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ORIGINAL ARTICLE

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Multicenter clinical experience with non-invasive cell-free DNA screening for monosomy X and related X-chromosome variants

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Abstract

Objective: We aimed to investigate how the presence of fetal anomalies and different X chromosome variants influences Cell-free DNA (cfDNA) screening results for monosomy X.

Methods: From a multicenter retrospective survey on 673 pregnancies with prenatally suspected or confirmed Turner syndrome, we analyzed the subgroup for which prenatal cfDNA screening and karyotype results were available. A cfDNA screening result was defined as true positive (TP) when confirmatory testing showed 45,X or an X-chromosome variant.

Results: We had cfDNA results, karyotype, and phenotype data for 55 pregnancies. cfDNA results were high risk for monosomy X in 48/55, of which 23 were TP and 25 were false positive (FP). 32/48 high-risk cfDNA cases did not show fetal anomalies. Of these, 7 were TP. All were X-chromosome variants. All 16 fetuses with high-risk cfDNA result and ultrasound anomalies were TP. Of fetuses with abnormalities, those with 45,X more often had fetal hydrops/cystic hygroma, whereas those with "variant" karyotypes had different anomalies.

Conclusion: Both, 45,X or X-chromosome variants can be detected after a high-risk cfDNA result for monosomy X. When there are fetal anomalies, the result is more likely a TP. In the absence of fetal anomalies, it is most often an FP or X-chromosome variant.

Key points

What is already known about this topic?

- Cell-free DNA (cfDNA) screening can detect monosomy X and other X chromosome variants associated with a Turner syndrome phenotype, but performance is poorer than for trisomy 21.
- Little is known about the performance of cfDNA screening for X chromosome abnormalities in the presence of fetal structural anomalies.

What does this study add?

- A positive cfDNA screening result for monosomy X is more often confirmed when fetal anomalies are detected.
- In the absence of fetal anomalies, a positive cfDNA screening result is more often a false positive or associated with mosaicism or other X-chromosome variants.

1 | INTRODUCTION

Turner syndrome is diagnosed in about 1:1700–1:2000 liveborn girls.[1,2] It is more common prenatally because most affected fetuses demise early in pregnancy.[3] The severity of the prenatal phenotype ranges from early fetal hydrops with high mortality to a phenotypically female fetus without congenital anomalies. Congenital anomalies associated with Turner syndrome that are detectable prenatally include left-sided heart lesions, renal and urinary tract abnormalities, lymphedema of the hands and feet, and fetal growth restriction with shortened long bones.[4,5]

A clinical presentation of Turner syndrome can be caused by a 45,X karyotype, different forms of 45,X mosaicism, or structural anomalies of one of the X chromosomes.[6] Fetuses with a non-mosaic 45,X karyotype typically have a more severe phenotype, including cystic hygroma (CH) and hydrops. Those with mosaic 45,X/46 XX karyotypes typically have milder clinical features, but their clinical phenotype is highly variable and cannot be fully predicted by the karyotype. Fetuses without sonographic abnormalities who are incidentally prenatally diagnosed with a 45,X karyotype have a milder postnatal phenotype.[7–10] It is also unclear if and how often low-level 45,X mosaicism (in <10% of cells) is associated with a prenatally detectable clinical phenotype since most studies on its phenotypic consequences were done in adult women.[11–14] In addition, data on prenatal phenotypes associated with other X chromosome abnormalities are scarce. Postnatally, patients with isochromosome Xq and ring X do not have a milder phenotype than girls with a 45,X karyotype[15] and girls with a ring X or marker X that lacks the XIST

region can have intellectual disability in addition to phenotypic features of Turner syndrome.[11,16]

Underdiagnosis and late diagnosis are the unresolved problems for girls with Turner syndrome and hinder the timely initiation of relevant health screening and therapy.[1,17–19] Cell-free DNA (cfDNA)-based non-invasive prenatal screening for common aneuploidies and additional sex chromosome aneuploidies (SCA), including monosomy X, is now offered by commercial laboratories and has resulted in a larger number of pregnancies identified to be at an increased risk for fetal Turner syndrome. However, the positive predictive value (PPV) for detection of 45,X by cfDNA screening is much lower than for trisomy 21, ranging from 9% to 40%, and various factors limit its performance.[20–26] Confined placental mosaicism (CPM) and true fetal mosaicism are more common for monosomy X than for other chromosomal abnormalities.[27] The performance of cfDNA testing for SCA can also be affected by undiagnosed maternal sex chromosome abnormalities, such as X-chromosome deletions or mosaic X-chromosome aneuploidy, including the well-described somatic maternal loss of the second X chromosome with increasing maternal age.[25,28] Finally, as with any other aneuploidy, cfDNA shed from the trophoblast of a vanishing twin could be the cause of abnormal cfDNA screening results for SCA.[25,29]

Robust data about cfDNA screening detection rates and false positive rates for mosaic 45,X and other X-chromosome variants are limited.[26,30–32] There is little information on which karyotypes are subsumed under the suspected diagnosis of monosomy X made by cfDNA tests, how they present prenatally, and on the outcome of affected pregnancies. These cytogenetic variants can be mosaics that along with the 45,X cell line contain cells with various combinations of XXX, XX, or XY complements of sex chromosomes or with structural X-chromosome abnormalities, such as ring X, isochromosome of the long or the short arm (Xq or Xp), and rarely subchromosomal deletions of X or unbalanced X/autosome translocations.[6,15,33] Consequently, the position of professional societies on the inclusion of cfDNA based screening for SCA is not uniformly consistent.[34–38] Concerns include its still-developing performance, the variable phenotype in individuals with 45,X, and the possibility that incidental maternal findings are revealed, which raises ethical issues.[38] The higher number of false positive (FP) results with screening for SCA also increases the burden for pre- and posttest counseling.[39]

To gain more data on the various karyotype findings after cfDNA screening results that indicate a high risk for monosomy X, we conducted a targeted analysis of a multicenter retrospective database of pregnancies with a prenatal diagnoses of monosomy X or an X chromosome variant. First, we analyzed the performance of cfDNA screening for Turner syndrome in general and depending on ultrasound findings in a mixed-risk population. Second, we examined whether other cytogenetic variants affecting the X chromosome—and if so, which ones—can be detected when cfDNA screening indicates an increased risk for monosomy X, along with the phenotypes of the affected fetuses and pregnancy outcomes. Third, we evaluated the rate of invasive testing after a high-risk cfDNA result for monosomy X. In addition, we analyzed the effect of maternal age on the FPR of cfDNA screening for monosomy X.

2 | METHODS

2.1 | Subjects and ethical approval

This study is a subgroup analysis of data from a multicenter retrospective survey study approved by the ethics committee of the Justus-Liebig-University, Gießen, Germany, AZ 119/19. We acquired data on 673 pregnancies between 2000 and 2021 with prenatally or neonatally diagnosed or prenatally suspected fetal 45,X or variant Turner syndrome through a detailed questionnaire sent between September 2019 and May 2021 to referral centers for fetal medicine in Germany and their associated genetic laboratories. The questionnaire can be provided upon reasonable request.

For this study, we only reviewed data on 274 pregnancies that occurred after July 2014 when cfDNA screening for SCA was introduced. From this group, we focused only on 58 pregnancies for which prenatal cfDNA screening that included analysis of sex chromosomes was performed. We reviewed data from: (1) pregnancies with a high-risk cfDNA screening result for monosomy X with or without ultrasound findings suggesting possible fetal Turner syndrome and for whom a pre- or postnatal karyotype analysis was performed, (2) pregnancies with a low-risk cfDNA screening result and ultrasound findings suggesting possible fetal Turner syndrome who received prenatal genetic diagnostic testing results indicating fetal 45,X, mosaic 45,X, or another X chromosome variant, and (3) one uncomplicated pregnancy with a low-risk cfDNA screening result and no fetal anomalies on prenatal ultrasound exam but with a postnatal diagnosis of Turner syndrome.

2.2 | Assessment of the fetal phenotype and pregnancy outcomes

We collected data on anomalies detected at the first specialist ultrasound and during follow-up appointments when available. These included presence or absence of fetal hydrops, CH, increased nuchal translucency (NT), pleural effusions, ascites, cardiac anomalies, kidney anomalies, and fetal growth restriction. Other anomalies could be added by the experts in a separate field. We also asked for the date of diagnosis and dates of the first expert ultrasound exam. For cases where the first ultrasound exam was done at <15 weeks and amniocentesis (AC) was done for diagnosis, we considered the earliest time point of AC, >15 weeks (the earliest recommended time point for AC), as the time of the follow-up expert scan if not otherwise specified. Data on pregnancy outcome and postnatal phenotype were listed where available.

2.3 | cfDNA screening and confirmatory genetic testing

Different assays for cfDNA screening were used, including chromosome-selective sequencing (Harmony®), massively parallel signature sequencing (Praenatalis®, Praena®, Fetalis®), or SNP-

based sequencing (Panorama®), which were used for cfDNA screening.

In Germany, G-banded karyotyping is the first-line test for prenatal diagnosis of chromosomal anomalies on chorionic villus or amniotic fluid samples, and chromosomal microarray (CMA) is carried out as a second-line test for specific indications. For our cohort, CMA was done only in one fetus with low-level mosaicism and a severe cardiac phenotype presenting in our own institution (case 14) using an Illumina HumanCytoSNP-12v2.1 SNP array on an iScan system (Illumina, Inc.). Briefly, 200 ng of genomic DNA were amplified, fractionated, hybridized, and fluorescence-tagged. After scanning of the slides, further analysis was carried out using the software KaryoStudio v1.4 provided by Illumina. Genotype and copy number were calculated by the determination of B-allele frequency and log2 ratio. Genome Build GRCh37 served as a reference database.

For some cases, fluorescence in situ hybridization (FISH) with chromosome-specific probes (X-centromere) for rapid aneuploidy or low-frequency mosaicism detection was performed in different diagnostic laboratories using the individual laboratory's standard conditions.

Prenatal genetic analysis was done on samples obtained by chorionic villus sampling (CVS) with either short-term culture (STC), long-term culture (LTC) or both, by AC, or by cordocentesis (only in case 50). Postnatal karyotype analysis was done on blood samples of the newborn or infant. If more than one diagnostic procedure was performed (e.g., CVS followed by AC), we choose the result "closest to the fetus" as the true fetal karyotype for calculation of the cfDNA screening performance in the following priority: postnatal karyotype = cordocentesis > AC > CVS LTC > CVS STC.

2.4 | Definition of TP, FP, and FN results

Pregnancies with a high-risk cfDNA result were classified as true positives (TP) when the prenatal karyotype on AC samples or postnatal karyotype identified 45,X, a mosaic 45,X, or other X-chromosome variants with or without phenotypic features of fetal Turner syndrome, or when the prenatal karyotype on CVS identified 45,X, a mosaic 45,X, or another X-chromosome variant with associated phenotypic features of Turner syndrome. False positives (FP) were those with high-risk cfDNA screening results that did not meet these criteria and false negatives (FN) included those with confirmed 45,X, a mosaic 45,X, or other X-chromosome variants that had low-risk cfDNA screening results.

2.5 | Data analysis and statistics

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 26. Continuous variables were represented as mean ± standard deviations (SD) and frequencies were listed for categorical variables. A p-value <0.05 was considered statistically significant. Approximately normally distributed continuous variables were analyzed using t-tests with Cohen's d as a standardized effect measure.

3 | RESULTS

3.1 | Data collection

Sixteen expert centers in prenatal ultrasound and associated genetic laboratories participated in the survey. cfDNA screening that includes analysis of SCA became available in Germany in July 2014. We collected data from 58 pregnancies that had been screened for SCA by cfDNA analysis between July 2014 and May 2021. All 58 women carrying these pregnancies received counseling by a Maternal-Fetal Medicine specialist and 41 of them (71%) received additional counseling by a geneticist. Three cases for which a confirmatory karyotype was not available were excluded, leaving 55 for further analysis (Figure 1).

Table 1 includes all collected data on cfDNA results, karyotypes (including sample source), FISH results and CMA results if performed, fetal phenotypic findings from prenatal ultrasound, and pregnancy outcomes on these 55 pregnancies. There were 19/55 (34.5%) women who were lost to follow-up after prenatal diagnostic testing, most of whom (12/19) sought no further specialist care after invasive testing because the cfDNA result was an FP (Table 1).

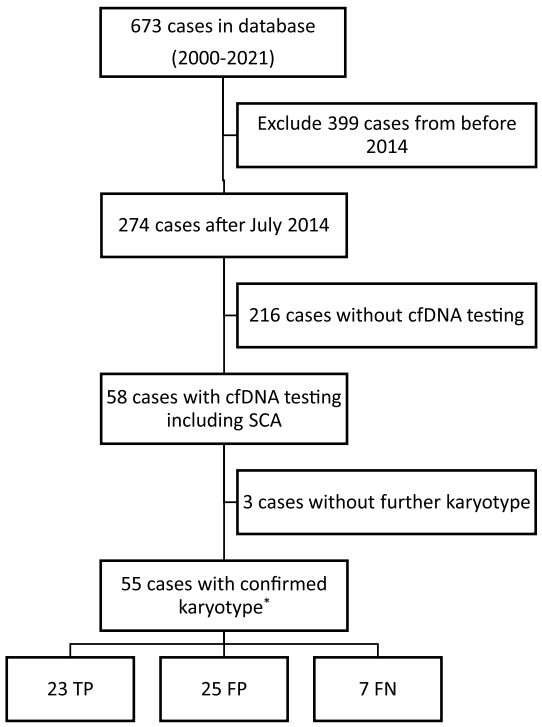

FIGURE 1 Study profile. * mosaic 45,X and other X chromosome variants included.

TABLE 1 cfDNA result, pre- and postnatal karyotypes, and phenotypes.

Nr.	cfDNA result	cfDNA classification	Prenatal karyotype	Postnatal karyotype	Fetal anomalies on US	First and follow-up expert US (weeks/days)	Outcome	Postnatal phenotype
1	45,X	TP	45,X[b]	–	NIHF	14 + 3	LTF	
2	45,X	TP	45,X[a]	–	NIHF, CH	10 + 5	TOP	
3	45,X	TP	45,X[b]	–	NIHF	14 + 0	AB	
4	45,X	TP	45,X (STC)[a]	–	NIHF	12 + 0	TOP	
5	45,X	TP	–	45,X	NIHF	12 + 3	TOP	
6	45,X	TP	45,X[a,b]	–	CHD	13 + 4	TOP	
7	45,X	TP	45,X (STC)[a]	–	NIHF, CH	11 + 5	TOP	
8	45,X	TP	45,X[a]	–	CH	10 + 5	TOP	
9	45,X	TP	45,X[a]	–	CH, pleural effusion	10 + 1	TOP	
10	45,X	TP	45,X[a]	45,X	NIHF, absent DV, dorsal pedal edema	13 + 0 24 + 5 31 + 6	LB	Dorsal pedal edema, BAV GA: 40 weeks W:3710 gr (71.P) H: 53 cm (72.P)
11	45,X	TP	45,X[a]	–	NIHF, CH	13 + 0	LTF	
12	45,X	TP	45,X[b]	–	Dorsal pedal edema	21 + 4	LB	Normal GA: 40 weeks W: 2890 gr (<10.P) H: 50 cm (22.P)
13	45,X	TP	nuc ish(DXZ1x1,DYZ3x0)[34/50],(DXZ1x1,DYZ3x2)[4/50],(DXZ1,DYZ3)x1[8/50],(DXZ1x2,DYZ3x0)[4/50][d] (FISH)[b]; 45,X[b,d]	–	NT > 95th percentile, phenotypic female	13 + 1 16 + 6	TOP	
14	45,X	TP	mos 45,X[8]/46,XX[58][d]; arr[hg19]Xp22.33(93118_838354)1x (SNP-array)[b]	mos 45,X[3]/46,XX[48]	VSD, CoA, short femur, IUGR	13 + 2 22 + 6 33 + 3 37 + 2	LB	VSD, hypoplastic aortic arch, BAV GA: 39 weeks W: 2785 gr (<10.P) H: 48 cm (<10.P)
15	45,X	TP	mos 45,X[8]/46,X?del(X)(q26)[10][b,d]	–	Duplex kidney	16 + 0	LTF	
16	45,X	TP	mos 45,X[25]/47,XXX[8][b,d]	–	Absent DV (no liver bypass)	10 + 6 16 + 1 23 + 6	LB	Normal GA: 39 weeks W: 3005 gr (21.P) H: 52 cm (66.P)
17	45,X	TP	mos 45,X[16]/46,Xr(x)[4][b,d]	–	None	>15	LTF	
18	45,X	TP	mos 45,X[19]/46,Xder(X)[5]/46,XX[6],ish der(X)(XIST-)[b,d]	–	None	18 + 1	LB	No data available
19	45,X	TP	mos 45,X[10]/46,Xder(X)[10][b,d]	–	None	15 + 3	TOP	
20	45,X	TP	–	mos 45,X[1]/46,XX[10]	None	12 + 0	LB	Normal GA: 40 weeks W: 3160 gr (23.P) H: 50 cm (22.P)

TABLE 1 (Continued)

Nr.	cfDNA result	cfDNA classification	Prenatal karyotype	Postnatal karyotype	Fetal anomalies on US	First and follow-up expert US (weeks/days)	Outcome	Postnatal phenotype
21	45,X	TP	mos 45,X[2]/46,X,der(X)[3]/46,XX [55][b,d]	–	None	>15	LB	No data available
22	45,X	TP	mos 45,X[4]/46,XX[16][d] (LTC)[a] 46,XX (STC)[a]	–	None	13 + 6	LB	Widely spaced nipples, GA: 38 + 4 weeks W: 3320 gr (42.P) H: 50 cm (25.P)
23	45,X	TP	–	mos 45,X [3]/46, XX[9]	None	12 + 0	LB	Widely spaced nipples, GA: 39 weeks W: 3420 gr (58.P) H: 47 cm (<10.P)
24	45,X	FP	46,XX (LTC)[a] mos 45,X[7]/46,XX[12][d] (STC)[a]	–	None	11 + 0	LB	
25	45,X	FP	46,XX[b]	–	None	>15	LTF	
26	45,X	FP	46,XX[b]	–	None	13 + 1 >15	LTF	
27	45,X	FP	46,XX[b]	46,XX	None	13 + 1 >15	LB	
28	45,X	FP	46,XX[b]	–	None	13 + 6 >15	LTF	
29	45,X	FP	46,XY[b]	46,XY	None	13 + 5 >15	LB	
30	45,X	FP	46,XX[b]	–	None	10 + 1 15 + 1	LTF	
31	45,X	FP	46,XX[a]		None	9 + 5	LTF	
32	45,X	FP	46,XY[b]	–	None	>15	LTF	
33	45,X	FP	46,XX[b]	–	None	13 + 2 >15	LB	
34	45,X	FP	46,XX[a]	–	None	11 + 6	LB	
35	45,X	FP	46,XX[b]	–	None	14 + 0	LB	
36	45,X	FP	46,XX[a]	–	None	13 + 3	LB	
37	45,X	FP	46,XX[b]	–	None	16 + 6	LTF	
38	45,X	FP	46,XX[b]	–	None	13 + 2 >15	LTF	
39	45,X	FP	46,XX[b]	–	None	13 + 2 >15	LTF	
40	45,X	FP	46,XX[b]	–	None	15 + 4	LTF	
41	45,X	FP	46,XX[b]	–	None	>15	LTF	
42	45,X	FP	46,XX[b]	–	None	>15	LTF	
43	45,X	FP	46,XX[b]	–	None	15 + 4	LB	

(Continues)

TABLE 1 (Continued)

Nr.	cfDNA result	cfDNA classification	Prenatal karyotype	Postnatal karyotype	Fetal anomalies on US	First and follow-up expert US (weeks/days)	Outcome	Postnatal phenotype
44	45,X	FP	–	46,XX	None	10 + 6	LB	
45	45,X	FP	46,XX[b]	–	None	11 + 6 >15	LB	
46	45,X	FP	46,XX[b]	–	None	15 + 2	LB	
47	45,X	FP	46,XX[b]	–	None	12 + 2 >15	LB	
48	45,X	FP	46,XX (STC)[a]	–	None	11 + 6	LB	
49	Normal (XY)	FN	45,X[a]	–	NIHF	13 + 2	LTF	
50	Normal	FN	mos 45,X[14]/46,Xr[16][c,d]	–	IUGR, short long bones	29 + 6	LB	>37 weeks GA (no exact data on GA available) W: 2240 gr H: 44 cm
51	Normal	FN	45,X[a]	45,X	Short long bones, pleural effusion	22 + 1	LB	Normal GA: 38 weeks W: 2320 gr (<10.P) H: 31 cm (<10.P)
52	Normal (XY)	FN	mos 45,X[28]/46,XY[7][b,d]	–	Increased NT	13 + 0 >15	LTF	
53	Normal	FN	45,X[b]	–	NIHF	13 + 5 >15	LTF	
54	Normal	FN	45,X (STC)[a]	–	NIHF	14 + 2	TOP	
55	Normal	FN	–	mos 45,X [11]/ 46,XX [20]	None	No data available	LB	Thrombocytopenia, petechiae, multiple hematomas GA: 40 weeks W: 3845 gr (81.P) H: 51 cm (37.P)

Abbreviations: AB, abortion; BAV, bicuspid aortic valve; cfDNA, cell-free DNA; CH, cystic hygroma; CHD, congenital heart defect; CoA, coarctation of the aorta; DV, Ductus venosus; FN, false negative; FP, false positive; GA, gestational age; H, height; IUGR, intrauterine growth restriction; LB, live birth; LTC, long-term culture; LTF, lost to follow-up; NIHF, non-immune hydrops fetalis; NT, nuchal translucency; P, Percentile; STC, short-term culture; TOP, termination of pregnancy; TP, true positive; US, ultrasound; VSD, ventricle septum defect; W, weight.

[a]Chorionic Villus Sampling (CVS).

[b]Amniocentesis (AC).

[c]Cordocentesis.

[d]Maternal cell contamination (MCC) was excluded.

3.2 | Overall cfDNA screening results and confirmation rate

Of the 55 included cases with cfDNA screening results, 48 (87.2%) were classified as high risk for monosomy X. Of these, 23 (41.8%) were TP and 25 (45.5%) were false positives (FP). There were 7/55 (12.7%) FN (Figure 1).

Cases 4 and 7 (classified as TP), case 48 (classified as FP), and case 54 (classified as FN) warrant further critical discussion. For these cases, we only have results from the CVS STC which, like cfDNA, analyzes the genome of the cytotrophoblast. The cytotrophoblast is embryologically more distant from the fetus than amniocytes or long-term CVS cultures, which contain cells from the mesenchymal core of chorionic villi.[40] Thus, it is possible that the

karyotype from these cultures only represents that of the placenta and not of the fetus. However, cases 4, 7, and 54 were presented with typical fetal anomalies that can be seen in Turner syndrome, non-immune hydrops fetalis and CH, and a non-mosaic 45,X karyotype, supporting that this is indeed the fetal karyotype. Grati et al. suggested that a cfDNA result indicating an increased risk for monosomy X should be interpreted in combination with ultrasound findings and that ultrasound anomalies alone could be enough to provide a PPV.[41] We therefore interpreted results for cases 4 and 7 as TP and for case 54, which had a low-risk cfDNA result, as an FN. Case 48 was classified as an FP based on the short-term CVS culture karyotype results of 46,XX and a normal fetal ultrasound exam, but we note that karyotype results from long-term CVS, AC, or postnatal samples as well as a maternal karyotype were not available. Thus, although this is likely an FP, we cannot fully exclude Turner syndrome. In the group of FP results, there is one case (case 24) with discordance between CVS STC (mos 45,X[7]/46,XX[12]) and LTC (46, XX) results. This supports that the false positive result is caused by CPM, but we unfortunately do not have follow-up results on AC or postnatal genetic diagnostic testing for this fetus.

For cases with a TP result, diagnostic testing showed a 45,X karyotype in 52% and a mosaic 45,X or other X chromosome variants in 48%. The detailed karyotypes are depicted in Table 1.

3.3 | Results for pregnancies with and without fetal anomalies

Ultrasound detected fetal anomalies in 22 of the 55 cases (40%). Of these 22, 16 (72.7%) had TP cfDNA screening results, 6 (27.3%) had FN results, and none had FP results (Figure 2). Of the 16 TP cases with ultrasound anomalies, 12 had monosomy X. Of these 12 fetuses with a non-mosaic 45,X karyotype, 10 had a CH and/or fetal hydrops and the remaining two had a cardiac anomaly (case 6) and dorsal edema of the foot (case 12). The four fetuses with mosaic 45, X or another X chromosome variant presented with other congenital anomalies. These included a duplex kidney in a fetus with a mos 45,X[8]/46,X?del(X)(q26)[10] (case 15), an absent ductus venosus in a fetus with a mos 45,X[25]/47,XXX[8] (case 16), and abnormal first-trimester biochemistry (PAPP-A 0.28 MoM, hCG 5.35 MoM), short long bones, and cardiac anomalies (ventricular septum defect and coarctation of the aorta) in a fetus with mos 45,X[3]/46,XX[48] detected in amniocytes (case 14). This case was particularly interesting because of the severe cardiac phenotype with a very low level of mosaicism. We therefore performed an SNP array to further delineate the cytogenetic findings. Although the B-allele frequencies of chromosome X were suspicious for mosaicism on visual inspection, they were interpreted as within normal limits, below our mosaic detection cutoff of 12.5%. Interestingly, a 745 kb Xp deletion, arr[hg19] Xp22.33(93118_838354)x1, was identified. This region includes the SHOX gene, which is not known to cause fetal congenital heart disease (Figure 3). Because neither the deletion or the low-level 45,X mosaicism could explain the cardiac phenotype, we also offered trio-based exome sequencing but it was declined by the parents. FISH studies on direct preparations of biopsies obtained after delivery from all four quadrants of the placenta showed locally restricted mosaicism: disomy X was found in two quadrants, monosomy X was found in 94 of 100 cells imaged in the third quadrant, and in 6 of 100 cells imaged in the fourth quadrant. Since the mosaicism was identified in amniocytes, we conclude that this most likely represents true fetoplacental mosaicism with possible unequal distribution of mosaic cells in fetal tissues. Whether this might explain the cardiac anomalies remains unclear and would require further evaluation, such as organ-specific assessment of mosaicism. Even if tissues could be obtained, for example, during cardiac surgery, results from such studies may not reflect tissue-specific levels of mosaicism at critical stages of development.

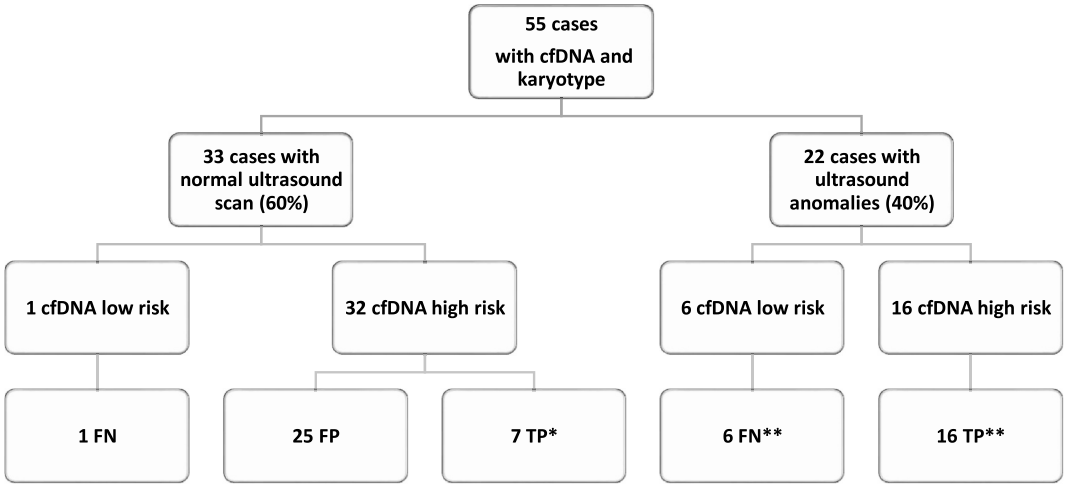

FIGURE 2 Performance of non-invasive prenatal testing depending on anomalies on fetal ultrasound. * All cases are mosaic 45,X and other X chromosome variants. ** 6 cases with mosaic 45,X and other X chromosome variants: 4 TP and 2 FP. cfDNA, cell-free DNA; FN, false negative; FP, false positive; TP, true positive.

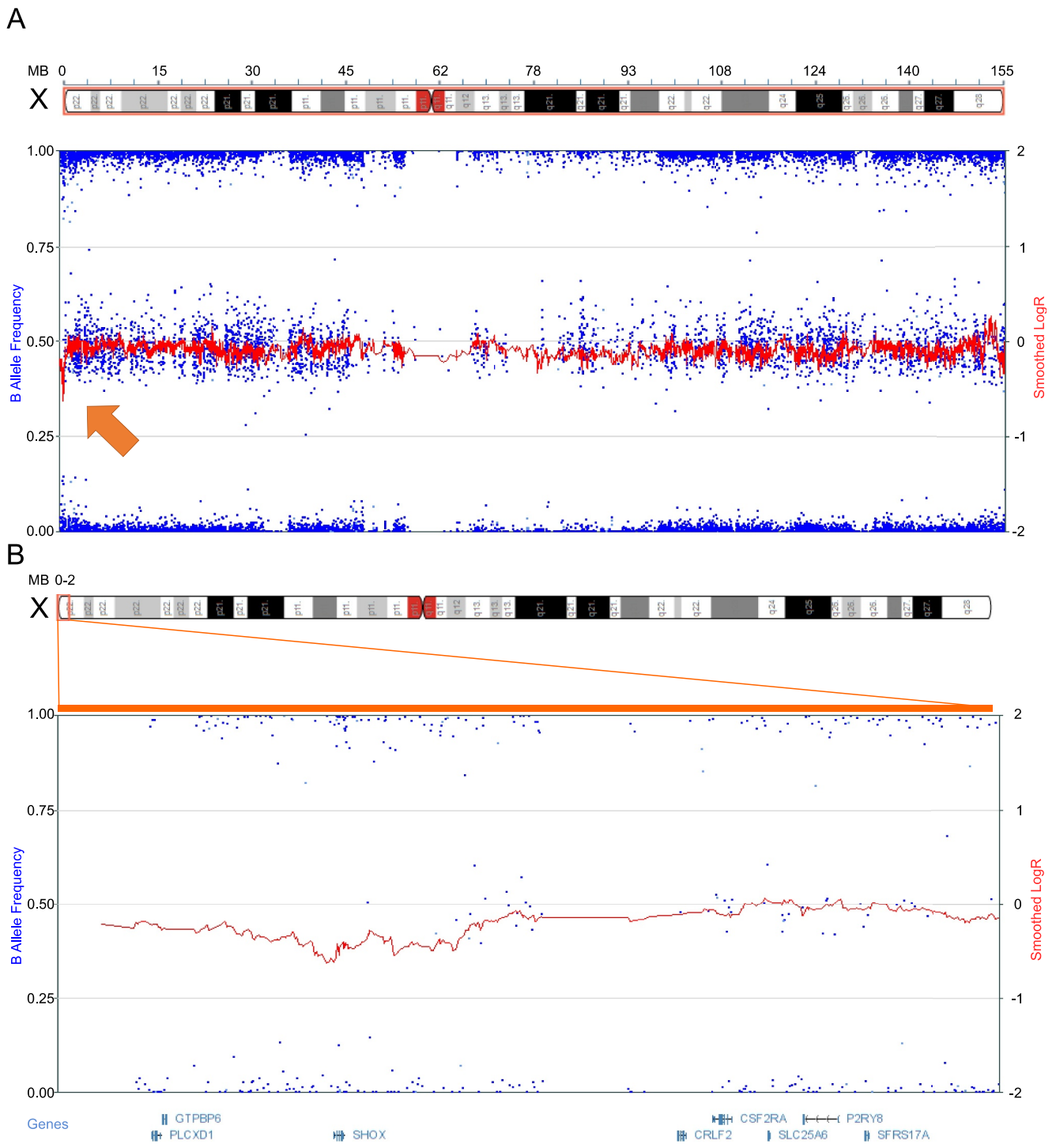

FIGURE 3 SNP-Array (case 14): smoothed LogR ratio (red line) and B-Allele frequency (blue dots) indicate a terminal 745 kb deletion at the telomeric site of the short arm of the X-chromosome arr[hg19] Xp22.33(93118_838354)x1 including *SHOX*. (A) Data of the complete X-chromosome. The orange arrow indicates the small microdeletion, presented in more detail in panel B. (B) Data of the first two Mb of the X chromosome. [Colour figure can be viewed at wileyonlinelibrary.com]

One phenotypically female fetus had an isolated NT > 95th percentile. The first US was done at 13 weeks gestational age (GA) and a follow-up ultrasound was performed at 16 weeks (case 13). Karyotype on cultured amniocytes (with evaluation of 33 mitoses) was 45,X, but the result of FISH on uncultured amniocytes was nuc ish(DXZ1x1,DYZ3x0)[34/50],(DXZ1x1,DYZ3x2)[4/50],(DXZ1,DYZ3)x1[8/50],(DXZ1x2,DYZ3x0)[4/50]. This indicated complex mosaicism of 45,X in 34 cells, 47,XYY in 4 cells, 46 XY in 8 cells, and 46,XX in 4 cells of the 50 cells that were analyzed. As FISH is a sensitive tool to identify low-frequency mosaic cell lines and uncultured amniocytes

are less prone to culture artifacts, we classified this case after extensive discussions with our geneticists as a mosaic monosomy X.[42–44] Ultrasound in the first trimester did not show evidence of a vanishing twin. The patient opted for pregnancy termination at 19 weeks GA (Table 1).

Ultrasound was normal in 33 of the 55 cases (60%) for which there were cfDNA screening results. In 32/33 (97%), the cfDNA screening result indicated an increased risk for monosomy X. Of those 32, there were 25 (78.1%) FP and 7 (21.9%) TP. All TP had a mosaic 45,X or another X chromosome variant, and none had a 45,X karyotype (Table 1). For one case (Table 1, case 55) without fetal anomalies and a low-risk DNA screening result (1/33; 3%), an incidental diagnosis of a mosaic 45,X (mos 45,X[11]/46,XX[20]) was made after birth during a genetic work-up for thrombocytopenia of unknown origin. Thus, the cfDNA screening result for this pregnancy was an FN (Figure 2).

3.4 | Diagnostic testing after a high-risk cfDNA result

Forty-four of the 48 women with a high-risk cfDNA screening result opted for prenatal genetic diagnostic testing. For 15 of those, fetal anomalies were also detected by prenatal ultrasound and the suspected diagnosis was confirmed for all 15. For 29, there were no prenatally detected fetal anomalies and the suspected diagnosis of Turner syndrome was only confirmed in 5.

In four of the 48 cases, genetic testing was deferred until after birth or termination of pregnancy (TOP). For one of these four, there was non-immune fetal hydrops and the parents opted for TOP without prenatal genetic diagnostic confirmation. Karyotype analysis on the product of conception was 45,X (case 5). For the three other cases (20,23,44), prenatal ultrasound did not show fetal anomalies and parents decided for postnatal genetic diagnostic testing. For cases 20 and 23, the postnatal karyotype showed a mosaic 45,X, while case 44 was an FP and the newborn had a 46,XX karyotype.

3.5 | Maternal age in TP and FP cases

The mean maternal age of the whole group was 34.8 years (range 24–47 years). There was no significant difference in maternal age between the group with TP results (34 ± 4 years; $n = 23$) and the group with FP results (35.2 ± 4 years; $n = 25$) (t-test: $d = 0.3$; $p = 0.305$). In the group of false positives, maternal karyotype was done in 3 cases; all were normal 46,XX.

3.6 | Postnatal confirmation of prenatal karyotype in liveborn females with Turner syndrome

International guidelines recommend postnatal confirmation of constitutional karyotype of prenatally diagnosed fetuses with Turner syndrome.[6] In our study, this was done only for three fetuses (cases 10, 14, and 51). In cases 10 (previously reported)[45] and 51, the pre- and postnatal karyotypes were 45,X. In case 14, the prenatal karyotype was mos 45,X/46,XX that was confirmed after birth. In cases 20, 23, and 55, karyotyping was deferred until after birth. Cases 20 and 23 had a high-risk cfDNA result in a fetus without ultrasound anomalies, and the postnatal karyotype of newborn blood leukocytes was mos 45,X/46,XX for both. For case 55, with a low-risk cfDNA result and no fetal anomalies on ultrasound, the postnatal karyotype also was mos 45,X/46,XX (Table 1).

4 | DISCUSSION

The primary goal of this retrospective survey study was to add data on the implications of cfDNA screening results indicating a high risk for monosomy X in fetuses with or without prenatally detected anomalies. Our key findings are that after a high-risk cfDNA screening result for fetal monosomy X, the diagnosis of monosomy X, mosaic 45,X, or other X-chromosome variants was confirmed for less than 50% of fetuses. When there were fetal anomalies detected by prenatal ultrasound, all were confirmed to be true positives. In contrast, when there were no fetal anomalies, only 22% were confirmed, and all had mosaicism or an X-chromosome variant, highlighting the importance of a meticulous prenatal ultrasound. This data adds valuable information for counseling of women undergoing cfDNA-based aneuploidy screening with a test that includes the analysis of sex chromosomes.

An increased risk for sex chromosome anomaly on cfDNA screening is detected for 0.3%–0.6% of pregnancies, but—as found in our data—the FPR is high, particularly when cfDNA results show an increased risk for monosomy X.[31,46–49] This potential for FP results should be addressed in pre-test counseling because it can cause parental anxiety while awaiting confirmatory diagnostic testing results and may influence emotional well-being for the remainder of the pregnancy.[38,50,51] The data from our study on the impact of fetal anomalies on the confirmation rate of high-risk cfDNA screening results for monosomy X are useful for pre- and posttest counseling, and knowledge about fetal anomalies can help direct the choice of confirmatory genetic testing.

A high-risk cfDNA result for monosomy X can indicate an affected fetus, an unknown maternal sex chromosome abnormality, fetoplacental mosaicism for SCA, or CPM for SCA, which is more common for monosomy X than for trisomy 18 and 21.[40,41,46] Because of the higher chance for CPM, AC is the confirmatory test of choice when the prenatal ultrasound is normal.[41,46,52] When there are fetal anomalies, the rate of CPM drops to about 2%, and CVS, ideally with long- and STC, can still be offered if a faster result is desired in the first trimester.[41,52] If prenatal diagnostic testing is declined, confirmatory testing can be done on cord or newborn blood after birth.[53]

Several factors complicate prenatal counseling when cfDNA screening finds an increased risk for monosomy X. The contribution of a fetal 45,X karyotype versus mosaic 45,X and other X chromosome variants and how they correlate with the presence, severity, or

absence of fetal anomalies on ultrasound or predict the postnatal phenotype have not been extensively studied.[54] It is not well known whether the prenatal detection of fetal anomalies by ultrasound affects the uptake of invasive testing after a high-risk cfDNA result for monosomy X, and data are also limited on the outcome of these pregnancies.[55,56] An increased risk for monosomy X on cfDNA screening associated with a normal fetal karyotype is sometimes caused by maternal X-chromosome abnormalities. Our survey-based multicenter retrospective study on 55 pregnancies with monosomy X on cfDNA screening aimed to add data that can help close some of these knowledge gaps.

First, we evaluated the performance of cfDNA screening for Turner syndrome in this mixed-risk population in general and in relation to prenatal ultrasound findings. We found that a high-risk cfDNA result was confirmed by diagnostic genetic testing more often than previously reported in most studies.[21–26,29,50,57,58] This is not surprising, since there was a relatively high number of fetuses in our cohort that had anomalies identified by ultrasound. This is also consistent with findings by Grati et al. and Sandow et al.[41,59] and has also been shown for trisomy 13 and 18.[60] It was also not surprising that in the absence of fetal anomalies, a high-risk cfDNA result for monosomy X was less often confirmed by diagnostic genetic testing (TP 7/32, 22%), but the finding that all TP in this group without fetal anomalies had mosaic 45,X or other X-chromosome variants and none had a non-mosaic 45,X karyotype is interesting and useful for counseling. Without the cfDNA screening findings, these cases would not have been ascertained prenatally and would likely also have evaded neonatal detection. Thus, cfDNA screening provided an opportunity for early detection and initiation of appropriate health surveillance. This information can inform pretest counseling and aid parental decision-making on inclusion of screening for SCA when cfDNA screening for common autosomal trisomies is considered. The benefit of early detection on timely treatment and reduction of long-term complications and co-morbidities in an affected girl should be balanced against the high rate of FP results.[61,62]

Second, we analyzed the detected karyotypes in more detail because it has been reported that approximately 50% of girls with Turner syndrome ascertained after birth have a 45,X karyotype and the remainder have mosaic forms with 46,XX, 47,XXX, or 46,XY cell lines or have structural anomalies on one of their X chromosomes.[6,11] Furthermore, in a retrospective review of 28 patients with an abnormal cfDNA result for monosomy X, additional discrepant structural sex chromosome abnormalities, including translocations, isochromosomes, deletions, rings, markers, and uniparental disomy, were found. Such findings may change the expected phenotype emphasizing the need of confirmatory genetic testing.[54] In a review of the literature on the frequency of prenatally ascertained karyotypes after a high-risk cfDNA result for Turner syndrome, the distribution of findings after confirmatory testing was 45,X in 67%, mosaic 45,X/46,XX in 20%, mosaic 45,X/46,XY in 10%, and X chromosome rearrangements in 3%.[31] The findings in our study are consistent with these results. Interestingly, we found that none of the fetuses with a mosaic 45,X or another X chromosome variant had fetal hydrops or CH, and for the majority, no fetal anomalies were detected by prenatal ultrasound. When congenital anomalies were present, they were relatively mild or not typical for Turner syndrome. Delayed diagnosis in girls with mosaic and variant forms of Turner syndrome is well described.[1,63] This impedes screening for significant complications that can occur later in life, such as aortic root dilatation, in girls and women with these forms of Turner syndrome. Yet, our data and that of others indicate that prenatal cfDNA screening has limited performance. Thus, further studies with larger numbers on a less-selected cohort will be needed.

Third, we were interested in the uptake of invasive testing after a high-risk cfDNA result for monosomy X and the outcome of pregnancies with confirmed Turner syndrome. Most women with a high risk for monosomy X result elected to have invasive diagnostic testing, even when the ultrasound exam was normal. In most cases with a normal ultrasound, the fetal karyotype was normal. However, for those with a confirmed diagnosis of fetal Turner syndrome, counseling is complex and information on the prognosis often cannot be precise due to the limited phenotype-genotype correlation, in particular for fetuses without congenital anomalies and/or with karyotypes other than 45,X.[9] In addition, other factors, such as epigenetic modifications, that could influence phenotypes are not currently included in prenatal testing and counseling.[61,64] Thus, the benefit of timely surveillance and treatment of affected girls after early detection because of cfDNA screening results must be balanced against the chance that the high-risk cfDNA screening result leads to unnecessary invasive testing, increase in time-consuming and complex counseling, and potentially termination of pregnancies with fetuses with mild phenotypes. We found that in our cohort, the overall rate of elective pregnancy termination was 39.1% for prenatally detected cases of Turner syndrome. Consistent with other studies,[65] it was more frequent (58.3%) with a 45,X karyotype and with "severe" fetal phenotypes. In contrast, only 2 of 11 (18.2%) pregnancies with an X-chromosome variant were terminated. We could not gather further information on parental decision making for one fetus with no anomalies, and a mos 45,X[10]/46,Xder(X)[10] karyotype (case 19). Another fetus with only an isolated enlarged NT > 95th percentile at 13 + 1 week GA and female external genitalia had a complex mosaic karyotype in chorionic villi and amniocytes (case 13). The parents for this pregnancy opted for termination, reportedly out of concern for decreased fertility, stigmatization, and an unpredictable future phenotype.

Finally, we examined if advanced maternal age and the associated physiological mosaic somatic loss of one maternal X chromosome influenced the FPR of cfDNA screening. Bianchi et al. reported on 18,000 samples with available results for SCA a significantly higher maternal age in discordant cfDNA screening results for monosomy X (36.7 vs. 31.7 years of age). Sandow et al., comparing two age groups (≤38 years of age and >38 years), found a more advanced maternal age in the false positive group that did not reach significance.[29,59] Our data showed that maternal age was higher for those with an FP cfDNA screening result, but the difference did not reach statistical significance. This may be due to a combination of the small sample size in our study, and as proposed by Mardy and Norton, the fact that the age-

related X chromosome loss is too small (2% at the age 40) to influence cfDNA results for monosomy X.[28,53] Other maternal X chromosome anomalies can also be the reason for an FP cfDNA result.[25,66,67] Samango-Sprouse found X chromosome anomalies in 1:1378 of reproductive age women with advanced maternal age.[68] It has been reported that SNP-based cfDNA testing can differentiate between maternal or fetal origin of the SCA but we were not able to ascertain this in our study.[69] Cell-based non-invasive prenatal testing has been proposed by others as a possible future option to avoid false positive results originating from the maternal fraction of cfDNA, but this has not yet been confirmed in large studies.[70] Currently, maternal chromosome analysis is recommended when there is an FP test result for SCA with a normal fetal ultrasound.[66] Unfortunately, this was done inconsistently in our cohort, and we cannot comment on the rate of abnormal maternal karyotype as a reason for false positive results. We conclude that our data add to those published and support the importance of including the possibility of maternal X chromosome anomalies in pre-test counseling for cfDNA for SCA.

Our study has several limitations. Calculations for this study are not based on a fully screened population but extracted from a database exclusively focused on cases with suspicion or diagnosis of Turner syndrome, limiting the ability to assess the PPV of cfDNA screening for SCA. Confirmation of the prenatal karyotype after birth was rarely reported, which may reflect practice patterns in Germany. Because the constitutional karyotype may differ from that obtained from chorionic villi or amniocytes, in particular for mosaic sex chromosome abnormalities, we are making efforts to raise awareness about the benefits of postnatal karyotype confirmation.[6,71,72] We know that CPM, which is reported in 2% of all CVS and is higher for monosomy X in the absence of fetal anomalies, is an important source of false positive results in cfDNA-based screening for SCA, particularly for monosomy X.[40,46] Unfortunately, because this was a retrospective survey, we do not have genetic information from placental biopsies for all cases and can therefore not comment on the rate of CPM as a source of FP results in our study. Maternal karyotype was offered inconsistently, and therefore, we could not calculate the contribution of abnormal maternal karyotype as a source of FP results in our study. Another limitation of our study is that we do not have information on the Z-scores used to interpret the various cfDNA screening results. One group investigated if a higher *Z* score improves the accuracy of the cfDNA screening assay and reported that the accuracy of monosomy X prediction was increased on a small number of cases, but this was not confirmed by others.[21,73] Thus, this issue requires more data. Our study is also limited by its retrospective nature, possible bias resulting from inclusion of a heterogenous, and selected cohort, small sample size, and incomplete data provided for some of the cases.

5 | CONCLUSION

It has been well reported that cfDNA screening for monosomy X has a lower PPV than for the common aneuploidies, and confirmatory genetic testing is always indicated when there is a high-risk cfDNA result for SCA, in particular monosomy X. In this retrospective cohort, the uptake of invasive diagnostic testing after a high-risk cfDNA screening result for monosomy X was high, and diagnostic findings varied based on the presence or absence of fetal anomalies, highlighting the importance of a detailed ultrasound that includes the evaluation of presence of "minor findings" for all such cases. We found that in cases with fetal abnormalities, a high-risk cfDNA result for monosomy X is more likely to be confirmed by diagnostic testing than for cases with a normal prenatal ultrasound. For those, the fetal karyotype is most often diploid 46,XX, and when there is an abnormal karyotype, it is most often either mosaic monosomy X or a structural variant of one of the X chromosomes. This information is valuable for genetic counseling since those often have a more favorable postnatal outcome, but ongoing prospective data collection should be done to confirm these observations in larger cohorts to confirm these results.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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