Genomic study of severe fetal anomalies and discovery of *GREB1L* mutations in renal agenesis

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Purpose: Fetal anomalies represent a poorly studied group of developmental disorders. Our objective was to assess the impact of whole-exome sequencing (WES) on the investigation of these anomalies.

Methods: We performed WES in 101 fetuses or stillborns who presented prenatally with severe anomalies, including renal agenesis or dysgenesis, VACTERL association (vertebral defects, anal atresia, cardiac defects, tracheoesophageal fistula, renal anomalies, and limb abnormalities), brain anomalies, suspected ciliopathies, multiple major malformations, and akinesia.

Results: A molecular diagnosis was obtained in 19 cases (19%). In 13 of these cases, the diagnosis was not initially suspected by the clinicians because the phenotype was nonspecific or atypical, corresponding in some cases to the severe end of the spectrum of a known disease (e.g., *MXN1*-, *RYR1*-, or *TUBB*-related disorders). In

addition, we identified likely pathogenic variants in genes (*DSTYK*, *ACTB*, and *HIVEP2*) previously associated with phenotypes that were substantially different from those found in our cases. Finally, we identified variants in novel candidate genes that were associated with perinatal lethality, including de novo mutations in *GREB1L* in two cases with bilateral renal agenesis, which represents a significant enrichment of such mutations in our cohort.

Conclusion: Our study opens a window on the distinctive genetic landscape associated with fetal anomalies and highlights the power—but also the challenges—of WES in prenatal diagnosis.

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INTRODUCTION

Birth defects are the main cause of mortality in infancy and represent an important source of morbidity at all ages, with a major proportion of cases being identified during pregnancy. The etiology of birth defects is complex. Copy-number variants (CNV) currently represent the most commonly recognizable cause of birth defects in fetuses and children. Indeed, the prevalence of submicroscopic pathogenic or likely pathogenic CNVs is 5.5–10.5% in fetuses with structural defects.^{1,2} The fact that CNVs represent a common cause of birth defects suggests that smaller genetic lesions, such as point mutations, may also contribute substantially to their pathogenesis.

Most pathogenic CNVs associated with birth defects occur de novo. A reciprocal relationship between the fitness effect of a mutation and the proportion of de novo mutations (DNMs)

is observed in a dominantly inherited disorder. Accordingly, DNMs may explain a larger proportion of severe cases of birth defects compared with milder ones. For instance, the contribution of de novo CNVs appears greater in cases with multiple malformations than those with isolated malformations.^{1,2} Similarly, de novo truncating mutations are significantly enriched in syndromic but not isolated forms of congenital heart defects.³ One potential corollary of these observations would be that DNMs contribute more to birth defects in fetuses than in newborns or older children because the former group includes a subset of cases that are so severely affected they cannot survive beyond the perinatal period.

Classical studies have established that recessive mutations also explain a large fraction of lethal developmental defects in animal models.⁴ Thus, as for DNMs, severe forms of fetal malformations in humans may involve inherited recessive

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mutations in genes that would not be identified by studying newborns or older children with malformations.

Based on these considerations, we hypothesized that inherited and de novo point mutations, as is the case for CNVs, explain a substantial subset of fetal cases with severe anomalies. We further hypothesized that some of these lesions disrupt genes that are specifically associated with fetal phenotypes. To explore these hypotheses, we performed whole-exome sequencing (WES) in a series of fetuses and stillborns investigated prenatally because of severe anomalies. We sought to measure the diagnostic utility of WES for various categories of anomalies while attempting to identify candidate genes associated with these disorders.

MATERIALS AND METHODS

This study was approved by the CHU Sainte-Justine's research ethics board. After obtaining informed consent from the parents, we prospectively recruited, over a 3-year period, 89 fetuses (from terminated pregnancies) and stillborns with: (i) at least two major malformations, (ii) severe ventriculomegaly (atria > 15 mm bilaterally) and/or structural brain malformations, or (iii) an anomaly associated with a high risk of perinatal lethality. We recruited from three other centers (Mount Sinai Hospital, Toronto, Canada; Hôpitaux Universitaires de Strasbourg, France, and the Children's Hospital of East Ontario, Ottawa, Canada) 12 additional cases that met these criteria for a total of 101 cases. These cases were unexplained and none of them was exposed to a known teratogen. Neither karyotyping ($n = 58$ cases) nor chromosomal microarray analysis ($n = 84$ cases) showed any candidate or pathogenic CNVs (**Supplementary Table S1** online). Autopsies were performed in 85 cases (**Supplementary Table S1**).

Genomic DNA was extracted from the umbilical cord, other fetal tissues, amniocytes or chorionic villi (**Supplementary Table S1**). For 83 cases WES was performed in trios, whereas for 18 cases it was performed in the affected cases but not in their parents, as indicated in **Supplementary Table S1**. The exome was captured using the Agilent SureSelect (V4 or V5) exome capture kit followed by 100 bp paired-end sequencing in a configuration of four exomes per lane on the Illumina HiSeq (2000 or 2500) system.

Exome sequence data processing, alignment (using a Burrows–Wheeler algorithm), and variant calling were performed according to the Broad Institute's GATK (v4) best practices (<http://www.broadinstitute.org/gatk/guide/topic?name=best-practices>), and variant annotation was performed using Annovar. The median coverage of the target bases was $110\times$ with 95% of the target bases being covered $\geq 10\times$. We focused on variants affecting the exome and intronic bases up to positions -3 and $+6$ from the exon boundaries. Only variants whose positions were covered at $\geq 10\times$ and supported by at least four variant reads constituting $\geq 20\%$ of the total reads for each called position were retained. This typically yielded an average of $\sim 22,000$ variants. This variant list was subsequently reduced to an average of ~ 400 rare

variants by filtering out those that were present in $\geq 0.5\%$ of in-house exome data sets ($n = 600$) from unrelated projects, as well as variants present in the 1000 Genomes Project or the Exome Variant Server (<http://evs.gs.washington.edu/EVS/>) with minor allele frequencies $\geq 0.5\%$. Putative DNMs (typically < 10 per exome) were then extracted from the rare variant list by further excluding those that were present in the exomes of the parents. The sequencing reads carrying putative DNMs were inspected visually in each trio, using the Integrative Genomics Viewer, to exclude obvious false positives. Interpretation of sequence variants was done according to the standards of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.⁵ Clinically significant results were returned to the parents.

RESULTS

We studied 101 fetal cases with severe anomalies who presented mainly during the second trimester of pregnancy. We classified these cases into six categories based on their phenotypes: (i) isolated bilateral renal agenesis or dysgenesis ($n = 11$), (ii) VACTERL (vertebral defects, anal atresia, cardiac defects, tracheoesophageal fistula, renal anomalies, and limb abnormalities) association ($n = 9$), (iii) cerebral anomalies ($n = 36$), (iv) suspected ciliopathies ($n = 5$), (v) miscellaneous patterns of multiple malformations ($n = 32$), and (vi) fetal akinesia ($n = 8$) (**Supplementary Table S1**).

In 16 families with a suspected autosomal recessive disorder because of parental consanguinity, the presence of multiple affected siblings and/or the nature of the phenotype (e.g., anomalies suggestive of ciliopathies, which are mainly autosomal recessive), WES was performed in the affected individuals ($n = 18$) but not in their parents. The remaining cases ($n = 83$), all sporadic, were investigated by WES in trios. We first aimed to systematically identify DNMs in these trios to determine whether our series was enriched for such mutations. We observed a high number of putative DNMs (between 12 and 40 DNMs) in the three probands for which genomic DNA was extracted from cultured amniocytes or chorionic villi (**Supplementary Table S1** online). Sanger validation of some of these variants using DNA extracted from another tissue suggested that the large majority of these putative DNMs are false positives, possibly related to culture artifacts. In DNA samples extracted from tissues, we identified and Sanger-validated a total of 116 DNMs, including 5 nonsense, 6 frameshift-causing indel, 77 missense and 2 canonical splice site variants (**Supplementary Table S2**). We documented the presence of at least one likely gene disrupting DNM (nonsense, frameshift or canonical splicing variant) in 11 of these cases (11%). The expected percentage of DNMs in a coding and splicing sequence that are synonymous is $\sim 29\%$.^{6,7} Among the SNV and canonical splice site DNMs identified herein ($n = 103$), 19 were synonymous (18%) and 84 were functional (77 missenses, 5 stopgains and 2 splicing variants). This distribution indicates a significant enrichment in functional DNMs ($P = 0.017$,

exact binomial test), thus suggesting that some of these are likely pathogenic.

For each category of anomalies, we next determined whether the WES data sets contained rare de novo or inherited variants in known disease genes associated or possibly associated with the reported phenotypes. We also sought to identify candidate genes based on their involvement in unrelated families or their known function in model organisms.

Renal a/dysgenesis

We studied eight sporadic cases with isolated bilateral renal agenesis (Supplementary Table S1 online). All these cases were males. Such a predominance of males has previously been documented in renal agenesis.⁸ In addition, we studied three cases (two females and one male) with isolated bilateral multicystic kidneys without any sign of lower urinary tract obstruction. In these cases, WES did not reveal any DNMs or rare potentially recessive variants in genes previously associated with congenital anomalies of the kidney and urinary tract (CAKUT).

Candidate gene

We identified DNMs in the *GREB1L* gene in two unrelated cases with bilateral renal agenesis (051-082-IEO and CONGE-070) (Supplementary Tables S1 and S3). Renal ultrasound was normal in the parents of these cases. One DNM is a predicted-damaging missense (NM_001142966; c.C2903T;p.A968V), whereas the other is a stopgain (NM_001142966; c.C293G;p.S98X) that truncates most of the protein (Supplementary Tables S1 and S3). Moreover, only one unvalidated loss-of-function variant in *GREB1L* is reported in the Exome Aggregation Consortium database, suggesting that this gene does not tolerate this type of variant. Using an established framework of gene-specific mutation rates,⁹ we observed an enrichment in functional DNMs in *GREB1L* in our series ($P = 0.00035$, two-tailed exact Poisson test), suggesting that damaging DNMs in this gene are pathogenic. *GREB1L* shares some identity with *GREB1*, a chromatin-bound estrogen receptor coactivator.^{10,11} The zebrafish homolog of *GREB1L* is induced by the Hedgehog pathway¹² and a truncating mutation in this gene causes abnormal swim bladder inflation (<http://zfin.org/ZDB-GENE-030131-4022>), which can be a sign of abnormal Hedgehog signaling.¹³ Interestingly, germ-line mutations in *Shh* cause renal aplasia in mice.¹⁴

VACTERL association

We studied nine cases with at least two features of the VACTERL association (OMIM 192350), including at least one core feature (anorectal or tracheoesophageal defects) (Supplementary Table S1). WES revealed a de novo truncating mutation in *CHD7* (OMIM 608892) (NM_017780; c.C5428T;p.R1810X) in a fetus (CONGE-79) with esophageal atresia and tetralogy of Fallot (Table 1 and Supplementary Tables S1 and S3). DNMs in *CHD7* have been reported to cause these defects in the context of the CHARGE syndrome (coloboma of the eye, heart defects, atresia of the choanae, retardation of growth and/or development, genital and/or urinary abnormalities, and ear abnormalities and deafness), which overlaps with the VACTERL association.¹⁵

WES also showed the presence of a truncating DNM (NM_015375; c.C2125T;p.R709X) in *DSTYK* (OMIM 612666) in a fetus (051-141-VWD) with left heart hypoplasia and esophageal atresia (Table 1 and Supplementary Tables S1 and S3). This mutation is located in an upstream exon, which suggests that it is likely inducing nonsense-mediated decay of the transcript (Supplementary Tables S1 and S3). Dominant variants in *DSTYK*, including truncating variants, have been involved in CAKUT.¹⁶ Our case did not show any kidney anomalies, raising the possibility that the mutation is not penetrant, as previously observed in some families with CAKUT.¹⁶ Alternatively, mutations in this gene might cause a broader spectrum of congenital anomalies than initially suspected, explaining the phenotype of our fetal case. *DSTYK* functions as a modulator of fibroblast growth factor signaling.¹⁶ Knockdown of *DstyK* in the zebrafish causes multiple defects of morphogenesis that are consistent with a disruption of fibroblast growth factor signaling.¹⁶ Interestingly, fibroblast growth factor signaling has been involved in esophageal development.¹⁷ Moreover, *DstyK* is expressed in the developing heart and a decrease in its expression causes heart failure in zebrafish larvae.¹⁶

In the other cases, WES did not reveal any DNMs or rare biallelic variants in genes previously associated with monogenic forms of the VACTERL association.

Cerebral anomalies

We studied a total of 36 fetal cases with major structural brain anomalies, eight of which also had at least one major peripheral anomaly (Supplementary Table S1). In total, we identified eight mutations in seven genes (*TUBA1A* (OMIM 602529), *TUBB3* (OMIM 602661), *TUBB* (OMIM 191130), *PEX1*

Table 1 Genetic findings in fetuses with VACTERL association

ID	Phenotype	Gene	Variant	Inheritance	Interpretation
CONGE-79	(20 weeks) persistence of the left superior vena cava, left heart hypoplasia, right lung with two lobes, type A esophageal atresia	<i>CHD7</i>	c.5428C>T: p.R1810X	Heterozygous, de novo	Pathogenic variant
051-141-VWD	(20 weeks) tetralogy of Fallot, esophageal atresia	<i>DSTYK</i>	c.2125C>T: p.R709X	Heterozygous, de novo	Pathogenic variant

VACTERL, vertebral defects, anal atresia, cardiac defects, tracheoesophageal fistula, renal anomalies, and limb abnormalities.

Table 2 Genetic findings in fetal cases with cerebral anomalies

ID	Phenotype	Gene	Variant	Inheritance	Interpretation
51-061-NWI	(34 weeks) polymicrogyria and heterotopias in both cerebral and cerebellar hemispheres	PEX1	c.3205C>T (p.Q1069X)	Compound	Likely pathogenic variant
		PEX1	c.2097_2098insT (p.I700fs)	heterozygous	Likely pathogenic variant
051-080-RLH	(26 weeks and 6 days) ventriculomegaly, simplified cortical gyration, pontocerebellar hypoplasia	TUBB3	c.862G>A (p.E288K)	Compound	Likely pathogenic variant
			heterozygous	heterozygous	
51-155-PES	(23 weeks) severe microlissencephaly with neuronal migration defects	TUBA1A	c.55G>A (p.A19T)	Heterozygous, de novo	Likely pathogenic variant
CONGÉ-043	(27 weeks) microlissencephaly, agenesis of the corpus callosum, pontocerebellar hypoplasia, circumferential skin folds	TUBB	c.920C>T (p.P307L)	Heterozygous, de novo	Likely pathogenic variant
051-140-ZGS	(27 weeks) microcephaly with simplified gyri	PDHA1	c.1134dupG (p.V378fs)	Heterozygous, de novo	Pathogenic variant
CONGÉ-052	(20 weeks) ventriculomegaly, agenesis of the corpus callosum, cerebellar hypoplasia	TUBB3	c.533C>T (p.T178M)	Heterozygous, de novo	Likely pathogenic variant
051 021 LIC	(20 weeks) triventriculomegaly, narrow aqueduct	ACTB	c.617G>A (p.R206Q)	Heterozygous, de novo	Likely pathogenic variant
051 039 KUY	(20 weeks) triventriculomegaly, occipital encephalocele	HIVEP2	c.2968_2971del (p.K990fs)	Heterozygous, de novo	Pathogenic variant
		DNAH7	c.8624G>A (p.R2875Q)	Compound	Candidate variant
		DNAH7	c.4787_4788insA (p.Y1596X)	heterozygous	candidate variant
				Compound heterozygous	

(OMIM 602136), *PDHA1* (OMIM 300502), *ACTB* (OMIM 102630), and *HIVEP2* (OMIM 616977)) that are pathogenic or likely pathogenic (Table 2). Interestingly, four of these mutations affect genes that code for tubulins (*TUBA1A*, *TUBB3*, and *TUBB*). Since the phenotypes observed in these eight fetuses were somewhat nonspecific, these mutations were not readily suspected by the clinicians. However, their phenotypes were consistent with what was reported for these genes with the exception of the cases with mutations in *TUBB*, *ACTB*, and *HIVEP2*, which are described hereafter.

Fetus CONGE-043 bears a missense DNM in *TUBB* (NM_001293212; c.C920T:p.P307L), which has not been reported in the Exome Aggregation Consortium database and which is predicted to be damaging by the tools used in this study (Table 2 and Supplementary Tables S1 and S3). DNMs in *TUBB* have been previously described in six children who displayed microcephaly and mild anomalies of the corpus callosum and basal ganglia.^{18,19} Some of these individuals also displayed circumferential skin creases.¹⁹ Fetus CONGE-043 showed a similar but much more severe phenotype with the presence of microlissencephaly, agenesis of the corpus callosum, dysmorphic basal ganglia, cerebellar hypoplasia, and circumferential skin creases (Table 2 and Supplementary Table S1). The cortex of our case was characterized by the presence of glomerular structures and a voluminous germinal area, which has been associated with tubulinopathies, further supporting the pathogenicity of the variant in *TUBB*.

Fetus 051-021-LIC bears a de novo missense in *ACTB* (NM_001101; c.G617A:p.R206Q), which has not been reported in the Exome Aggregation Consortium database and which is predicted to be damaging (Table 2 and Supplementary Tables S1 and S3). DNMs in *ACTB* cause Baraitser–Winter syndrome, which includes intellectual disability and neuronal migration defects of the cortex.²⁰ The variant identified in fetus 051-021-LIC has been reported in ClinVar (SCV000322073) as pathogenic, but without any description of the associated phenotype. This variant clusters with several other variants that have been associated with Baraitser–Winter syndrome.²⁰ Fetus 051-021-LIC presented with severe ventriculomegaly of the lateral and third ventricles with a narrow aqueduct, but there was no sign of neuronal migration defects on pathological examination at 35 weeks of gestation (Table 2 and Supplementary Table S1 online). Thus, this *ACTB* variant appears pathogenic but the associated phenotype is atypical.

Fetus 051-039-KUY presented at 20 weeks of gestation with severe triventriculomegaly and occipital encephalocele. WES showed that this fetus carried a truncating DNM in *HIVEP2* (OMIM 143054) (NM_006734; c.2968_2971del:p.K990fs), which is represented by only one RefSeq isoform (Table 2 and Supplementary Tables S1 and S3). This variant is located in an upstream exon and is thus predicted to induce nonsense-mediated decay of the transcript. Nine children with DNMs in *HIVEP2*, including eight truncating mutations, have been reported.^{21,22} All of these children displayed

intellectual disability with minimal or no structural changes on the magnetic resonance images. One possibility is that DNMs in *HIVEP2* are associated with a more variable phenotype than previously suspected, explaining the structural defects observed in our case. Alternatively, this fetus might be affected by two different genetic disorders with the DNM in *HIVEP2* causing a neurodevelopmental disorder that was not readily apparent in the fetus and mutation(s) in another gene causing her brain structural defects. We found that the fetus was a compound heterozygote for predicted-damaging variants in *DNAH7*, which has been associated with primary ciliary dyskinesia (Table 2 and Supplementary Tables S1 and S3).²³ Interestingly, ciliary dysfunction can disrupt the ependymal flow through the aqueduct, causing its closure and subsequent triventricular hydrocephalus.^{24,25}

Malformations suggestive of ciliopathies

We studied five fetuses with at least two of the following features of ciliopathies: occipital meningocele, large/hyperechogenic kidneys and vermis hypoplasia (Table 3). Four of these fetuses were compound heterozygous for pathogenic or likely pathogenic variants in known ciliopathy genes (*CEP290* (OMIM 610142), *TCTN1* (OMIM 609863), *TMEM67* (OMIM 609884), and *BBS10* (610148)), whereas the remaining one was a compound heterozygote for variants of unclear significance in *TMEM67*, which is also associated with a ciliopathy (Table 3 and Supplementary Tables S1 and S3).

Multiple malformations

We studied 32 cases with at least two major malformations (with patterns not corresponding to those included in the other categories) (Supplementary Table S1). We considered that six of them (19%) were likely explained by mutations in known disease genes, including *FRAS1* (OMIM 607830), *FLNB* (OMIM 603381), *FAM20C* (OMIM 611061), *TGFBR1* (OMIM 190181), *EP300* (OMIM 602700), and *MNX1* (OMIM 142994) (Table 4 and Supplementary Tables S1 and S3). The phenotype of these cases was consistent with that previously associated with these genes, except for fetus 051-008-TNK with a mutation in *EP300* and fetus CONGE-005 with a mutation in *MNX1*. We describe these two fetuses in more detail hereafter.

Fetus 051-008-TNK presented during the second trimester with intrauterine growth restriction, spina bifida, and postaxial polydactyly. WES showed the presence of a truncating DNM in *EP300* (NM_001429; c.102_105del:p.G34fs) (Table 4 and Supplementary Tables S1 and S3). Dominant mutations in *EP300* cause Rubinstein–Taybi syndrome. The autopsy of fetus 051-008-TNK showed signs of Rubinstein–Taybi syndrome, including the typical facial features and broad halluces (Supplementary Table S1). However, postaxial polydactyly and neural tube defects are atypical features of *EP300*-related Rubinstein–Taybi

Table 3 Genetic finding in fetal cases with suspected ciliopathies

ID	Phenotype	Gene	Variant	Inheritance	Interpretation
051 038 OZX ^a	(21 weeks) molar tooth sign, occipital meningocele, hyperechogenic kidneys, postaxial polydactyly	<i>CEP290</i>	c.6401T > C (p. Ile2134Thr)	Compound heterozygous	Pathogenic variant
		<i>CEP290</i>	c.4195-1G > A	Compound heterozygous	Pathogenic variant
051-115-RKO ^a	(27 weeks) postaxial polydactyly, vermician hypoplasia	<i>TCTN1</i>	c.342-2A > C	Compound heterozygous	Pathogenic variant
		<i>TCTN1</i>	c.898C > T: (p.R300X)	Compound heterozygous	Pathogenic variant
CONGE-001	(16 weeks) large and hyperechogenic kidneys, vermician hypoplasia, mild ventriculomegaly	<i>TMEM67</i>	c.149_151del (p.N52del)	Compound heterozygous	Variant of unclear significance
		<i>TMEM67</i>	c.1674G > A (p.Q558Q)	Compound heterozygous	Variant of unclear significance
CONGE-030	(20 weeks) microcephaly, occipital encephalocoele, bilateral nephromegaly	<i>TMEM67</i>	c.233G > A (p.C78Y) ^b	Compound heterozygous	Likely pathogenic variant
		<i>TMEM67</i>	c.1717delA (p.N573fs)	Compound heterozygous	Pathogenic variant
CONGE-006	(20 weeks) postaxial polydactyly, large and hyperechogenic kidneys	<i>BBS10</i>	c.1241T > C (p.L414S)	Compound heterozygous	Likely pathogenic variant
		<i>BBS10</i>	c.909_912del (p.S303fs)	Compound heterozygous	Pathogenic variant

^aPreviously published in ref. 41. ^bHas been reported in a fetus with Meckel syndrome in trans with another missense variant.⁴²

Table 4 Genetic findings in fetal and neonatal cases with multiple malformations

ID	Phenotype	Gene	Variant	Inheritance	Interpretation
051 008 TNK	(19 weeks) IUGR, broad halluces, sacral spina bifida, postaxial polydactyly of left foot	<i>EP300</i>	c.102_105del (p.G34fs)	Heterozygous, de novo	Pathogenic variant
051 022 CYB	(25 weeks) brachycephaly, exophthalmos, short nose with depressed root, broad clavicles, belt-shaped thorax, increased density of long bones and base of the skull, brain calcifications	<i>FAM20C</i>	c.956G > A (p.R319K)	Compound heterozygous	Likely pathogenic
		<i>FAM20C</i>	c.1528C > T (p.R510C)	Compound heterozygous	variant Likely pathogenic variant
051-089- QUI	(22 weeks) clubfeet, short limbs, large thumbs	<i>FLNB</i>	c.4732G > C (p.A1578P)	heterozygous, de novo	Likely pathogenic variant
051-102- OHU	(20 weeks) cryptophthalmos, syndactyly, short pharynx with hypoplasia of the epiglottis, renal agenesis, vaginal atresia	<i>FRAS1</i>	c.370C > T (p.R124X)	Homozygous	Pathogenic variant
CONGE-005	(38 weeks) IUGR, cleft palate, anal imperforation, scrotal agenesis, agenesis of the lumbar and sacral spine and spinal cord	<i>MNX1</i>	c.2T > C (p.M1T)	Homozygous	Pathogenic variant
CONGE-021	(25 weeks) heart defects, posterior cleft palate with microretrognathia, clubfeet	<i>TGFBR1</i>	c.605_606insGAGAACTATTGT (p.A202delinsARTIV)	Heterozygous, de novo	Likely pathogenic variant
CONGE-074	(24 weeks) IUGR, phocomelia, diaphragmatic hernia, vertebral segmentation defects	<i>VEGFA</i>	c.789C > A (p.C263X)	Heterozygous, de novo	Candidate variant
051 043 BEV	(19 weeks) severe thoracolumbar scoliosis with segmentation defects, absence of visible ribs	<i>FKBP8</i>	c.572C > T (p.P191L)	Homozygous	Candidate variant

IUGR, intrauterine growth restriction.

syndrome, having been described only in single cases with this disorder.^{26,27} Interestingly, the loss of *Ep300* disrupts neurulation in mice.²⁸

Fetus CONGE-005 presented with akinesia and a short spine at 12 weeks of gestation. The fetus was delivered at term but died within minutes of birth with several anomalies including lumbosacral agenesis, anal imperforation, and scrotal agenesis (**Supplementary Figure S1**). The parents were consanguineous and previously had three newborns who died in the immediate neonatal period with a similar phenotype characterized by a short spine and the variable presence of sacrococcygeal teratoma and anal imperforation. The conjunction of caudal agenesis with a presacral mass, such as a teratoma, is consistent with Currarino syndrome, which is caused by dominant mutations in *MNX1* (OMIM 176450). Interestingly, we found that fetus CONGE-005 is homozygous and his parents heterozygous for the variant c. T2C/p.M1T in *MNX1* (NM_005515) (**Table 4** and **Supplementary Tables S1** and **S3**). This gene has two isoforms—a longer isoform 1 of 401 amino acids (NP_005506.3) and a shorter isoform 2 of 189 amino acids (NP_001158727.1) that lacks the N-terminus present in isoform 1 where this variant is located. Specifically, p.M1T alters the initiation methionine of the longer isoform of the protein, likely impairing its production as the second in-frame methionine is far downstream at amino acid 221. Three cases, all with neonatal diabetes, have been reported with biallelic

missense variants in *MNX1*.^{29,30} One of these cases also had Currarino syndrome²⁹. Our case has a much more severe phenotype than that found in any of the previously reported cases with mutations in *MNX1*. We postulate that the p.M1T variant is hypomorphic, being tolerated in the heterozygotes, but causing severe defects in the homozygotes.

Candidate genes

Fetus 051-043-BEV presented with scoliosis associated with vertebral segmentation defects. As this fetus was the product of a consanguineous union, we focused our attention on homozygous variants (**Table 4** and **Supplementary Table S1**). Among these, we identified a rare, predicted-damaging missense variant in *FKBP8* (OMIM 604840) (NM_012181; c.C572T:p.P191L) (**Table 4** and **Supplementary Tables S1** and **S3**). Interestingly, *Fkpb8* mutant mice show extensive fusion of vertebrae, suggesting that the segmentation defects observed in the fetus are also caused by disruption of this gene.^{31,32}

Fetus CONGE-074 presented during the second trimester with multiple anomalies, including phocomelia (**Table 4** and **Supplementary Table S1**). This fetus carried a truncating DNM in *VEGFA* (OMIM 192240) (NM_003376; c.C789A:p. C263X), which is likely inducing nonsense-mediated decay of the transcript, as it is located in an upstream exon that is present in all RefSeq isoforms (**Table 4** and **Supplementary Table S3**). *VEGFA* codes for a critical regulator of blood

vessel development. Remarkably, the loss of a single *Vegfa* allele is lethal in mouse embryos between days 11 and 12.^{33,34} These embryos show impaired early vascular development and multiple structural anomalies, which remain poorly characterized because of the early lethality. At least some of the defects observed in our fetus might be explained by a defect of blood vessel development. For instance, it appears that the maternal administration of thalidomide during pregnancy causes fetal phocomelia by disrupting angiogenesis.³⁵

Fetal akinesia

We studied eight akinetic fetuses from seven unrelated families (Supplementary Table S1). We identified pathogenic biallelic mutations in *RYR1* (OMIM 180901) in two siblings from a consanguineous union (MALFO 1.13 and MALFO 2.13) and in an unrelated case (CONGE-060) (Table 5 and Supplementary Tables S1 and S3). Recessive mutations in *RYR1* are mainly associated with myopathies presenting in childhood, but have previously been described in a few fetuses with akinesia.³⁶ We did not find any rare variants in genes previously associated with akinesia in the remaining cases.

DISCUSSION

The discovery of severe anomalies in a fetus raises the critical question of the prognosis and recurrence risk. Clinicians typically order prenatal chromosomal microarray analysis testing following the discovery of major fetal anomalies with the aim of identifying the cause of the disorder and establishing a precise prognosis. Although the diagnostic yield of chromosomal microarray analysis is relatively low, it currently represents the most powerful test for the investigation of fetal structural defects. The goal of this study was to evaluate the added value of WES for the investigation of these anomalies. By mainly recruiting cases from terminated pregnancies, we aimed to enrich our series with severe anomalies while complementing their phenotypic characterization with pathological data.

The diagnostic yield (19%) observed in our study is similar to those recently obtained in similar studies, with yields of 10% (3/35),³⁷ 20% (17/84)³⁸, and 21% (5/24)³⁹. Our yield was higher for ciliopathies (80%), cerebral anomalies (19%), multiple malformations (19%), and fetal akinesia (25%), but lower for renal a/dysgenesis (0%) and the VACTERL association (11%). The diagnosis was initially suspected by

the clinicians in 6 of these families (those with mutations in *FRAS1*, *TCTN1*, *CEP290*, *TMEM67*, and *BBS10*) but not in the remaining 13 families (those with mutations in *CHD7*, *TUBA1A*, *TUBB3*, *TUBB*, *PEX1*, *PDHA1*, *EP300*, *FLBN*, *MXN1*, *TGFBR1*, and *RYR1*). In some of these latter cases, the phenotypes were not specific enough to invoke the diagnoses of interest. This lack of phenotypic specificity represents a major challenge of prenatal diagnosis and is explained by several factors, including the immaturity of the fetuses and our limited ability to phenotype them prenatally. For instance, single-gene disorders can be difficult to distinguish prenatally from common disorders that are genetically more complex, as illustrated by fetus CONGE-79, who presented with known features of *CHD7*-related disorders, such as esophageal atresia and tetralogy of Fallot, that can also be associated with the more common VACTERL association.

In other instances, the molecular diagnosis was not suspected because the phenotype was atypical and not corresponding to the phenotype usually found in children and adults. Some of these cases might represent the severe end of the phenotypic spectrum associated with known disease genes. These include the fetuses with mutations in *TUBB*, *MXN1*, and *RYR1* who displayed a similar but much more severe phenotype than that previously described in individuals with mutations in these genes. Other cases might represent presentations of known disease genes that are substantially different from those previously documented. This might be the case of the fetus with a DNM in *EP300* who presented with features such as spina bifida and postaxial polydactyly that are not usually found in *EP300*-related disorders. Similarly, DNMs in *DSTYK* (051-141-VWD), *ACTB* (051-021-LIC), and *HIVEP2* (051-039-KUY) were found in fetuses with phenotypes that have not been previously associated with these genes. It remains unclear whether these DNMs explain the anomalies of these last three fetuses. This clinical heterogeneity associated with fetal anomalies and our limited ability to correlate genotypes and phenotypes in ongoing pregnancies represent challenges for the integration of WES in prenatal diagnosis.

The overall diagnostic yield of 19% is slightly lower than that obtained with the use of WES for the investigation of rare disorders in children or adults, which is ~25–31%.⁴⁰ The lower yield of WES for some groups of anomalies could be explained by the involvement of epigenetic mechanisms or more complex genetic models (see, for example, ref. 3).

Table 5 Genetic findings in fetal cases with akinesia

ID	Phenotype	Gene	Variant	Inheritance	Interpretation
MALFO 1.13	(17 weeks) cystic hygroma and akinesia	<i>RYR1</i>	c.784G>T (p.E262X)	Homozygous	Pathogenic variant
MALFO 2.13	(12 weeks) cystic hygroma and akinesia	<i>RYR1</i>	c.784G>T (p.E262X)	Homozygous	Pathogenic variant
CONGE-060	(20 weeks and 6 days) hydrops, flexion contractures of the elbows and atrophy of the muscle in the upper limbs	<i>RYR1</i>	c.328C>T (p.H110Y)	Compound heterozygous	Likely pathogenic variant
			c.14927T>C (p.L4976P)	Compound heterozygous	Likely pathogenic variant

Alternatively, some of these severe anomalies could be caused by mutations in sets of genes other than those involved in disorders presenting after birth. For instance, we identified a novel candidate gene for renal agenesis, *GREB1L*, which is associated with a lethal phenotype that would not be ascertained beyond the immediate neonatal period. Very little is known about the genetic basis of the rare and severe disorders specifically encountered in fetuses. Systematic genomic studies of fetal anomalies will thus provide insight into the distinctive function of numerous genes acting during embryonic or fetal life while enhancing the diagnostic utility of genomic sequencing for prenatal diagnosis.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/gim>

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DISCLOSURE

The authors declare no conflict of interest.

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