2). The greatest proportion of diagnostic genetic variants (subgroups with ≥10 cases) were found in fetuses presenting with multiple (34%, n = 27/79) and cerebral (28.5%, n = 4/14) anomalies (Figure 3B).

The average number of HPO terms used was 5, and most of the cases were described using 3 or 4 terms (Figure 3C). As seen in the prospective cohort, there is a trend suggesting that a detailed fetal phenotype characterization enhances the likelihood of a diagnosis (Figure 3D).

VUS that may have contributed to the fetal phenotype were reported in 7% of prospective (12/183) and retrospective (8/120) cases (Figures 2A and 3A, Supplemental Table 8). The analysis of the gene panel for congenital anomalies (Figure 1B) allowed VUS identification in 4 of 12 prospective and 3 of 8 retrospective cases. In 5 of 12 prospective and 5 of 8 retrospective cases, VUS were detected after analysis of all fCES genes. In the prospective cohort, additional simplex gene panel analyses identified the 3 remaining VUS.

Fetal incidental findings were reported in 2 prospective cases (Supplemental Table 9), and 2 of the 3 variants reported were found after the analysis of all the fCES gene content. Parental incidental findings were reported in 5 cases (1 retrospective and 4 prospective cases), and they were mostly detected through whole clinical exome analysis (Supplemental Table 10).

The average TAT, defined as the number of days between the request for fCES and the final report validation by a clinical geneticist, was calculated for both cohorts. The average TAT was 4 and 2.5 months for the retrospective and prospective cohorts, respectively. For the latter, TAT improved to 29 working days (range 17-43 working days) during the course of our study.

Discussion

The use of fCES in fetuses presenting with anomalies detected using ultrasound allowed the identification of the underlying genetic cause in 13% prospective and 29% retrospective cases by using a multistep variant analysis. One factor that could explain the difference might be the case recruitment procedure because interrupted pregnancies more often displayed a severe multisystem phenotype and were selected by clinical geneticists. In addition, detailed phenotypic information was available, offering valuable support for variant interpretation. Consequently, the diagnostic rate of the retrospective cohort was similar to that of

Table 2 Overview of the diagnoses identified in the retrospective cohort

Case Number	Phenotypic Category	Prenatal Phenotype	Post-mortem Exams/ Postnatal Findings	Overall HPO Terms	Consanguinity	Recurrence of the Disorder	Gene	Transcript	Variant(s) and Protein Effect(s)	Variant Classification Phenotype's Contribution	Inheritance	fCES Interpretation Strategy that Identified the Pathogenic Variant(s)	Disorder	OMIM	Pregnancy Outcome
R3	Multiple	Bilateral talipes equinovarus, clenched hands	Low-set ears, cystic hygroma	HP:0001776; HP:0001188; HP:0000369; HP:0000476	N	Y	*NEB*	NM_ 001164508.1	c.13134_ 13135delAG p.(Arg4378fs*10) c.6805C>T p.(Gln2269*)	IV Full	Compound heterozygote	*In silico* gene panel - Congenital anomalies (design 1)	Nemaline myopathy 2, autosomal recessive	256030	TOP
R6	Multiple	Bilateral talipes equinovarus, polyhydramnios, hydrops fetalis	Slight low-set ears, pericardial effusion, pleural effusion, pulmonary hypoplasia, increased variability in muscle fiber diameter	HP:0001776; HP:0001561; HP:0001789; HP:0000369; HP:0001698; HP:0002202; HP:0002089; HP:0003557	N	N	*ACTA1*	NM_ 001100.4	c.49G>A p.(Gly17Ser)	V Full	De novo	*In silico* gene panel - Congenital anomalies (design 1)	ACTA1-related disorders	161800/ 255310	TOP at 24w
R14	Multiple	Clenched hands, hypospadias	Micropenis, slight low-set ears	HP:0001188; HP:0000047; HP:0000054; HP:0000369	N	N	*MYH3*	NM_ 002470.4	c.2014C>T p.(Arg672Cys)	V Full	De novo	*In silico* gene panel - Congenital anomalies (design 1)	Arthrogryposis, distal, type 2A	193700	TOP
R16	Multiple	Hydrops fetalis, polyhydramnios, increased nuchal translucency, hyperechogenic kidneys, short long bone	NA	HP:0001789; HP:0001561; HP:0010880; HP:0004719; HP:0003026	N	N	*HRAS*	NM_ 001130442.2	c.38G>A p.(Gly13Asp)	V Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	Costello syndrome	218040	TOP at 24w
R23	Multiple	Fetal akinesia sequence, bilateral talipes equinovarus, clenched hands, hydrops fetalis, polyhydramnios	NA	HP:0001989; HP:0001776; HP:0001188; HP:0001789; HP:0001561	N	N	*ASCC1*	NM_ 001198799.3	c.157dupG p.(Glu53fs*19)	V Full	Recessive homozygote	*In silico* gene panel - Congenital anomalies (design 2)	Spinal muscular atrophy with congenital bone fractures 2	616867	TOP
R24	Abnormality of the nervous system	Microcephaly, cerebellar hypoplasia	NA	HP:0000252; HP:0001321	N	N	*ASPM*	NM_ 018136.5	c.3811C>T p.(Arg1271*) c.2975C>G p.(Ser992*)	V, IV Full	Compound heterozygote	*In silico* gene panel - Congenital anomalies (design 2)	Microcephaly 5, primary, autosomal recessive	608716	TOP at 24w
R27	Multiple	Fetal akinesia sequence, abnormal cardiac ventricle morphology (asimmetry: L>R), distal arthrogryposis (clenched hands, hyperflexed legs, bilateral talipes equinovarus)	Hypertelorism, protruding tongue, long face, pulmonary hypoplasia, abnormal lung lobation (2 on the R rather than 3), skeletal muscle atrophy (muscular hypotrophy), single transverse palmar crease (right)	HP:0001989; HP:0001713; HP:0005684; HP:0000316; HP:0010808; HP:0000276; HP:0002089; HP:0002101; HP:0003202; HP:0000954	N	N	*GBE1*	NM_ 000158.4	c.2081T>A p.(Ile694Asn) c.783C>A p.(Ser261Arg)	IV Full	Compound heterozygote	*In silico* gene panel - Congenital anomalies (design 2)	Glycogen storage disease IV	232500	TOP

(continued)

Table 2 Continued

Case Number	Phenotypic Category	Prenatal Phenotype	Post-mortem Exams/ Postnatal Findings	Overall HPO Terms	Consanguinity	Recurrence of the Disorder	Gene	Transcript	Variant(s) and Protein Effect(s)	Variant Classification Phenotype's Contribution	Inheritance	fCES Interpretation Strategy that Identified the Pathogenic Variant(s)	Disorder	OMIM	Pregnancy Outcome
R34	Multiple	Encephalocele, echogenic intracardiac focus	Hypertelorism, broad neck	HP:0002084; HP:0010942; HP:0000316; HP:0000475	N	N	*B3GALNT2*	NM_ 152490.5	c.261-1G>A c.824_825dupTT p.(Ile276fs*26)	IV, V Full	Compound heterozygote	*In silico* gene panel - Congenital anomalies (design 2)	Muscular dystrophy-dystroglycanopathy (congenital with brain and eye anomalies, type A, 11	615181	TOP at 17w
R35	Multiple	Increased nuchal translucency (7mm)	Hypertelorism, protruding tongue, broad forehead, anteverted ears, short neck, pes valgus (left), pleural effusion, pulmonary hypoplasia	HP:0010880; HP:0000316; HP:0010808; HP:0000337; HP:0040080; HP:0000470; HP:0008081; HP:0002202; HP:0002089	N	N	*PTPN11*	NM_ 002834.5	c.206A>T p.(Glu69Val)	V Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	Noonan syndrome 1	163950	Fetal demise at 30w
R36	Multiple	Encephalocele, polydactyly	NA	HP:0002084; HP:0010442	N	N	*ISPD*	NM_ 001101426.4	c.627_628delAG p.(Arg209fs*3)	IV Full	Recessive homozygote	*In silico* gene panel - Congenital anomalies (design 2)	Muscular dystrophy-dystroglycanopathy (congenital with brain and eye anomalies), type A, 7/ Muscular dystrophy-dystroglycanopathy (limb-girdle), type C, 7	614643/ 616052	TOP at 17w
R39	Multiple	Oligohydramnios, ventriculomegaly, occipital encephalocele, lemon sign, cerebellar atrophy, polycystic kidney dysplasia,	Hypertelorism, microretrognathia, hypoplasia of the thymus, malformation of the hepatic ductal plate	HP:0001562; HP:0002119; HP:0002085; HP:0032269; HP:0001272; HP:0000113; HP:0000316; HP:0000308; HP:0000778; HP:0006563	N	N	*CEP290*	NM_ 025114.4	c.5012+5G>T	V Full	Recessive homozygote	*In silico* gene panel - Congenital anomalies (design 2)	Meckel syndrome 4	611134	TOP at 23w
R41	Abnormality of the fluid regulation	Hydrops fetalis, edema, pleural effusion	NA	HP:0001789; HP:0000969; HP:0002202	N	Y	*PIEZO1*	NM_ 001142864.4	c.1965C>G p.(Tyr655*) c.635-1G>A	IV Full	Compound heterozygote	Clinical Exome	Lymphatic malformation 6	616843	TOP at 24w
R46	Abnormality of the nervous system	Microcephaly, simplified gyral pattern, hypoplasia of the corpus callosum	NA	HP:0000252; HP:0009879; HP:0002079	N	N	*TUBGCP6*	NM_ 020461.4	c.1753C>T p.(Pro585Ser) c.1115A>G p.(Gln372Arg)	IV Full	Compound heterozygote	*In silico* gene panel - Congenital anomalies (design 2)	Microcephaly and chorioretinopathy, autosomal recessive, 1	251270	TOP at 32w
R51	Multiple	NA	Talipes equinovarus, retrognathia, abnormality of the cheeks, narrow palate, overlapping fingers, polydactyly (feet), hand clenching, renal duplication	HP:0001762; HP:0000278; HP:0004426; HP:0000189; HP:0010557; HP:0001829; HP:0001188; HP:0000075	Y	N	*TNNT3*	NM_ 006757.4	c.82+1G>A	IV Full	Recessive homozygote	*In silico* gene panel - Congenital anomalies (design 2)	Arthrogryposis, distal, type 2B2	618435	Not available

(continued)

Table 2 Continued

Case Number	Phenotypic Category	Prenatal Phenotype	Post-mortem Exams/ Postnatal Findings	Overall HPO Terms	Consanguinity	Recurrence of the Disorder	Gene	Transcript	Variant(s) and Protein Effect(s)	Variant Classification Phenotype's Contribution	Inheritance	fCES Interpretation Strategy that Identified the Pathogenic Variant(s)	Disorder	OMIM	Pregnancy Outcome
R53	Multiple	Bilateral cleft lip and palate, coarse facial features, hypertelorism, preauricular pit (bilateral), thoracic hypoplasia, limb undergrowth, clinodactyly, micropenis, broad thumbs, broad hallux	Ulnar bowing, delayed ossification of the hand bones, abnormal foot bone ossification, short first metatarsals, hypoplasic terminal phalanges	HP:0002744; HP:0000280; HP:0000316; HP:0004467; HP:0005257; HP:0009826; HP:0030084; HP:0000054; HP:0011304; HP:0010055; HP:0009882; HP:0003031; HP:0004052; HP:0010675; HP:0010105	N	N	*DVL1*	NM_ 004421.3	c.1562del p.(Pro521Hisfs*128)	V Full	De novo	Clinical Exome	Robinow syndrome, autosomal dominant 2	616331	TOP at 16w
R54	Multiple	NA	Macrocephaly, hemifacial hypoplasia, proptosis, short nose, long philtrum, cleft palate, low-set ears, short neck, thoracic hypoplasia, protuberant abdomen, umbilical hernia, decreased skull ossification, absent or minimally ossified vertebral bodies, phocomelia, deficient ossification of hand bones	HP:0000256; HP:0011332; HP:0000520; HP:0003196; HP:0000343; HP:0000175; HP:0000369; HP:0000470; HP:0005257; HP:0001538; HP:0001537; HP:0004331; HP:0004599; HP:0009829; HP:0004274	N	N	*FLNB*	NM_ 001164317.2	c.512T>C p.(Leu171Pro)	V Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	FLNB-Related Disorders	108720/ 108721/ 112310/ 150250	TOP at 16w
R62	Multiple	Occipital encephalocele, occipital meningocele, fetal pyelectasis (unilateral)	NA	HP:0002085; HP:0002436; HP:0010945	N	N	*IDS*	NM_ 000202.8	c.818G>A p.(Arg273Gln)	IV Full	X-linked	*In silico* gene panel - Congenital anomalies (design 2)	Mucopolysac -charidosis II	309900	TOP at 18w
R66	Multiple	Severe intrauterine growth retardation (-6w at 30w), proptosis, volvulus, single umbilical artery, cerebellar hemisphere hypoplasia	Clinodactyly, thin ribs, abnormal bone ossification (delayed)	HP:0008846; HP:0000520; HP:0002580; HP:0001195; HP:0100307; HP:0030084; HP:0000883; HP:0011849	N	N	*DDX11*	NM_ 001257144.2	c.918del p.(Arg307Glyfs*28) c.1403dup p.(Ser469Valfs*32)	IV Full	Compound heterozygote	Clinical Exome	Warsaw breakage syndrome	613398	TOP

(continued)

Table 2 Continued

Case Number	Phenotypic Category	Prenatal Phenotype	Post-mortem Exams/ Postnatal Findings	Overall HPO Terms	Consanguinity	Recurrence of the Disorder	Gene	Transcript	Variant(s) and Protein Effect(s)	Variant Classification Phenotype's Contribution	Inheritance	fCES Interpretation Strategy that Identified the Pathogenic Variant(s)	Disorder	OMIM	Pregnancy Outcome
R69	Multiple	NA	Low-set ears, hypertelorism, thin vermilion border, single transverse palmar crease, hepatomegaly, dilatation of the renal pelvis, abnormal cardiac ventricle morphology (pronunced interventricular groove, dilatation of the ventricules)	HP:0000369; HP:0000316; HP:0000233; HP:0000954; HP:0002240; HP:0010946; HP:0001713	N	N	*RIT1*	NM_ 006912.6	c.270G>A p.(Met90Ile)	V Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	Noonan syndrome 8	615355	NA
R76	Multiple	NA	Partial agenesis of the corpus callosum, ventriculomegaly, cavum septum pellucidum, adrenal gland agenesis, aplasia/ hypoplasia of the optic tract, optic nerve aplasia	HP:0001338; HP:0002119; HP:0002389; HP:0011743; HP:0011000; HP:0012521	Y	N	*HESX1*	NM_ 003865.3	c.509C>T p.(Ser170Leu)	IV Full	Paternal	*In silico* gene panel - Congenital anomalies (design 2)	Septooptic dysplasia	182230	TOP
R77	Multiple	NA	Cerebellar hypoplasia, micrognathia, hypertelorism, bell shaped chest, clinodactyly, abnormal lung lobation	HP:0001321; HP:0000347; HP:0000316; HP:0001591; HP:0030084; HP:0002101	N	N	*BRAF*	NM_ 004333.6	c.1574T>G p.(Leu525Arg)	IV Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	BRAF-related disorders	115150/ 613707/ 613706	TOP
R84	Abnormality of the cardiovascular system	Ventricular septal defect, Pulmonary artery stenosis	NA	HP:0001629; HP:0004415	N	N	*JAG1*	NM_ 000214.3	c.1720+2T>C	V Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	Alagille syndrome 1	118450	TOP at 24w

(continued)

Table 2 Continued

Case Number	Phenotypic Category	Prenatal Phenotype	Post-mortem Exams/ Postnatal Findings	Overall HPO Terms	Consanguinity	Recurrence of the Disorder	Gene	Transcript	Variant(s) and Protein Effect(s)	Variant Classification Phenotype's Contribution	Inheritance	fCES Interpretation Strategy that Identified the Pathogenic Variant(s)	Disorder	OMIM	Pregnancy Outcome
R90	Multiple	Polyhydramnios, decreased fetal movement, short long bone, microretrognathia, hand clenching, cerebellar hypoplasia, pes cavus, overlapping toe, dilatation of the bladder, microcephaly, cerebellar vermis hypoplasia, polymicrogyria, small posterior fossa, widened subarachnoid space, congenital diaphragmatic hernia, syringomyelia	Brachycephaly, thickened ears, abnormally folded helix, pulmonary hypoplasia, dextrocardia, longitudinal vaginal septum	HP:0001561; HP:0001558; HP:0003026; HP:0000308; HP:0001188; HP:0001321; HP:0001761; HP:0001845; HP:0010955; HP:0000252; HP:0001320; HP:0002126; HP:0040010; HP:0012704; HP:0000776; HP:0003396; HP:0000248; HP:0009894; HP:0008544; HP:0002089; HP:0001651; HP:0008740	Y	N	*ALG3*	NM_ 005787.6	c.667_ 669delCTC p.(Leu223del)	IV Full	Recessive homozygote	Clinical Exome	Congenital disorder of glycosylation, type Id	601110	TOP at 34w
R97	Multiple	Fetal akinesia sequence, fetal ascites, anasarca	NA	HP:0001989; HP:0001791; HP:0012050	Y	Y	*ASCC1*	NM_ 001198799.3	c.157dupG p.(Glu53Glyfs*19)	V Full	Recessive homozygote	*In silico* gene panel - Congenital anomalies (design 2)	Spinal muscular atrophy with congenital bone fractures 2	616867	Fetal demise in utero at32w
R100	Multiple	Talipes, polyhydramnios, short philtrum, R kidney not visualised	Atrial septal defect, cleft palate, horseshoe kidney (L), pelvic kidney (R), gray matter heterotopia	HP:0001883; HP:0001561; HP:0000322; HP:0001631; HP:0000175; HP:0000085; HP:0000125; HP:0002282	N	N	*KMT2D*	NM_ 003482.4	c.10180C>T p.(Gln3394*)	IV Full	De novo	*In silico* gene panel - Congenital anomalies (design 2)	Kabuki syndrome 1	147920	Fetal demise in utero 37w
R108	Abnormality of the skeletal system	Dolichocephaly, abnormal parietal bone morphology, short long bones, short ribs, bell-shaped thorax, skeletal dysplasia, bowing of the long bones	NA	HP:0000268; HP:0002696; HP:0003026; HP:0000773; HP:0001591; HP:0002652; HP:0006487	N	N	*COL1A1*	NM_ 000088.4	c.1777G>A p.(Gly593Ser)	V Full	De novo (maternal mosaicism)	*In silico* gene panel - Congenital anomalies (design 3)	Osteogenesis imperfecta, type III/ type IV	259420/ 166220	Fetal demise in utero at 21w

(continued)

Table 2 Continued

Case Number	Phenotypic Category	Prenatal Phenotype	Post-mortem Exams/ Postnatal Findings	Overall HPO Terms	Consanguinity	Recurrence of the Disorder	Gene	Transcript	Variant(s) and Protein Effect(s)	Variant Classification Phenotype's Contribution	Inheritance	fCES Interpretation Strategy that Identified the Pathogenic Variant(s)	Disorder	OMIM	Pregnancy Outcome
R109	Multiple	N	Partial absence of cerebellar vermis, abnormality of neuronal migration, pachygyria, coarctation of aorta, hepatic steatosis, ectopic parathyroid, ectopic thymus tissue, hydrocele testis	HP:0002951; HP:0002269; HP:0001302; HP:0001680; HP:0001397; HP:0011769; HP:0010517; HP:0000034	N	N	*ETFA*	NM_ 000126.4	c.251dupA p.(Tyr84*) c.494T>C p.(Val165Ala)	IV, V Full	Compound heterozygote	*In silico* gene panel - Congenital anomalies (design 3)	Glutaric acidemia IIA	231680	Newborn dead after 2d
R111	Abnormality of the urinary system	Bilateral renal agenesis	NA	HP:0010958	N	N	*GREB1L*	NM_ 001142966.2	c.5074G>T p.(Asp1692Tyr)	IV Full	De novo (paternal mosaicism)	*In silico* gene panel - Congenital anomalies (design 3)	Renal hypodysplasia/ aplasia 3	617805	Not available
R116	Abnormality of the nervous system	Ventriculomegaly, cerebellar hypoplasia, cerebral cortical atrophy	NA	HP:0002119; HP:0001321; HP:0002120	N	N	*PDHA1*	NM_ 001173454.1	c.1035_ 1050dupTCA GGAAGTA AGAAGT p.(Lys351 SerfsTer8)	IV Full	De novo, XLD	*In silico* gene panel - Congenital anomalies (design 3)	Pyruvate dehydrogenase E1-alpha deficiency	312170	Newborn, neonatal death
R118	Multiple	Intrauterine growth retardation, choroid plexus cyst, single umbilical artery, suspicion of congenital heart defects	Secundum atrial septal defect, ventricular septal defect, hepatic necrosis, enlarged kidneys, adrenal hypoplasia, gray matter heterotopia, jaundice, edema	HP:0001511; HP:0002190; HP:0001195; HP:0001684; HP:0001629; HP:0002605; HP:0000105; HP:0000835; HP:0002282; HP:0000952; HP:0000969	N	Y	*ANKRD11*	NM_ 001256183.2	c.2408_ 2412delAAAAA p.(Lys803Argfs*5)	V Full	Maternal	*In silico* gene panel - Congenital anomalies (design 3)	KBG syndrome	148050	Fetal demise at 25w

Details are provided only when the patients agreed with personal data publication (30/35).

the postnatal series.[10,15] Conversely, the prospective cohort included unselected cases, notably some with fetal anomalies weakly associated with monogenic findings (ie, increased NT or neural tube defects). Furthermore, variant interpretation was hampered by the limited knowledge of in utero phenotypes. Results from the unselected cohort are comparable with the ones described in other ES prospective studies in which diagnostic variants were detected in 8.5% to 10.3% of cases.[16,17] Our slightly higher diagnostic yield may be explained by a greater proportion of consanguineous couples (12/171, 7%).

The highest diagnostic rates were achieved for fetuses presenting with multisystem and cerebral anomalies in the retrospective cohort. In the multisystem subgroup, a conclusive molecular diagnosis was reached for most cases with a fetal akinesia sequence (9/11, 82%) or a Meckel-Gruber–related phenotype (5/6, 83%), indicating that fCES is highly recommended for these ultrasound findings. In the prospective cohort, diagnostic rates were highest in fetuses with multiple systems, skeletal, urinary, and cerebral anomalies. These results are in agreement with previous studies,[16,17] although higher rates for fetuses with cardiac[16] and lymphatic[17] anomalies were also described. Because the proportion of cases belonging to these phenotypic categories was small in our cohort, further studies are required to draw final conclusions. Similarly, the absence of diagnostic variants in rarely explored phenotypic categories (ie, isolated intrauterine growth restriction and anomalies of the amniotic fluid) needs to be further investigated in larger series. In line with other studies, no NGS diagnostic variants were found in fetuses with either neural tube defects or isolated increased NT.[16,23] More data, more exploratory variant selection, and more complex heritability investigations (eg, oligogenic, noncoding, polygenic) will be necessary to assess the diagnostic yield of NGS in these anomalies.

Interestingly, our study shows that the proportion of AD and AR diagnoses was the same, which is in contrast with reports related to postnatal series in which de novo variants accounted for most of the cases.[24] Remarkably, our study shows the importance of investigating compound heterozygous variants (especially in nonconsanguineous cases) because they represent 54.5% and 26% of positive AR diagnoses in our 2 cohorts. This result may be explained by the fact that AR diseases are more often responsible for the interruption of pregnancy or perinatal lethality. Other fetal series observed similar proportions,[12,16,17,25] suggesting that AR disorders play an important role in severe fetal phenotypes.

On the basis of the experience accumulated in this and other studies,[11,26,27] it seems beneficial to perform a trio/duo-based analysis involving all the genes of the clinical exome. Such an analysis strategy carries a diagnostic gain of 16.7% (prospective cases) and 14% (retrospective cohort) when compared with a trio/duo analysis focused on a comprehensive congenital anomalies gene panel designed using literature (Figures 2A and 3A). In complement to this strategy, we found that, when appropriate, a simplex analysis of in silico panels comprising genes specific for the fetal phenotype with adapted variant selection criteria also increased the diagnostic yield (8.3% in the prospective cohort) (Figure 2A).

In addition to expanding our understanding of fetal presentations for known genetic conditions (as in case P6, described in[28]) and identifying new types of pathogenic variants in association with them (as in case P32, reported in[29]), phenotypes resembling conditions not previously reported prenatally may be identified, leading to challenges in data interpretation and assessment of the variants' pathogenicity (represented here by case P131). Additional challenges arise with recessive pathologies for which only 1 pathogenic variant is detected, such as in prospective case P136. Finally, further analysis of specific gene panels using simplex analysis allowed the detection of inherited pathogenic variants responsible for AD disorders with incomplete penetrance or the discovery of 1 diagnostic variant in genes responsible for AR disorders consistent with the fetal phenotype, which would otherwise have been missed if only trio-based analysis was performed (P136).

In most of the reported VUS, the variants were heterozygous in an asymptomatic parent, and further clinical examinations and segregation analysis were recommended to assess pathogenicity. Incomplete penetrance and variable expression among family members (eg, variants within the *COL4A2* gene and risk of porencephaly) complicate evaluation, and these variants often remain of unknown significance until similar cases with the same variant are identified in independent families. VUS may also contribute to some phenotypic features that a diagnostic variant could not explain (as found in case P158) (Table 1). Although smaller, focused gene panels limit the incidental identification of VUS[16] and true diagnoses may also be missed,[25] making the selection of the most appropriate analysis method challenging.

As fCES/ES become more widely implemented, it is crucial to share phenotypic and molecular data in international databases to improve variant interpretation and recognition of novel fetal genotype–phenotype correlations. The absence of a statistically significant correlation between the number of HPO terms and the percentage of conclusive diagnoses may be explained by multiple factors, such as the reduced number of cases presenting with a high number of HPO terms, the presence of cases with multiple anomalies with a low-level association with monogenic diseases, and/or the fact that some genetic anomalies or variants in genes not studied in our design are not detected. Further analyses on larger series will be needed to draw final conclusions on the importance of the HPO terminology usage. Nonetheless, we highly recommend that clinicians requesting fetal NGS provide detailed clinical information and family history to genetics laboratories.[18] Moreover, in the context of interrupted pregnancies, the complementary information identified by postmortem examination strongly contributes to a higher diagnostic yield. Of note, we encountered difficulties in describing some fetal phenotypes because HPO terms were missing for a portion of prenatal anomalies detected using ultrasound (eg, antenatal hyperechoic colon) or subtypes of anomalies. We thus believe that efforts should be

made to expand the existing fetal HPO terminology. Similarly, we found that variant classification could be particularly challenging in a prenatal setting because classical criteria[21] were not always applicable given the inevitably limited phenotypic characterization in utero.

Our study has limitations regarding the diagnosis of some phenotypic categories that will require further clinical evaluations. Another limitation of our study is that exonic CNVs were not routinely investigated and were only analyzed when 1 diagnostic variant was already found in a gene causing an AR phenotype. In contrast to other studies, ES was not performed. Although our approach limits the discovery of new disease-causing genes and the data reanalysis power, it is suitable for a prenatal clinical setting, allowing very high-quality coverage data in well-known genes causing Mendelian disorders with prenatal onset. In addition, clinical exome sequencing (CES)/ES-based methods are limited to the detection of coding variants and misses some genetic defects (eg, deep intronic variants, nucleotide repeat expansions). This limitation can be overcome by genome sequencing (GS). However, because of the greater cost of GS over CES/ES-based methods, it is likely that combined CMA and CES/ES-based analysis will become more widely implemented before the advent of GS in the prenatal clinical setting. The refined prenatal phenotype–genotype correlations expected to be obtained from CES/ES-based methods will likely facilitate the subsequent implementation of fetal GS.

In conclusion, this study showed that the overall diagnostic yields of CES using a multistep variant analysis were 13% and 29% in prospective and retrospective cases, respectively. In particular, trio/duo-based analysis involving all the genes of the clinical exome and simplex analysis (ie, in silico panels on fCES data comprising genes specific for the fetal phenotype) were complementary to achieve the highest diagnostic rate possible, and compound heterozygous genotypes were not rare. fCES-based diagnosis was efficient in fetuses presenting with cerebral, skeletal, urinary, or multiple anomalies. The comparison between a retrospective and a prospective cohort highlighted the importance of providing detailed phenotypic information to genetic laboratories performing fetal NGS for better interpretation and reporting of genetic variants. Finally, selected cases illustrate some interpretation challenges faced during the analysis of genome-wide data and widen the knowledge of the prenatal presentation of genetic syndromes.

Data Availability

Clinical and genetic data of 198 of 303 patients are described in detail in Supplemental Tables 6 and 7). For the remaining 105 of 303 cases for whom no formal informed consent for the sharing of personal data was provided, only limited information was included in the general statistics (ie, only their phenotypic categories and the presence/absence of diagnostic variant(s)/variants of uncertain significance were shared). Further details about our methods are available upon request.

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Ethics Declaration

This study was approved by the ethical committee of the Hôpital Erasme, Brussels, Belgium, under the reference P2016/236. Informed consent was obtained from all participants. This study adheres to the principles set out in the Declaration of Helsinki.

Conflict of Interest

The authors declare no conflict of interest.

Additional Information

The online version of this article (https://doi.org/10.1016/j.gim.2021.09.016) contains supplementary material, which is available to authorized users.

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