SUPPLEMENTARY MATERIALS

Prenatal Genome-Wide Cell-free DNA Screening: Three Years of Clinical Experience in a Hospital Prenatal Diagnostic Unit in Spain

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Supplementary Appendix A

One case in the rare autosomal aneuploidies (RAA) cohort was a trisomy 20 case that had an initial low-risk result with fetal sex XY on cell-free DNA (cfDNA) analysis. An ultrasound scan noted a female fetus, and a second cfDNA analysis was performed giving a high-risk result for trisomy 20 (fetal sex XY). In this case the log likelihood ratio (LLR) was 16.5 (close to the reference 10.6), the mosaic ratio was 25%, and the fetal fraction (FF) was 14%. An amniocentesis with array was performed in a private center with the karyotype result mos 46,X,+mar[25]/45,X[5] and the array showed two different deletions on chromosome Y (and trisomy 20 was discordant). One of these was a terminal deletion of region Yp11.32p11.2 (chrY: 118552-3082444; Hg19) of 2.96 Mb with 28 OMIM® genes (118552-3082444), and this deletion affected the *SHOX* and *SRY* genes: arr [GRCh37]Yp11.32p11.2(118,552-3,082,444)x0,Yq11.221q12(18,292,329-59,336,104)x0. The other deletion was a terminal deletion of region Yq11.221q12 (chrY: 18292329-59336104; Hg19) of 41.04 Mb encompassing 27 OMIM® genes (18292329-59336104). With regards to the first deletion, pathogenic deletions or point mutations of the *SHOX* (short stature homeobox) gene give rise to its haploinsufficiency. The phenotypic spectrum ranges from Leri-Weill dyschondrosteosis (with the classic clinical triad of short stature, mesomelia, and Madelung deformity) at the severe end of the spectrum to nonspecific short stature at the mild end of the spectrum. The deletion of the *SRY* gene in a patient with the male genetic sex is associated with complete gonadal dysgenesis or Swyer syndrome (OMIM® 400044). It is a disorder of sexual development associated with anomalies in gonadal development. Individuals with 46,XY gonadal dysgenesis may present from ambiguous genitalia to phenotypically female genitalia; however, they do not develop secondary sexual characteristics at puberty and have primary amenorrhea. The gonads are usually made up of fibrous tissue and are associated with an increased risk of developing tumors (mainly dysgerminomas), they usually present a uterus and fallopian tubes, and the external genitalia are female. This deletion of the *SRY* gene explains the discrepancy observed between the cfDNA result (male) and ultrasound sex (female). With regards to the second deletion, deletions of the long arm of the Y chromosome (Yq) represent one of the known causes of failure in spermatogenesis. It is produced by the loss of one or more of three loci called azoospermia factor (AZF)a, AZFb, and AZFc causing infertility in males. In general, the symptoms associated with the detected chromosomal alteration (45,X/46,XY) are very similar to those observed in patients with Turner syndrome, although because it is a chromosomal mosaic, they may present greater variability of symptoms. The most characteristic symptoms presented by the majority of patients with complete Turner syndrome (chromosomal set 45,X in all cells) are short stature and primary amenorrhea, with ribbon gonads and absence of ovarian follicles. In this case, we would recommend consultation with a pediatric endocrinologist.

Abbreviations: Mb, megabase; OMIM®, Online Mendelian Inheritance in Man®.

Table S1. Cell-free DNA screening results and concordance with clinical outcomes for SCA cases.

Case	GA, wk	FF, %	cfDNA Screening Result	Confirmatory Testing	Genetic Result
1	12	16	45,X	Amniocentesis	46,XX
2	12	11	45,X	Amniocentesis	Concordant: 45,X
3[a]	12	6	45,X	No confirmatory testing	N/a
4	13	13	45,X	Amniocentesis	46,XX
5	13	7	45,X	Amniocentesis	46,XX
6[a]	14	15	45,X	CVS	Concordant: 45,X
7	13	7	45,X	Amniocentesis	Concordant: 45,X[18%]
8	12	15	45,X	Amniocentesis	46,XX
9[b]	13	6	45,X	No confirmatory testing	N/a
10	13	10	45,X	Amniocentesis	46,XX
11	12	7	45,X	Amniocentesis	46,XX
12	16	8	45,X	Amniocentesis	46,XX
13	13	4	45,X	Amniocentesis	Concordant: 45,X[60%]
14[a]	15	8	45,X	Amniocentesis	Discordant: XXX
15	12	8	45,X	Amniocentesis	Concordant: 45,X
16	13	10	47,XXY	No confirmatory testing	N/a
17	13	12	47,XXY	Amniocentesis	Concordant: 47,XXY
18	26	9	47,XXY	No confirmatory testing	N/a
19	12	6	47,XXY	CVS	Concordant: 47,XXY
20	12	11	47,XYY	Amniocentesis	Concordant: 47,XYY
21	21	15	47,XYY	No confirmatory testing	N/a
22	16	8	47,XYY	No confirmatory testing	N/a
23	12	4	47,XYY	Amniocentesis	Concordant: 47,XYY
24	14	11	47,XXX	Amniocentesis	Concordant: 47,XXX
25	13	20	47,XXX	Amniocentesis	Concordant: 47,XXX
26	13	11	47,XXX	Amniocentesis	Concordant: 47,XXX
27	12	5	47,XXX	Amniocentesis	Concordant[c]: 47,XXX
28	13	5	47,XXX	Amniocentesis	Concordant: 47,XXX
29	14	7	47,XXX	No confirmatory testing	N/a

In case of amniocentesis or chorionic villus sampling (CVS), some laboratory techniques are performed as quantitative fluorescent PCR (QF-PCR), karyotype or both. SCA, sex chromosome aneuploidies; GA, gestational age; wk, weeks; FF, fetal fraction; cfDNA, cell-free DNA; N/a, not available. [a]Patient had an elective termination. [b]Newborn died at 18 days of life. A homozygous deletion of the *GBE1* gene was detected associated with glycogenosis type IV. [c]arr[GRCh37] Xp22.33q28(168547_155233731)x3.

Table S2. Cell-free DNA screening results and clinical outcomes for RAA cases.

Case	GA, wk	FF, %	cfDNA Screening Result	Confirmatory Testing	Genetic Result	Pregnancy Outcomes	Birth Outcomes	LLR Threshold	LLR Sample	Mosaic Ratio (%)
1	12	7	T3	Amniocentesis	46,XX	Gestational diabetes	Normal	5	65.67	84.32
2	13	5	T3	Amniocentesis	46,XY	Normal	Normal	5	12.51	37.69
3	14	10	T4	Amniocentesis	46,XY	Obstetric metrorrhagia in the third trimester	Normal	7	675.88	100
4	12	19	T7	Amniocentesis	46,XY	PAPP-A was 0.18; late FGR (36 wk)	Emergency C-section at 36+5 wk; reduced birth weight (1,980g; 2nd percentile); neonatal hypoglycaemia	6.6	2772.04	82
5	13	7	T7	Amniocentesis	46,XX	Preeclampsia	Reduced birth weight at 38+2 wk (2,480g, 8th percentile)	6.6	26.81	40
6	14	14	T7	Amniocentesis	46,XY	Normal	Normal	6.6	130	38
7	13	14	T7	Amniocentesis	46,XX	Normal	Cerebral haemorrhage due to arteriovenous malformation	6.6	876.46	82
8	13	5	T7	Amniocentesis	46,XY	Normal	Normal	6.6	12.77	36.89
9	13	11	T8	Amniocentesis	46,XY	Normal	Normal	6.6	17.12	21
10	12	9	T8	No confirmatory testing	N/a	N/a	N/a	5.8	19.52	21.88
11	12	8	T8	Amniocentesis	46,XY	Obstetric metrorrhagia and uterine hypertonus	Spontaneous preterm birth at 24+6 wk; low Apgar score; admission to NICU; neonatal death at 23 h	5.8	130	38
12	12	5	T8	Amniocentesis	46,XX	Suspicion of late preeclampsia	Normal	5.8	100.19	100
13	13	5	T9	Amniocentesis	46,XY	Preeclampsia; FGR before 32 wk	Delivery by C-section >37 wk; baby had short stature and short long bones; exome sequencing was performed with a homozygous VOUS in *NPR2* gene suggesting Aarskog syndrome	8	157.72	100
14[a]	16	15	T9	Postnatal	46,XX	Fetal echogenic bowel and choroid plexus cysts. Mild preeclampsia and oligoamnios.	Normal	8	609.88	6.2
15	13	5	T10	Amniocentesis	46,XX	Normal	Reduced birth weight at 40+2 wk (2,730g, 5th percentile)	8.8	41.32	62
16	12	12	T10	Amniocentesis	46,XX	Normal	Normal	8.8	168.41	44.43
17	12	7	T10	Amniocentesis	46,XX	Normal	N/a	8.8	74.59	51.44

18	13	11	T12	Amniocentesis	46,XY	Chronic hypertension	Emergency C-section at 30+4 wk; baby was admitted to the NICU with respiratory distress	11.6	324.35	73.42
19	10	10	T12	CVS	46,XY	Unknown	Emergency C-section >37 weeks	11.6	15.8	19.74
20	12	20	T16	Amniocentesis	46,XX	Normal	N/a	10.7	966.76	71
21	12	5	T16	Amniocentesis	Concordant: 47,XX,+16[1]/46, XX[84]	Spontaneous abortion at 17 wk	N/a	10.7	N/a	N/a
22	12	15	T16	Amniocentesis	46,XX	Spontaneous abortion at 16 wk	Autopsy noted intestinal and hepatic gastroschisis	10.7	889.47	94.15
23	11	11	T16	Amniocentesis	46,XX	Hypothyroidism, gestational diabetes, isolated FGR <32 wk	Baby had mucocutaneous jaundice that did not require phototherapy and weight loss that required supplemental breastfeeding	10.7	64.9	59.63
24[b]	15	3	T16	Amniocentesis and postnatal	Concordant: [arr(16)x3(0,14)] and [arr[GRCh37] 16p13.3p12.3(94 808_18843684)x 2 hmz]	FGR and preeclampsia. Patient was followed until 36 wk but had delivery in a private center	Newborn was seen at our hospital at the age of 20 days (weight at birth: 1,840 g, 1st percentile); baby was followed at the hospital because of digestive problems, stools with mucus, irritability after feedings.	10.7	14.07	100
25	12	15	T20	Amniocentesis	46,XY	Late FGR (36 wk); premature rupture of membranes	Spontaneous preterm birth at 37+2 wk; low birth weight (2,580g, 16th percentile)	10.6	428.59	86.6
26	20	14	T20	Amniocentesis	arr[GRCh37] Yp11.32p11.2 (118,552-3,082,444)x0,Yq1 1.221q12 (18,292,329-59,336,104)x0	Normal	Normal	10.6	369.01	100
27	12	6	T20	Amniocentesis	46,XY	Gestational diabetes	Low birth weight (2,760g, 10th percentile)	10.6	30.37	63.82
28	13	7	T22	Amniocentesis	46,XY	Normal	Preterm birth at 33+2 wk; baby was admitted to the NICU due to prematurity	13.5	67.91	75
29	12	8	T22	Amniocentesis	46,XY	Gestational diabetes; Isolated FGR <32 wk	Emergency C-section at 37 wk; very low birth weight (1,750g, <1st percentile)	13.5	84.23	72.9
30	12	5	T22	Amniocentesis and POC	Concordant: 47,XX,+22(9)	Spontaneous abortion at 16 wk	N/a	13.5	45.78	100
31	12	11	T22	Amniocentesis	46,XY	Normal	Normal	13.5	15.3	36.11

In case of amniocentesis, chorionic villus sampling (CVS), products of conception (POC), or postnatal studies, some laboratory techniques are performed as quantitative fluorescent PCR (QF-PCR), karyotype, or both and array. RAA, rare autosomal aneuploidies; GA, gestational age; wk, weeks; FF, fetal fraction; cfDNA, cell-free DNA; LLR, log likelihood ratio; T, trisomy; PAPP-A, pregnancy associated plasma protein-A; FGR, fetal growth restriction; g, grams; C-section, caesarean section; N/a, not available; NICU, neonatal intensive care unit; h, hours; VOUS, variant of uncertain significance. [a]Maternal mosaic of trisomy 9. [b]Trisomy 16 confirmed as 14% mosaic following amniocentesis with array [arr(16)x3(0,14)]. Postnatal genetic study was performed in peripheral blood resulting a maternal partial uniparental isodisomy [arr[GRCh37] 16p13.3p12.3(94808_18843684)x2hmz].

Table S3. Cell-free DNA screening results and clinical outcomes for CNV cases.

Case	GA, wk	FF, %	cfDNA Screening Result	Confirmatory Testing	Genetic Result	Pregnancy Outcomes	Birth Outcomes
1	12	8	dup(1)(q21.1q32.2)	Amniocentesis	46,XX	Normal	Normal
2	13	5	dup(1)(p22.3q25.3)	No confirmatory testing	N/a	Gestational diabetes	Emergency C-section >37wk due to premature rupture of membranes
3	16	6	dup(1)(p32.3q31.2)	Amniocentesis	46,XX	Normal	Emergency C-section at 32+2 wk due to threatened premature birth; admission to NICU due to prematurity; baby had slight retrognathia; inguinal hernia operated on 4 months post-birth; baby girl was born at 32+2 wk, weight:1,960 g (72nd percentile).
4	12	7	dup(1)(p35.1p31.3)	Amniocentesis	46,XY	Placental hydrops; spontaneous abortion at 16 wk	N/a
5	14	4	dup(1)(p36.22p33)	Amniocentesis	46,XX	Normal	Normal
6	13	14	del(5)(q14.3q32)	Amniocentesis	46,XY	Pregnancy in progress; ultrasound scan at 32 wk noted fetal ventricular asymmetry which was confirmed with an MRI	N/a
7[a]	14	4	del(5)(p15.33p13.3)	Amniocentesis	46,XY but array showed LOH chr 5 arr[GRCh38] 5p15.33p13.3(113462_30725947)x2 hmz	Preeclampsia; isolated FGR <32 wk	Emergency C-section >37 wk; baby was admitted to the NICU with early hypoglycemia; neonatal death occurred at 21 days; baby had microcephalus and mild right pyelectasis
8[b]	12	3	dup(5)(p15.1p13.3)	No confirmatory testing	N/a	Gestational diabetes	Normal
9[c]	15	4	del(7)(q21.11q31.32)	Amniocentesis	46,XX	Gestational diabetes	Preterm birth
10	12	6	del(8)(p23.3p22)	Postnatal	46,XY	Normal	Normal
11	12	12	del(8)(p23.3p11.21)	Amniocentesis	46,XX	Gestational hypertension; late FGR (37 wk)	Normal
12	18	16	dup(9)(p24.3p13.1)	Amniocentesis	46,XX	Chronic hypertension; isolated FGR <32 wk	Emergency C-section after 37 wk; reduced birth weight for 37+1 wk (1,950g, 1st percentile)

13	18	10	dup(10)(q11.21q26.13)	Amniocentesis	46,XY	Gestational diabetes; intrauterine fetal demise at 28+3 wk	Weight was 836g; no autopsy was carried out but an anatomic pathology exam noted a mature placenta with signs of chorioamnionitis
14	13	18	dup(10)(p15.3q11.21)	Amniocentesis	46,XX	Normal	Spontaneous preterm birth at 36+2 wk
15	13	3	del(10)(p15.3p12.31)	Amniocentesis	46,XX	Severe FGR in one twin; spontaneous abortion of both twins at 19+3 wk	Pathological anatomy noted hydropic fetuses without congenital malformations
16	13	7	del(10)(q25.1q26.3)	Amniocentesis	46,XX	Normal	Normal
17	12	9	del(10)(q25.2q26.3)	Amniocentesis	46,XY	Normal	Normal
18	12	6	del(10)(q25.2q26.3)	Amniocentesis	46,XX	Pregnancy in progress	N/a
19[c]	12	7	del(13)(q12.11q12.2)	Amniocentesis	46,XX	Gestational hypertension	Emergency C-section after 37 wk
20	13	7	del(13)(q13.3q14.3)	Amniocentesis	Concordant: 46,XY, del(13)(q13.3q14.3)	Termination of pregnancy	N/a
21	14	7	dup(13)(q14.3q21.33)	Amniocentesis	46,XY	Normal	Normal
22	13	8	dup(13)(q14.3q34)	Amniocentesis	46,XY	During gestation, ultrasound scan revealed mild bilateral renal pyelectasia	N/a
23	13	4	del(13)(q31.1q33.3)	Amniocentesis	46,XY	Normal	Normal
24	17	14	del(13)(q31.1q31.3)	Amniocentesis	Concordant: 46,XX, del(13)(q31.1q31.3)	FGR	A baby girl was born who developed alterations in the carpus and tarsus with anomalous phalanges
25	12	8	del(14)(q31.1q32.33)	No confirmatory testing	N/a	Ultrasound at 12 wk noted fetal hydrops and an nuchal translucency of 4.2; termination of pregnancy at 15 wk	Autopsy detected small saddle nose, accentuated retrognathia, and low implantation of the auricle but no major malformations were detected
26	14	18	dup(15)(q14q26.3)	Amniocentesis	46,XY	Normal	Normal
27	12	11	del(18)(p11.32p11.22)	CVS	Concordant: 46,XX,del(18)(p11.32p11.22)	Intrauterine fetal demise	N/a
28	14	11	dup(20)(p13q11.21)	Amniocentesis	46,XY	Normal	Normal
29	13	12	dup(21)(q21.1q21.3)	Amniocentesis	46,XX	Normal	Emergency C-section >37 wk
30	13	10	dup(21)(q21.1q22.3)	Amniocentesis	46,XY	Normal	Emergency C-section >37wk
31	12	15	dup(22)(q11.21q13.31)	Amniocentesis	46,XX	Metrorrhagia in the third trimester	Spontaneous preterm birth at 36+3 wk

In case of amniocentesis or chorionic villus sampling (CVS), some laboratory techniques are performed as quantitative fluorescent PCR (QF-PCR), karyotype or both. CNV, copy-number variation; GA, gestational age; wk, weeks; FF, fetal fraction; cfDNA, cell-free DNA; del, deletion; dup, duplication; N/a, not available; C-section, caesarean section; NICU, neonatal intensive care unit; g, grams; MRI, magnetic resonance imaging; LOH, loss of heterozygosity; FGR, fetal growth restriction. [a]The patient had a controlled pregnancy and gave birth to a child with early hypoglycemia. At 24 hours of life, he presented hyperbilirubinemia and phototherapy was maintained for 2 days. The newborn also presented a

microcephaly but brain ultrasound scan was normal. Following neonatal death at 21 days, a clinical exome study was performed and was not informative. [b]cfDNA screening was repeated and a low-risk result was obtained. [c]Follow-up of newborn in another hospital.

Table S4. Cell-free DNA screening results and clinical outcomes for multiple anomalies cases.

Case	GA, wk	FF, %	cfDNA Screening Result	Confirmatory Testing	Genetic Result	Pregnancy Outcomes	Birth Outcomes
1	14	8	T4; dup(12)(q13.13q24.32)	Amniocentesis	46,XY	Normal	Normal
2	13	5	T13; T21	Amniocentesis	47,XY,+13	Termination of pregnancy	N/a
3	12	12	T7; T21	Amniocentesis	47,XX,+21	Normal	Newborn compatible with Down syndrome phenotype
4	12	11	del(18)(q21.33q23); T22	POC tissue	46,XY,del(18)(q21.33q23)[a]	Termination of pregnancy	N/a
5	13	9	T12; T15	Amniocentesis	46,XY	Fetal structural cardiopathy leading to hospitalization; FGR <32 wk; premature abruption of a normally inserted placenta/premature rupture of membranes	Emergency C-section at 26 wk; baby admitted to NICU due to prematurity; baby had congenital cardiopathy
6[b]	12	10	T7; T13	Amniocentesis	46,XX	Normal	N/a
7[c]	22	17	dup(1)(p36.11p34.1); MX	Amniocentesis	46,XX	Normal	Normal
8[d]	12	9	del(1)(q42.12q42.3); dup(1)(q31.1q31.3)	Amniocentesis	46,XX	Pregnancy ongoing (at 22 wk is normal)	N/a

9[e]	12	11	1st cfDNA screen:
			T2; M4;
			dup(7)(p22.1p15.2); T8;
			M9
			2nd cfDNA screen:
			dup(2)(p21p16.2);
			del(3)(p14.2p13); M4;
			del(5)(q23.1q31.2);
			del(6)(p12.3q27);
			T7; T8; M9; M10; T11; M12; M13; M14; M15; M17; T18; T20; M21; T22
				Amniocentesis	46,XX or 46,XY	Termination of pregnancy	N/a

In case of amniocentesis or products of conception (POC), some laboratory techniques are performed as quantitative fluorescent PCR (QF-PCR), karyotype, or both. GA, gestational age; wk, weeks; FF, fetal fraction; cfDNA, cell-free DNA; T, trisomy; M, monosomy; del, deletion; dup, duplication; N/a, not available; FGR, fetal growth restriction; NICU, neonatal intensive care unit; C-section, caesarean section; Mb, megabases; kb, kilbases. [a]cfDNA screening called a deletion on chromosome 18 (q21.33- q23) that was 18.8 Mb. On array, a deletion on chromosome 18 was confirmed with a deletion size of 1.161 kb that was probably pathogenic. [b]Patient was followed up in another hospital. [c]At 36 wk the pregnancy was normal. Delivery was carried out in another center and no further follow-up was available. [d]At 22 wk gestation the pregnancy was normal. [e]Maternal malignancy case: at 23 weeks of gestation an advanced gastric carcinoma was detected and pregnancy was terminated.

Table S5. Overview of high-risk twin pregnancies.

Case	Type of Pregnancy	Maternal Age, yr	GA, wk	FF, %	cfDNA Screening Result	Clinical Follow-up
1	Twin	29	13	8	Trisomy 21	Normal amniocentesis
2	Twin	39	12	9	Trisomy 21	Trisomy 21 confirmed in one of the fetuses by amniocentesis
3	Twin	30	13	3	del(10)(p15.3p12.31)	Normal amniocentesis
4[a]	Vanishing twin	35	12	7	Trisomy 13	No diagnostic testing; normal pregnancy
5[b]	Vanishing twin	31	17	9	Trisomy 18	Normal amniocentesis
6[c]	Vanishing twin	43	14	13	Trisomy 18	No diagnostic testing
7[d]	Vanishing twin	30	10	10	Trisomy 12	Normal CVS
8[e]	Vanishing twin	41	13	9	Trisomy 12 and trisomy 15	Normal amniocentesis
9[f]	Vanishing twin	39	13	10	dup(21)(q21.1q22.3)	Normal amniocentesis

yr, years; GA, gestational age; wk, weeks; FF, fetal fraction; cfDNA, cell-free DNA; CVS, chorionic villus sampling. [a]One fetus arrested in week 8 of pregnancy. A repeat cfDNA screen carried out later in the pregnancy was normal. [b]Suspicion of a vanishing twin at 16 weeks of gestation. [c]At 13+5 weeks of gestation, two embryos are present (one fetus had no cardiac activity). [d]Twin gestation at beginning of pregnancy but at week 10+4 one fetus was not visualized. [e]One fetus arrested in week 8 of pregnancy. Structural heart defects were detected in the surviving fetus. [f]Suspicion of a vanishing twin at 13 weeks of gestation.