frontiers | Research Topics

Unravelling the basis of non-invasive prenatal screening results

Edited by
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Unravelling the basis of non-invasive prenatal screening results

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Editorial: Unravelling the basis of non-invasive prenatal screening results

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prenatal diagnosis, non-invasive prenatal screening, discordant results, fetoplacental chromosomal mosaicism, twin pregnancies

Editorial on the Research Topic
Unravelling the basis of non-invasive prenatal screening results

The presence of circulating cell-free DNA (cfDNA) from the placenta in the maternal circulation was first demonstrated by Lo et al. (Lo et al., 1997). Since its commercial launch in 2011, cfDNA-based non-invasive prenatal testing (NIPT) has permitted screening for T21, T18, and T13 with high specificity and sensitivity in both high-and low-risk populations (La Verde et al., 2021).

Circulating cell-free DNA in pregnant women is a mixture of maternal and placental cell-free DNA, in which the maternal fraction is on average ten times the fetal one (fetal fraction, FF). Hence, false-positive, false-negative as well as non-reportable cases exist and may due to technical issues or may be attributable to biological causes such as low fetal fraction, feto-placental mosaicism, or vanishing twin (Grati, 2014; 2016; Bianchi and Chiu, 2018; Deng and Liu, 2022). This Research Topic Unravelling the basis of non-invasive prenatal screening results collect some recent papers focused on discordances between non-invasive prenatal screening result and fetal karyotype with emphasis on chromosomal mosaicisms. Chromosomal mosaicism (CM) is a biological phenomenon in human and is found in approximately 1%–4% of prenatal diagnosis performed by chorionic villus sampling and in about 0.1%–0.3% of amniocentesis (Hsu et al., 1996; Grati et al., 2017; Lund et al., 2020). As reported by Li et al., CM is still one of the main difficult Research Topic in prenatal diagnosis due to the uncertainty outcome, especially when fetal ultrasonographic features appear normal and the use of multiple methods, such as a combination of karyotyping, and fluorescent *in situ* hybridization (FISH) was recommended. Moreover, CMA combined with karyotyping can be recommended as the preferred method of prenatal diagnosis for cases where NIPS results indicate a high risk in pregnancy as suggested by Bu et al. In this context the classic karyotype analysis and NIPT analysis are limited in determining the mosaic sex chromosomal abnormalities (Ma et al., 2021). On the contrary, single nucleotide polymorphism (SNP) array is validated in detecting the chromosomal syndromes, mosaic chromosomal syndromes as well as chromosomal deletions/duplications with high accuracy and high resolution (Samango-Sprouse et al., 2013). Wang et al. reported a retrospective investigation of sex chromosomes anomalies in Fujian Province cohort by SNP array, showing the importance of

using different technologies to define segmental aneuploidies. False negative NIPT results, that have the highest clinical impact on patients and clinicians, are mainly due to placental mosaicisms. Feresin et al., reported two cases of feto-placental mosaicism of trisomy 21, both with a low-risk NIPT result, identified by ultrasound signs and a subsequent amniocentesis consistent with a trisomy 21. In both cases, cytogenetic and/or cytogenomic analyses were performed on the placenta and fetal tissues, showing in the first case a mosaicism of trisomy 21 in both the placenta and the fetus, but a mosaicism in the placenta and a complete trisomy 21 in the second case. In addition, Bonanni et al., reported a case of CPM in which a NIPT false-positive result for trisomy 13 required two further invasive diagnostic tests–an amniocentesis and a cordocentesis–to rule out the fetal aneuploidy. In this paper the authors showed that given the trophoblastic origin of cf-DNA, NIPT is a screening test and the real benefit of cfDNA analysis lies, therefore, in its complementary use with ultrasound scan, Therefore, NIPT remains a powerful tool allowing non-invasive access to the cytotrophoblast. In this regard, Kleinfinger et al. showed that genome-wide NIPT can be used to characterize the supernumerary marker chromosomes (SMCs) revealed by karyotyping of chorionic villi, effectively guiding the choice of further genomic analyses and reducing the period of uncertainty for the patient. They were able to carry out targeted FISH resulting in rapid, effective, and accurate characterization of the SMCs and their distribution in the fetoplacental unit, ultimately allowing determination of their clinical significance. In contrast to chorionic villus sampling (CVS), an invasive diagnostic technique that samples a small region of the placenta, NIPT noninvasively assesses the genetic status of the cytotrophoblast as a whole. These cases emphasize the need for accurate and complete pre-test NIPT counselling, as well as for molecular studies of placenta and fetal tissue in order to discriminate between placental, fetal and feto-placental mosaicism, and between complete or mosaic fetal chromosomal anomalies.

As the cfDNA in the maternal plasma fraction originates from the cytotrophoblast of chorionic villi (CV), a high-risk call for a rare autosomal aneuploidy (RAA) may be indicative of confined placental mosaicism (CPM) and not true fetal aneuploidy. In more recent years, the use of cfDNA screening has been expanded to genome-wide screening for RAAs and partial deletions and duplications (i.e., copy number variants, including selected microdeletions) and an increasing number of studies have described the test performance and the clinical validity of these applications (Pescia et al., 2017; Pertile et al., 2021; Soster et al., 2021; van Prooyen Schuurman et al., 2022). The screen-positive rate for RAAs has been shown to range from 0.12% (Scott et al., 2018) to 1.1% (Van Opstal et al., 2020). In this Research Topic Mossfield et al. described a cohort of pregnancies with a NIPT high risk result for the presence of a RAA. Follow up information was available in 68% (74/109) of the patients with a concordance rate of 20.3%, i.e., the presence of a RAA was confirmed in 15/74. Intrauterine fetal demise, fetal growth restriction, and preterm birth, were observed both in patients with fetal or placental confirmation of the presence of a RAA, as well as patients that did not undergo fetal and/or placental diagnostic testing. Furthermore, the Authors proposed that genome-wide cfDNA screening for RAA can in some cases provide useful information for pregnancy management and counselling giving a possible explanation for adverse pregnancy outcome.

Although the recent ACMG guidelines note that at this time there is insufficient evidence to either recommend or not recommend NIPT for the identification of RAA and CNV (Dungan et al., 2023), and the ISPD position statement not recommend NIPT for the identification of RAA and CNV for the routine care of unselected populations (Hui et al., 2023), some studies explored the attitudes and preferences of patients regarding expanded NIPT. In this Research Topic Dubois et al. examined the attitudes and preferences on expanded NIPT of pregnant women having first-tier cfDNA screening at a private prenatal clinic in Canada, including the main factors influencing the decision-making process undergoing expanded cfDNA screening. Their findings suggest that with appropriate pre-test counseling, pregnant women may choose NIPT for an expanding list of conditions, even if, they should be made aware of both the benefits and limitations of expanded NIPT and the possibility of discordant/inconclusive results.

Therefore, development of reliable synthetic materials available for NIPS is necessary for validation steps and quality assessment in laboratories providing this test. Although synthetic positive plasmas are commercially available, they are usually insufficient for the initial validation due to limited abnormality types and sample quantity. In the paper Qi et al., described a simple method of making synthetic positive plasmas that are reliable and excellent alternatives of positive maternal plasmas for validation and monitoring NIPS performance.

Another interesting topic is the application of NIPT in multiple pregnancies. The rates of twin pregnancies have increased over the last four decades in many countries, likely due to several factors including increased maternal age at birth and the increased use of assisted reproductive techniques (Pison et al., 2015; Palomaki et al., 2021). Multifetal pregnancies are at increased risk for a broad range of pregnancy complications and adverse outcomes, and the primary associated risk factor for a poor pregnancy outcome in twin pregnancies is the chorionicity. Zygosity can be established using NIPT and this can be particularly useful when there are concerns about chorionicity or determining whether one *versus* two fetuses are affected (Norwitz et al., 2019; Benn and Rebarber, 2021). Guo et al., presented a rare case in which an IVF-ET twin pregnancy gave birth to a partial trisomy 21 chimera girl in which both Nuchal translucency (NT) and NIPT had limitations in detecting the trisomy 21 mosaicism in a twin pregnancy. Hence, the results from this case report indicate that IVF-ET pregnancies should be strictly monitored by ultrasound and obstetric follow up also to exclude false negative results.

Author contributions

LD, EP, ZQ, and AN contributed equally to the conceptualization, methodology, and writing (original draft and editing) of this editorial. All authors contributed to the article and approved the submitted version.

Conflict of interest

Author LD is employed by AMES.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Case Report: Twin Pregnancy Gives Birth to a Girl with Partial Trisomy 21 Mosaicism after *in vitro* Fertilization and Embryo Transfer

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Objective: To report a rare case in which an IVF-ET twin pregnancy gave birth to a partial trisomy 21 chimera girl.

Design: Case report.

Setting: University hospital.

Patient: A girl with partial trisomy 21 mosaicism after *in vitro* fertilization and embryo transfer.

Interventions: *In vitro* fertilization (IVF) and embryo transfer (ET).

Main Outcome Measure: Karyotype analysis, Copy Number Variation sequencing (CNV-seq), stLFR-WGS, and Short Tandem Repeat (STR) analysis.

Results: Being assisted with IVF and EF technology, the couple successfully gave birth to twin sisters at 37 weeks of gestational age. The NonInvasive Prenatal Testing (NIPT) and Nuchal Translucency (NT) examination showed no detectable genetic abnormalities during pregnancy. However, the younger infant displayed growth retardation and feeding difficulties after birth, which was not observed in her twin sister. Further genetic counseling and diagnosis suggested that she is a Chimera with complex partial trisomy 21. The stLFR-WGS assay showed multiple CNV variations in Chr21 and STR analysis confirmed the paternal origin of the additional fragments.

Conclusion: It is rare for IVF-ET-assisted twin pregnancy to give birth to a girl with a complex combination of abnormal Chr21, which might result from paternal chromosome rearrangement during meiosis and mitosis.

Keywords: IVF-ET, NIPT, chromosome defects, twin pregnancy, down syndrome, case report

INTRODUCTION

After the first "test-tube baby," Louise Brown was born after conception by *in vitro* fertilization experiment (IVF) in 1978 (Steptoe and Edwards, 1978). IVF- and intracytoplasmic sperm injection (ICSI)-based Assisted Reproductive Technology (ART) development has rapidly soared (Bonduelle et al., 2005; Dyer et al., 2016; Fishel, 2018; Saito et al., 2018). Since 1978, millions of babies were born by ART, marking the technology a widespread alternative for treating human infertility in the past decades (Meldrum, 2013; Sunderam et al., 2015; Johnson, 2019). However, despite the prevalence of ART, it is unclear if IVF or ICSI increased the risk of congenital disabilities in newborns. Aneuploidy is the most common genetic abnormality and considered the leading cause of implantation failure, miscarriage, and congenital disabilities (Nagaoka et al., 2012; MacLennan et al., 2015). The high frequency of aneuploidy in IVF-produced embryos was thought to be one of the main reasons affecting the implantation and pregnancy rates (Franasiak et al., 2014).

To address the issue, researchers developed the preimplantation genetic testing for aneuploidies (PGT-A), which can analyze the embryonic chromosomal status, do embryo selection prior to transfer, and thereby allow implantation of genetically normal embryos (Brezina and Kutteh, 2015). Many methods have been successfully applied in the PGT-A process, such as fluorescence *in situ* hybridization (FISH) on fixed cells, array comparative genomic hybridization (aCGH), digital polymerase chain reaction (dPCR), single-nucleotide polymorphism (SNP) array, real-time quantitative PCR (qPCR), and next generation sequencing (NGS) (Sato et al., 2019). Nevertheless, PGT-A was mainly proposed for advanced maternal age (AMA), defined as ≥ 37 years; repeated implantation failure (RIF); history of recurrent miscarriage (RM); and severe male factor infertility (Rubio et al., 2019).

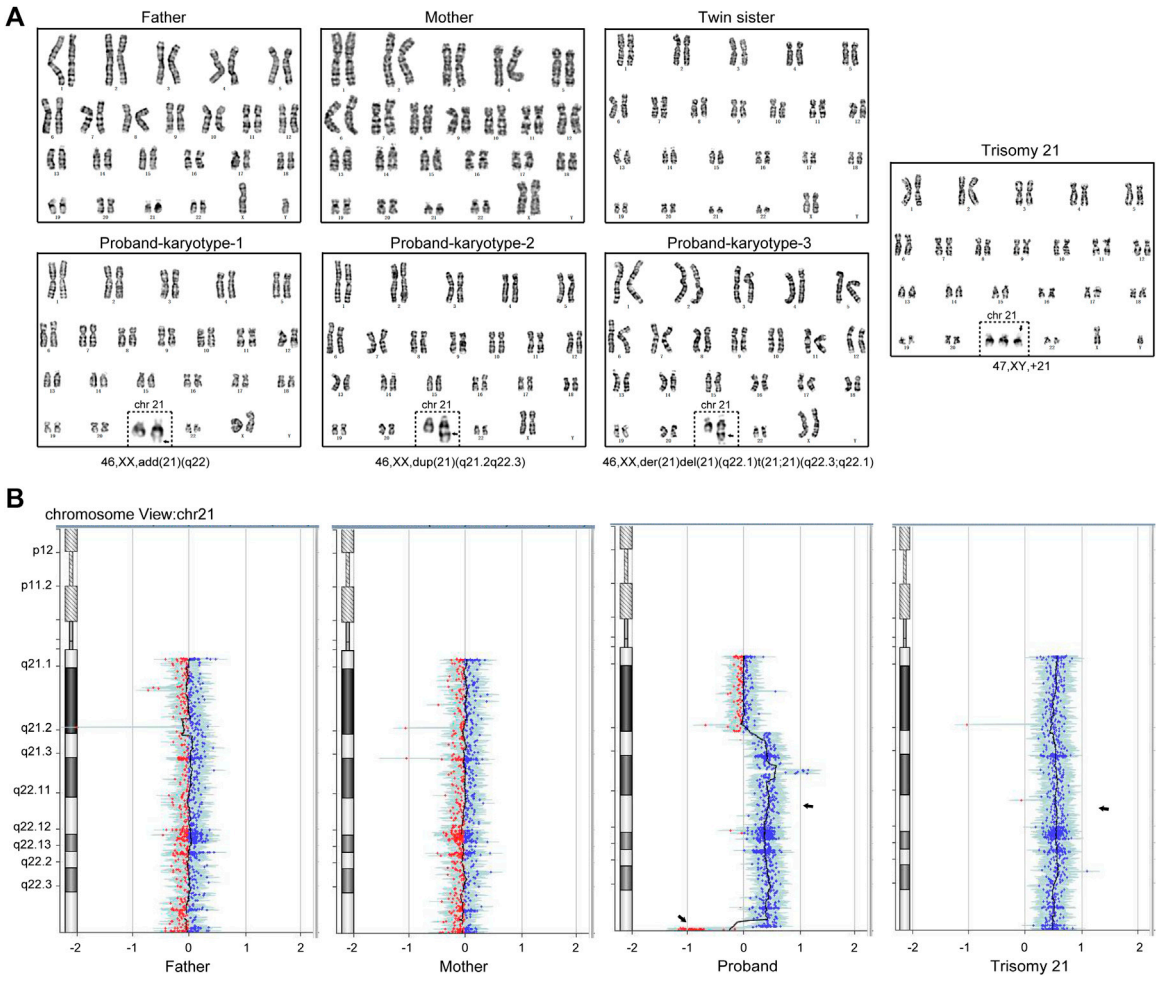
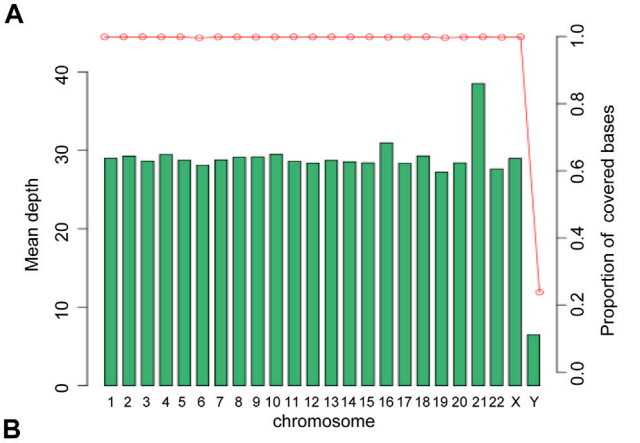


FIGURE 1 | A couple gave birth to a Chimeric child with a partial trisomy 21 after IVF and EF. **(A)** Giemsa staining and analysis of the cultured peripheral blood mononuclear cells in the family members, in which the proband exhibits the combination of three karyotypes: 46,XX, add (21) (q22), 46,XX, dup (21) (q21.2q22.3) and 46,XX, der (21)del (21) (q22.1)t (21; 21) (q22.3; q22.1). **(B)** Copy number variation sequencing (CNV-seq) showed the copy number of Chr21 (q21.2-q22.3) was 2–3. The Trisomy 21 patient karyotype and CNV-seq results were used as positive control.



B

stLFR-CNV

Chr	SegStart	SegEnd	Type	SegLen
>chr21	15460410	15461714	DEL	1305
>chr21	23654972	23666251	DEL	11280
>chr21	23689282	23717881	DEL	28600
>chr21	24508355	30045081	DUP	5536727
>chr21	30250089	42366171	DUP	12116083
>chr21	42413890	42845910	DUP	432021
>chr21	42845911	42847630	DEL	1720
>chr21	44713162	47054760	DUP	2341599
>chr21	47158521	47159520	DEL	1000
>chr21	47387959	47389558	DEL	1600
>chr21	47389559	47842328	DUP	452770

FIGURE 2 | The stLFR whole-genome sequencing result showed multiple duplications and deletions in the proband's Chr21. **(A)** The mean depth distribution in different chromosomes of the proband. **(B)** Detailed copy number variations (CNVs) in the proband's Chr21 summarized from the genome sequencing comparison.

Trisomy 21, or Down syndrome, is one of the most commonly occurring aneuploidies caused by the presence of all or part of an extra chromosome 21. Trisomy 21 is manifested by multiple phenotypes, including intellectual disability, congenital heart defects, and muscle hypotonia (Hecht and Hook, 1996; Antonarakis et al., 2004; Mazurek and Wyka, 2015; Antonarakis, 2017; de Graaf et al., 2017; Reeves et al., 2019; Bull, 2020; de Graaf et al., 2021). Advanced maternal age, environmental factors, as well as meiotic and mitotic errors are the main risk factors for trisomy 21 (Antonarakis et al., 1992; Torfs and Christianson, 2003; Allen et al., 2009; Ghosh et al., 2009; Nagaoka et al., 2012; Hunter et al., 2013; Coppede, 2016; Gruhn et al., 2019; Keen et al., 2020). Here, we present a rare case about a young couple with normal karyotypes who underwent IVF and ET assisted pregnancy. The twin pregnancy gave birth to a partial trisomy 21 female with a complex karyotype and multiple CNVs. Further analysis suggested a paternal origin of the extra chromosome 21 fragment.

MATERIALS AND METHODS

In Vitro Fertilization and Embryo Transfer

In vitro fertilization (IVF) was carried out based on the standard long protocol. First, pituitary suppression was achieved by administering 3.75 mg triptorelin acetate (Ipsen Pharma Biotech, Paris, France). When the patient reached the criteria for pituitary suppression (Fang et al., 2020; Hu et al., 2020), ovarian stimulation was initiated with gonadotropin (Gonal-F, Merck Serono, Geneva, Switzerland; Puregon, Organon, Oss, The Netherlands). When one primary follicle diameter was ≥20 mm and at least two follicles reached 18 mm, hCG (Ovitrelle, Merck Serono) was injected to trigger oocyte maturation. Follicle aspiration was conducted through transvaginal ultrasound 36–38 h after hCG administration. Retrieved eggs were fertilized and checked after incubation with the sperm from the husband, in which two good-quality cleavage embryos were picked up and transferred transcervical under ultrasound guidance. Biochemical pregnancy was determined by the serum β-Hcg concentration increase at 14 days after ET, and clinical pregnancy was defined by the presence of a gestational sac by abdominal ultrasound at 35 days after ET.

Copy Number Variation Sequencing

Fifty nanograms of DNA extracted from peripheral blood was fragmented. DNA libraries were constructed by end filling, adapter ligation, and PCR amplification. DNA libraries were then subjected to massively parallel sequencing on the NextSeq 500 platform (Illumina, San Diego, CA) to generate approximately 5 million raw sequencing reads with genomic DNA sequences of 36 bp in length. The hg19 genomic sequence was used as a reference. A total of 2.8–3.2 million reads were mapped using the BurrowseWheeler algorithm. Mapped reads were allocated progressively to 20-kb bin sizes from the p to q arms of the 24 chromosomes. Counts in each bin were compared between all test samples run in the same flow cell to evaluate copy number changes using previously described algorithms. Sprinkle, a comprehensive tool developed by BerryGenomics, was used for CNV calling. The CNVs were interrogated against publicly available databases, including Decipher, Database of Genomic Variants (DGV), 1,000 genomes, and Online Mendelian Inheritance in Man (OMIM), and their pathogenicity was assessed according to the guidelines outlined by the American College of Medical Genetics (ACMG) for interpretation of sequence variants.

Karyotype Analysis

Peripheral blood samples from the proband were cultured using the standard technique. G banding assay was used for analysis, and more than 100 metaphase chromosome images were captured and investigated for the Chimera.

stLFR Whole Genome Sequencing

Peripheral blood genomic DNA extracted from the proband was quantified using the dsDNA BR assay on a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The stLFR technology used Tn5 transposase to construct DNA libraries according to the standard protocol using the MGIEasy stLFR Library Preparation kit v1.1 (PN: 1000005622)*. In brief, the transposon integrated DNAs were hybridized with magnetic beads containing multi-copy molecular barcodes by the principle of DNA double-strand complementation adapter and sequenced on the BGISEQ-500 sequencer. The original data was

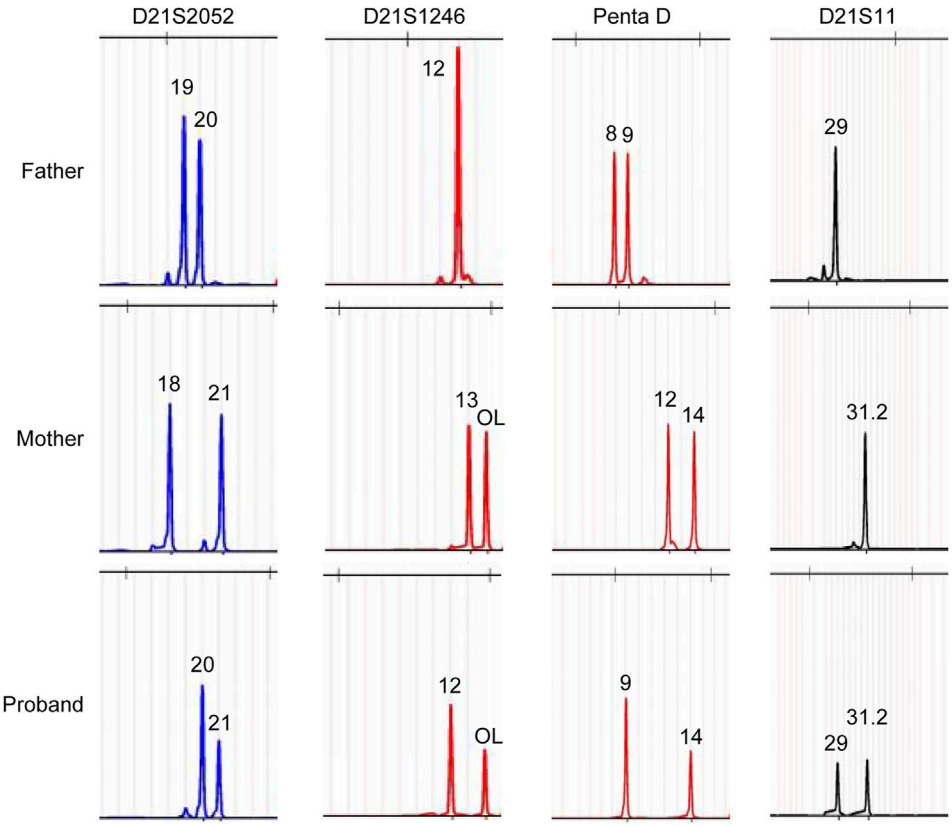


FIGURE 3 | Polymorphic Chr21 specific DNA marker analysis showed a paternal origin of the extra chromosome 21 fragment.

filtered and compared to the human reference genome (GRCh37/Hg19) to obtain the initial alignment result. Picard tool was applied to remove duplicate reads, and GATK (v4.0.3)'s HaplotypeCaller was used for base quality recalibration. Based on the comparison results, the evaluation indexes such as the sequencing depth, coverage, and comparison ratio of each sample were counted. Copy number variation (CNV) was detected using the self-developed LFR-CNV software.

Short Tandem Repeat Analysis

To identify the origin of the extra Chr21 fragment, peripheral blood-derived DNA samples of the father, mother, and proband were prepared. Four Chr21 specific STR markers (D21S2052 in 21q21.3, D21S1246 in 21q22.2, D21S11 in 21q21.1, and Penta D in 21q22.3) were analyzed using PowerPlex 21 HS genotyping system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The Sequence Information for Selected STR Systems could be found on the STRBase (https://strbase.nist.gov/seq_info.htm). Amplification mixture with 5 μl of PowerPlex 21 5× Master Mix, 5 μl of PowerPlex 21 5× Primer Pair Mix, and 5 ng of DNA were conducted in GeneAmp PCR System 9,700 Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA). PCR conditions were as follows: 96°C for 1 min; 94°C for 10 s; 59°C for 1 min; 72°C for 30 s, 30 cycles in total; and 60°C for 10 min. PCR products were detected by capillary electrophoresis and analyzed using the GeneMapper ID ver. 3.2 software (Applied Biosystems).

Case Report

In 2019, a 26-year-old woman diagnosed with infertility caused by tubal obstruction was assisted with reproductive technology (ART) and *in vitro* fertilization (IVF) (two embryos were transferred). Her husband was 28 years old, and the routine examination of semen quality showed normal results. The karyotype analysis showed that the couple had normal karyotypes (46, XX, and 46, XY) without any detectable deletion, duplication, translocation, or inversion. Genetic counseling results showed no family history of genetic diseases. Combined, the couple was suggested to do the IVF-ET without PGT-A. *In vitro* fertilization (IVF) was carried out based on the standard long protocol mentioned in the method. After embryo transfer, serum β-Hcg and abdominal ultrasound demonstrated successful clinical pregnancy. The mother received NIPT (Berry Genomics, Beijing, China) and Nuchal Translucency (NT) examination at 12 + 4 weeks gestation, which was generally applied to screen chromosome aneuploidies, especially for Trisomy 21 (T21). In this case, ultrasound results showed two viable fetuses with normal NT value, in which the F1 fetus was 1.4 mm and F2 fetus was 2.4 mm. Meanwhile, NIPT indexes were below 0.5, indicating a low risk for T21, T18, and T13. At 22 + 2 weeks of gestation, color Doppler ultrasound was used to detect the development of the two fetuses, and no abnormality was observed.

The twin pregnancy gave birth to two girls at 37 + 1 week of gestation by cesarean section in 2020, with no hypoxia or birth trauma. However, one neonate weighed 2.9 kg, and was observed to exhibit hypotonia and feeding difficulties 10 days after birth. Further ultrasonic examination demonstrated a 4.0-mm ventricular septal defect, patent foramen ovale, and tricuspid regurgitation in the heart. On the contrary, the sister did not show any abnormalities. Full karyotyping was performed on the twin sisters after genetic counseling. The result showed that the proband is a Chimera with a partial trisomy 21 karyotype, while the sister has a normal 46, XX karyotype. G-banding from peripheral blood cells of the patient showed a complicate karyotype of 46,XX,add (21) (q22)[25]/46,XX,der (21)del (21) (q22.1)t (21; 21) (q22.3; q22.1)[36]/46,XX,dup (21) (q22.1q22.3)[32] (**Figure 1A**). The CNV-seq result consistently showed the copy number of Chr21 from q22.1 to q22.3 was 2–3 (**Figure 1B**).

To better understand the Chr21 re-arrangement in this case, we applied the stLFR-WGS developed by BGI to detect the precise breakpoints and CNVs of Chr21. **Figure 2** listed the candidate CNVs of Chr21, including multiple larger duplication and small deletion from q22.1 to q22.3. To trace the origin of the additional Chr21 fragment, a Short Tandem Repeat (STR) analysis with four Chr21-specific DNA markers was performed using the DNA extracted from the girl and parental blood samples. The result showed a paternal origin of the extra chromosome 21, indicating the paternal origin of the partial trisomy 21 (**Figure 3**).

To track the development of the affected twin and establish the relationship between the Chr21 mosaicism and disease phenotype, we suggest a long-term follow-up of the patient, especially for the Chr21 aneuploidy-related manifestations, such as intellectual development and nervous and cardiovascular system function, which would be helpful for the clinical management of similar cases.

DISCUSSION

Emerging evidence has suggested the increased risk of congenital disabilities, including chromosome abnormalities and development disorders, in fetuses conceived with ART methods (Chen and Heilbronn, 2017; Sammel et al., 2018; Yu et al., 2018; Luke et al., 2020; Wen et al., 2020; Luke et al., 2021; Wang et al., 2021). The results from our case report indicate that IVF-ET pregnancies need more attention and careful prenatal screening. Fetal nuchal translucency (NT) thickness, maternal serum biochemical markers (such as PAPP-A and free β-hCG), as well as NIPT utilizing high-throughput methods for detecting free placental DNA (cfDNA) are the most commonly applied prenatal screening methods, which could provide a risk assessment for common autosomal aneuploidies (Giroux et al., 2021; Kimelman and Pavone, 2021; Merriel et al., 2021; Qi et al., 2021; Suzumori et al., 2021). Although there is a high prediction rate of NIPT in singletons, twin pregnancies are dizygotic (DZ), which reduces the serum markers and cfDNA concentrations, increasing the risk of a missed diagnosis (Struble et al., 2014).

In this case, the young couple was assisted with *in vitro* fertilization (IVF) and embryo transfer (IVF) because of tubal obstruction induced infertility. Unexpectedly, successful twin pregnancy gave birth to a partial trisomy 21 mosaicism and a normal child. It is worth mentioning that nuchal translucency (NT) examination and NIPT failed to screen this mosaicism fetus, which was finally confirmed through karyotyping assay and CNV-seq analysis. The result showed a complex combination of duplication from q22.1 to q22.3 and distal absence and fused chromosome in Chr21. It suggests that the present prenatal diagnosis regulations may not meet the detection of the Chr21 chimera in this case.

The stLFR-WGS was applied to analyze the potential breakpoints in the Chr21 (Wang et al., 2019). Multiple candidate breakpoints and formed CNVs from q22.1 to q22.3 were discovered, suggesting that IVF-introduced breakpoints may be enlarged during the meiotic and mitotic process *in vitro*, thus attributing to the complex Chr21 mosaicism in the proband. Short tandem repeat (STR) analysis with Chr21-specific DNA markers demonstrated that the extra Chr21 segments originated from the father. The proband exhibited hypotonia and feeding difficulties, and further ultrasonic examination identified Cardiac dysplasia, which the extra Chr21 may cause. We will continuously track the development of the child, including her nervous, immune, cardiovascular, and other bodily systems.

Considering some reported cases of twin pregnancy gave birth to children with trisomy 21 following IVF, the abnormal Chr21 may result from the IVF-ET process (Chaliha et al., 1999; Dunn et al., 2001; Zeng et al., 2003; Lavery et al., 2008). It seems like traditional methods of nuchal translucency (NT) and NIPT have limitations in detecting the trisomy 21 mosaicism in a twin pregnancy. Meanwhile, some articles have revealed a high incidence of aneuploidy and mosaicism in embryos from young couples undergoing IVF (Baart et al., 2006). Therefore, the preimplantation genetic testing for aneuploidies (PGT-A) in young couples taking IVF-ET should be considered, although more data is needed to make a definitive statement.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical approval was obtained from the Ethics Committee of Henan Provincial People's Hospital, China, with NO.2020-75. The informed consent was obtained from the proband's parents, and the parents agreed and signed the consent document.

AUTHOR CONTRIBUTIONS

ZG designed the project; ZG performed most of the experiments. BK and DW helped with the clinical patient's information collection. HX helped with the karyotype analysis. The final version was edited by LH, BH and ZG. ZG, BH, and SL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Case Report: Challenges of Non-Invasive Prenatal Testing (NIPT): A Case Report of Confined Placental Mosaicism and Clinical Considerations

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Since the introduction of cell-free (cf) DNA analysis, Non-Invasive Prenatal Testing (NIPT) underwent a deep revolution. Pregnancies at high risk for common fetal aneuploidies can now be easily identified through the analysis of chromosome-derived components found in maternal circulation, with the highest sensitivity and specificity currently available. Consequently, the last decade has witnessed a widespread growth in cfDNA-based NIPT use, enough to be often considered an alternative method to other screening modalities. Nevertheless, the use of NIPT in clinical practice is still not devoid of discordant results. Hereby, we report a case of confined placental mosaicism (CPM) in which a NIPT false-positive result for trisomy 13 required not only amniocentesis but also cordocentesis, to rule out the fetal aneuploidy, with the additional support of molecular cytogenetics on placental DNA at delivery. Relevant aspects allowing for precision genetic diagnosis and counselling, including the number of analysed metaphases on the different fetal cells compartments and a repeated multidisciplinary evaluation, are discussed.

Keywords: NIPT, cfDNA, confined placental mosaicism, prenatal diagnosis, aneuploidies

INTRODUCTION

Cell-free (cf) DNA-based Non-Invasive Prenatal Testing (NIPT) is widely considered to be the most sensitive and specific screening option for trisomy 21, 18, and 13. However, some concerns regarding its clinical role in routine obstetric care persist. These include, *inter alia*, the reliability of Positive Predictive Value (PPV) estimates. According to the most recent metanalyses (Gil et al., 2015; Taylor-Philips et al., 2016; Iwarsson et al., 2017; Mackie et al., 2017), the combined False-Positive Rate (FPR) in successful tests is 0.15%. In this sense, most studies on NIPT performance still suffer from a high risk of bias, in particular, the reported FPRs are likely to be underestimated.

As it is well known, circulating cfDNA derives from both the mother and the fetal-placental unit. Consequently, the main sources of unreliability of NIPT are Confined Placental Mosaicism (CPM), maternal copy number variants, vanishing twin, and maternal cancer (Grati et al., 2014).

Despite these shortcomings, obstetric care providers are increasingly prone to prescribe cfDNA analysis as an alternative or stand-alone screening method compared to ultrasound examinations. Moreover, there is still some controversy concerning the standard protocols that would best investigate fetal anomalies during the first trimester.

With this in mind, we report a case of CPM in which a NIPT false-positive result for trisomy 13 required two further invasive diagnostic tests–an amniocentesis and a cordocentesis - to rule out the fetal aneuploidy. Molecular cytogenetics performed on placental DNA at the delivery could add relevant data for the unequivocal diagnosis of CPM.

Case Presentation

A 31-year-old, gravida 1, para 0, Caucasian woman was referred to our hospital (Agostino Gemelli University Policlinic, Rome, RM, Italy) at 19 2/7 weeks of gestation for evaluation of suspected trisomy 13. Her previous medical and obstetric history had been unremarkable. Screening and diagnostic steps are presented in **Figure 1**. The first-trimester ultrasound findings were normal. At 12 2/7 weeks of gestation, she underwent the PrenatalSAFE® 5 Test (Eurofins Genoma Group Srl, Rome, Italy) through ILLUMINA VeriSeq NIPT sequencing systems, which revealed a suspected aneuploidy in chromosome 13 with a Fetal Fraction (FF) of 11% and a PPV of 92,86%. At 14 6/7 weeks of gestation, she underwent amniocentesis to confirm the positive NIPT result. By analysing 77 metaphases, we found that all but one had a normal male chromosome constitution, 46 XY. The unique cell with trisomy 13 we observed was first consistent with CPM, or, alternatively, with a very low mosaicism for trisomy 13 in the fetus.

A detailed fetal ultrasound was carried out at 19 2/7 weeks of gestation with normal results.

At 19 2/7 weeks of gestation, upon genetic counselling, the couple also decided to undergo cordocentesis to rule out a True Fetal Mosaicism (TFM) for trisomy 13. The analysis of 200 fetal lymphocytes confirmed a normal male karyotype 46, XY in the totality of cells. Accordingly, CPM was considered to explain the previous results.

Subsequent ultrasound scans proved to be within normal limits.

Labor induction was performed at 39 2/7 weeks of gestation for reduction of the fetal growth trend (from 64th to 23rd percentile), that was considered to reflect the final functional dysfunction of the trisomic placenta. A male neonate with a birth weight of 3430 g and Apgar scores of 9–10 at 1–5 min, respectively, was born by vaginal delivery. Physiological newborn jaundice was present, and the infant did not present any phenotypic anomaly. Molecular cytogenetic examination (Array-CGH) of the placenta, revealed a complete trisomy of chromosome 13 in about 20% of the analysed genome, allowing to definitively establish the diagnosis of CPM. During a final genetic counselling, no risk for phenotypic abnormalities was given to the newborn, and amniocentesis for fetal chromosomal examination was suggested in subsequent pregnancies of the parents.

DISCUSSION

In a seminal paper, Lo and Wainscoat (Lo et al., 1997) described for the first time the presence of fetal DNA in maternal plasma. Since then, several studies (Sekizawa et al., 2000; Bianchi, 2004; Tjoa et al., 2006) have been carried out to investigate cfDNA mechanisms of release during pregnancy, demonstrating both the maternal and the fetal-placental unit origin. Despite the high sensitivity and specificity of currently available PCR and MPS analytical techniques for the study of cfDNA (Bianchi et al., 2012), the primary trophoblastic origin of the latter is a known driver of the relatively large number of false positive and false negative results (Grati et al., 2014). The upshot of this is the designation of NIPT as a screening - and not a diagnostic - test. In this context, the present case highlights the shortcomings of NIPT when used as an alternative to the first trimester ultrasound scan for the screening of the most common aneuploidies. We aim at evaluating some critical aspects that should be considered in protocol decision-making practices and which could best investigate fetal anomalies during the first trimester.

First, it is interesting to note that Fetal Blood Sampling (FBS) through cordocentesis can sometimes be diriment to rule out a fetal aneuploidy after a NIPT positive result. In our case, normal ultrasound findings against the background of a positive result for trisomy 13 at NIPT led initially to consider amniocentesis the best diagnostic tool to avoid erroneous results due to CPM. Despite this, cordocentesis was then deemed necessary to exclude a fetal mosaicism. This means not only exposure to all the potential risks of FBS - including bleeding from the puncture site, fetal bradycardia, pregnancy loss and vertical transmission of maternal infection (Berry et al., 2013) - but also a 47-days delay in the final response, resulting in substantial psychological stress over a long period. Such an emotional strain should be avoided since it could lead, in extremis, to an improper decision to have a first-trimester abortion for the sake of the mother's health.

This emphasizes, above all, how important both pre- and post-test counselling are, allowing patients to understand the difference between a screening and a diagnostic test. In this sense, we believe that the best prenatal practice encompasses the interpretation of both positive and negative NIPT results in view of other screening modalities' findings (Salomon et al., 2017). Conversely, most laboratories report the average risk in the screen-positive patient as a PPV, disregarding the prior-test risk based on age, ultrasound, prior history and screen-positive serum test. Abnormal findings at NIPT, contrasting with normal fetus development at ultrasound scan, could disclose other biological causes (Hartwig et al., 2017), such as maternal Copy Number Variations (CNVs) and Confined Placental Mosaicism (CPM) (Mardy & Wapner, 2016). In this context, even if the performance of NIPT is higher, the first trimester ultrasound scan has been proved to potentially change clinical management in almost one in 10 women if performed prior to cfDNA screening (Brown et al., 2020). This is especially the case of trisomies 18 and 13, for which a detailed ultrasound examination can detect characteristic defects.

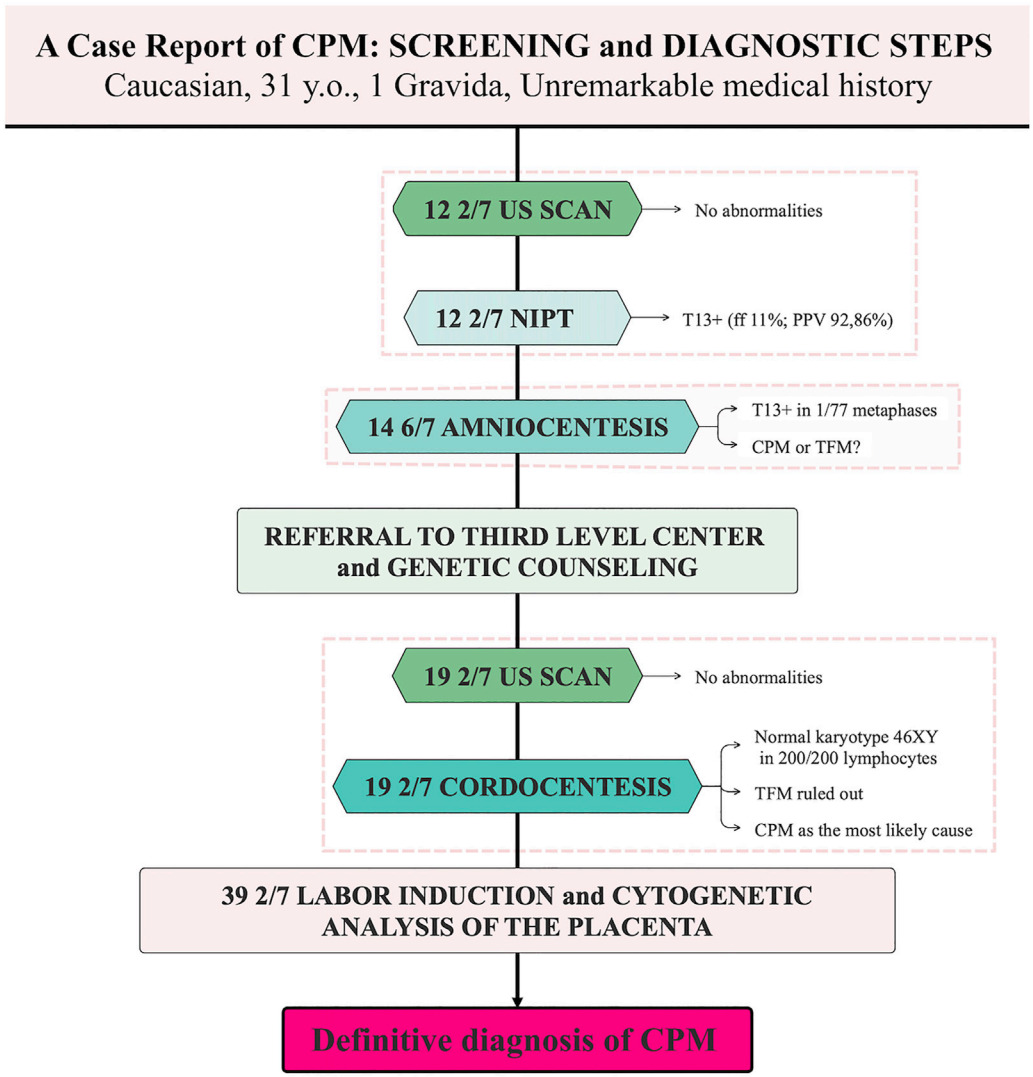

FIGURE 1 | Screening and diagnostic steps in a case of Confined Placental Mosaicism. CPM, Confined Placental Mosaicism; TFM, True Fetal Mosaicism; ff, Fetal Fraction; PPV, Positive Predictive Value.

The present case also maintains the need of a careful perinatal management when CPM is suspected. After ruling out recognized risk factors such as constitutional chromosomal abnormalities, the rate of infants with Intrauterine Growth Restriction (IUGR) associated with CPM has been estimated to be 10 times higher than in the appropriately grown controls infants (Wilkins-Haug et al., 2006). Notwithstanding this, a recent retrospective cohort study did not confirm any significant association between CPM and adverse pregnancy outcomes except for CPM for trisomy 16 (Grati et al., 2020).

In conclusion, this case provides significant clinical considerations on using NIPT in daily practice. In particular, it presents the major pitfalls of interpreting screening tests' findings in the absence of a mutual work of integration. In recent years, we have witnessed an uncontrolled spread of cf-DNA analysis for which the economic interest of the industry has certainly contributed. In this context, a responsible integration of similar technological innovations should always be sought in clinical experience. Our clinical experience confirms that, given the trophoblastic origin of cf-DNA, NIPT cannot but be a screening test. The real benefit of cfDNA analysis lies, therefore, in its complementary use with ultrasound scan, which helps to shed light on the most likely risk of fetal aneuploidy. We suggest that, in case of cfDNA testing positive for T21, T18, and T13 during first trimester screening, in the absence of anomalies detected during the ultrasound examination, an invasive procedure by Chorionic Villus Sampling (CVS) could be recommended only for T21, since in such a case the risk of confined placental mosaicism is about 1–2%, which is comparable to the risk of mosaicism in the general population. Conversely, for T18 or T13, the best management would be to offer an amniocentesis because the risk of confined placental mosaicism is high: 3–4% for T18 and 22% for 13 (Grati et al., 2014; Grati et al.,

2015; Malvestiti et al., 2015). However, based on our observation, cordocentesis can be also required to definitively rule out the fetal aneuploidy. Analysis of a larger number of metaphases from fetal blood cells, with respect to amniocytes, is recommended in these cases.

Finally, we strongly emphasize the importance of an adequate education of all obstetrical providers in order to maximize the benefit brought by cfDNA analyses. Moreover, we notice that further research is needed to examine the extent to which maternal risk factors (e.g., age, obesity, hypertension, diabetes) influence the incidence of IUGR associated to CPM.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The written consent of the patient for the publication of the case was obtained according to the guidelines.

AUTHOR CONTRIBUTIONS

BG: conceptualization, data curation, writing—original draft, project administration TV: review; editing; ZM: writing—review; editing; DSM: review; editing, RF: review; editing, LA: review; editing BE: validation, writing—review; editing, supervision.

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Prenatal Diagnosis of Chromosomal Mosaicism in Over 18,000 Pregnancies: A Five-Year Single-Tertiary-Center Retrospective Analysis

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Background: Chromosomal mosaicism (CM) is a common biological phenomenon observed in humans. It is one of the main challenges in prenatal diagnosis due to uncertain outcomes, especially when fetal ultrasonographic features appear normal. This study aimed to assess the phenotypic features of CM detected during prenatal diagnosis and the risk factors affecting parents' pregnancy decisions.

Materials and methods: A retrospective cohort study involving 18,374 consecutive pregnancies that underwent prenatal diagnosis by karyotyping, fluorescence *in situ* hybridization (FISH), or chromosome microarray analysis (CMA) was conducted. The association of risk factors with malformations detected by ultrasound and pregnancy outcomes was assessed using the chi-square test and binary logistic regression. Discordant results between the different methods were identified and further analyzed.

Results: During this five-year period, 118 (0.6%) patients were diagnosed with CM. The incidences of CM in the chorionic villus, amniotic fluid, and umbilical cord blood were 3.2, 0.5, and 0.7%, respectively. The frequency of ultrasound malformations in individuals with a high fraction of autosomal CM was significantly higher than that in other groups (62.5% vs. 21.4–33.3%, all p <0.05). Inconsistent results between karyotyping and CMA/FISH were observed in 23 cases (19.5%). The risk of pregnancy termination in cases with ultrasound malformations, consistent results, autosomal CM, or a high CM fraction increased with an odds ratio of 3.09, 8.35, 2.30, and 7.62 (all p <0.05). Multiple regression analysis revealed that all four factors were independent risk factors for the termination of pregnancy.

Conclusion: Patients with a high fraction of autosomal CM are more likely to have ultrasound malformations. Inconsistent results between different methods in CM are not rare. Ultrasound malformations, consistent results between different methods, autosomal CM, and a high CM fraction were independent risk factors for the choice to terminate pregnancies.

Keywords: chromosomal mosaicism, prenatal diagnosis, ultrasound malformations, inconsistent results, termination of pregnancy

INTRODUCTION

Chromosomal mosaicism (CM) refers to the presence of two or more chromosomally different cell lines in an individual derived from a single zygote (Eggermann et al., 2015). It is a biological phenomenon in humans that may occur through a variety of mechanisms, including chromosome non-disjunction, anaphase lagging, trisomy rescue, and endoreplication (Taylor et al., 2014). It has been reported that CM occurs frequently during human pre-implantation development, with a prevalence of 15–75% in cleavage-stage embryos and 3–34% in blastocysts (Harton et al., 2017). However, with embryo development, CM is assumed to be less pervasive (Popovic et al., 2020). Previous studies demonstrated that CM was found in approximately 1–4% of prenatal diagnoses performed by chorionic villus sampling (CVS) and in approximately 0.1–0.3% of amniocentesis (Hsu et al., 1996; Carey et al., 2014; Grati et al., 2017; Gu et al., 2018; and Lund et al., 2020). Nevertheless, CM is still one of the main challenges in prenatal diagnosis due to uncertain outcomes, especially when fetal ultrasonographic features appear normal (Wallerstein et al., 2015).

In recent years, karyotyping, fluorescence *in situ* hybridization (FISH), and chromosome microarray (CMA) analysis have been widely used in the prenatal diagnosis for chromosomal analysis. Karyotyping, which requires cell culture, is a conventional cytogenetic test with a resolution of 5–10 Mb. Unlike karyotyping, FISH and CMA are performed in uncultured cells or DNA extracted from uncultured cells, respectively. Generally, FISH is used to detect numerical aberrations of chromosomes 13, 18, 21, X, and Y rapidly, whereas CMA can detect aneuploidy, microduplications, and microdeletions throughout the genome. Unlike karyotyping and FISH, which require manual counting of the chromosome composition, the result of CMA can be achieved automatically through a bioinformatic analysis. All the aforementioned differences in these methods can lead to inconsistencies in the results, especially in the case of CM. The discordant results further aggravate the challenges of genetic counseling for CM in prenatal diagnosis.

This study aimed to assess the incidence and characteristics of CM detected by karyotyping, FISH, and/or CMA in more than 18,000 consecutive pregnancies referred to our center for prenatal diagnosis over a five-year period, with a focus on their phenotypic features and the risk factors affecting the parents' pregnancy decisions, and further comparing the discordant results identified by different methods.

MATERIALS AND METHODS

Patients

This retrospective single cohort study was conducted in the reproductive genetic center of the International Peace Maternal and Child Health Hospital (IPMCH) of Shanghai Jiao Tong University School of Medicine. From January 2016 to December 2020, 18,374 fetuses were consecutively referred to our center for invasive prenatal diagnosis. According to the gestational age, fetal samples were obtained using CVS (n = 823), amniocentesis (n = 16,419), or umbilical cord blood (UCB) sampling (n = 1,132). All fetal samples were analyzed by karyotyping (n = 340, 1.8%), karyotyping and CMA (n = 13,966, 76.0%), or karyotyping and FISH (n = 4,068, 22.1%). Among them, 118 cases diagnosed with CM using at least one method were selected and further analyzed in this study, including 113 singleton pregnancies and five twin pregnancies. In twin pregnancies, only one fetus was affected in each pair. Among the 118 cases, 104 were diagnosed by karyotyping and CMA, whereas 14 were diagnosed by karyotyping and FISH (**Figure 1**). At our center, all cases diagnosed with CM were recommended for a more detailed ultrasonographic examination to further identify structural abnormalities in the fetuses. All cases were further consulted regarding prognosis and were additionally followed up for clinical outcomes.

Karyotyping Analysis, Fluorescence *In Situ* Hybridization, and Chromosomal Microarray

Cells were cultured and prepared for conventional G-banding karyotyping (550-band resolution), according to the standard protocol for all 18,374 fetal samples. Generally, at least 15 metaphase cells were assessed for numerical abnormalities of chromosomes, and five metaphase cells were carefully examined by experienced technicians to detect structural chromosomal abnormalities. If abnormal karyotypes were identified within the 15 metaphase cells, additional images were captured, and a total of 50 metaphases were karyotyped for each sample if possible. CM was diagnosed when ≥2 cells with the same abnormality were observed in two independent culture vessels.

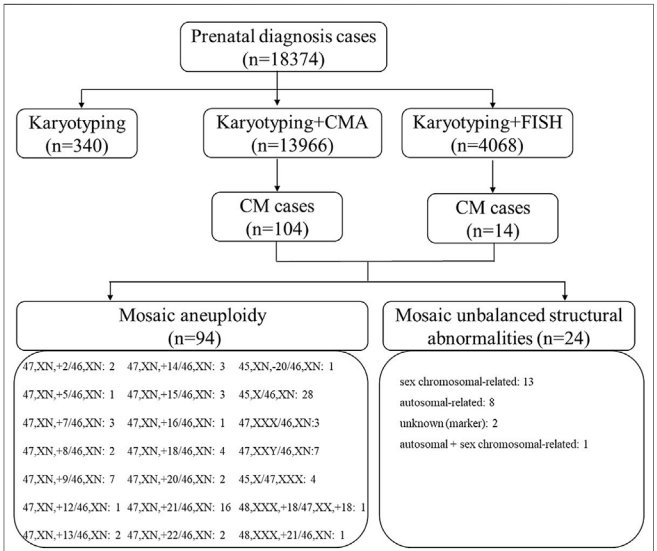

FIGURE 1 | Flow chart of fetuses with CM at our center in a five-year period between January 2016 and December 2020. CM, chromosomal mosaicism; CMA, chromosomal microarray; FISH, fluorescence *in situ* hybridization; T, trisomy.

FISH was performed in uncultured cells using commercially available probes for chromosomes 13, 18, 21, X, and Y, according to the manufacturer's instructions (Beijing GP Medical Technologies, Beijing, China). At least 50 interphase nuclei per target probe were evaluated for each sample. If 90% of the detected cells were normal, the sample was classified as normal. If 60% of the cells were abnormal, the sample was considered abnormal. In cases in which there was any doubt, the number of cells evaluated increased to 100. Mosaic was suspected when 10–60% of the cells were aberrant, and the results were reported as uninformative through FISH.

Genomic DNA was isolated according to standard procedures (Li et al., 2019). Quantitative fluorescent (QF)-PCR (R1004T; GENESKY, Shanghai, China) was used for a potential maternity contamination analysis in suspicious samples. Approximately all CMA analyses were performed on direct (uncultured) specimens, except those with maternal cell contamination. CMA was performed using Affymetrix CytoScan 750 K Array (Affymetrix, Inc., Santa Clara, CA, United States). Results were analyzed by Affymetrix Chromosome Analysis Suite software (ChAS) version 3.1. Genomic coordinates were based upon the UCSC human Genome Browser release of February 2009 (GRCh37/hg19). CNVs of a region of at least 100 kb with a minimum of 50 markers were analyzed carefully. The interpretations of CNVs were performed according to the guidelines (Kearney et al., 2011; Riggs et al., 2019). The level of mosaicism was obtained from the median Log2Ratio value calculated by the piece of software, and CM was reported when the level was between 20 and 70%.

Statistical Analysis

Continuous variables are presented as the mean ± standard deviation or median (range). Categorical variables are summarized as numbers (percentage). The association of risk factors with ultrasound malformations and pregnancy outcomes was assessed using the chi-square test and binary logistic regression. Increased nuchal translucency (NT), echogenic intracardiac focus, choroid plexus cysts, echogenic bowel, mild ventriculomegaly, thickened nuchal fold, mild pyelectasis, single umbilical artery, hypoplastic nasal bone, and enlarged cisterna magna were defined as ultrasound soft markers (Li et al., 2020). Both ultrasound structural anomalies and ultrasound soft markers were defined as ultrasound malformations. All CMs related to autosomal chromosomes were classified into the group of autosomal abnormalities, whereas CMs related to sex chromosomes were classified into the group of sex chromosomal abnormalities. With respect to the CM fraction, we defined a fraction greater than or equal to 50% as high and others as low (Bellil et al., 2020; Capalbo et al., 2021). As the results of karyotyping which were counted manually may introduce human error, the classification of the CM fraction was performed based on the results of CMA. In cases with normal CMA but an abnormal karyotype, the fraction of karyotyping results was used. For cases analyzed by karyotyping and FISH, the fraction of the FISH results was used. Inconsistent results between karyotyping and CMA/FISH were also analyzed. All analyses were performed by SPSS statistical software ver. 22.0 (IBM, Armonk, NY, United States). A p value <0.05 (two sides) was statistically significant.

TABLE 1 | Clinical characteristics of 118 patients with CM in this study.

	n **(%)/median (range)**
N	118
Maternal age, years	32 (18, 45)
Gestational age, weeks	18 (12, 30)
Invasive procedures	
CVS	26 (22.0)
AF	84 (71.2)
UCB	8 (6.8)
Indications	
Positive non-invasive prenatal test	32 (27.1)
Ultrasound structural anomalies	30 (25.4)
Advanced maternal age (≥35)	20 (16.9)
Ultrasound soft markers	14 (11.9)
Abnormal biochemical screening	9 (7.6)
Adverse history of pregnancy	6 (5.1)
Others	7 (5.9)
Type of CM	
Mosaic aneuploidy	94 (79.7)
Autosomal trisomy * [a]	49 (41.5)
Sex chromosomal monosomy [b]	28 (23.7)
Sex chromosomal trisomy [b]	10 (8.5)
Sex chromosomal monosomy + trisomy [b]	4 (3.4)
Autosomal trisomy + sex chromosomal trisomy [a]	2 (1.7)
Autosomal monosomy [a]	1 (0.8)
Mosaic unbalanced structural abnormalities	24 (18.6)
Sex chromosomal–related [b]	13 (11.0)
Autosomal-related [a]	8 (6.8)
Unknown (marker)	2 (1.7)
Autosomal + sex chromosomal–related [a]	1 (0.8)

**Include one fetus with mosaic trisomy 15 and 22q11.2 deletion.*
[a]classified into the group of autosomal abnormalities.
[b]classified into the group of sex chromosomal abnormalities.
CM, chromosomal mosaicism; CVS, chorionic villus sampling; AF, amniotic fluid; UCB, umbilical cord blood.

RESULTS

Clinical Characteristics

The clinical characteristics of 118 patients with CM are presented in **Table 1**. The incidences of CM in CVS, amniotic fluid (AF), and UCB were 3.2% (26/823), 0.5% (84/16,419), and 0.7% (8/1,132), respectively. Among the 26 CVS cases, five were subjected to amniocentesis, and the AF results were normal, indicating confined placental mosaicism (CPM). Ultrasound malformations were observed in 44 fetuses (37.2%). Among the 14 cases with ultrasound soft markers, increased NT (n = 12) was the most common type.

Types of Chromosomal Mosaicism and Their Associations With Ultrasound Malformations

Overall, 94 (79.7%) mosaic aneuploidy cases and 24 (20.3%) mosaic unbalanced structural abnormalities were identified (**Table 1** and **Figure 1**). Of the 94 mosaic chromosomal

TABLE 2 | Associations of CM types and CM fractions with ultrasound malformations.

	Cases with ultrasound malformations, *n* (%)	Cases without ultrasound malformations, *n* (%)	*p*
CM types			
Autosomal	27 (44.3)	34 (55.7)	0.06
Sex chromosomal	15 (27.3)	40 (72.7)	
CM fraction			
High (≥50%)	23 (45.1)	28 (54.9)	0.06
Low (<50%)	19 (28.4)	48 (71.6)	
CM types + CM fraction			0.006[a]
Autosomal high	15 (62.5)	9 (37.5)	
Autosomal low	12 (32.4)	25 (67.6)	0.02[b]
Sex chromosomal high	9 (33.3)	18 (66.7)	0.04[b]
Sex chromosomal low	6 (21.4)	22 (78.6)	0.003[b]

[a]p *for trend.*
[b]*Compared to the group of autosomal high.*
CM, chromosomal mosaicism.

aneuploidy cases, mosaic autosomal trisomy was the most common type (n = 49). Among the 49 cases of autosomal trisomy CM, the most common type was trisomy 21 (n = 16), followed by trisomy 9 (n = 7) and trisomy 18 (n = 4) (**Figure 1**). With respect to mosaic unbalanced structural abnormalities, 13 were sex chromosomal–related abnormalities, while eight were autosomal–related abnormalities. Notably, two fetuses with mosaic marker chromosomes but an unknown source and one fetus with partial chromosome 8 duplication and Y chromosome deletion were identified.

To explore the effects of CM types and CM fractions on fetal phenotypes, we further compared the frequencies of ultrasound malformations among fetuses with different types and fractions of CM. As shown in **Table 2**, the frequency of ultrasound malformations in cases with mosaic autosomal abnormalities was higher than that in cases with sex chromosomal abnormalities, although the difference was not statistically significant, partially because of the small sample size (44.3 vs. 27.3%; p = 0.06). The same result was observed when comparing cases with a high fraction of CM and those with a low fraction of CM (45.1 vs. 28.4%; p = 0.06).

We further compared the frequency of ultrasound malformations among groups with a high fraction of autosomal CM, a low fraction of autosomal CM, a high fraction of sex chromosomal CM, and a low fraction of sex chromosomal CM. The frequency of ultrasound malformations in individuals with a high fraction of autosomal CM was significantly higher than that in the other groups (62.5% vs. 21.4–33.3%, all p <0.05). A significant trend was observed for the frequency of ultrasound malformations among the four groups (p for trend = 0.006).

Inconsistent Results Between Karyotyping and Chromosomal Microarray /Fluorescence *In Situ* Hybridization

Inconsistent results between karyotyping and CMA/FISH were identified in 23 cases (19.5%), including seven cases (26.9%) derived from CVS and 16 cases (19.0%) derived from AF (**Table 3**).

Among the seven CVS cases with inconsistent results, four had underwent amniocentesis, and the results were normal (cases 1–4), indicating CPM. Of the 16 AF cases, a subsequent genetic analysis was performed in only four cases (8, 9, 12, and 23). In case 8, a second amniocentesis was performed, and both karyotyping and CMA were normal. AF and UCB were redrawn in case 9; the results of CMA and FISH in uncultured AF were still mosaic trisomy 15, whereas the results of karyotyping, CMA, and FISH in UCB were normal. The fetus was delivered prematurely at 33 weeks because of maternal antepartum hemorrhage. After birth, copy number variation sequencing of peripheral blood, urine, oral mucosal cells, umbilical cord root, and umbilical cord (approximately 3 cm away from the root of the umbilical cord root) was performed. As shown in **Table 3**, a low fraction of mosaic trisomy 15 was detected in the urine, oral mucosal cells, and umbilical cord root, whereas no abnormalities were detected in the umbilical cord and peripheral blood. UCB puncture was performed in case 12, and the karyotype was normal. The karyotyping result of the placenta in case 23 was normal after birth. Cases 13–22 were AF cases where karyotyping was abnormal, but the CMA was normal. The discordant results may be due to the low mosaic fraction (<20%) for which the CMA cannot detect (cases 13–20) or may indicate that the marker chromosome was heterochromatin (cases 21–22). No further genetic testing was performed in these patients.

Fifteen of them were born. Except for a short stature below the 10th percentile of case 1 (at 2 years of age), the development of all other cases has been normal until the time of publication (6 months–4 years of age).

Risk Factors Affecting the Parent's Pregnancy Decision

Pregnancy outcomes were available for 115 cases [115 of 118 (97.5%)]. Overall, 81 (77.1%) cases had a termination of pregnancy (TOP). The effects of ultrasound malformations, inconsistent results of different methods, different types of CM, and CM fractions on the choice of TOP were examined. As shown in **Table 4**, the rate of TOP in cases with ultrasound

TABLE 3 | Inconsistent results in fetuses with CM between the karyotype and CMA/FISH.

Case ID	Sample	UM	1st results		2nd results			Pregnancy outcome
			Karyotype	CMA	Sample	Karyotype	CMA and/or FISH	
1	CVS	No	46, XN	arr (X) × 1–2	AF	46, XN	arr (1–22) × 2, (X, N) × 1	Full-term delivery
2	CVS	Increased NT (3.5 mm)	46, XN	arr (X) × 1, (Y) × 0–1	AF	46, XN	arr (1–22) × 2, (X, N) × 1	Full-term delivery
3	CVS	Increased NT (3.1 mm)	46, XN	arr (7) × 2–3	AF	46, XN	arr (1–22) × 2, (X, N) × 1	Full-term delivery
4	CVS	Hydrops	46, XN	arr (X) × 1, (Y) × 0–1	AF	46, XN	arr (1–22) × 2, (X, N) × 1	TOP
5	CVS	anencephaly	46, XN	arr (7) × 2–3				TOP
6	CVS	No	45, X (9)/46, XX (41)	arr (1–22) × 2, (X, N)×1				Full-term delivery
7	CVS	Hydrops	46, XN, der (10) (pter→q26:: ?)(7)/46, XN (46)	arr (1–22) × 2, (X, N) × 1				TOP
8	AF	No	46, XN	arr (X) × 1–2	AF/PB	46, XN	arr (1–22) × 2, (X, N) × 1	Full-term delivery
9	AF	No	46, XN	arr (15) × 2–3	AF	46, XN	arr (15) × 2–3 nuc ish (CSP15 × 3) (21/100)[a]	Preterm delivery (33 weeks)
					UCB	46, XN	arr (1–22) × 2, (X, N) × 1 Normal[a]	
					Urine		seq (15) × 2–3[b]	
					OMC		seq (15) × 2–3[b]	
					UCR		seq (15) × 2–3[b]	
					UC		Normal[b]	
					PB		Normal[b]	
10	AF	MM	46, XN	arr (14) × 2–3				TOP
11	AF	CHD	46, XN	arr (16) × 2–3				TOP
12	AF	echogenic bowel	46, XN	arr (7) × 2–3	UCB	46, XN		Lost to follow-up
13	AF	Increased NT (3.0 mm)	45, X (4)/46, XN (26)	arr (1–22) × 2, (X, N) × 1				Full-term delivery
14	AF	No	47, XN.+20 (9)/46, XN (57)	arr (1–22) × 2, (X, N) × 1				Full-term delivery
15	AF	No	47, XN,+20 (5)/46, XN (45)	arr (1–22) × 2, (X, N) × 1				Full-term delivery
16	AF	No	45, XN,-20 (3)/46, XN (47)	arr (1–22) × 2, (X, N) × 1				Full-term delivery
17	AF	No	47, XN,+?8 (3)/46, XN (15)	arr (1–22) × 2, (X, N) × 1				Preterm delivery (33 weeks)
18	AF	CCAM	45, X (3)/46, XN (37)	arr (1–22) × 2, (X, N) × 1				Full-term delivery
19	AF	MM	45, X (3)/46, XX (32)	arr (1–22) × 2, (X, N) × 1				TOP
20	AF	MM	47, XN,+5 (4)/46, XN (46)	arr (1–22) × 2, (X, N) × 1				TOP
21	AF	Increased NT (3.3 mm)	47, XN,+mar (5)/46, XN (45)	arr (1–22) × 2, (X, N) × 1				Full-term delivery
22	AF	VSD	47, XN,+mar (4)/46, XN (55)	arr (1–22) × 2, (X, N) × 1				Full-term delivery
23	AF	No	47, XX + 21 (3)/46, XX, (7)	Normal[a]	placenta	46, XN		Full-term delivery

[a]*Analyzed by FISH.*

[b]*Copy number variation sequencing was performed in these samples obtained after birth.*

CM, chromosomal mosaicism; CMA, chromosomal microarray; FISH, fluorescence in situ *hybridization; UM, ultrasound malformations; CVS, chorionic villus sampling; AF, amniotic fluid; PB, peripheral blood; UCB, umbilical cord blood; TOP, termination of pregnancy; OMC, oral mucosal cells; UCR, umbilical cord root; UC, umbilical cord; CCAM, congenital cystic adenomatoid malformation; VSD, ventricular septal defect; MM, multiple malformations; CHD, congenital heart disease.*

malformations was significantly higher than that in cases without ultrasound malformations (83.7 vs. 62.5%, p = 0.02). Similar results were observed in cases with consistent results, autosomal CM, or a high CM fraction in comparison to those with inconsistent results, sex chromosomal CM, or a low CM fraction (79.6 vs. 31.8%, $p = 1 \times 10^{-5}$; 79.3 vs. 63.0%, p = 0.049; and 90.2 vs. 54.7%, $p = 3 \times 10^{-5}$). Compared to cases without ultrasound malformations, or with inconsistent results, sex chromosomal CM or a low fraction of CM, the risk of the TOP in those with ultrasound malformations, consistent results, autosomal CM, or a high CM fraction was increased with an odds ratio of 3.09 (95% CI: 1.21–7.90, p = 0.02), 8.35 (95% CI: 1.15–7.52, $p = 5 \times 10^{-5}$), 2.30 (95% CI: 1–5.34, p = 0.049), and 7.62 (95% CI: 2.68–21.70, $p = 1 \times 10^{-4}$), respectively. To further clarify

TABLE 4 | Effect of ultrasound features, CM types, CM fractions, and consistent or inconsistent results on pregnancy outcomes.

	N	TOP, *n* (%)	*p*	Or (95% CI)	*p*	Adj-OR*	Adj-*p**
Ultrasound malformations							
Yes	43	36 (83.7)	0.02	3.09 (1.21, 7.90)	0.02	6.02 (1.48, 24.53)	0.01
No	72	45 (62.5)					
Consistent results							
Consistent	93	74 (79.6)	1×10^{-5}	8.35 (2.98, 23.36)	5×10^{-5}	14.0 (3.08, 63.59)	0.001
Inconsistent	22	7 (31.8)					
CM types							
Autosomal	59	47 (79.3)	0.049	2.30 (1, 5.34)	0.049	4.41 (1.41, 13.76)	0.01
Sex chromosomal	54	34 (63.0)					
CM fractions							
High (≥50%)	51	46 (90.2)	3×10^{-5}	7.62 (2.68, 21.70)	1×10^{-4}	6.18 (1.86, 20.58)	0.003
Low (<50%)	64	35 (54.7)					

**Covariates listed in the table were mutually adjusted.*

CM, chromosomal mosaicism; TOP, termination of pregnancy; OR, odds ratio; CI, confidence interval.

the independent effects of these factors on pregnancy outcomes, multiple logistic regressions were conducted. The results showed that all four factors were independent risk factors for the TOP (**Table 4**).

DISCUSSION

In this study, we retrospectively analyzed 118 cases of CM detected at our center in a five-year period between January 2016 and December 2020 and found that approximately 62.8% of fetuses with CM had no abnormalities detected on ultrasound, further confirming that genetic counseling for CM is indeed a great challenge in the clinical setting (Zhang et al., 2021). To provide valuable information for genetic counseling and management of prenatal mosaic cases for clinicians and patients, we focused on the factors related to ultrasound malformations and pregnancy outcomes.

Our study demonstrated that compared to cases with autosomal CM, those with sex chromosomal CM were more likely to exhibit normal ultrasound finding and lead to a normal birth. In our study, 20 cases of sex chromosomal CM were born, including 10 cases of 45, X/46, XX; four cases of 45, X/46, XY; three cases of 47, XXY/46, XY; two cases of 47, XXX/46, XX; and one case of mosaic X chromosomal unbalanced structural abnormality. Except for one case that exhibited a short stature, the development of all other cases (6 months–3 years of age) was normal. Long-term follow-up and management of these cases is warranted as some of them may encounter abnormal sexual development (Dendrinos et al., 2015; Wong et al., 2021), autism spectrum disorder, or cognitive problems later in life (Vorsanova et al., 2021). Recently, Tuke et al. demonstrated that mosaic monosomy X showed reduced penetrance, and the management of women with 45, X/46, XX should be minimal in an adult population study (Tuke et al., 2019). Studies have also shown that compared to non-mosaic 47, XXY, the phenotypic symptoms in cases of XXY/XY mosaicism may present more mildly, and many cases fail to be identified (Samplaski et al., 2014; Davis et al., 2016). More population-based studies are needed to demonstrate the prevalence of abnormal phenotypes in individuals with sex CM to better guide clinical management and genetic counseling.

Unlike sex CM, there is no strong correlation between karyotypic and phenotypic abnormalities in autosomal CM (Wallerstein et al., 2015). We found that the frequency of ultrasound malformations in cases with a high fraction of autosomal CM was higher (62.5%) than that in cases with a low fraction of autosomal CM (32.5%; $p = 0.02$), indicating that a high mosaic fraction was a risk factor for adverse outcomes. Trisomy 9 mosaicism (T9M) is a rare chromosomal abnormality with a significant clinical variability (Pejcic et al., 2018). To date, no more than 200 cases of T9M have been reported (Li et al., 2021); surprisingly, seven cases with T9M were detected in 11,834 cases (0.06%) at our center, indicating that it was not rare in fetuses. Six of the seven cases chose TOP, whereas one fetus was born without any clinical defects, and no evident abnormalities were identified to date (3.5 years old). As the long-term growth trends of T9M vary widely, follow-up and management of the case is warranted. It is also worth mentioning that although the risk of abnormal outcomes of 47,+20/46 was very high (>60%) in a review conducted by Wallerstein et al. (2015), others reported that approximately 90–93% of cases with mosaic trisomy 20 (T20M) at the prenatal diagnosis have been associated with a normal phenotype (Hsu et al., 1987; Chen et al., 2020). In our study, two T20M cases were detected in AF by karyotyping, whereas the results of CMA were normal. Both patients were born normally, suggesting that the prognosis of T20M, which CMA cannot detect, was good, consistent with previous studies (Hsu et al., 1987; Chen et al., 2020).

Inconsistent results between different methods in CM were not rare and were observed in 19.5% of the cases in our study. The selective growth of the cells during culture and the detection ability of different methods for the low fraction of CM may be responsible for these inconsistent results (Cheung et al., 2007). Although we recommended the use of multiple methods, such as a combination of karyotyping, CMA, and FISH to further confirm the results before any irreversible decision was made, it was very difficult to implement, hindered by the uncertainty of clinical outcomes even after the second results were normal, as well as the high cost of genetic testing. Four of the seven CVS cases with

inconsistent results underwent amniocentesis, and the results were normal, indicating CPM. In our case series, the incidence of CM in CVS was 3.2%, similar to the results reported by others using CMA (Gu et al., 2018; Lund et al., 2020). However, as most of these cases exhibited increased NT and/or fetal structural anomalies (23/26, 88.4%), the detected CM can partially explain the abnormal phenotypes (i.e., increased NT); amniocentesis was not performed in most of them. The ratio of CPM in our study could not be achieved. Recently, associations of CPM with negative developmental outcomes, including fetal growth restriction (71.7%), preterm birth (31.0%), and structural fetal anomalies (24.2%), have been reported, especially when chromosomes 2, 3, 7, 13, 15, 16, and 22 are involved (Eggenhuizen et al., 2021). In this circumstance, the CM detected in the CVS needed to be counseled with caution, even if it was found to be CPM. Unlike CVS, AF is considered to be the optimal specimen for fetal confirmation as it includes cells primarily from fetal anatomical districts, including the urogenital tract, respiratory tract, and epithelial systems, representing different embryological layers (Cremer et al., 1981). Partly because of the application of a high-resolution CMA, the incidence of CM in AF was 0.5% in our case series, which was slightly higher than previous reports (0.1–0.3%) (Hsu et al., 1996; Carey et al., 2014). It is generally accepted that UCB puncture is not required when CM is identified in AF as UCB cells are primarily derived from the mesoderm and can only reveal the mosaicism state of the mesoderm (Wieczorek et al., 2003). The negative UCB result could not negate the CM results of AF. However, in our study, two patients (case 9 with mosaic trisomy 15 and case 12 with mosaic trisomy 7) required UCB puncture due to anxiety. The UCB results in these two cases were normal. Although the outcome of case 12 was unknown, the development of case 9 was normal, indicating that a negative UCB result may be more prone to a good prognosis. More case evidence is required to demonstrate the significance of the UCB puncture in the case of CM identified in AF.

In our hospital, counseling on CM is provided by a geneticist at the prenatal diagnosis center. We observed that 77.1% of the patients decided to proceed with the TOP. Cases with ultrasound malformations, consistent results between karyotyping and CMA/FISH, autosomal CM, or a high fraction of CM were more likely to result in a TOP, whereas those with normal ultrasound, inconsistent results, sex chromosomal CM, or a low fraction of CM were likely to continue with the pregnancy and lead to normal birth. However, as CM is associated with many other abnormalities, including neuropsychiatric disorders, long-term monitoring and follow-up of these carriers are necessary.

It is worth mentioning that the sensitivity and specificity of ultrasound in the detection of malformations were important factors in our study. However, autopsy was not performed in the cases of TOP; therefore, we could not determine whether there were other structural abnormalities that were not detected by the prenatal ultrasound. However, for almost all live-birth cases, the results were consistent with the prenatal ultrasound findings, suggesting that the results of the prenatal ultrasound in our patients were reliable. In addition, although it does not affect our conclusions, it should be mentioned that mosaic unbalanced structural abnormalities with a fragment size smaller than 5 Mb would be missed in 14 cases of CM diagnosed by karyotyping and FISH.

In conclusion, our study demonstrated that patients with a high fraction of autosomal CM were more likely to have ultrasound malformations. Inconsistent results between different methods in CM are not rare. All four factors, including ultrasound malformations, consistent results between different methods, autosomal CM, and a high CM fraction, were independent risk factors for the choice of TOP. Further studies are warranted to provide more information for genetic counseling during prenatal diagnosis.

DATA AVAILABILITY STATEMENT

The data presented in the study can be found in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/, SUB11294080).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the International Peace Maternal and Child Health Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YW and SL designed the study. SL, XH, YC, YhS, and WH performed the genetic experiments and analyzed the data. YrS and XZ conducted the genetic counseling and follow-up of the patients. SL and YW contributed to the writing and revision of the manuscript. All authors read and approved the manuscript.

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Multisite assessment of the impact of cell-free DNA-based screening for rare autosomal aneuploidies on pregnancy management and outcomes

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Cell-free (cf) DNA screening is a noninvasive prenatal screening approach that is typically used to screen for common fetal trisomies, with optional screening for sex chromosomal aneuploidies and fetal sex. Genome-wide cfDNA screening can screen for a wide variety of additional anomalies, including rare autosomal aneuploidies (RAAs) and copy number variants. Here, we describe a multi-cohort, global retrospective study that looked at the clinical outcomes of cases with a high-risk cfDNA screening result for a RAA. Our study cohort included a total of 109 cases from five different sites, with diagnostic outcome information available for 68% (74/109) of patients. Based on confirmatory diagnostic testing, we found a concordance rate of 20.3% for presence of a RAA (15/74) in our study population. Pregnancy outcome was also available for 77% (84/109) of cases in our cohort. Many of the patients experienced adverse pregnancy outcomes, including intrauterine fetal demise, fetal growth restriction, and preterm birth. These adverse outcomes were observed both in patients with fetal or placental confirmation of the presence of a RAA, as well as patients that did not undergo fetal and/or placental diagnostic testing. In addition, we have proposed some suggestions for pregnancy management and counseling considerations for situations where a RAA is noted on a cfDNA screen. In conclusion, our study has shown that genome-wide cfDNA screening for the presence of rare autosomal aneuploidies can be beneficial for both patients and their healthcare practitioners. This can provide a possible explanation for an adverse pregnancy outcome or result in a change in pregnancy management, such as increased monitoring for adverse outcomes.

KEYWORDS

noninvasive prenatal testing, rare autosomal aneuploidy, pregnancy outcome, mosaicism, genetic counseling, genome-wide

Introduction

Clinical availability of cell-free (cf) DNA screening (also known as noninvasive prenatal testing [NIPT] or noninvasive prenatal screening [NIPS]) has resulted in a paradigm shift in chromosomal prenatal screening, with testing options quickly expanding from trisomy 21 screening only to the inclusion of screening for trisomies 13 and 18 (Nicolaides et al., 2012; Palomaki et al., 2012; Nicolaides et al., 2013), as well as optional screening for fetal sex and sex chromosomal aneuploidies (Mazloom et al., 2013; Samango-Sprouse et al., 2013). However, this cfDNA screening approach will still miss about 17% of clinically relevant fetal chromosomal abnormalities (Wellesley et al., 2012). In more recent years, the scope of cfDNA screening has broadened to encompass genome-wide screening for rare autosomal aneuploidies (RAAs) and partial deletions and duplications (i.e., copy number variants, including select microdeletion syndromes) as an option for some clinicians and their patients. Several studies have shown strong test performance for the detection of these additional anomalies by genome-wide cfDNA screening (Pescia et al., 2017; Pertile et al., 2021; Soster et al., 2021).

The screen-positive rate for RAAs in large genome-wide cfDNA screening studies has been shown to range from 0.12% (Scott et al., 2018) to 1.1% (Van Opstal et al., 2018). Benn *et al.* (Benn et al., 2019) pooled data from a number of studies and found that rare autosomal trisomies (RATs; i.e., an autosomal trisomy other than trisomy 21, 18, or 13) were present in 0.32% of cfDNA samples compared to 0.41% of trophoblast samples from chorionic villus sampling (CVS). Rare autosomal aneuploidies can be associated with a number of adverse pregnancy outcomes, including early miscarriage, intrauterine fetal demise (IUFD), fetal growth restriction (FGR), structural fetal anomalies, and preterm birth (Pertile et al., 2017; Eggenhuizen et al., 2021); as well as a proportion of cases in which there are no adverse pregnancy outcomes and birth of a healthy child. As cfDNA screening analyzes DNA released by apoptotic placental trophoblasts present in maternal plasma, a high-risk call for a RAA may be indicative of confined placental mosaicism (CPM) and not true fetal aneuploidy. A recent study (Van Opstal et al., 2020) found that cell-free DNA screening may be more sensitive than CVS for detection of CPM involving the cytotrophoblast. Although CPM cases are typically called as false positives for cfDNA screening, some studies have shown that CPM for autosomal aneuploidies can lead to adverse pregnancy outcomes, particularly for CPM involving chromosome 16 (Vaughan et al., 1994; Zimmermann et al., 1995; Sánchez et al., 1997; Van Opstal et al., 1998; Eggenhuizen et al., 2021), as well as chromosomes 2, 3, 7, 13, 15, and 22 (Eggenhuizen et al., 2021). In addition, a high-risk cfDNA result can be a marker for uniparental disomy (UPD, i.e., two copies of a whole chromosome derived from one parent (Benn, 2021)), especially if the CPM involves chromosomes that carry imprinted genes associated with defined syndromes (Mardy and Wapner, 2016; Grati et al., 2020).

With the increasing availability and uptake of genome-wide cfDNA screening, information on the clinical impact of rare autosomal aneuploidies will help guide pregnancy management and counseling. The majority of studies to date have had either small data sets or have not detailed the pregnancy and birth outcomes of patients in their study cohort. Here, we describe a global multi-site study that looked at the pregnancy and clinical outcomes following a high-risk RAA call in a large number of cases. We also wanted to determine if having a high-risk screening result for a rare autosomal aneuploidy was beneficial for management of the pregnancy in our cohort, and whether this was useful information for the healthcare provider and the pregnant patient. Results from our study, together with the experience of our Consortium members, were considered in an attempt to provide suggestions for pregnancy management and appropriate counselling of pregnant patients when these aneuploidies are detected.

Materials and methods

Study and patient details

All members of the Global Expanded NIPT Consortium were invited to submit details from their laboratory/clinic of cases reported as a rare autosomal aneuploidy following genome-wide cfDNA screening. Cases had to involve RAAs on a single whole chromosome only. All known cases from the time each site commenced genome-wide cfDNA screening, up to and including cases reported in 2021, were considered for inclusion in the study. The end date for cases reported in 2021 varied by contributing sites from May 2021 to October 2021, with one site not contributing any cases for 2021. The patient samples in this retrospective data analysis study were collected as part of routine cfDNA screening in either general or high-risk populations, dependent on the protocols and standards of care of each contributing site. The study included both singleton and twin pregnancies; however, not all sites performed genome-wide cfDNA screening for twin

pregnancies. Referral indications for cfDNA screening were collected as noted on the Test Requisition Form (TRF).

Information on human chorionic gonadotropin levels, PAPP-A levels, inhibin levels, and nuchal translucency (NT) were available for some of the patients in our study cohort. These were collected either as part of conventional screening, as part of the first trimester anatomy scan, or as part of preeclampsia screening.

All data was de-identified before analysis was carried out. The study received an IRB exemption from WCBIRB as it does not meet the definition of human subject research as defined in 45 CFR 46.102; specifically, the research involves analysis of retrospectively collected de-identified data only.

Genome-wide cfDNA screening

Genome-wide cfDNA screening and analysis was carried out at each site following site-specific routine laboratory procedures. All of the sites used a massively parallel whole-genome next-generation sequencing approach. Four of the five sites used the VeriSeq™ NIPT Solution v2 assay (Illumina, Inc.) (Pertile et al., 2021); the other site used the TruSeq™ Nano 16 sample protocol (Illumina, Inc.) for sequencing of the cfDNA (Illumina, 2017).

Where available, the fetal fraction (FF) was provided for each RAA case that was included in the study. A subset of cases at one of the sites had non-interpretable FF results. Presence of a RAA was thought to be the underlying etiology for interference with the bioinformatics analysis. In these cases, the result was considered non-interpretable, and FF was not reported. However, this was considered an indication that a RAA was present, and another bioinformatic software was then utilized to establish which chromosome was involved. These non-interpretable FF cases were denoted as "FF unavailable" in the Results section of the manuscript.

Clinical outcomes collection

Follow-up was attempted for all cases and was carried out according to the individual procedures of each laboratory or clinic. Clinical outcome information included diagnostic procedures, namely chorionic villus sampling (CVS) and amniocentesis, products of conception (POC) testing, findings from ultrasound examinations, newborn physical exam information, and/or any testing performed on the newborn. Desired clinical outcome data included baseline demographic details, adverse pregnancy outcomes, birth weight, or outcomes from the ongoing pregnancy (such as serial growth and any newborn complications). Cases were considered to have had confirmatory fetal testing if amniocentesis, POC testing, or newborn testing (blood test or umbilical cord test) had been carried out. Cases were considered to have undergone confirmatory placental testing if CVS or placental testing at birth had been carried out. Cases were deemed to be concordant if they involved a full or mosaic RAA or UPD on the chromosome of interest.

Results

Patient and sample details

In our study, a total of five sites provided details on 109 patients that received a high-risk screening result for a rare autosomal aneuploidy following genome-wide cfDNA screening, including 20 cases from Site A, 6 from Site B, 66 from Site C, 12 from Site D, and 5 cases from Site E. The five study sites were from multiple geographical regions, namely Australia, Canada, Argentina, and South Africa. Patients that underwent cfDNA screening from 2015 to 2021 were included, with 2020 having the highest percentage of cases included in the study cohort (23.9%; Supplementary Figure S1). Patient demographics are shown in Table 1; mean maternal age was 36.1 years, and mean gestational age was 11.9 weeks. The vast majority of patients (107, 98.2%) had a singleton pregnancy; both ongoing twin cases were dichorionic twin pregnancies. As can be seen from Table 1, maternal age was the most common referral indication (58.7%) followed by patient preference (32.1%). For a number of cases, we reassigned the referral indication based on additional information as detailed in Table 1 footnotes. Information on whether conventional screening was performed was available for 95 (87.2%) patients in the study cohort; of these, 19 (17.4%) had conventional screening and 76 (69.7%) did not. Of the 76 cases where conventional screening was not carried out, PAPP-A results were provided for 17 cases and NT results were provided for 20 cases. Overall, NT results were available for 29 (26.6%) patients in our cohort, with 27 (93.1%) of these cases having a normal NT result (<3.5 mm).

Genome-wide cfDNA screening results

Figure 1 shows the distribution of RAAs (i.e., aneuploidies that were not trisomy 21, 18, or 13) across chromosomes in our patient population; no RAAs were found for chromosomes 1, 12, 17, and 19. There was only one monosomy case in our cohort (monosomy 18); the rest were all trisomy cases. Trisomy 7 was the most common RAA (n = 20; 18.3%), followed by trisomy 22 (n = 17; 15.6%) and trisomy 16 (n = 14; 12.8%). For the twin pregnancies, a trisomy 15 and a trisomy 22 were reported. There were four cases that were listed as singleton pregnancies that had

a demised twin prior to the first cfDNA screening blood draw. All four of these cases (2x T15, 1x T22, and 1x T16) had a repeat blood draw with resolution of the aneuploidy, and normal pregnancy/birth outcomes. Diagnostic testing was only performed in one case and was normal for the ongoing twin. For additional details on these four cases, please *see* Appendix A (Data Sheet 1) in the Supplementary Materials.

TABLE 1 Demographics of the study cohort (n = 109).

Variable	Value
Maternal Age, yr	
Mean	36.1
Median	37
Range	25–47
Gestational Age, wk	
Mean	11.9
Median	11.1
Range	10–22.1
Basis of Gestational Age, *n* (%)	
Based on LMP	9 (8.3%)
Based on USS	100 (91.7%)
Type of Pregnancy, *n* (%)	
Singleton[a]	107 (98.2%)
Twins	2 (1.8%)
Referral Indications, *n* (%)	
Abnormal Ultrasound	3 (2.8)
Maternal Age[b]	64 (58.7)
Family History[c]	2 (1.8)
Patient Preference[d]	35 (32.1)
Multiple Indications[e]	5 (4.6)

LMP, last menstrual period; USS, ultrasound scan.
[a]Includes four cases of demised twin which occurred prior to the cfDNA screening blood draw.
[b]Thirty-eight of these cases had no known indication on the test requisition form (TRF). As the maternal age was over 35 years, we reassigned them as Maternal Age.
[c]For one case, family history details listed sensory motor neuropathy with or without agenesis of the corpus callosum. For the second case, there was a previous affected child/pregnancy (the child is being investigated for Prader-Willi syndrome and other genetic syndromes).
[d]Twenty-four of these cases had no indication listed on the TRF. As the maternal age was less than 35 years, we reassigned them as Patient Preference cases. For the remaining 16 cases, the TRF listed the referral indication as Other, with primary screening test as the detail provided. We reassigned these cases as Patient Preference cases as well.
[e]Two of these cases were originally entered as advanced maternal age on the TRF. As one of the cases also had an NT of 4.6 mm following ultrasound, and the other case had a previous affected child/pregnancy we reassigned both cases as Multiple Indications. The other three cases were listed as Other on the TFF. As one patient had a maternal age over 35 years of age and had a previous failed cfDNA screen at a different provider, we reassigned this case as a Multiple Indications case. The other two patients had a positive conventional screening result and advanced maternal age.

It is therefore reasonable to assume that in all four cases, the initial RAA noted on cfDNA screening may be attributed to the demised twin.

The fetal fraction (FF) was available for 67 (61.5%) cases in our study cohort and ranged from 3% to 27%, with both an average and a median of 9%. The fetal fraction distribution for these 67 cases across each of the affected chromosomes is shown in Supplementary Figure S2. We have categorized these 67 cases into cases that were concordant with the confirmatory fetal testing, concordant with the confirmatory placental testing, and the unconfirmed cases (includes cases where the RAA was not detected in the fetus but the placenta was not tested). We also highlighted the two discordant cases that underwent both confirmatory fetal and placental testing; further information on diagnostic outcomes is provided in the following section. There were seven chromosomes that had at least three RAA cases with FF information (Figure 2). Although the range of FF varied between these chromosomes, all cases reported a FF between 3% and 15% (except for the outlier of 27% for one of the trisomy 7 cases). It should be noted that all cases included in Figure 2 were singleton cases that underwent cfDNA screening in the first trimester. When we looked at these seven affected chromosomes only (chromosomes 7, 8, 9, 15, 16, 20, and 22), we found that there were also five RAA cases with known FFs from patients with singleton pregnancies that had undergone cfDNA screening in the second trimester (not shown in Figure 2). These included two trisomy 7 cases (13% FF; 15% FF), one case of trisomy 8 (9% FF), one case of trisomy 16 (14% FF), and one case of trisomy 22 (7%).

Diagnostic outcome data

Figure 3 shows a flowchart of the outcomes for all 109 RAA cases, with details in Supplementary Table S1. Overall, 74/109 patients (67.9%) underwent some type of diagnostic testing, with most undergoing amniocentesis (Supplementary Table S2). The RAA was confirmed in 10/72 (13.9%) patients where fetal testing was performed. Six patients had both fetal and placental testing, and an additional two patients had placental testing only. The RAA was confirmed in five of these eight patients (62.5%), indicating CPM as the underlying etiology for positive NIPT. Thus, based on these findings, fifteen cases could be considered to be concordant (either fetal or placental) with the diagnostic testing; the diagnostic yield was therefore 20.3% (15/74; *see* Table 2 for details of these cases). There were no cases in our cohort where the RAA was found to be present in both the fetus and the placenta following diagnostic testing.

In addition, UPD testing was carried out for 18 cases in our study cohort, including eight trisomy 7 cases, one trisomy 14 case, six trisomy 15 cases, two trisomy 16 cases, and one trisomy 20 case. There was one case of maternal UPD 15 and one

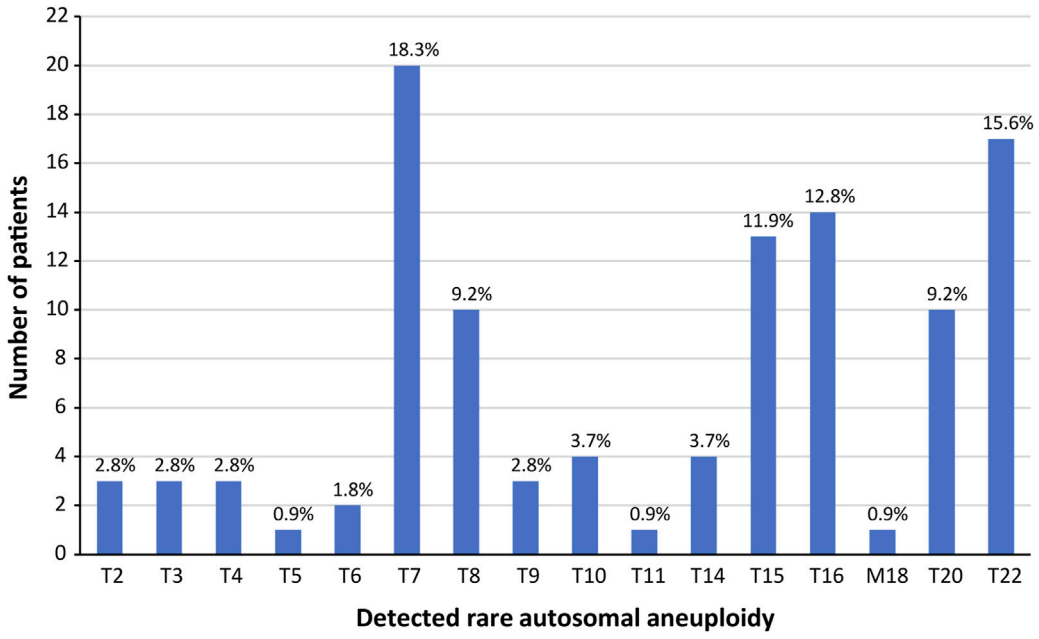


FIGURE 1
Detected rare autosomal aneuploidies in the study cohort. There were no rare autosomal aneuploidies observed on chromosomes 1, 12, 17, or 19; trisomies observed on chromosomes 13, 18, or 21 were not included.

case of maternal UPD 16 reported; these two cases are included in the 15 confirmed concordant cases.

Moreover, there are a number of other cases in the total cohort (109 cases) where it is possible that a RAA was present in either the fetus or the placenta (*see* Figure 4). This includes the four demised twin cases [*see* Appendix A (Data Sheet 1) of the Supplementary Materials], where the initial cfDNA screen reported a RAA but follow-up cfDNA screening was normal. In one of the dichorionic twin cases, which reported a trisomy 22 by cfDNA screening, there was a selective termination of one of the twins following cfDNA screening but prior to diagnostic testing. That twin had cystic hygroma on ultrasound at 13 weeks as well as delayed growth and possible brain anomalies. Amniocentesis was carried out on the surviving twin only, which was found to be normal and there was a normal birth outcome. Amongst the singleton cases with no diagnostic testing, there were three cases of a spontaneous miscarriage (one trisomy 9 and two trisomy 22), twelve cases with an IUFD (one trisomy 7, three trisomy 15, two trisomy 16, one trisomy 20, and five trisomy 22 cases), and one case of an elective termination of pregnancy (TOP) due to fetal anomalies. If we include these additional 21 cases (Figure 4, category 2), then the concordance would increase to 33.0% (36/109). There were also 17 singleton cases that underwent diagnostic fetal testing (RAA not detected in the fetus), but not placental testing, that experienced adverse pregnancy outcomes (such as FGR, preeclampsia, or preterm birth) or that ended with a termination of the pregnancy. Both of the elective TOP cases had anomalies noted on ultrasound (Figure 4, category 3). If we include these additional 17 cases, then the concordance could be as high as 53/109 (48.6%).

Pregnancy outcomes

Pregnancy outcome data was available for 84 cases in our cohort (Supplementary Table S1 and Figure 5). This included eight of the ten fetal concordant cases; further details of these cases are provided in Table 2. There were also 52 cases with pregnancy outcomes where the cfDNA screening result was not confirmed in the fetus. Of these 52 cases, three patients had preeclampsia, ten experienced FGR, and in one other case the placenta was reported as being "grossly abnormal." Although the majority of these cases delivered at term, there were a number of cases that underwent an induced preterm delivery (Supplementary Table S1), and three of these patients had an elective termination of pregnancy. All three cases that underwent an elective TOP had anomalies noted on ultrasound or autopsy. The vast majority of these cases (46/52; 88.5%) had not undergone confirmatory placental testing.

In addition, there were 24 cases with pregnancy outcomes where fetal confirmatory testing had not been carried out. Of these 24 cases, 12 (50%) experienced IUFD (one trisomy 7, three trisomy 15, two trisomy 16, one trisomy 20, and five trisomy

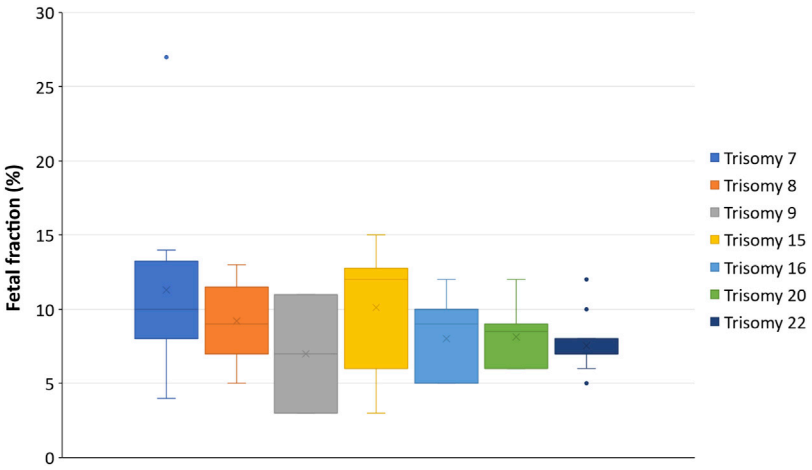


FIGURE 2
Relationship between fetal fraction and RAAs per chromosome. The box plots represent the first and third quartile (upper and lower margins of the box, respectively), the minimum and maximum FF values (lower and upper whiskers, respectively), the median FF (horizontal line within the box), and the mean FF (X within the box). The dots are outliers.

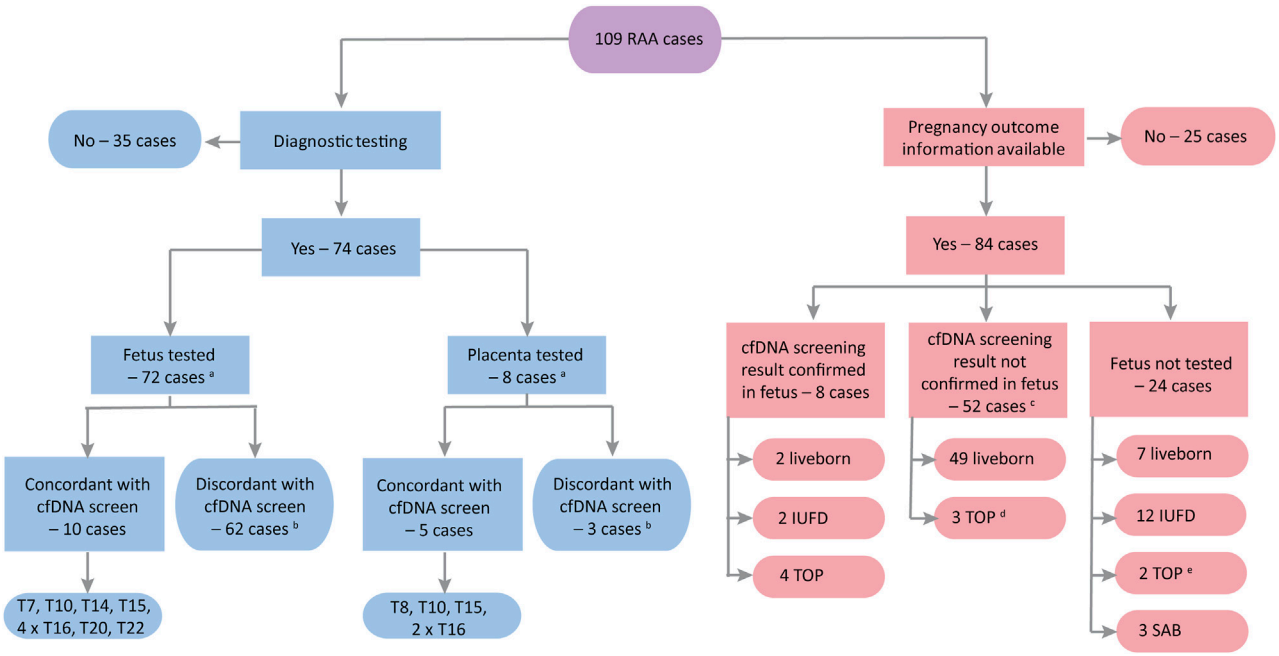


FIGURE 3
Flowchart of outcomes for the study cohort (*n* = 109). IUFD, intrauterine fetal demise; PX, pregnancy; SAB, spontaneous abortion; TOP, termination of pregnancy. [a]Six cases underwent both fetal and placental testing. [b]In two cases the cfDNA screening result was discordant with both fetus and placenta. [c]The cfDNA screening result was confirmed in placental tissue in four of these cases: three liveborn (trisomy 8 case with FGR and premature birth; trisomy 16 case with spontaneous premature birth; trisomy 16 case with induced premature birth for preeclampsia) and one selective TOP (trisomy 15 with severe FGR). [d]The other two TOP cases were a trisomy 20 case with multiple abnormalities noted on autopsy and a trisomy 7 case with cleft lip/palate identified on ultrasound following the cfDNA screen. [e]One case (trisomy 7) had a mutation in SLC12A6; the other case (trisomy 8) had abnormalities but no further details are available.

TABLE 2 Details of concordant cases.

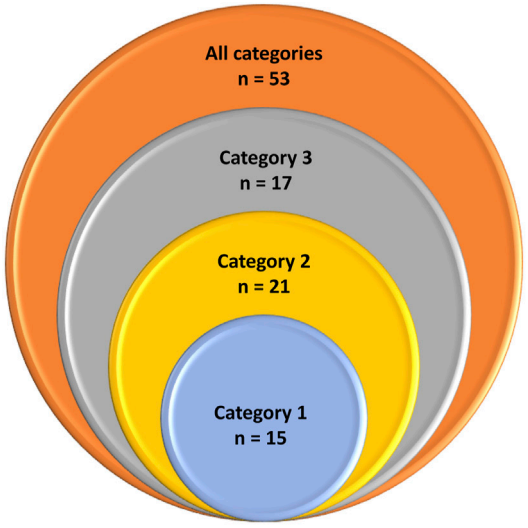
Observed concordance	Case no.	cfDNA screening result	Fetal fraction (%)	Interventions prompted	Pregnancy outcome	Pregnancy complications	Newborn physical exam
Fetus (mosaic)	1	Trisomy 7	13	TOP	Elective TOP	—	—
Fetus (mosaic)	2	Trisomy 10	N/a	TOP	Elective TOP	—	—
Fetus	3	Trisomy 14	N/a	Fetal anatomy scan	IUFD	—	—
Fetus (mosaic/UPD)	4	Trisomy 15	N/a	TOP	Elective TOP	—	—
Fetus (mosaic)	5	Trisomy 16	5	TOP	Elective TOP	—	—
Fetus (UPD)	6	Trisomy 16	9	Alteration of pregnancy monitoring[a]	Liveborn[b]	Preeclampsia	FGR, prematurity, cleft palate
Fetus (mosaic)	7	Trisomy 16	N/a	Alteration of pregnancy monitoring[a]	Unknown (lost to follow-up)	Unknown	Unknown
Fetus (mosaic)	8	Trisomy 16	9	Alteration of pregnancy monitoring[a]	Unknown (lost to follow-up)	Unknown	Unknown
Fetus (mosaic)	9	Trisomy 20	N/a	Alteration of pregnancy monitoring[a]	Liveborn[c]	None	Normal. Baby required breathing support at birth but was otherwise well
Fetus	10	Trisomy 22	N/a	Other	IUFD	—	—
Placenta (mosaic)	11	Trisomy 8	N/a	Alteration of pregnancy monitoring[a]	Liveborn[d]	Severe FGR	Normal
Placenta (mosaic)	12	Trisomy 10	N/a	Alteration of pregnancy monitoring[a]	Unknown (lost to follow-up)	Unknown	Unknown
Placenta	13	Trisomy 15	N/a	Alteration of pregnancy monitoring[a]	Elective TOP	Severe FGR	Hypospadias
Placenta (mosaic)	14	Trisomy 16	N/a	Alteration of pregnancy monitoring[a]	Liveborn[e]	Preeclampsia	No information provided
Placenta (mosaic)	15	Trisomy 16	10	—	Liveborn[f]	None	No information provided

FGR, fetal growth restriction; IUFD, intrauterine fetal demise; N/a, not available; TOP, termination of pregnancy.
[a]Monitoring for fetal growth and adverse pregnancy outcomes.
[b]Induced premature birth (27–32 weeks).
[c]Delivery at term (38–42 weeks).
[d]Induced premature birth at 36 weeks due to FGR.
[e]Induced premature birth at 34 weeks due to preeclampsia.
[f]Spontaneous premature birth (33–37 weeks).

22 cases), three cases (13%) experienced a spontaneous miscarriage (one trisomy 9 and two trisomy 22 cases), and two cases had an elective termination of pregnancy (one case had anomalies noted on ultrasound and the other case had a mutation in the SLC12A6 gene on prenatal diagnosis). The remaining seven cases were liveborn; of these seven liveborn cases, three were co-twin demise cases (discussed previously), one was an induced premature case with FGR, and another was a spontaneous premature case. Therefore, only two of the cases in this subcohort had no confirmed pregnancy complications.

Relationship between RAAs, low PAPP-A, and pregnancy outcomes

PAPP-A results were available for 29 patients in our cohort, of which 10 (34.5%) had low (<0.5 MoM) or very low (<0.2 MoM) PAPP-A values (Table 3). Two of these cases had a confirmed RAA (trisomy 16) in the fetus, with both reporting very low PAPP-A values (0.04 and 0.12). None of the 10 cases had placental testing for presence of aneuploidy. Fetal growth restriction was observed in four of these patients (one trisomy 2, one trisomy 7, one trisomy 15, and one trisomy 16). Overall, six of the ten cases noted pregnancy complications and one other case was an elective TOP (confirmed trisomy 16) which may have had complications that were not noted for the study. Of the 19 cases with normal PAPP-A values, fifteen underwent diagnostic testing—fourteen had amniocentesis with one case of confirmed RAA in the fetus (mosaic trisomy 16 [20%–30%]). The other case had postnatal testing of newborn blood (no aneuploidy detected) and placental tissue (which confirmed mosaic Trisomy 16 in the placenta). Two of these 19 cases experienced IUFD, one had a low-lying placenta, two had preeclampsia, and one had presence of fibroma.



Concordance category	Outcome
1	Cases in which diagnostic testing confirmed the cfDNA screening result in the placenta or fetus
2	Twin pregnancies with co-twin demise (n=4) or selective TOP of one twin with anomalies (n=1) and singleton pregnancies with no diagnostic testing but pregnancy loss or TOP (n=16; 3 SAB, 12 IUFD, 1 TOP)
3	Singleton pregnancies with evidence of CPM AND either: a) no diagnostic testing; or b) normal amniocentesis with no placental testing/CVS (n=17)
4	Total of all concordance categories

FIGURE 4
Estimation of concordance in the study cohort.

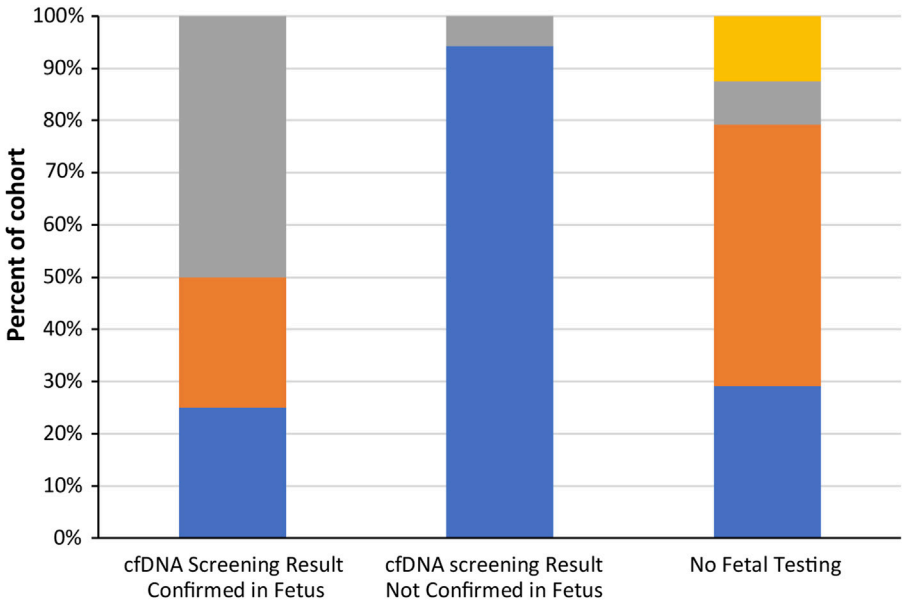
Discussion

Here, we discuss a multi-site global study looking at the clinical implications of prenatal screening for RAAs by genome-wide cfDNA screening. Our study showed the wide array of situations and outcomes that can occur when a patient receives a high-risk call for a RAA following cfDNA screening, reflecting the real-world experiences of prenatal screening. We found that early identification of these aneuploidies by genome-wide cfDNA screening was beneficial in a variety of clinical situations. It allowed for a change in pregnancy management in a number of cases (e.g., alteration of pregnancy monitoring for fetal growth and adverse pregnancy outcomes) and also provided a possible explanation for cases of miscarriage, co-twin demise, and fetal death as well as other pregnancy complications.

Trisomies 7, 22, and 16 were the most commonly observed trisomies in our study cohort. Trisomy 7 was also found to be the most commonly observed RAA in several other studies (Pertile et al., 2017; Scott et al., 2018; van der Meij et al., 2019; Van Den Bogaert et al., 2021). Trisomy 16 was the most common trisomy to be confirmed in either the fetus or the placenta (40% of all cases) in our cohort, with one case confirmed as UPD and the rest confirmed as mosaic trisomy 16. This is not surprising given that trisomy 16 is believed to be the most common trisomy, occurring in at least 1% of clinically recognized pregnancies (Hassold et al., 1995). Trisomy 16 is associated with a high probability of fetal death, fetal growth restriction, fetal anomalies, and preterm delivery (Benn, 1998; Peng et al., 2021). A study by Yong *et al.* (Yong et al., 2003) found that the level of trisomy in the different fetal or placental tissues was an indicator of the severity of outcomes. Unfortunately, we did not have complete outcomes for a number of our confirmed trisomy 16 cases.

In our study, ten of the 72 cases (14%) that underwent fetal diagnostic testing had a confirmed fetal RAA, with the majority of these cases ending in either an elective termination of pregnancy or fetal demise, highlighting the importance of this knowledge to the patient. As noted above, the true concordance could not be determined due to the fact that approximately a third of patients did not undergo confirmatory fetal testing and over 90% of patients did not have confirmatory placental testing. However, based on adverse pregnancy outcomes observed in many of the patients, there were many other cases where the RAA may have been present in either the fetus or the placenta. When we included the placental concordant cases, our overall rate of concordance was 20.3%. A recent study by Soster et al. (Soster et al., 2021) noted a positive predictive value of 22.4% for rare autosomal trisomies in a large cohort of genome-wide cfDNA screening samples. Although the PPV for rare autosomal aneuploidies is not as high as that for common trisomies, it is still approximately four-fold higher than the PPV observed (3.4%) using conventional screening for trisomies 21 and 18 (Gregg et al., 2016). Of the five confirmed CPM cases in our cohort, two had severe FGR and one had a spontaneous premature birth (outcomes for the remaining two cases are unknown), illustrating the impact that a placental RAA can have on pregnancy outcomes. A recent literature review by Eggenhuizen *et al.* (Eggenhuizen et al., 2021) looking at the association between CPM and adverse pregnancy outcomes found that CPM was associated with fetal growth restriction, preterm birth, structural fetal anomalies, and pregnancy complications such as preeclampsia. A number of these adverse outcomes were also noted in many of our study patients.

Our study clearly illustrates there can be value in genome-wide cfDNA screening for many patients, particularly in cases of pregnancy loss. Even in cases where diagnostic testing is not carried out, having a high-risk assessment for a RAA based on cfDNA screening may provide some explanation in the event of a pregnancy loss, which could be of value to patients. In our study cohort, we reported four cases of a co-twin demise, three cases of spontaneous miscarriage, and fourteen cases of IUFD. As noted above, in all four of the co-twin demise cases a follow-up cfDNA screen at a later gestational date did not show presence of a RAA,



Liveborn	2 (25%)	49 (94%)	7 (29%)
Intrauterine fetal demise	2 (25%)	0 (0%)	12 (50%)
Elective termination	4 (50%)	3 (6%)	2 (8%)
Spontaneous miscarriage	0 (0%)	0 (0%)	3 (13%)
Total N per cohort	**8**	**52**	**24**

FIGURE 5
Pregnancy outcomes for the study cohort (n = 84).

providing a likely explanation to the patient for loss of that twin. None of the spontaneous miscarriage cases underwent diagnostic testing. Although none of the IUFD cases had diagnostic testing prior to loss of the fetus, two of these 14 cases had follow-up POC analysis which showed presence of the RAA (trisomy 14 and trisomy 22, respectively) in the fetus. Genome-wide cfDNA screening can also be beneficial in altering the pregnancy management of the patient with the potential to offer increased and earlier ultrasounds and diagnostic procedures. In addition, a high-risk call for a RAA on a cfDNA screen can provide a possible explanation for pregnancy complications such as fetal growth restriction and preeclampsia.

Detailing outcomes for RAA cases can be very challenging in a study such as this one. This is due to the varying number of RAA cases per affected chromosome, the different approaches taken for pregnancy management of these patients, the differences in the outcome data collected and available at each of the contributing sites, and the low prevalence of RAAs in general. This has also been reported in single site studies of RAAs identified by cfDNA screening, with studies frequently reporting incomplete outcomes and varied clinical management practices (Liang et al., 2018; Kleinfinger et al., 2020; Soster et al., 2021). Whilst there is a myriad of ways that these outcomes could be grouped and analyzed, we chose to group patients into three different tiers as noted in Figure 4. The first tier (category 1) in our system represents patients where diagnostic testing found presence of the RAA in the fetus and/or placenta. The second tier (category 2) denotes cases where the high-risk RAA cfDNA screening result provided potentially valuable information for the patient, with outcomes that include pregnancy loss and elective termination of pregnancy following the identification of ultrasound anomalies. The final third tier (category 3) represents cases where the cfDNA screening result could be beneficial for the healthcare professional, as it indicated the need to monitor the pregnancy closely for complications such as fetal growth restriction and preeclampsia. Although our

TABLE 3 Relationship between RAAs, low PAPP-A, and Pregnancy Outcomes.

PAPP-A	cfDNA screening result	Confirmed in fetus[a]	Pregnancy complications	Pregnancy outcomes
0.04	Trisomy 16	Yes	Preeclampsia	Induced (premature at 27–32 weeks); FGR
0.12	Trisomy 16	Yes	None reported	Elective TOP
0.14	Trisomy 7	No	FGR	Liveborn (spontaneous premature 33–37 weeks)
0.15	Trisomy 8	No	None reported	Liveborn (term)
0.15	Trisomy 2	No	FGR	Liveborn (spontaneous premature 33–37 weeks)
0.24	Trisomy 9	No[b]	Gestational diabetes	Liveborn (term)
0.25	Trisomy 16	No[c]	None reported	Liveborn (term); child has developmental delay
0.39	Trisomy 3	No	None reported	Liveborn (term)
0.4	Trisomy 15	No	FGR	Liveborn (term)
0.48	Trisomy 7	No	Irritable uterus, multiple admissions for preterm labor, induced for reduced fetal movement	Liveborn (term)

FGR, fetal growth restriction; NBS, newborn screening; PAPP-A, pregnancy associated plasma protein-A; TOP, termination of pregnancy.
[a]None of the cases underwent placental testing.
[b]2p22.1 duplication in fetus (305 kb).
[c]Deletion in chromosome region 16p13.11 in the fetus.

approach is subjective, this tiered system could be a useful framework for healthcare professionals in determining the clinical utility of screening for RAAs.

One of the strengths of our study is that it is a global multi-centre study where a broad spectrum of care was received by patients undergoing cfDNA screening. This includes the use of different patient protocols and techniques for follow-up testing and analysis, as well as varying clinical practices for pregnancy management in these patients. We also had a large number of patients with clinical outcome information, including diagnostic outcomes and/or pregnancy outcomes. A limitation of our study is that we only had a small number of rare autosomal aneuploidies on some chromosomes and no aneuploidies observed on other chromosomes, preventing us from drawing conclusions regarding the clinical impact of RAAs on individual chromosomes. Due to the low prevalence of RAAs, this will be a limitation of all studies looking at RAAs. As our study was a retrospective analysis and full outcomes were not available for all of the cases, we were also not able to make an accurate determination of the true rate of pregnancy complications and adverse pregnancy outcomes in our study population. Both of these limitations could be addressed in future studies that focus on either a particular rare autosomal aneuploidy or a particular type of outcome observed in these patients such as FGR or IUFD. In addition, there may have been ascertainment bias regarding which cases had placental testing, which may have also been influenced by the clinical protocols in place at each site. Due to the small number of cases that did undergo placental testing, it was not possible to make any statistical comparisons between known CPM cases and suspected CPM cases, or cases where CPM was ruled out. Finally, a large number of patients were of a slightly higher maternal age than would be observed in a general pregnancy cohort, with over 60% of study participants listing maternal age as the referral indication for genome-wide cfDNA screening.

A large number of studies have been published in recent years looking at the identification of RAAs by genome-wide cfDNA screening. There is a lot of variation in these studies, including type of patient population, number of samples tested, and availability of outcome information (such as diagnostic testing for presence of RAAs, UPD testing, and adverse pregnancy outcomes). We attempted to capture the large amount of data that currently exists in the literature by compiling a table (Supplementary Table S3) that provides an overview of many of these recent studies. We carried out this multi-site global study to not only add to this growing body of evidence, but also to provide more information on the pregnancy and birth outcomes experienced by patients with a high-risk call for a RAA following genome-wide cfDNA screening, as these outcomes are not provided in many of the other studies to date.

Based on the results of our study, information from previous publications identified through the literature review that we carried out, and the experiences of members of our Consortium, some options for pregnancy management and patient counseling have been considered, with recognition that further research in this area is required to confidently establish an

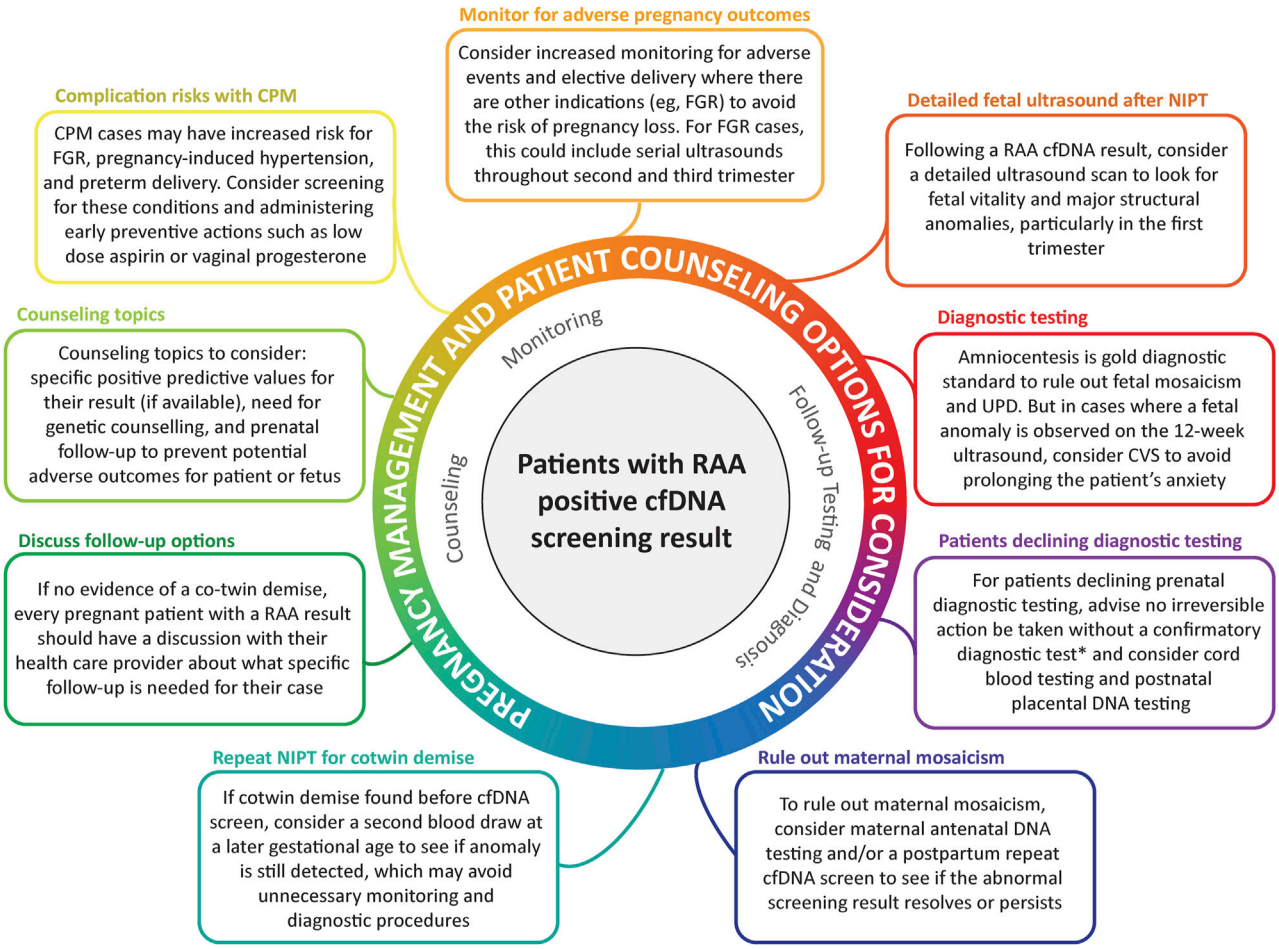


FIGURE 6
Pregnancy management and patient counselling options for consideration. *NIPT is a screening test. No irreversible clinical decisions should be made based on these screening results alone.

appropriate approach. These considerations are outlined in Figure 6.

In conclusion, our study has shown that genome-wide screening for presence of rare autosomal aneuploidies can be beneficial in a number of clinical situations, such as providing a possible explanation for an adverse pregnancy outcome or resulting in a change in pregnancy management. These interventions and possible explanations for pregnancy outcomes are of great benefit to pregnant patients, allowing for increased monitoring throughout the pregnancy or potentially alleviating any feelings of perceived personal responsibility for adverse outcomes. It can also be valuable for future pregnancies to determine if there is a recurrence risk for the anomaly in question. The recurrence risk may be low in many cases, which in itself can be valuable information in terms of the patient's anxiety and future pregnancy planning. This multi-site global study adds to the growing body of evidence regarding genome-wide cfDNA screening, and also adds valuable information regarding the clinical outcomes of patients that receive a high-risk screening call for a rare autosomal aneuploidy.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the corresponding author. The clinical outcome data in this study was obtained from patient records and therefore will not be made available through a database because of privacy concerns as well as ethical restrictions. However, deidentified data that underlie the results reported in this article (text, figures, tables, and appendices) are available by reasonable request within 12 months of publication. Proposals must include a detailed and sound methodological approach, including a statistical analysis

plan, as would be reasonably required for the purposes of publication in a peer-reviewed scientific journal.

Ethics statement

The studies involving human participants were reviewed and approved by WCBIRB. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

All authors contributed to the initial conception of the study. TM, MM, GA, MLD, JG, TH, KL, and PM contributed to data collection and entry or curation. ES assisted in data curation. TM, ES, and MM performed data analysis. ES, GA, MJ, PK, KL, and RS contributed to the background literature review. TM, ES, JG, and TH contributed to writing of the original draft of the manuscript. All authors contributed to review and editing of the manuscript, and all authors have read and approved the manuscript.

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Conflict of interest

TM is an employee of Genea and has been a paid speaker for Illumina. ES is an employee of Laboratory Corporation of America with option to hold stock and has also been a paid speaker for Illumina. MM and TH are employees of Monash IVF Group. GA and KL are employed by Next Biosciences. JG previously received a research grant from Illumina, Inc. MJ is employed by Genomed SA. PK is employed by Laboratory CERBA. PM is employed by Veragen. RS is employed by AMES, Centro Polidiagnostico Strumentale, Srl.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.975987/full#supplementary-material

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A retrospective single-center analysis of prenatal diagnosis and follow-up of 626 chinese patients with positive non-invasive prenatal screening results

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This study explored the diagnostic efficiency of different prenatal diagnostic approaches for women with positive non-invasive prenatal screening (NIPS) results by analyzing their clinical information and pregnancy outcomes. We collected data on 626 NIPS-positive pregnant women from January 2017 to June 2021 and arranged subsequent prenatal diagnostic operations for them after genetic counseling, along with long-term intensive follow-up. A total of 567 women accepted invasive prenatal diagnosis (IPD) (90.58%), and 262 cases were confirmed as true positives for NIPS. The positive predictive values for trisomies 21 (T21), 18 (T18), and 13 (T13); sex chromosome aneuploidies (SCAs); rare autosomal trisomies (RATs); and microdeletion and microduplication syndromes (MMS) were 81.13%, 37.93%, 18.42%, 48.83%, 18.37%, and 41.67%, respectively. Discordant results between NIPS and IPD were observed in 48 cases, with the discordance rate being 8.47%. Additionally, there were 43 cases with discordant results between karyotyping and chromosomal microarray analysis (CMA)/copy number variation sequencing. Additional reporting of RATs and MMS with routine NIPS that only detects T21/T18/T13 and SCAs can yield more accurate diagnoses. However, NIPS cannot be used as a substitute for IPD owing to its high false positive rate and discordance with other diagnostic methods. Therefore, we recommend CMA combined with karyotyping as the preferred method for accurately diagnosing NIPS-positive women.

KEYWORDS

karyotyping, positive predictive value, invasive prenatal diagnosis, chromosomal microarray analysis, non-invasive prenatal screening

Introduction

Approximately 900,000 new cases of congenital disabilities, including congenital structural, functional, and/or biochemical-molecular defects, are recorded yearly in China, with a prevalence rate of approximately 56.0 per 1,000 live births (Dai et al., 2011). Approximately 80% of congenital disability cases have unknown causes; however, strong evidence suggests that genetic conditions contribute to their etiologies (Feldkamp et al., 2017; Sun et al., 2018). Chromosomal abnormalities such as trisomies 21 (T21), 18 (T18), and 13 (T13) and sex chromosome aneuploidies (SCAs) are the main causes of congenital disabilities (Xie et al., 2021). In addition, multiple lines of evidence indicate that copy number variants (CNVs) in submicroscopic chromosomal structures can also play an important role in the etiology of some congenital disability cases (Lupo et al., 2019) (Hobbs et al., 2014). Congenitally disabled infants with chromosomal or genetic abnormalities are often diagnosed with varying degrees of intellectual disabilities, multiple malformation syndrome, growth retardation, and multiple dysfunction syndrome (Jackson et al., 2020), resulting in a considerable economic burden for families and society, thus highlighting the importance of prenatal genetic screening and diagnosis.

Non-invasive prenatal screening (NIPS), introduced into clinical practice in 2012, has gained popularity in recent years as a screening test for genetic abnormalities during pregnancy. NIPS identifies genetic abnormalities by analyzing maternal blood during pregnancy by employing next-generation sequencing (NGS) techniques to detect highly fragmented circulating cell-free fetal DNA (cffDNA), which is derived from placental tissues and has rapid post-delivery clearance profiles (Breveglieri et al., 2019; Chiu and Lo, 2021). Therefore, the risks associated with conventional invasive techniques are avoided, making it more acceptable to pregnant women as a preferred diagnostic method than conventional methods. In the last 10 years, numerous studies have focused on the clinical applicability of NIPS, mostly for detecting common autosomal trisomies (T21, T18, and T13) and SCAs (Bedei et al., 2021). Nevertheless, with the rapid development of NGS technologies, such as whole genome sequencing, the applicability of NIPS has been gradually extended to rare autosomal trisomies (RATs) and microdeletion and microduplication syndromes (MMS). NIPS involves the direct examination of DNA derived from the placenta, which has the same origin as the fetus, and has been shown to have much higher specificity and sensitivity than that of traditional serum analyte screening, which requires considering additional biochemical indicators as well as maternal age, race, and weight (D'ambrosio et al., 2019). However, NIPS-based identification of enhanced risk is susceptible to false positives; therefore, invasive prenatal diagnostic approaches such as amniocentesis, chorionic villi sampling, and/or percutaneous umbilical cord blood sampling are recommended to identify false positive findings (Liang et al., 2018; La Verde and De Falco, 2021).

TABLE 1 Demographics of the 626 women with NIPS positive results.

Characteristics	*N*	Constituent ratio (%)
Gestational age at NIPS (weeks)		
First trimester (6–13 weeks)	10	1.60
Second trimester (14–27 weeks)	615	98.24
Third trimester (≥28 weeks)	1	0.16
Maternal age (years)		
<30 years	287	45.85
30–34 years	217	34.66
35–39 years	87	13.90
≥40years	35	5.59
Pregnancy		
Singleton pregnancy	623	99.52
Twin pregnancy	3	0.48
Pregnancy method		
Natural conception	611	97.60
Assisted reproductive conception	15	2.40

In this study, we present the clinical data of 626 NIPS-positive cases detected based on whole genome sequencing of patients at a tertiary medical center in China from January 2017 to June 2021. The confirmatory invasive test results and detailed follow-up information were summarized to assess the performance of NIPS in detecting common autosomal trisomies, SCAs, RATs, and MMS and to analyze the clinical outcomes following high-risk results. In addition, we analyzed and compared the invasive test results with those of karyotyping and chromosomal microarray analysis (CMA)/copy number variation sequencing (CNV-seq) to evaluate the accuracy, efficacy, and incremental yield of CMA/CNV-seq compared with those of karyotyping for routine prenatal diagnosis.

Materials and methods

Ethics approval and consent to participate

The study design and protocol were reviewed and approved by the ethics committee of Changsha Hospital for Maternal and Child Health Care (No. 2021004). All methods and clinical procedures were performed in accordance with the relevant guidelines and regulations. All pregnant women received genetic counseling and provided informed consent before testing.

Subjects

From January 2017 to June 2021, 53,437 pregnant women underwent NIPS at our hospital, and 626 received positive results. The average age of the pregnant women who received

TABLE 2 Performance of NIPS in detecting trisomies and MMS in the 626 positive samples

Type of abnormalities		NIPS (*n*)	Prenatal diagnosis (*n*)		Diagnostic Rate (%)	With diagnosis results		PPV [% (95% CI)]
			Accepted (*n*)	Refused (*n*)		Accordance (*n*)	Discordance (*n*)	
Common autosomal trisomies	T21	110	106	4	96.36	86	20	81.13 (73.6–88.7)
	T18	59	58	1	98.31	22	36	37.93 (25.1–50.8)
	T13	40	38	2	95.00	7	31	18.42 (5.5–31.3)
SCAs	45, X	95	90	5	94.74	19	71	21.11 (12.5–29.7)
	47, XXY	54	49	5	90.74	40	9	81.63 (70.4–92.9)
	47, XXX	51	44	7	86.27	23	21	52.27 (36.9–67.6)
	47, XYY	38	30	8	78.95	22	8	73.34 (56.5–90.1)
RATs		114	98	16	85.96	18	80	18.37 (10.6–26.2)
MMS		71	60	11	84.51	25	35	41.67 (28.8–54.5)
Total		632*	573*	59	90.57	262	311	45.28 (41.6–49.8)

CI, confidence interval; *, Six cases suggested abnormalities on two chromosomes. Therefore, 6 more than the total of 626 and 567.

positive NIPS results was 31.0 ± 5.7 years. Among the study participants, 122 women were of advanced maternal age (≥35 years), accounting for 19.49% of the study population. Maternal blood was collected at gestational ages approximately between 12 and 28 weeks. Table 1 lists the demographic characteristics of these women.

On receiving a positive NIPS result, the pregnant women received prenatal genetic consultation with a professional geneticist and were informed of the importance of prenatal diagnosis to ascertain the true positives identified by NIPS. In our research, 567 women accepted the prenatal genetic diagnosis, while 59 women refused. The prenatal genetic diagnosis was carried out according to our routine experimental method and was completed at our prenatal diagnosis center. Five hundred and sixty-five women underwent amniocentesis at a suitable gestational stage (16–28 weeks), while two underwent percutaneous umbilical cord blood sampling (>28 weeks).

Non-invasive prenatal screening

We collected 5 ml of peripheral blood from the pregnant women using EDTA anticoagulant tubes and stored them at 4°C. The blood sample was treated as follows: centrifuged at 4°C, 1600 g for 10 min and the plasma was collected carefully and dispensed into 2.0 ml Eppendorf tubes. The plasma was centrifuged again at 4°C, 16,000 g for another 10 min. The upper plasma was carefully divided into new 2.0 ml Eppendorf tubes and each contained approximately 600 ml plasma, - 80 C refrigerator to save. Thereafter, plasma-free cell DNA (cfDNA) was extracted by magnetic bead extraction using a DNA extraction kit (BGI, Wuhan, China). The extracted DNA library was constructed using a fetal chromosome aneuploidy (T21/T18/T13) detection kit (BGI), and high-throughput sequencing (0.5×) was performed using the combinatorial probe-anchor synthesis-based BGISEQ-500 platform (BGI). We mainly analyzed T21-, T18-, and T13-positive cases, along with an additional positivity analysis for SCAs, RATs, and MMS.

Prenatal diagnosis by G-banded karyotyping

Amniocytes or cord blood cells were transferred to amniotic cell culture (Biosan, Zhejiang, China) and T cell culture media (Biosan), respectively, on an ultra-clean workbench for *in vitro* cell culture. When a specified number of cells were in the metaphase of active division, colchicine was added to inhibit mitosis. After the cells were digested by trypsin to isolate amniocytes, treated with hypotonic solution, fixed, and subjected to G-banded karyotyping, the karyotype was captured using an automatic scanner (Leica Microsystems,

TABLE 3 Cases showing discordance between NIPS and positive IPD results.

NO.	Categories	NIPS results	Diagnosis results		Cases (*n*)	Total (*n*)
			Primary classification	Secondary classification		
1	Multiple-to-one	Abnormality of multiple chromosomes	Abnormality only on one of those chromosomes	Trisomy (n = 3)	3	
				Mosaicism (n = 21)		
2	One-to-one	Abnormality of one chromosome	Abnormality of the same chromosome	Partial deletion or duplication (n = 8)	32	
				From monosomy to trisomy (n = 3)		
3	One-to-multiple	Abnormality of one chromosome	Multiple chromosomal abnormalities that included the target chromosome	Trisomy of two or more (n = 2)	9	48
				Trisomy + sSMC (n = 1)		
				Unbalanced structural rearrangement (n = 6)		
4	One-to-another one	Abnormality of one chromosome	Abnormality on another chromosome	Trisomy of another (n = 1)	4	
				Microdeletion (n = 3)		

sSMC, small supernumerary marker chromosomes.

Wetzlar, Germany). We then manually counted 30 or more integrity cleavage phases and analyzed five or more for description according to the principles stated in *An International System for Human Cytogenetic Nomenclature, ISCN 2020*.

Prenatal diagnosis by chromosomal microarray analysis

Amniocyte genomic DNA (250 ng) or umbilical cord blood cells was extracted using a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), after which it was digested, ligated, PCR-amplified, purified, fragmented, labeled, and hybridized to the Affymetrix CytoScan 750K array. The raw data were analyzed using the Chromosome Analysis Suite (ChAS) 4.2 (Affymetrix, Santa Clara, CA, United States). Interpretation and reporting of constitutional CNVs were performed according to the standards and guidelines released by the American College of Medical Genetics (Riggs et al., 2020). We described the clinical significance of CNVs under a five-tiered system: pathogenic, likely pathogenic, variants of uncertain significance, likely benign, and benign. In accordance with the aforementioned standards, we did not report microdeletions less than 500 kb, microduplications less than 1 Mb, and some CNVs with low penetrance (Rosenfeld et al., 2013; Armour et al., 2018). In addition, regions of homozygosity (ROH) with a size of more than 10 Mb were reported.

Prenatal diagnosis by CNV-seq

Genomic DNA was extracted from amniocytes or umbilical cord blood cells using a Qiagen DNeasy Blood & Tissue Kit (Qiagen). Genomic DNA (50 ng) was prepared as a template to construct a sequencing library and sequenced using a NextSeq CN500 System (Illumina, San Diego, CA, United States). The sequencing results were subjected to bioinformatics analysis and annotated by the chromosome aneuploidy and gene microdeletion analysis software (Berry, Inc., Beijing, China). The whole experiment process was commissioned by Berry, Inc. The clinical evaluation of results showing CNVs (>100 kb) was based on the aforementioned guidelines.

Statistical analysis

The positive predictive value (PPV) was calculated as the number of cases for which NIPS screening and confirmatory diagnostic testing were concordant (including mosaicism) divided by the number of cases with IPD results multiplied by 100. SPSS 26.0 software (SPSS Inc., Chicago, IL, United States) was used to determine the confidence interval of PPV.

Results

Positive predictive value of non-invasive prenatal screening

Within the study period, 53,437 pregnant women underwent NIPS examination at our institute, and 626 received positive results, with an overall positive rate of 1.17%. Among the 626 positive cases recorded at Changsha Hospital for Maternal and Child Health Care from January 2017 to June 2021, 59 patients refused prenatal genetic diagnosis, while 567 patients underwent IPD, with a diagnostic rate of 90.58%,

which included 115 confirmed cases of common autosomal trisomies, 104 of SCAs, 18 of RATs, and 25 of MMS (Table 2). Moreover, the PPV for T21, T18, T13, SCAs, RATs, and MMS was 81.13% (86/106), 37.93% (22/58), 18.42% (7/38), 48.83% (104/213), 18.37% (18/98), and 41.67% (25/60), respectively. In addition, among the different types of SCAs, 47, XXY had the highest PPV (40/49, 81.63%); followed by 47, XYY (22/30, 73.34%); 47, XXX (23/44, 52.27%), and 45, X (19/90, 21.11%).

Discordance between non-invasive prenatal screening and positive invasive prenatal diagnosis results

Among the 567 NIPS-positive samples, 48 cases were discordant with the positive IPD results except for cases of balanced structural rearrangement. We divided these cases into the following four categories according to the number of chromosomes considered for the evaluation based on NIPS and IPD (Table 3): 1) Multiple-to-one: NIPS results suggested multiple chromosome abnormalities, whereas IPD identified abnormality on only one of those chromosomes; 2) One-to-one: NIPS results suggested abnormality of one chromosome; IPD results also suggested abnormality of the same chromosome but were discordant with the NIPS result in terms of the location/type of the chromosomal aberration. This included mosaicism in 21 cases, partial deletion/duplication in 8 cases, and from monosomy to trisomy in 3 cases; 3) One-to-multiple: NIPS results suggested abnormality of one chromosome, whereas IPD results revealed multiple chromosome abnormalities that included the target chromosome; 4) One-to-another one: NIPS results suggested abnormality of one chromosome, whereas IPD identified the abnormality on another chromosome. In types "one-to-multiple" and "one-to-another one," IPD reported several additional findings involving other chromosomes compared with those of NIPS, which included trisomy/partial trisomy, microdeletions/microduplications, and unbalanced structural rearrangements. Details are shown in Supplementary Table S1.

Discordance between results of karyotyping and CMA/CNV-seq

Among the pregnant women who chose to proceed with the diagnostic procedures, 512 cases were diagnosed at our prenatal diagnosis center; 308 pregnant women opted for both karyotyping and CMA/CNV-seq. Discordant results between karyotyping and CMA/CNV-seq were found in 43 cases, accounting for 13.96% of the study population (Table 4 and Supplementary Table S2). This excluded chromosome polymorphisms, such as inv (9)(p12q13), inv (1)(p13q21), and inv(Y)(p11.2q11.2); seven discordant cases were associated with mosaicism, including four cases of sex chromosome mosaicism and three cases of autosomal mosaicism. Among these, six cases were successfully detected by karyotyping but not by CMV/CNV-seq, and for Case 304, while a normal karyotype was observed, the CMA result was arr (2) × 3 [0.52] hmz. Case 108 showed positive results for both karyotyping and CMA, with the CMA identifying the source of the small supernumerary marker chromosomes (sSMCs) detected by karyotyping. Karyotyping detected reciprocal translocation and inversion in cases 140 and 437, respectively; however, these balanced chromosome rearrangements were not identified by CMA. Moreover, 10 cases with MMS and 6 with ROH were detected by CMA in 193 samples with normal karyotypes, thus having improved diagnostic rates of 5.18% and 3.11%, respectively, compared with those for karyotyping. In addition, chromosome breakpoints in 17 cases with unbalanced rearrangements were detected relatively accurately by CMA/CNV-seq (Supplementary Table S2).

Analysis of pregnancy follow-up

We followed up on all the NIPS-positive cases (Figure 1). Among the 567 pregnant women who underwent IPD, 262 were confirmed as true positive cases. Tracking the pregnancy outcomes of 260 pregnant women among them led to the following observations: mothers of all fetuses diagnosed with T21, T13, T18, RATs, Klinefelter syndrome, and Turner syndrome terminated their pregnancies, excluding one T21 case (Supplementary Table S1; Case 439) and two cases of haploid chromosome X with a low rate of mosaicism and normal ultrasound findings throughout pregnancy (Supplementary Table S1; cases 309 and 512); five cases diagnosed as having fetuses with Triple X syndrome and eight cases diagnosed as having fetuses with 47, XYY syndrome terminated their pregnancies, with birth rates of 77.27% (17/22) and 63.64% (14/22), respectively. Among the MMS cases detected by NIPS, the clinical significance of most cases was unknown, and due to the presence of pathogenic CNVs, only 45.83% (11/24) cases terminated their pregnancies. Additionally, among the 305 cases confirmed to be false positives, pregnancy outcome tracking of 296 pregnant women showed the following: two patients underwent spontaneous abortion; six patients terminated their pregnancies due to other genetic abnormalities; two patients had abortions due to abnormal ultrasound findings; three patients terminated their pregnancies for unknown reasons, and the remaining 283 mothers had infants that were born healthy.

Among the 59 pregnant women who refused prenatal genetic diagnosis, the pregnancy outcomes of 42 women were tracked: eight patients terminated their pregnancies due to multiple malformations found by ultrasound, and 34 underwent

TABLE 4 Cases showing discordance between karyotyping and CMA/CNV-seq results.

No	Case number	Maternal Age (Years Old)	Gestational Age (Weeks *)	NIPS	Karyotype	CMA/CNV-seq results	Size (Mb)	Ultrasound findings	Pregnancy outcome
1	Case 309	30	16^{+5}	XO	45, X [6]/46, XX [75]	N	—	*N*	Born
2	Case 312	27	18^{+1}	XO	45, X [41]/47, XXX [20]	N	—	Single umbilical artery	TOP
3	Case 353	29	19^{+1}	XO	45, X [6]/46, XX [84]	N	—	N	TOP
4	Case 493	28	16^{+3}	XO	47, XXX [18]/46, XX [37]	N	—	N	Born
5	Case 122	37	20^{+}	T13, T20	47, XN, +20 [28]/46, XN [22]	N	—	N	TOP
6	Case 386	25	14^{+3}	T4	47, XX, +4 [19]/46, XX [71]	N	—	N	TOP
7	Case 304	31	17^{+3}	T2	N	arr (2)x3 [0.52] hmz	—	FGR, Oligohydramnios	TOP
8	Case 108	28	18^{+}	T16	47, XN, +mar [14]/46, XY [18]	arr [GRCh37] 16p11.2q22.1 (33,766,659_67,589,639)x3 [0.52]	33.8	—	TOP
9	Case 140	48	20^{+}	T16	46, XY, t (4;9) (q12; q22)[9]/46, XY [31]	N	—	N	Born
10	Case 437	33	20^{+1}	XXX	46, XX, inv (6) (p21q13) mat	N	—	N	Born
11	Case 109	28	17^{+}	MMS	N	arr [GRCh37] 5p15.33 (113,576_2,835,831)x1	2.7	N	TOP
12	Case 121	36	22^{+}	MMS	N	arr [GRCh37] 3q23q25.31 (141158071_155492129)x3	14.3	N	TOP
13	Case 123	24	27^{+}	MMS	N	arr [GRCh37] 2q24.1q31.1 (158448403_174291185)x1 dn	15.8	NT was 3.3 mm at 12 gestational age	TOP
14	Case 172	33	19^{+}	MMS	N	arr [GRCh37] 16p13.11p12.3 (15319277_18172468)x1	2.8	N	Born
15	Case 242	30	17^{+3}	T16	N	arr [GRCh37] 16p13.11p12.3 (15325072_18242713)x3 mat	2.9	N	Born
16	Case 347	31	17^{+3}	T15	N	arr [GRCh37] 1p36.33 (849,466_1996635)x1 dn	1.15	Fetal tetralogy of Fallot, PLSVC, Thoracic vertebral abnormality	TOP
17	Case 64	28	26^{+}	T21	N	arr [GRCh37] 13q33.3q34 (107382604_115107733)x1	7.7	FGR	TOP
18	Case 376	28	18^{+4}	MMS	N	arr [GRCh37] 15q13.1q13.3 (28635057_32444261)x1 mat	3.81	N	Born
19	Case 164	27	20^{+}	T15	N	arr [GRCh37] 15q11.2q13.1 (23281885_28526905)x4	5.2	N	TOP
20	Case 500	33	19^{+4}	MMS	N	arr [GRCh37] 22q13.33 (50207711_51197766)x1	0.99	Normal indicators at 12 weeks	TOP
21	Case 86	38	18^{+}	MMS	N	arr [GRCh37] 5p15.33p15.1 (113,576_16203210)x2 hmz	16.0	Missed follow-up	
22	Case 146	36	19^{+}	MMS	N	arr [GRCh37] 2q31.1q37.3 (174605494_242773583)x2 hmz	68.1	FGR, Placental thickening, Oligohydramnios	TOP
23	Case 156	31	18^{+}	T16	N	arr [GRCh37] 16p13.3p12.3 (94,807_17705580)x2 hmz, 16q22.3q24.3 (73772289_90146366)x2 hmz	17.6, 16.3	N	Born
24	Case 240	32	20^{+}	T13	N	arr [GRCh37] 18p11.23q12.2 (7131233_34755544)x2 hmz	27.6	N	Born
25	Case 477	27	16^{+5}	MMS	N	arr [GRCh37] 18q21.32q23 (56947979_77997606) hmz	21.05	N	Born
26	Case 552	30	16^{+4}	CNV	N	arr [GRCh37] 18p11.32p11.21 (136,305_11807701)x2 hmz	11.67	N	Born

XO, 45, X high risk; XXX, 47, XXX high risk; N, Normal;/: No; PLSVC, persistent left superior vena cava; NT, nuchal translucency; TOP, termination of pregnancy; *, weeks + days.

delivery. Among the cases that resulted in live births, a confirmed occurrence of T21 was found in an infant from a twin pregnancy, and the remaining 33, which included one case of T13, reported healthy births that were confirmed by long-term follow-up.

Discussion

From 2012 onwards, NIPS for fetal aneuploidies has been broadly implemented for detecting common autosomal trisomies and SCAs owing to the advantages associated with it, such as non-invasiveness, zero risk for the unborn baby, capability to acquire diagnostic hints as early as the 10th week of gestation onwards, immediate results within as early as 2 weeks, as well as high sensitivity (99.3% for T21, 97.4% for T18, and 97.4% for T13) and specificity (pooled specificity was 99.9% for all three trisomies) (Taylor-Phillips et al., 2016; Liehr, 2021). However, this approach identifies only 75%–85% of clinically relevant aneuploidies (Pescia et al., 2017). Therefore, additional screening based on identifying RATs and MMS is necessary. Here, we assessed a series of 626 NIPS-positive cases with low genomic coverage and detected a broad range of aneuploidy classes, namely the common autosomal trisomies, SCAs, RATs, and MMS. The PPV of T21 (81.23%) observed using our platform in the present study was within the range of values reported in published literature (between 80% and 90%) (Junhui et al., 2021). The PPVs of T18 and T13, presented as the main positive results, were 37.93 and 18.42%, respectively, slightly lower than those reported by previous studies using the same platform (Lu et al., 2020). The PPVs of SCAs, RATs, and MMS, presented as additional positive results, were 48.83, 18.37, and 41.68%, respectively, slightly higher than those reported by previous studies using the same platform (Wang et al., 2021). PPVs obtained via NIPS, excluding that of T21, are known to have large variations associated with prior risk factors, such as maternal age and individual trisomies (Petersen et al., 2017; Skrzypek and Hui, 2017). NIPS results are affected by an insufficient or absent fetal fraction, fetoplacental mosaicism, the presence of a vanishing twin, maternal mosaicism, maternal CNVs, and maternal malignancy, leading to false positives that are discordant with results obtained by other methods (Hartwig et al., 2017; Samura and Okamoto, 2020). Moreover, technical factors such as testing procedures, sequencing algorithms, and depths, as well as Z-scores, may also be important in terms of their effect on NIPS results (Junhui et al., 2021). This makes the fluctuation of the PPV of NIPS in different study populations a common occurrence. In our research, we found that RATs have a PPV of 18.37%, similar to that of T13 presented as the main positive results and could therefore act as an extension of NIPS screening. MMS had a higher PPV than that of T18 presented as positive results, but most of the CNVs were identified as hereditary and of unknown significance. Disclosure of these results to pregnant women did not provide them any substantial help with pregnancy-related decisions and had a negative psychological impact on them. Therefore, for cases of MMS suggested by NIPS results, it is recommended to only present the diagnoses to pregnant women if the CNVs are in genomic regions that have definite associations with certain syndromes or after pathogenicity has been identified.

Discordant results associated with NIPS often occur during screening and diagnosis. At present, the discordant cases reported in literature mainly focus on false positive and false negative NIPS cases [26]. In this research, we focused on true positive cases and identified 48 discordant cases (which accounted for 8.47% of the total cases) between the positive results of NIPS and IPD. Assessment of the cases in our analysis confirmed the importance of testing by IPD in addition to NIPS. There are four main reasons for the discordance. First, there was a certain degree of false positivity in NIPS, so it can not accurately determine abnormalities as being on one or two chromosomes in type of "Multiple-to-one". Second, NIPS has high detection rates coupled with high sensitivity for common fetal aneuploidies (trisomies 13, 18, and 21), but the screening accuracy for SCAs, RATs, and CNVs is lower than that for the common autosomal aneuploidies (Taylor-Phillips et al., 2016; Liehr, 2021; Wang et al., 2021). Therefore, some aneuploidies and CNVs have been found unexpectedly in IPD. Third, it must be considered that NIPS, which is based on second-generation sequencing technologies, is not sensitive to some DNA fragments with a high average content of guanine and cytosine bases, and the sequencing depth of NIPS also means that NIPS cannot give more genetic information about some sSMCs and CNVs (Ye et al., 2021). Last, NIPS is cffDNA-based non-invasive prenatal screening. In pregnant women, the small amount of plasma cffDNA is believed to be a contribution from the cytotrophoblast cells of the chorionic villi in the placenta (Lun et al., 2008). NIPS identifies fetal genetic abnormalities under the assumption that the cytogenetic constitution of the placenta matches that of the fetus. However, during embryonic development, mitotic error and trisomy, monosomy, and deletion rescue can lead to two (or more) genetically different cell lines that differentiate into different parts. As a result, the karyotype of cytotrophoblast cells does not always represent fetal chromosome constitution (Van Opstal and Srebniak, 2016). Besides, the different occurrence times of mitotic non-disjunction of different chromosomes in early embryo development results in varying levels of chromosomal mosaicism in different placental and fetal tissues. Among our discordant cases, we found that NIPS suggested trisomy/monosomy in 21 cases where IPD results indicated mosaicism. This accounted for the largest proportion of discordance observed between NIPS and IPD results. Our observations show that in some cases diagnosed with very low rates of mosaicism confirmed by multiple detection methods, pregnant women choosing to continue with pregnancy had fetuses that developed well after birth (Supplementary Table S1; Case 439). Therefore, it is advised that pregnant women who receive positive NIPS results should not hasten to adopt a negative attitude and should actively undergo

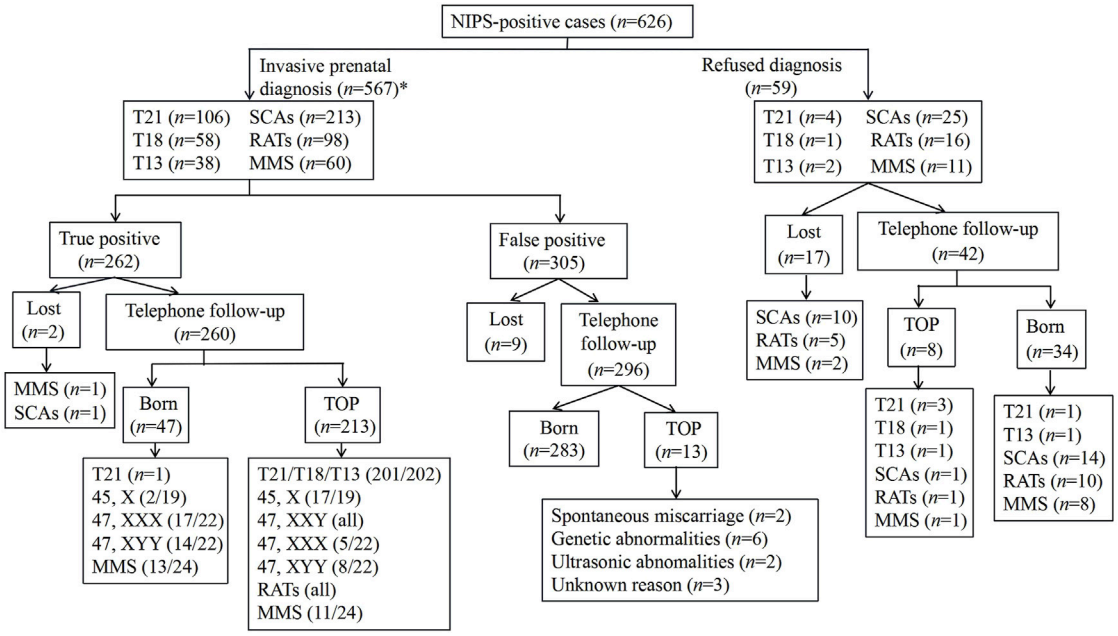

FIGURE 1
Outcomes of all NIPS-positive cases.TOP, termination of pregnancy; *, six cases suggested abnormalities on two chromosomes. Therefore, the sum in the box is six more than 567.

follow-up consultations to identify the abnormality by means of IPD; only then can they make decisions regarding the continuation or termination of pregnancy. Accordingly, NIPS should not be regarded as a diagnostic tool for conclusive diagnoses, and positive NIPS results must be further assessed using invasive prenatal genetic diagnostic and ultrasonic diagnostic approaches.

G-banded karyotyping, which has limited resolution (5–10 Mb), is a common diagnostic technique and the gold standard for diagnosing chromosomal disorders. It can detect chromosomal aneuploidy or polyploidy, large chromosomal deletions/duplications, and balanced chromosomal rearrangement. Other commonly used prenatal diagnostic techniques, namely CMA and CNV-seq, can be used to analyze aneuploidy as well as microdeletion and microduplication (≥100 kB) (Armour et al., 2018; Zhao and Fu, 2019). In our study, 43 discordant cases were found in the chromosomal analysis of 308 patients performed using karyotyping and CMA/CNVseq. Four instances of sex chromosome mosaicism were detected by karyotyping but not by CMA. For cases of sex chromosome abnormality indicated by NIPS, karyotyping was seen to be more effective than CMA in confirming true positive detection of sex chromosome mosaicism. Additionally, two cases of autosomal mosaicism were detected by karyotyping but not by CMA, whereas one case of autosomal mosaicism was detected by CMA but not by karyotyping.

Karyotyping and CMA each have certain advantages and disadvantages for their use in detecting autosomal mosaicism. Although karyotyping requires cell culture, it can detect mosaics of different types, including those of a very low proportion. However, multiple factors, such as aberration of the primary amniotic cells themselves and cell aberration resulting from *in vitro* culturing, may lead to pseudomosaicism, a loss or increase in the abnormal cell line resulting in a change in the proportion of mosaic cells, or even to missing the detection of autosomal mosaicism (Fan et al., 2021). Conversely, CMA can only stably detect mosaicism in cells with larger proportions (>30%) of it and can directly detect the amniotic fluid genome, thus being capable of reflecting the proportion of true mosaicism in the sample. Additionally, CMA has the unique advantage of being able to detect CNVs and ROH, which cannot be detected by karyotyping. Our results show that compared with CNVs detected by karyotyping, 10 more cases of pathogenic CNVs were detected by CMA, indicating an improved diagnostic rate of 5.18% compared with that of karyotyping. In addition, for NIPS-positive samples showing normal karyotypes, a total of 3.11% ROH was detected by SNP-based microarrays. The presence of large fragments of ROH in the fetus is associated with the risk of uniparental disomy (UPD), which is the result of the successful rescue of cells from aneuploidy to euploidy after germ cell fertilization. A UPD diagnosis should be considered when NIPS suggests trisomy, especially on chromosomes 6, 7, 11, 14, 15, and 20 (Benn, 2021). Thus, it can be seen that a single detection method can easily lead to misdiagnosis. Therefore, combining karyotyping with CMA seems preferable for obtaining accurate diagnoses of chromosomal abnormalities.

At the later stages of follow-up, most women with fetuses diagnosed with autosomal trisomies had terminated pregnancy, excluding one case of T21 with a low rate of mosaicism. SCAs are the most frequent chromosomal abnormalities encountered in NIPS. In true positive cases, the overall termination of pregnancy rate was 22.7% (5/22) for Triple X syndrome and 36.36% (8/22) for 47, XYY syndrome, which was significantly lower than those for other chromosomal syndromes. The prevalence of Triple X and 47, XYY syndromes among newborns is high at 11 per 100,000 females and 18 per 100,000 males, respectively (Gruchy et al., 2016). Although an increased risk of psychosocial problems or psychiatric disorders (such as autism) during childhood has been associated with the 47, XYY syndrome, long-term, unbiased follow-up studies have concluded that Triple X and 47, XYY syndromes do not cause postnatal development disorders. Children with these conditions have IQs in the normal range despite physical abnormalities being occasionally observed (Berglund et al., 2019). The acceptance of fetuses with SCAs tends to be affected by many factors, such as social and cultural background, disease type, genetic counseling methods, and the economic status of the family. In China, an increasing number of people are accepting children with Triple X and 47, XYY syndromes. Therefore, the exclusion of Triple X and 47, XYY syndromes from the NIPS process is expected in the near future. Moreover, the true or false positive nature of ultrasound findings is also an important factor in determining the decision to continue a pregnancy.

Conclusion

NIPS has a high positive rate for detecting common trisomies and SCAs in the general testing of pregnant women, and testing for RATs and MMS can be additionally conducted with the informed consent of pregnant women to obtain a more accurate diagnosis. However, NIPS cannot be used as a substitute for amniocentesis and prenatal diagnosis techniques owing to its high rate of false positives and discordance with diagnoses provided by IPD. CMA combined with karyotyping can be recommended as the preferred method of prenatal diagnosis for cases where NIPS results indicate a high risk in pregnancy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The study design and protocol were reviewed and approved by the Ethics Committee of Changsha Maternal and Child Health Hospital, China (May 7, 2021; 2021004). The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors have materially participated in the study and manuscript preparation. SD, HL, and JH carried out all the molecular genetic analysis and participated in the design of the work; SL and SL collected all clinical data and participated in conceiving the work; XB, SZ, and XL designed the work and drafted and revised the manuscript. All authors have approved the final article.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.965106/full#supplementary-material

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Case Report: How whole-genome sequencing-based cell-free DNA prenatal testing can help identify a marker mhromosome

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A supernumerary marker chromosome (SMC) is a structurally abnormal chromosome that cannot be characterized by conventional banding cytogenetics. Marker chromosomes are present in 0.075% of prenatal cases. They are associated with variable phenotypes, ranging from normal to severely abnormal, and the prognosis is largely dependent on the results of further cytogenomic analysis. Here, we report the identification and characterization of a marker chromosome following prenatal screening in a 39-year-old pregnant patient. The patient had a normal first trimester ultrasound but was high-risk for fetal chromosome anomalies based on the results of maternal serum parameters. Chorionic villus sampling was performed, and analysis of chorionic villi revealed the presence of two identical marker chromosomes. In the interest of a rapid identification of the markers, we performed noninvasive prenatal testing (NIPT) together with chorionic villus sampling. A pericentromeric 29 Mb duplication of chromosome 20: dup (20) (p13q11.21) was identified and thereafter confirmed by targeted metaphasic FISH. Whole-genome sequencing-based NIPT was instrumental in rapid characterization of the SMCs and allowed us to obviate the need for multiple expensive and time-consuming FISH analyses.

KEYWORDS

case report, supernumerary marker chromosome, noninvasive prenatal testing, fish, array

Introduction

A supernumerary marker chromosome (SMC) is a supplementary chromosome that cannot be characterized using conventional banding cytogenetic analysis (ISCN 2020). SMCs are usually equal in size or smaller than a chromosome 20 of the same metaphase spread (Liehr and Weise, 2007). Marker chromosomes have been shown to be present in 0.075% of unselected prenatal cases but only in 0.044% of consecutively studied postnatal cases (Liehr and Weise, 2007). The clinical phenotypes associated with marker chromosomes can be highly variable, ranging from normal to severely abnormal (Paoloni-Giacobino et al., 1998; Jang et al., 2016). The prognosis in pregnancies with marker chromosomes depends on whether euchromatin is present, if the marker chromosome is inherited or *de novo*, if it is homogeneous or mosaic, whether it is confined to the placenta, and on the presence or absence of uniparental disomy (UPD) if the marker is derived from a chromosome subjected to imprinting (Starke et al., 2003). Thus, to determine the prognosis, it is essential to characterize the SMC. There are two primary molecular cytogenetic methods used for identification and characterization of SMCs: Centromeric fluorescence *in situ* hybridization (FISH) and chromosomal microarray. Centromeric FISH allows characterization of markers originating from acrocentric chromosomes and is readily available, fast, and affordable. For markers originating from non-acrocentric chromosomes, it is an expensive and time-consuming method. Array allows only euchromatin detection, and low-level mosaicism can cause false-negative results. Thus, a normal array result is not always reassuring because of the risk for mosaicism and the implications of an undetected imprinted chromosome.

Cell-free DNA (cfDNA)-based noninvasive prenatal testing (NIPT) can screen for a range of fetal chromosome anomalies, with some approaches reporting aneuploidies on all chromosomes and large autosomal deletions/duplications (Fiorentino et al., 2017; Pescia et al., 2017; Pertile et al., 2021; Soster et al., 2021). As cfDNA originates from the cytotrophoblast, it is interrogating the genetic status of the placenta as a proxy for the fetus (Taglauer et al., 2014). The high sensitivity of NIPT implies that it can detect mosaic chromosome anomalies. In contrast to chorionic villus sampling (CVS), an invasive diagnostic technique that samples a small region of the placenta, NIPT noninvasively assesses the genetic status of the cytotrophoblast as a whole.

We report a case of a 39-year-old pregnant patient at high-risk for fetal chromosomal anomalies based on the results of maternal serum parameters. CVS and karyotyping of chorionic villi revealed two supernumerary marker chromosomes. NIPT allowed us to characterize the nature of the markers and effectively guide the choice of further genomic analyses of the chorionic villi. NIPT is a screening test that is usually carried out prior to invasive diagnostic testing. Here, on the contrary, NIPT was used as a follow-up tool to identify marker chromosomes primarily detected through invasive diagnostic testing.

Case description

The patient was a 39-year-old pregnant woman with no relevant family history. Her obstetrical history included one voluntary termination pregnancy and two miscarriages. No medical analyses were performed to explain the miscarriages. The first trimester ultrasound at 12.6 weeks' amenorrhea was normal (Crown Rump Length of 67.7 mm; Nuchal translucency of 2.2 mm) but maternal serum screening results from blood drawn on the same day reported the patient as being at a risk of 1:10 for trisomy 21 (β-hCG of 3.28 MoM; PAPP-A of 0.44 MoM). The patient elected to have diagnostic testing and CVS was performed at 13.1 weeks' amenorrhea. Direct analysis of short-term cultured chorionic villi with conventional RHG banding revealed two homogeneous, supernumerary and identical SMCs: 48,XX,+marx2 (Figure 1). A genome-wide array Cytoscan® 750K (SNP Affymetrix, 750K markers) performed according to the Affymetrix protocol on whole villi (cytotrophoblast and mesenchyme) showed normal results.

While waiting for results of the long-term culture, the patient was offered whole-genome sequencing-based NIPT to try to identify the marker chromosomes. A blood sample was obtained at 14.1 weeks of amenorrhea and NIPT was carried out using the VeriSeq™ NIPT Solution v2 assay (Illumina, Inc.) in the genome-wide mode as previously described (Kleinfinger et al., 2020). Following bioinformatic sequencing analysis, the NIPT results indicated a pericentromeric 29 Mb duplication of chromosome 20: dup (20) (p13q11.21) (Table 1), with a fetal fraction at 11%. As can be seen from Table 1, the "region_llr_trisomy" value was 509.27, which far exceeded the threshold value for CNVs of 15.1. In addition, a mosaic ratio of 2.06 was observed which is consistent with the presence of two extra copies and therefore suggestive of the possible presence of a tetrasomy. Based on the log-likelihood ratios, the markers appeared to be homogeneous which was concordant with the conventional cytogenetic study of the short-term culture. Subsequent targeted interphase and metaphase FISH on a short-term culture preparation of cytotrophoblast with a chromosome 20 centromeric probe [Vysis, CEP 20 (D20Z1) SpectrumOrange Probe] confirmed the segmental tetrasomy 20 in 100% of investigated cells (100/100 nuclei and 15/15 mitoses). Parental karyotypes were also performed at that time and no chromosomal anomalies were identified.

In long-term cultured villi all metaphases analyzed with conventional cytogenetics were normal (46,XX). Metaphase

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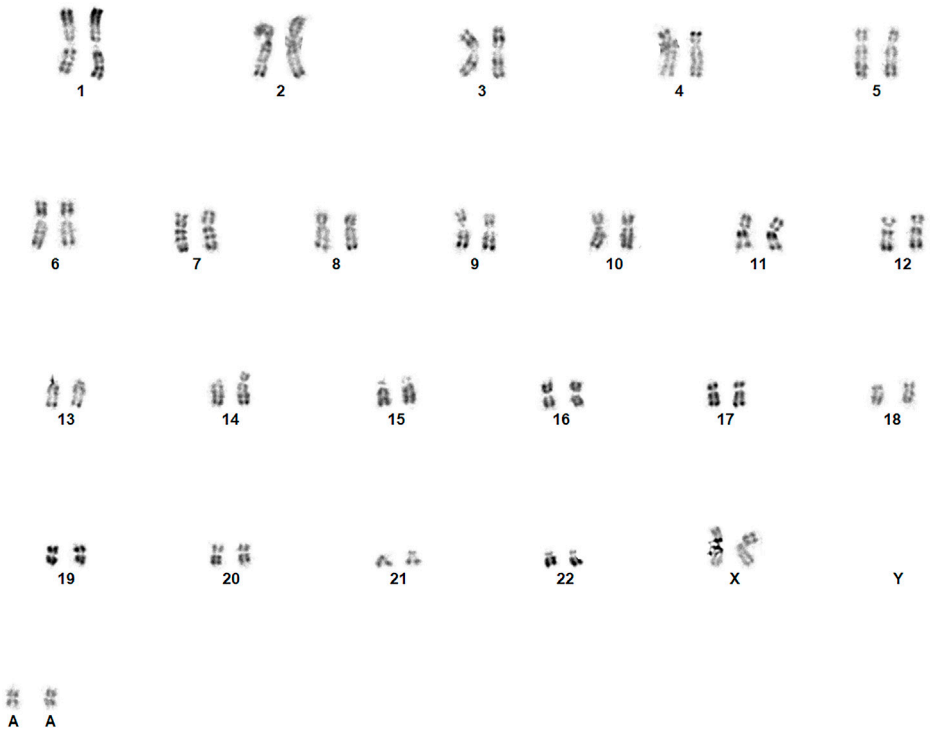

FIGURE 1
Identification of the marker chromosomes with direct examination of the cytotrophoblast following CVS [revealed two supplementary and identical SMCs (48,XX,+marx2)].

TABLE 1 NIPT result indicating a pericentromeric 29 Mb duplication of chromosome 20: dup (20) (p13q11.21).

Variable	Description or value
Region classification	DETECTED: dup (20) (p13q11.21)
Chromosome	Chr 20
Fetal fraction	11%
Start base	600,001
End base	29,700,000
Start cytoband	p13
End cytoband	q11.21
Region size (Mb)	29.1
Region LLR Trisomy	509.2733426
Region LLR monosomy	NA
Region t stat long reads	34.33013067
Region mosaic ratio	2.059591718
Region mosaic LLR trisomy	521.6461786
Region mosaic LLR monosomy	NA

Abbreviations: Chr, chromosome; Mb, megabase; LLR, log likelihood ratio; NA, not applicable.

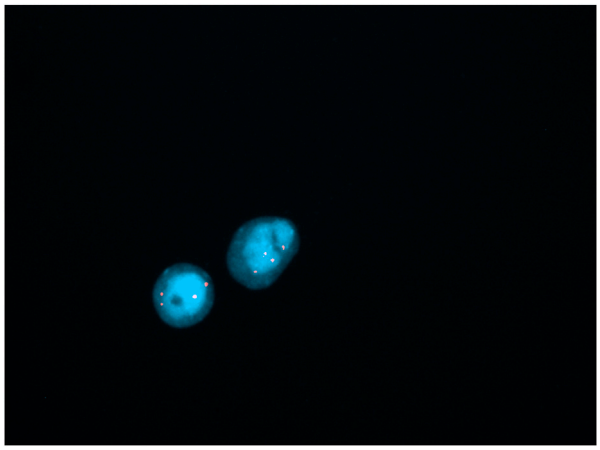

FIGURE 2
Identification of the marker chromosomes with interphasic FISH using centromeric probe of chromosome 20 (showing tetrasomy 20 in 20% of nuclei; lens 100X).

FISH with the 20 centromeric probe was normal in 25/25 mitoses, but interphase FISH found segmental tetrasomy 20 in 20% of the 100 examined nuclei (Figure 2). These results allowed us to conclude that this was either a case of type III confined placental mosaicism (CPM; anomaly in both the placental cytotrophoblast and

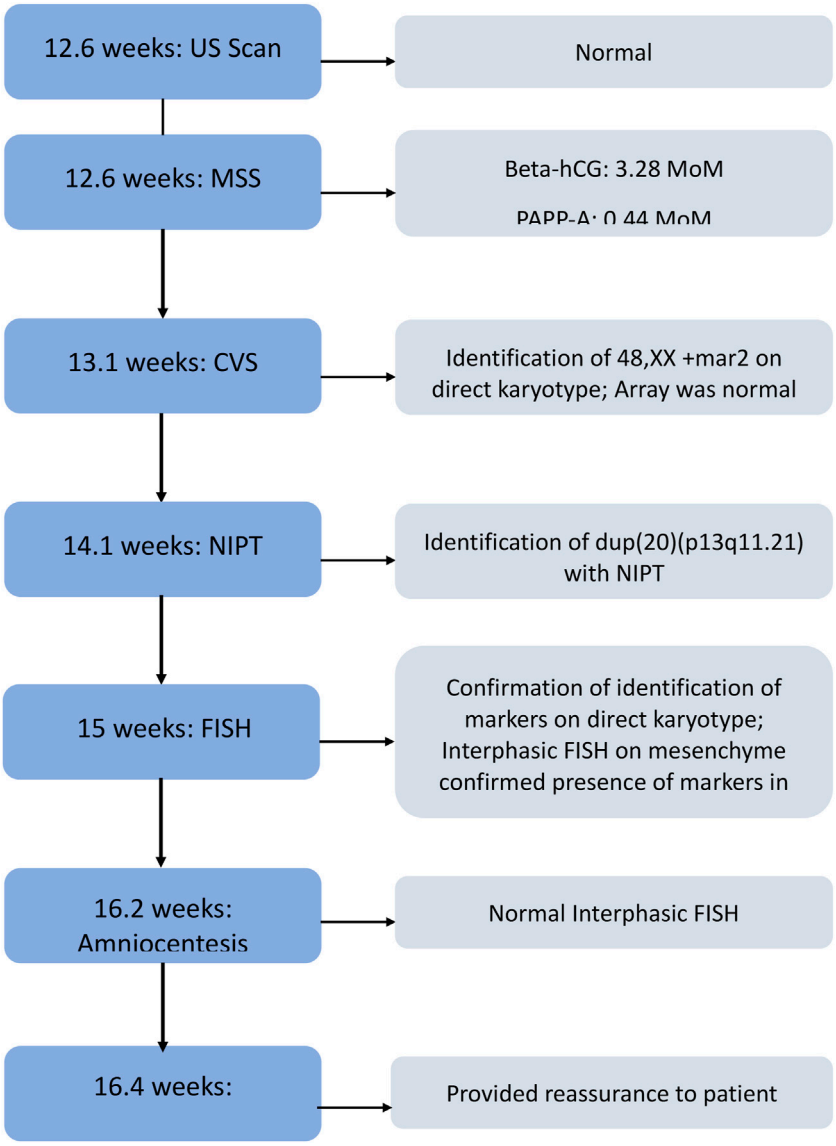

FIGURE 3
Patient's analysis workflow by weeks of amenorrhea. US, ultrasound; MSS, maternal serum screen, CVS, chorionic villus sampling, NIPT, noninvasive prenatal testing; FISH, fluorescence *in situ* hybridization.

the mesenchyme but not in the fetus) or type VI true fetal mosaicism (TFM; anomaly in the cytotrophoblast, mesenchyme, and the fetus).

As both the cytotrophoblast and mesenchyme were affected, the risk was increased that the anomaly may not be confined to the placenta. To determine whether the fetus was affected, amniocentesis was performed at 16.2 weeks' amenorrhea. Interphase FISH with the 20 centromeric probe revealed a normal result in 100/100 nuclei, allowing us to reassure the patient within 24 h of the procedure. Metaphase FISH in cultured cells was normal on 31/31 mitoses (13 clones *in situ*, 18 mitoses after trypsinization). A analysis flowchart for the patient is shown in Figure 3.

Ultrasounds carried out at 23 and 32 weeks' amenorrhea did not show any anomalies. A normal female baby with a birth weight appropriate for gestational age was born at 40 weeks' amenorrhea (APGAR score of 10). She presented with torticollis which spontaneously disappeared within a few days. At 1 year, she was a healthy girl, except for a G6pD deficiency (a disease with a X linked dominant transmission).

Discussion

Small supernumerary marker chromosomes are rare; it is estimated that there are ~3.3 million SMC carriers worldwide, of which ~2.2 million are asymptomatic (Liehr, 2021). These marker chromosomes can originate from any of the human chromosomes. About 70% of SMCs are caused by a *de novo* event whilst 30% are inherited (Jafari-Ghahfarokhi et al., 2015). A 2007 study by Liehr and Weise found marker chromosomes to be present in 0.075% of unselected cases where prenatal diagnosis had been carried out (Liehr and Weise, 2007), and a 2014 study by Malvestiti *et al.* reported an overall *de novo* small SMC frequency of 0.072% in prenatal samples (Malvestiti et al., 2014). In addition, the clinical phenotype of SMC carriers is highly variable. It is therefore important, and also very challenging, that SMCs are characterized as soon as possible in pregnant patients to facilitate a change in pregnancy management and allow patients to make informed decisions about their pregnancy. Here, we discuss a case of a 39-year-old pregnant patient with two identical supernumerary marker chromosomes diagnosed through CVS at 13.1 weeks' amenorrhea where additional analysis by genome-wide NIPT allowed for targeted FISH resulting in rapid, effective, and accurate characterization of the marker chromosomes and their distribution in the fetoplacental unit, ultimately allowing determination of their clinical significance.

In our case, the usual methods for identification of the markers would not have been helpful. The vast majority of SMCs are derived from acrocentric chromosomes (chromosomes 13, 14, 15, 21, and 22), with most originating from chromosome 15. Therefore, the centromeric FISH for these chromosomes takes precedence over other centromeric probes. Because the markers in our study were not from an acrocentric-derived chromosome, FISH would have been a very time-consuming approach. In addition, the SNP array failed to identify the markers. Even though array is supposed to examine both the cytotrophoblast and mesenchyme, it is not unusual for one of these tissues to be dominant. In this case, the normal result of SNP array can probably be explained by the fact that the array mainly examined the mesenchyme. Here, NIPT characterized the marker chromosomes to be pericentromeric 29 Mb duplications of chromosome 20. The risk for an abnormal phenotype in prenatally-characterized *de novo* SMC cases that are derived from a non-acrocentric autosome (such as chromosome 20) is 28% (Crolla, 1998; Liehr and Weise, 2007).

A second factor that is important in determining the clinical significance of a chromosomal anomaly is the distribution in the fetoplacental unit and the presence of mosaicism, i.e., the presence of two or more chromosomally different cell lines (Grati, 2014). As outlined above, this was a mosaic case because direct examination of CVS cytotrophoblasts showed the presence of two identical SMCs, but long-term cultures showed a normal karyotype. Identification of the markers by NIPT allowed targeted FISH analysis which found the markers in the mesenchyme, leading us to reinterpret the mosaic as either CPM type III (where the abnormal cell line is present in both the trophoblast and mesenchyme but not in the amniocytes) or TFM type VI (where the abnormal cell line is present in the trophoblast, mesenchyme, and amniocytes) (Grati, 2014).

The risk for fetal involvement is higher when mosaicism is present in both layers of the placenta compared to when it is present only in the trophoblasts (CPM type I) or only in the mesenchyme (CPM type II) (Grati, 2014). In addition, presence of the marker chromosomes in both layers of the placenta suggested that the anomaly was more likely to have originated from a meiotic error rather than a mitotic error, which puts the patient at a greater risk for pregnancy complications and UPD (Grati et al., 2021). It also increases the risk of this anomaly occurring in other pregnancies. In our case, amniocentesis was carried out at 16.2 weeks' amenorrhea to determine the fetal karyotype. This confirmed that the marker chromosomes identified by CVS and NIPT were confined to the placenta and were not present in the fetus, allowing us to provide timely reassurance to the patient. The presence of UPD needs to be taken into consideration following prenatal identification of a marker chromosome. Although there have been a few cases reported of UPD with SMCs derived from this chromosome (Liehr et al., 2011), there is currently little to no evidence showing that UPD of chromosome 20 is associated with an abnormal phenotype and we therefore did not include UPD as a risk factor for our patient.

NIPT analyzes placental cfDNA to screen for the presence of chromosomal anomalies. This noninvasive prenatal screening test has been available for over a decade, with earlier versions of this assay typically screening for common trisomies (trisomy 21, 18, and 13) only (Nicolaides et al., 2012; Palomaki et al., 2012). Nowadays, some NIPT assays offer optional testing for a range of additional conditions including sex chromosomal aneuploidies (Mazloom et al., 2013; Samango-Sprouse et al., 2013), select microdeletion and microduplication syndromes (Helgeson et al., 2015; Martin et al., 2018), and genome-wide anomalies such as rare autosomal aneuploidies and copy number variants (Kleinfinger et al., 2020; Pertile et al., 2021; Soster et al., 2021). As shown here, genome-wide NIPT can have additional utility such as directing the choice of genetic tests/probes. Another recent case study illustrates this as well (Zhang et al., 2022). In a woman with previous failed pregnancies, results of genome-wide NIPT prompted the performance of a diagnostic test and the choice of CMA as opposed to classic karyotyping. Silver-Russell syndrome associated with a 11p15.5 duplication of maternal origin was identified; this was relevant both for decisions on additional testing in the ongoing pregnancy and also for the parents in diagnosing the cause of loss in previous pregnancies and

establishing the recurrence risk. Genome-wide NIPT can play a role in identifying unbalanced chromosomal rearrangements due to parental balanced reciprocal translocations (Flowers et al., 2020).

One of the strengths of our case study was the speed at which the diagnosis was completed. In total, it took only 3 weeks from identification of the marker chromosomes on CVS, to characterization of the SMCs via NIPT and FISH, and finally analysis of the amniotic fluid to confirm that this anomaly was not present in the fetus. This prevented unnecessary extended patient anxiety. Identification and characterization of the marker chromosomes via NIPT also allowed us to avoid the high cost of multiple FISH analyses by enabling a targeted FISH approach with the appropriate probes. Finally, early identification of the type of mosaicism involved (i.e., whether this involved the cytotrophoblast, the mesenchyme, or both) was important, as this allowed us to adjust the genetic counselling that the patient received. A limitation of this study was that newborn karyotyping to confirm the absence of the markers was not performed. However, the prenatal tests on CVS and amniotic fluid allowed us to be reassured of the absence of these markers in the fetus, and the baby was healthy at 1 year of age.

In conclusion, this case illustrates that whole-genome sequencing-based cfDNA prenatal testing does not only contribute to prenatal care as a highly accurate screening test for chromosome ploidy. It can also serve as a molecular prenatal test that obviates the shortcomings of classic karyotyping and chromosomal microarray, in this case by characterizing marker chromosomes in a time- and cost-effective manner. Generating accurate and rapid results allowed for shortening the period of uncertainty for the patient and for comprehensive counseling.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MB performed the genetic counseling. PK, AL, LL, DT, AB, MV, J-MC, and SS performed the analyses. PK wrote the manuscript. All authors revised the manuscript and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Case Report: Two cases of apparent discordance between non-invasive prenatal testing (NIPT) and amniocentesis resulting in feto-placental mosaicism of trisomy 21. Issues in diagnosis, investigation and counselling

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The sequencing of cell-free fetal DNA in the maternal plasma through non-invasive prenatal testing (NIPT) is an accurate genetic screening test to detect the most common fetal aneuploidies during pregnancy. The extensive use of NIPT, as a screening method, has highlighted the limits of the technique, including false positive and negative results. Feto-placental mosaicism is a challenging biological issue and is the most frequent cause of false positive and negative results in NIPT screening, and of discrepancy between NIPT and invasive test results. We are reporting on two cases of feto-placental mosaicism of trisomy 21, both with a low-risk NIPT result, identified by ultrasound signs and a subsequent amniocentesis consistent with a trisomy 21. In both cases, after the pregnancy termination, cytogenetic and/or cytogenomic analyses were performed on the placenta and fetal tissues, showing in the first case a mosaicism of trisomy 21 in both the placenta and the fetus, but a mosaicism in the placenta and a complete trisomy 21 in the fetus in the second case. These cases emphasize the need for accurate and complete pre-test NIPT counselling, as well as to identify situations at risk for a possible false negative NIPT result, which may underestimate a potential pathological condition, such as feto-placental mosaicism or fetal trisomy. Post-mortem molecular autopsy may discriminate between placental, fetal and feto-placental mosaicism, and between complete or mosaic fetal chromosomal anomalies. A multidisciplinary approach in counselling, as well as in the

interpretation of biological events, is essential for the clarification of complex cases, such as feto-placental mosaicisms.

KEYWORDS

NIPT, false negative cffDNA, feto-placental mosaicism, trisomy 21, SNP array, autopsy

Introduction

In humans, the most common aneuploidies are trisomies, which represent about 0.3% of all live births and make up an even higher proportion in products of conception (Hassold and Hunt, 2001). The most common human trisomies involve chromosome 21 (T21) and may consist of either a complete trisomy or a mosaic trisomy. T21 is known as Down's syndrome (DS) and is associated with a specific phenotype, which typically includes brachycephaly, epicanthus, narrow and up-slanted palpebral fissures, flat nose, micrognathia, single palmar fusion crease and sandal gap, as well as systemic clinical manifestations that vary in severity, usually milder in mosaic T21, but always involving mild to moderate cognitive impairment and the possibility of life-threating comorbidities, caused in particular by cardiac or gastrointestinal malformations (Bull, 2020).

The prenatal presentation of T21 is also extremely heterogenous, ranging from precocious miscarriages, early severe malformations, associations of multi-organ conditions and soft markers to milder forms with isolated soft markers, but where there are no major or specific signs or even altered biochemical markers.

Women in their first 3 months of pregnancy are offered first trimester combined screening (FTCS), based on maternal age, fetal nuchal translucency thickness (NT) and serum markers such as beta human chorionic gonadotropin (β-HCG) and pregnancy-associated plasma protein A (PAPP-A), with a detection rate for T21 of 90–95% and a false positive rate of 2.5–5% (Kagan et al., 2019), as well as a positive predicted value of 3.4% (Norton et al., 2015).

Non-invasive prenatal testing (NIPT) is a screening method for the early identification of the most frequent autosomal aneuploidies (trisomies 21, 18 and 13) in the fetus during pregnancy, with high sensitivity and specificity and very high negative predictive values (NPV) (Bianchi and Wilkins-Haug, 2014). NIPT performance is demonstrably superior to FTCS in high-risk cases and also among the general population, particularly in the detection of T21 (Bianchi and Wilkins-Haug, 2014; Gil et al., 2017).

NIPT on cell free fetal-DNA (cffDNA) circulating in the maternal blood has been increasingly used since 2011, and today it is an integral part of clinical practice in many countries. Different policies concerning the proposal and administration of NIPT are currently applied across the world. In Europe, NIPT is currently offered as a first-tier universal screening method in the Netherlands (van der Meij et al., 2019) and in Belgium (Willems et al., 2014) and, in other countries, as a contingent test for women considered to be high-risk after FTCS. In most countries, however, the ease of use and the extensive adoption of NIPT may contribute to the lack of optimal pre and post-test counselling about the potentially controversial results determined by the current technical limitations in the method and in the way data can be interpreted and managed (Gadsbøll et al., 2020). CffDNA analysed by NIPT originates from apoptosis of placental (cytotrophoblast and syncytiotrophoblast) cells and thus represents the molecular identity of extraembryonic tissue (Lo et al., 1997; Flori et al., 2004).

NIPT does not produce a diagnostic result, due to technical and computational limits and also to biological issues. Among the test accuracy limitations are the occurrence of false positive and false negative results, which may occur in the case of multiple pregnancies, vanishing twins, maternal malignancies or mosaicisms. Rarely, mosaicism may reflect a maternal constitutional mosaicism, most frequently related to sexual chromosome aneuploidies (Zhang et al., 2017), or a somatic mosaicism, in the case of an eventual maternal malignancy (Bianchi et al., 2015). More frequently the mosaicism diagnosed through invasive analysis during pregnancy involves the placenta, the fetus or both (Ledbetter et al., 1992; Smidt-Jensen et al., 1993; Malvestiti et al., 2015).

We are reporting on two cases of feto-placental mosaicism of T21. In both cases, the discrepancy between the NIPT and amniocentesis results was consistent with a mosaicism: a feto-placental mosaicism in the first case and a confined placental mosaicismin the second case.

Clinical reports

Case 1

The case involves the first spontaneous pregnancy of a healthy couple of Caucasian ancestry, with unremarkable personal and family history of both the partners. The woman's age at conception was 31 and she was a smoker (about 10 cigarettes/day). The first trimester echography performed at the 11th week of gestation (WG) was normal, with a NT measurement of 1.8 mm and nasal bone visualisation. The PAPP-A level was slightly low, at 0.34 MoM, and the FTCS indicated a low risk for the main trisomies and a specific risk of 1: 1,326 for trisomy 21. The couple performed NIPT at 12 WG for

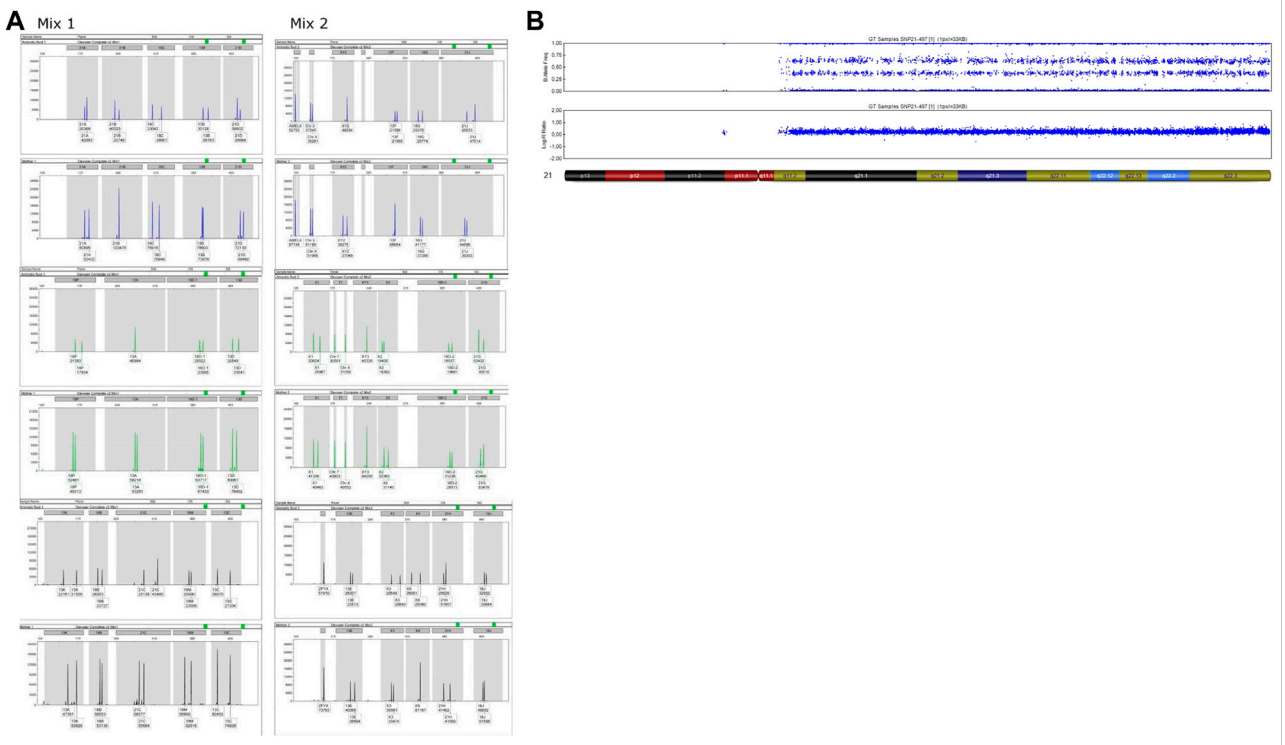

FIGURE 1
Analyses performed on amniotic fluid of Case 1. **(A)** QF-PCR results. For each channel, the first lane represents the amniotic fluid and the second is the maternal blood sample. Markers 21A, 21B, 21D and 21C show a complete T21. **(B)** SNP-array results. Complete T21.

their own choice in accordance with the referring physician, which confirmed low risks for the trisomies 13, 18 and 21, with a fetal fraction of 6%. The second trimester ultrasound (US) evaluation at 20 + 2 WG identified fetal growth restriction, an aberrant right subclavian artery (ARSA) and increased utero-placental resistance in the uterine arteries. An amniocentesis was proposed, and both the QF-PCR and the SNP-array on DNA extracted from the amniotic fluid cells were compatible with a T21 (Figures 1A,B). A second NIPT analysis was offered by the NIPT provider, which was performed at 20 + 3 WG, with a fetal fraction of 8%, and the results indicated a low risk for the main autosomal trisomies. The couple was given counselling by a multidisciplinary team for possible feto-placental mosaicism, after which they asked for a second amniocentesis, which confirmed the T21. The couple asked for termination of the pregnancy according to Italian legislation (Law 194/78).

After expulsion at 21 + 5 WG, the fetal dysmorphological examination showed a flat nasal bridge, prominent philtrum, mild macroglossia, ear asymmetry, slightly low-set ears, mild retrognathia, bilateral clinodactyly of fifth fingers (Figures 2A–F). Post-mortem imaging also revealed the presence of 11 rib pairs (Figure 2G). The autopsy confirmed both the presence of ARSA and the radiologic findings, without other malformations. The placenta examination was unremarkable.

Three placental samples were analysed via SNP-array, showing different results: two of the samples indicated the likelihood of a very low level of T21 mosaicism (Figures 3A,B), while a chromosome 21 disomy was evident in one sample (Figure 3C). DNA from fresh fetal skin showed a T21 (Figure 3D). DNA from the fetal liver showed the presence of euploidy (Figure 3E).

Case 2

The case involves the first spontaneous pregnancy of a healthy couple of Caucasian ancestry, with unremarkable personal and family history. The woman's age at conception was 33. The first trimester echography performed a slightly increased NT (NT = 3.35 mm). The couple performed a NIPT at 10+5WG, which confirmed low risks for the trisomies 13, 18 and 21 and the absence of Y chromosome, with a fetal fraction of 8%. Increased NT value persisted at the following US evaluation and an amniocentesis was proposed and performed at 17 WG. Cytogenetic and SNP-array analyses showed 46,XX,+21,der (21;21)(q10;q10) (Figure 4A) and a trisomy 21

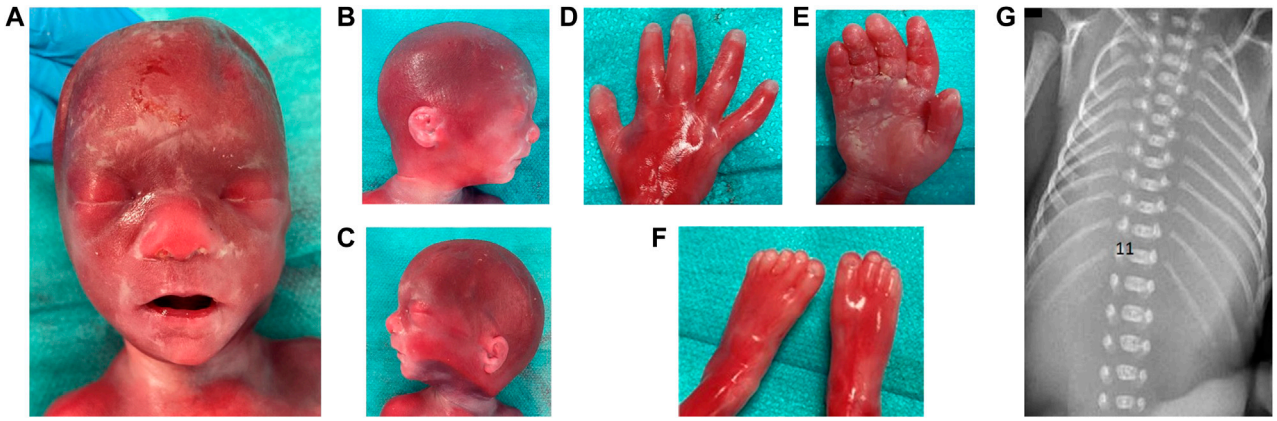

FIGURE 2
Dysmorphological examination of fetus of Case 1 is shown in **(A)** facial view: flat nasal bridge, prominent philtrum; **(B)** facial right; **(C)** facial left profile: ear asymmetry, mildly low-set ears, right lobar hypoplasia, mild retrognathia; **(D)** dorsal face of right hand: clinodactyly of 5th finger; **(E)** palmar face of right hand; **(F)** feet; **(G)** 11 bilateral and complete rib pairs and costal sketch of the twelfth vertebra on the right.

(Figure 4B), respectively. A second cffDNA was performed at 18 WG and the results again showed the absence of common chromosomal trisomies. The couple was given genetic counselling and finally asked for termination of the pregnancy according to Italian legislation (Law 194/78).

After expulsion at 20 + 5 WG, the fetal dysmorphological examination showed an eutrophic female fetus with macroglossia, low set ears, hypertelorism and micrognathia. The autopsy confirmed normal intrathoracic, intraabdominal and pelvic organs. The placenta showed no macroscopic or histologic anomalies. The placenta and fetal tissues were further examined via cytogenetic and cytogenomic analyses. Karyotype and SNP-array analyses revealed a placental mosaicism at about 60% (Figure 5A,B), while the SNP-array on DNA fetal skin showed a complete T21 (Figure 5C).

The QF-PCR patterns on placental tissue were consistent with the fetus' disomy for 13, 18, trisomy for 21, and the presence of two X chromosome and absence of the SRY gene (Figure 5D). In particular, all information carrying autosomal short tandem repeats markers demonstrated a normal 1:1 marker ratio, while sexual chromosome markers resulted compatible with a female genotype. The chromosomes markers for T21 (21B and 21H for example, as shown in Figure 5D) denote the presence of two cell lines, i.e., one disomic and one trisomic cell line for T21. An investigation of the two cell lines showed that placental mosaicism was present at about 50%.

Materials and methods

Case 1 and case 2 were referred to the Unit of Fetal Medicine and Prenatal Diagnosis and the Medical Genetics Laboratory at the Institute for Maternal and Child Health, IRCCS "Burlo Garofolo" in Trieste and to AMES laboratory in Naples and Medical Genetics in Avellino for prenatal consultation and analysis, respectively. Fetal autopsy was performed at the Pathological Anatomy and Histology Department of ASUGI (Trieste, Italy) and at Hospital of Avellino, for case 1 and case 2, respectively. Written informed consent for genetic analysis, clinical research and scientific publication were obtained according to the ethical standard defined by the Helsinki declaration.

NIPT analysis

For NIPT analysis, about 10 ml of peripheral blood was collected from the pregnant women in Streck blood collection tubes. For plasma isolation, the blood sample was first centrifuged at 1,600 g for 10 min at 4°C to separate the plasma from peripheral blood cells. Cell-free DNA from 900 µL of maternal plasma was extracted using the QIAamp DNA Blood MiniKit (Qiagen, Hilden, Germany) following the manucfacturer's protocol. NIPT analysis was performed using the VeriSeq NIPT Solution v2 bioinformatic pipeline (Illumina Inc., San Diego, CA, United States) based on the paired-end sequencing technique. The assay can report the results as Basic, with reporting for common trisomies and sex chromosomes (if selected), and Genome-wide analysis if the detection of the genome-wide fetal anomalies were included (including rare autosomal aneuploidies and partial deletions and duplications ≥ 7 Mb) (Borth et al., 2021; Pertile et al., 2021). The VeriSeq NIPT Assay Software v2 (www.illumina.com/NIPTsoftware) was used for data analysis of the aneuploidy

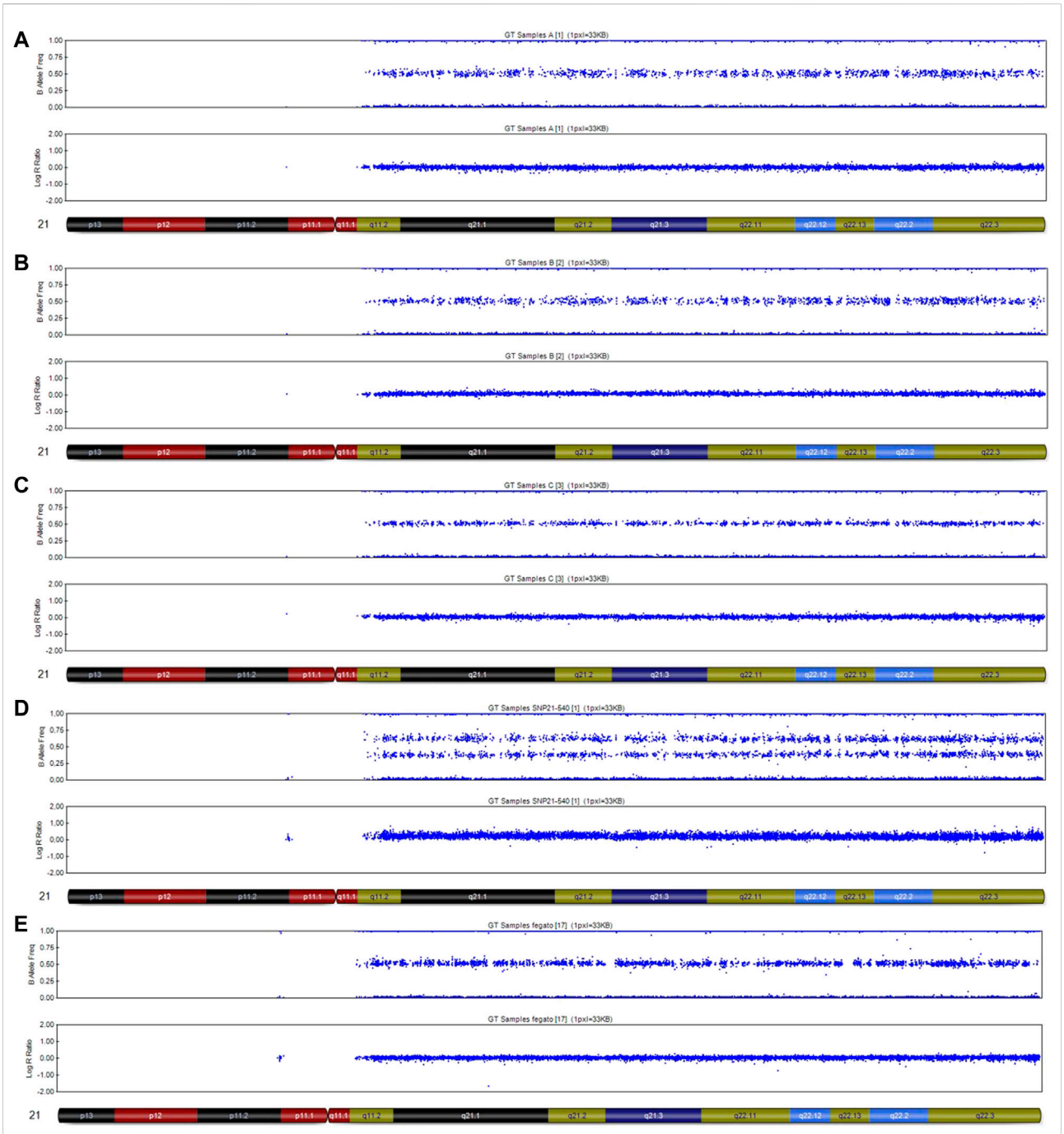

FIGURE 3
SNP-array analysis on placenta and fetal tissues of Case 1. **(A)** placenta sample 1 DNA, very low level of mosaicisms T21; **(B)** placenta sample 2 DNA, very low level of mosaicims T21; **(C)** placenta sample 3 DNA, disomy chromosome 21; **(D)** fetal skin DNA, complete T21; **(E)** fetal liver DNA, disomy chromosome 21.

status and fetal fraction from cffDNA. Sample results were classified using the VeriSeq NIPT Solution v2 Assay Software and analysis of "raw data" as reported previously (Borth et al., 2021; La Verde et al., 2021).

DNA extraction

Amniotic fluid, women's blood samples, placenta and fetal tissues were collected. Genomic DNA was extracted using the EZ1 (QIAGEN

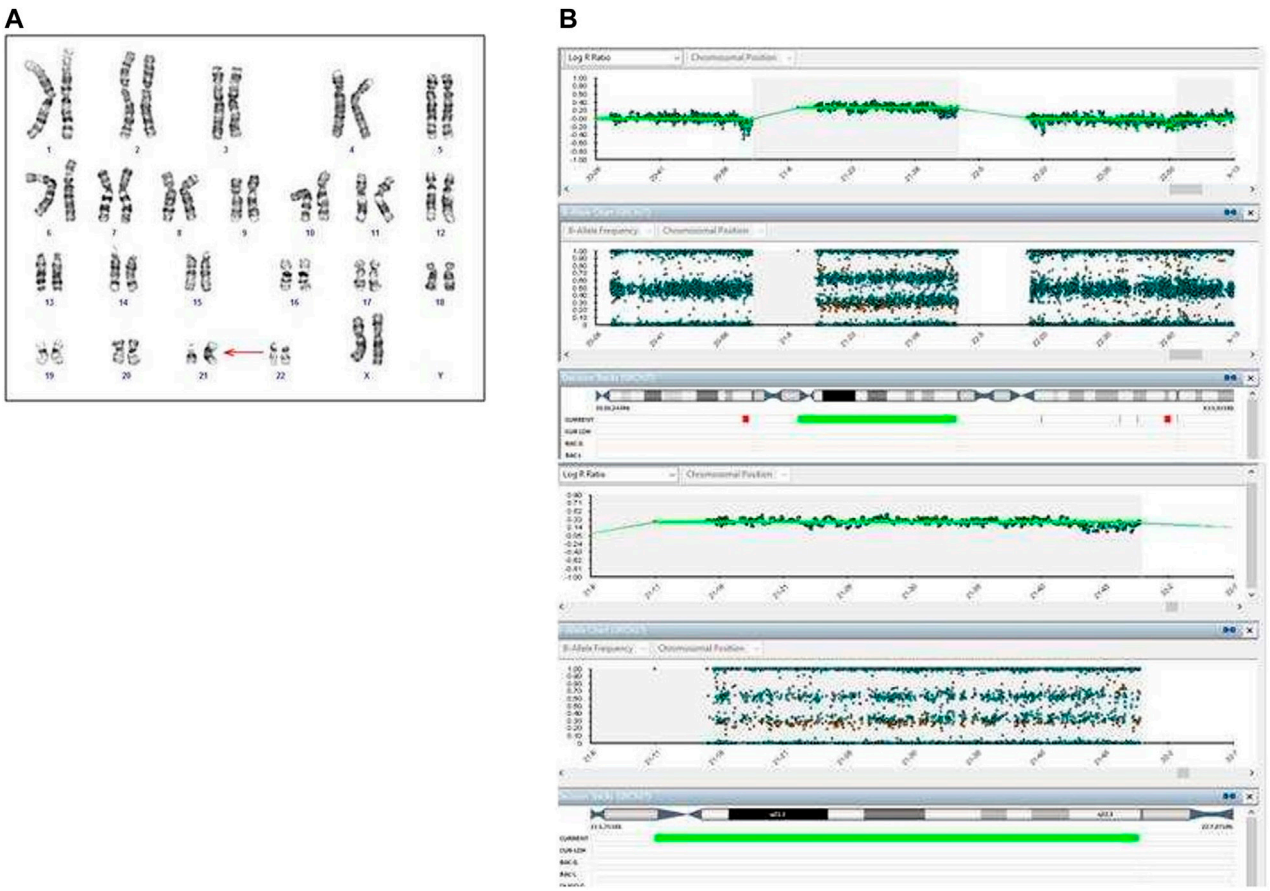

FIGURE 4
Cytogenetic and cytogenomic analyses on amniotic fluid of Case 2. **(A)** GTG banding karyotype shows a complete T21; **(B)** SNP-array shows a complete T21. Log ratio >0 indicates a copy number gain for chromosome 21.

Hilden, Germany) automated system. The extraction process was performed according to the instructions on the kit. After extraction, the quality and quantity of DNA were analysed using QIAxpert spectrophotometry.

QF-PCR

In the case 1 the test was performed using the Multiplex PCR Devyser kit (Devyser, Stockholm, Sweden), which was tested on 26 markers, including five STRs from chromosome 13 (D13S742, D13S634, D13S628, D13S305, D13S1492), five from chromosome 18 (D18S978, D18S535, D18S386, D18S976, GATA178F11), six from chromosome 21 (D21S1435, D21S11, D21S1411, D21S1444, D21S1442, D21S1437), and ten STRs from chromosomes X and Y (DXS1187, XHPRT, DXS2390, SRY, DXYS267, DXYS218, AMELX, AMELY, ZFY, ZFX). All the markers and the labelling information are included in the Devyser user manual. The PCR reaction was carried out using the SimpliAmp Thermal Cycler (Thermo Fisher, Waltham, MA, United States). Fragment analysis was performed through capillary electrophoresis using the Applied Biosystems 3,500 Dx DNA sequencer (Thermo Fisher, Waltham, MA, United States) after calibration, according to the instructions on the kit. The samples were run on a POP7 polymer. The results were analysed using GeneMapper™ Software (Thermo Fisher, Waltham, MA, United States). In the case 2 the quantitative fluorescent polymerase chain reaction test for rapid aneuploidy detection was performed using the Devyser Compactv3 QF-PCR kit (QF-PCR; Devyser Compactv3, Devyser). The amplified DNA samples were separated through electrophoresis using the ABI 3130xl Genetic Analyzer, and each allele was analysed for specific markers using GeneMapper Software ver. 4.0 (Applied Biosystems).

Cytogenetic analysis

For case 2, chromosomal analysis was performed on long-term amniotic fluid cultures from two separate culture flasks. GTG-banding 40 metaphases were analysed using CytoVision software (CytoVision, AB Imaging).

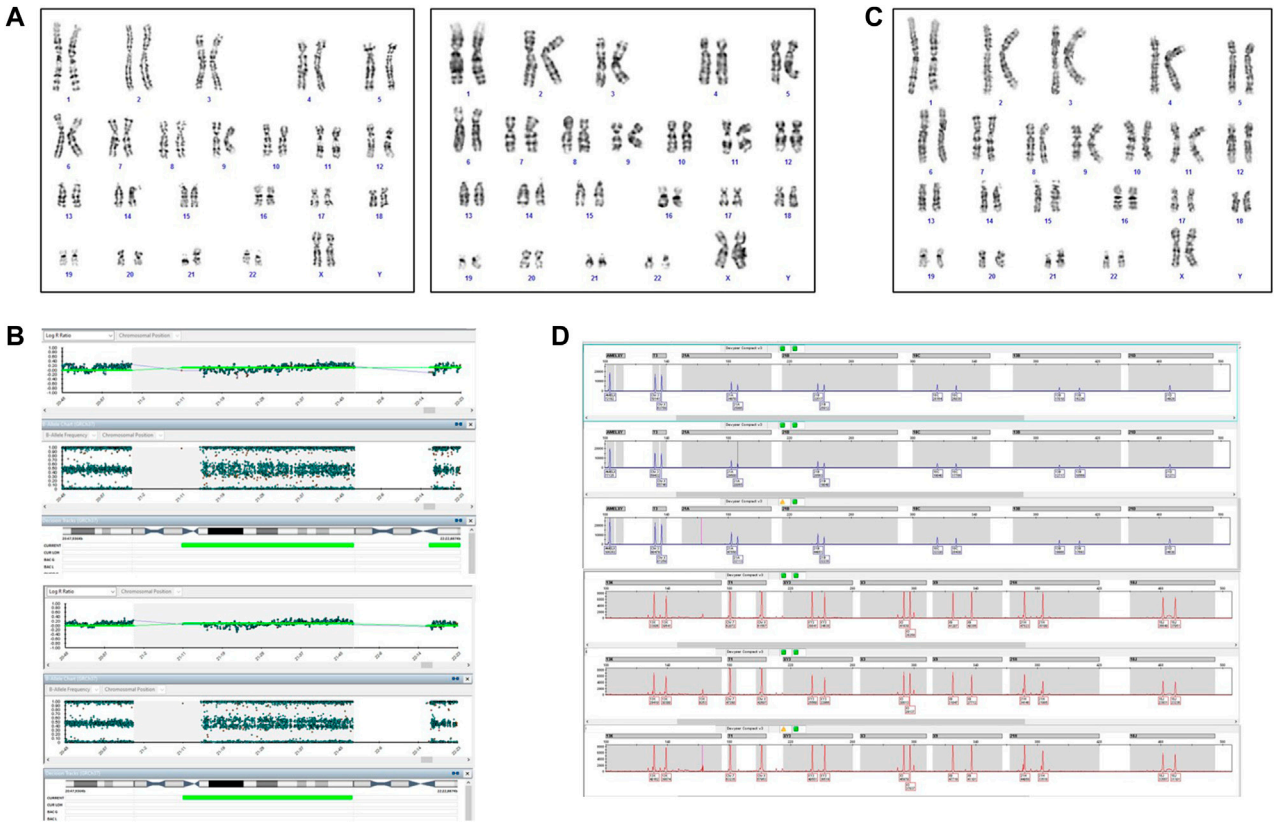

FIGURE 5
Cytogenetic and cytogenomic analyses on placenta and fetal tissues of Case 2. **(A)** GTG banding karyotype on placenta shows a mosaic T21; **(B)** SNP-array on two samples of placenta shows a mosaic T21. Log ratio >0 indicate a copy number gain for chromosome 21; **(C)** GTG banding karyotype on fetal skin shows a complete T21; **(D)** QF-PCR analysis on placenta. For each channel, the three lanes represent the different biopsies from the placenta.

SNP-array

In the case 1 the SNP-array analysis was performed on the genomic DNA using the Human OmniExpress Exome-8 Bead Chip (Illumina Inc., San Diego, CA, United States), which contains 960,919 loci derived from phases I, II and III of the International HapMap project. The array contains over 274,000 functional exonic markers, delivering unparalleled coverage of putative functional exonic variants selected from 12,000 individual exome and whole-genome sequences. In the case 2 the SNP-array analysis was performed on the genomic DNA using the HumanCytoSNP-12 v12.1 BeadChip Kit (Illumina Inc., San Diego, CA, United States), which contains ~300,000 SNPs targeting regions.

A total of 200 ng of gDNA (50 ng/μl) for each sample was processed according to Illumina's Infinium HD Assay Super protocol. The normalization of raw image intensity data, genotype clustering and individual sample genotype calls were performed using Illumina's GenomeStudio software v2.0 (cnvPartition 3.2.1). The CNVs were mapped to the human reference genome hg19 and UCSC refGene was used to annotate the gene variation. Allele detection and genotype calling were performed using GenomeStudio and NxClinical software.

Formalin-fixed paraffin-embeded (FFPE) samples

During fetal autopsy, several organ samples were collected and fixed in formalin. The only tissue eligible for analysis in case 1 was a liver biopsy, which was retrieved from the paraffine sample using a scalpel and DNA was then extracted using the QIAamp® DNA FFPE kit (QIAGEN, Hilden, Germany). After extraction, the quality and quantity of DNA were analysed using Quiaxpert spectrophotometry coupled with the Qubit dsDNA BR Assay Kit (Invitrogen) fluorimetry.

Prior to the SNP-array analysis, the sample was restored with the Infinium HD FFPE DNA Restore Kit (Illumina, San Diego, CA, United States) and the quality was newly assessed using the Qubit dsDNA BR Assay Kit. This kit enhances the quality of FFPE-extracted DNA, which is known to be fragmented, and enables an optimized whole-genome amplification strategy.

Discussion

Feto-placental mosaicism

Mosaicism is a biological condition in which two or more cell lines with different karyotypes derived from a single zygote coexist in a single individual (Strachan and Read, 2019). Most frequently, one of the cell lines may present a complete or partial aneuploidy and/or a chromosomal structural rearrangement (Porter et al., 1999; Brisset et al., 2003; Soler et al., 2003). Chromosomal mosaicism is one of the primary interpretative issues in prenatal diagnosis and it is diagnosed through villocentesis and amniocentesis, in 1–2% and 0.1–0.3% of pregnancies, respectively (Ledbetter et al., 1992; Smidt-Jensen et al., 1993; Malvestiti et al., 2015). Apart from the type of chromosome mechanism, the distribution of the different cell lines in the fetus and/or the placenta depends also on the timing when the mosaicism occurred and, on the embryo/fetal localization. Mosaicism could involve 1) only the placenta, where the condition is known as "confined placental mosaicism"; 2) both the placental and the fetus, where the condition is "feto-placental mosaicism" (Grati, 2014; Grati et al., 2017); 3) the fetus only. Thus, a complete fetal T21 may coexist with a normal placenta, a placenta with a complete trisomy or a placenta with a placental mosaicism (Eggenhuizen et al., 2021). These conditions may result in different clinical manifestations and diseases (Thorpe et al., 2020).

In that it is a screening test, the NIPT result requires confirmation through an invasive analysis (Hartwig et al., 2017). Chorionic villus sampling, or villocentesis, examines the cytotrophoblast and syncytiotrophoblast cells of the placenta, while amniocentesis analyses the fetal amniotic fluid cells, representing fetal tissues. Amniocentesis is the gold standard to confirm or exclude the NIPT result (Grati, 2014) in case of a high risk of T21. NIPT and the invasive test can potentially give discordant results, for various reasons, including false negative NIPT results as a consequence of mosaicism. The presence of feto-placental mosaicism can affect the interpretation and management of NIPT results, an issue given that the test is generally taken to determine the risk of fetal chromosome aneuploidies. Indeed, feto-placental mosaicism can generate discordance between the results from cffDNA testing and amniocentesis, producing a "false negative" or "false positive" NIPT result (Grati, 2014). The NIPT detection rate for T21 typically exceeds 99% with a low false-positive (FP) rate (<0.1%) (Mackie et al., 2017) and rare false-negative (FN) cases reported in some clinical studies (Zhang et al., 2015). To date, various FN T21 cases have been reported (Huijsdens-van Amsterdam et al., 2018), indicating that FN NIPT results may occur through biological mechanisms rather than through technical limitations.

Therefore, distinguishing between these embryologically and biologically different situations is challenging, and requires specific sampling and cytogenetic and cytogenomic analyses in various tissues. With the wide-spread implementation of prenatal non-invasive and invasive testing and the emergence of discrepant results, it may be helpful to set up strategies for investigating and understanding the mosaicism mechanism, and so back the counselling of couples and management of pregnancies with the right information. Genetic and chromosomal conditions of the placenta may differ from those of the fetus for different reasons (Hartwig et al., 2017). Thus, a negative NIPT result can only exclude the majority of adverse copy number changes in the fetus and/or the placenta, and a positive NIPT for a trisomy can be a false positive in up to ~2% of cases. Confined placental mosaicism should be presented as a real and serious condition to couples, who should be properly informed about the interpreting of NIPT and screening findings preferably before taking the tests, and they should be offered pre- or post-test counselling (Lau et al., 2014; Hartwig et al., 2017; Liehr et al., 2017). More recent developments in studying fetal and placental cell trafficking into the maternal circulation includes fetal cell based NIPT (cbNIPT), consisting in the examination of specifically extravillous trophoblasts originating from the placenta. This strategy is actually experimentally performed, having different limitations mainly due to accessibility and costs (Vossaert et al., 2021). Despite further studies are needed to assess its validity, it has proved, for example, a potential role in the screening of maternal mosaicism of sex-chromosomes anomalies, being superior to cell-free NIPT, which could fail to discriminate between maternal or fetal mosaicism (Jeppesen et al., 2021). In the context of false negative results at cell-free NIPT, analysing the total amount DNA, the results from cbNIPT, analysing only single cells or pool of cells harvested from maternal blood miming a placenta biopsy, could be candidate in the detection of confined placental mosaicism.

We have presented here two cases of feto-placental mosaicism, which were postulated after a discordant result between cell-free NIPT and amniocentesis and confirmed through cytogenetic and cytogenomic analyses of placental and fetal tissues. One of the main challenges in investigating mosaicism is to establish at which point during the embryo-fetal development does the mitotic error occur (Grati et al., 2017).

The issue of diagnosis

In the first case, the clinical presentation was not specific of aneuploidy, while in the second case, the increased NT was an early sign of suspect. However, in both cases, the diagnosis of T21 was clearly confirmed in the second trimester. In the second case, although the increased NT found in the first trimester of pregnancy suggested that an invasive test was advisable, the pregnant woman preferred to undergo a NIPT test, which recorded low-risk results for common trisomies. The NT

measurement in combination with serum biomarkers and maternal age meant that she was offered FTCS programme (Snijders et al., 1998). The introduction of non-invasive prenatal testing (NIPT) makes it possible to obtain information for common trisomies from as early as 10 weeks into gestation, proposing an alternative to traditional FTCS (Hui and Bianchi, 2017). Despite the superiority of NIPT to the combined test in the detection of T21, the fetal sonographic assessment is crucial for the determination of NT (Bardi et al., 2020). In the second case, the persistence of an increased NT measurement with a low-risk NIPT result was a challenge for both clinicians and the couple. In this context, an enlarged NT could be a marker for genetic conditions and fetal anomalies that would not be detected through NIPT (Yagel, 2021), as well as a condition of a feto-placental mosaicism. In our case the findings of the ultrasound subsequent to low risk cffDNA screening resulted in the woman deciding to undergo amniocentesis, and a non-mosaic isochromosome 21 was found (Oepkes et al., 2016).

Various elements characterizing the first case, such as the low PAPP-A value with a low-risk FTCS, mild ultrasound signs and ARSA, together with a few syndromic dysmorphisms and skeletal features with no major malformations, are valuable discussion points and clues for the diagnosis of T21 mosaicism.

A reduced PAPP-A level, which is the primary biochemical marker for the most common trisomies, in particular for T21 (Fialova and Malbohan, 2002), was the only altered marker at FTCS, when no suspicion of a possible fetal concern had emerged. Retrospectively, this serum marker may have been evocative of a placental mosaicism, and have contributed both to fetal growth restriction and reduced placental weight (Yong et al., 2009; Eggenhuizen et al., 2021).

Arguments supporting a possible fetus concern emerged during the second trimester US evaluation, highlighting fetal growth restriction, increased uterine arteries resistance and ARSA. In this case, maternal smoking could have primarily contributed to reducing the PAPP-A value, considering the periconceptional and pregnancy exposition to tobacco (Spencer, 1999), as well as to increasing arterial uterine resistance (Pintican et al., 2019) and directly and consequently to determining a fetal growth restriction (Abraham et al., 2017). Increased uterine resistance is more common in pregnancies with T21 than in normal (euploidy) pregnancies (Kaur et al., 2021). Fetal growth restriction is a common complication of pregnancies and could be a sign of a number of pathological conditions, including T21 (Fetal Growth Restriction: ACOG Practice Bulletin, Number 227, 2021). ARSA is the most common abnormality of the aortic arch, both in the general population and in normal (euploid) fetuses, affecting respectively 1–1.5% and 0.4–1.5%, with about a 16% prevalence in fetuses with T21 (Scala et al., 2015; Martínez-Payo et al., 2022). Isolated markers during the second trimester US evaluation, such as isolated ARSA, have a small effect on modifying the screening pre-test odds for T21, but all the most recent evidence suggests that ARSA should be considered as a soft marker of T21 (Agathokleous et al., 2013; Martínez-Payo et al., 2022).

Despite the low FTCS risk, with the nasal bone visualization and a negative NIPT result, the additional association of minor signs, as likely soft markers, which emerged in the second trimester, led to the multidisciplinary consultation and the indication for performing an amniocentesis, which demonstrated the presence of T21.

The issue of post-mortem analysis

In both cases, post-mortem examinations brought up other elements, including phenotypic features (Radhakrishnan et al., 2018), typical craniofacial anomalies (Guihard-Costa et al., 2006) and minor skeletal findings consistent with the diagnosis of T21. The dysmorphological examination revealed and supported the diagnosis of T21 in the fetus, while the total body imaging showed the presence of 11 pairs of complete ribs, which is a skeletal finding in 11% of fetuses with T21 investigated by autopsy (Grangé et al., 2006) and 33% of radiologically investigated newborn infants with T21 (Edwards et al., 1988). When there is a T21 diagnosis, there will also be a discussion as to whether a fetal autopsy should or should not be performed, in that it is complementary to the prenatal US investigation (Papp et al., 2007). Additionally, when there is such a T21 diagnosis, post-mortem cytogenomic analyses are uncommon, and DNA tests on fetal or placental tissues are, in general, never carried out. However, the indication for an autopsy was discussed with both the reported couples, and they gave their consent for classical and molecular autopsy (DNA analyses from fetal tissues). Cytogenetic and/or cytogenomic analysis were performed in placenta and fetal tissues in both cases, providing the confirmation of mosaicism in both the placenta and the fetus in the first case, and only in the placenta with complete T21 in the fetus in the second case. It is likely that the generation of the mosaicism was an early event in both cases. Indeed, in a feto-placental mosaicism, the postzygotic mitotic error, being itself a nondisjunction event in a somatic cell of an euploid conceptus or a trisomy rescue after a meiotic nondisjunction, will probably occur very early after the zygote has formed, that is, before the separation between embryonic and extraembryonic tissues (Grati, 2014). It has been shown that among the FN cases of T21 at NIPT, previously mentioned, about 22% had an isochromosome 21q, 44% had T21 and only one case had a feto-placental mosaicism (Huijsdens-van Amsterdam et al., 2018). As the cytotrophoblast is the primary source of the "fetal" cffDNA, a *de novo* isochromosome 21q formation which occurs in the inner cell mass precursors will be seen in the mesenchymal core and fetus, but, since the cytotrophoblast cells would remain predominantly euploid, the chromosomal aberration could not be detected via NIPT (Flori et al., 2004). A further difficulty is due in the absence, in many cases, of any studies on placenta and/or fetal tissue. A post-mortem

molecular autopsy has thus revealed to be an effective strategy to explain the molecular identity of placental and fetal tissues allowing to better define the typology of mosaicims. Finally, it could be an effective method to discriminate between placental, fetal or feto-placental mosaicism, and between complete or mosaic fetal chromosomal anomalies in cryptic prenatally uninvestigated conditions, as well as in antenatally undiagnosed cases.

The issue of counselling to couples

Both the cases reported here emphasize that there is the need for accurate and complete pre-test NIPT counselling. Couples should be informed about the meaning of NIPT as an accurate screening test, about its technical and biological limitations and about the possibility of false positive and false negative results (Liehr, 2021). Positive NIPT results always lead to a consultation being offered and require prompt investigations for their confirmation ("true positive") or confutation ("false positive"). On the contrary, a false negative NIPT result may be more difficult to find and investigate, or worse, it may falsely reassure both clinicians and couples, who may underestimate the risk of potential pathological conditions, such as feto-placental mosaicisms or fetal trisomy.

A multidisciplinary approach in counselling, as well as in the interpretation of biological events, is essential to explain complex cases, such as feto-placental mosaicisms.

In case of feto-placental mosaicisms, the lethality of a chromosomal aneuploidy is expected to be attenuated and related to the amount of trisomic cells and their distribution in different organs. In the prenatal setting, the mosaicism is quantified basing on fetal cells in the liquid amniotic, and thus of a partial proportion of fetal systems. A precise prediction about the postnatal clinical presentation is not possible. In particular, the severity of intellectual disability and eventual associated neuropsychiatric concern, as well as the prediction of sensorial deficits, which are typical of T21 is never feasible. US examination gives accurate description of structural anomalies, contributing to orient the prognostic outcome in terms of possible life-threating concerns at birth and indication of dedicated setting for delivery. The significance of mosaicism as a "Variant of Uncertain Outcome" (Levy et al., 2021) is one of the main challenging messages to provide during counselling of couples in the prenatal setting.

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

CA and AF were responsible for testing strategy design and manuscript preparation. SC, SU, SS, and BB carried out the SNP array and QF-PCR analyses for case 1. RR and PS carried out the SNP-array and QF-PCR analyses for case 2, PS and GS performed data analysis and the interpretation of NIPT. AP and MP performed cytogenetic analysis. FF and AF conducted genetic counselling for case 1. TS performed Ultrasound Screening and was involved in the counselling for case 1. FM and RB were involved in postmortem clinical investigation for case 1. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Retrospective analysis of the sex chromosomal copy number variations in 186 fetuses using single nucleotide polymorphism arrays

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Sex chromosomal abnormalities are associated with multiple defects. However, the types of sex chromosomal abnormalities during pregnancy in Fujian Province, China, are not recorded. In this retrospective analysis, we showed the sex chromosomal abnormalities of 186 fetuses, including 162 cases of X chromosomal abnormalities and 22 cases of Y chromosomal abnormalities in Fujian Province. We detected 73 cases of Turner syndrome, 24 cases of triple X syndrome, 37 cases of Klinefelter syndrome, and 14 cases of XYY syndrome. It was observed that 67.3% fetuses with classic Turner syndrome had their growth arrested. Moreover, we found 21 cases of mosaic Turner syndrome, 3 cases of mosaic Triple X syndrome, 2 cases of mosaic Klinefelter syndrome, and 1 case of mosaic XYY syndrome. Furthermore, 37 cases of large scales of sex chromosomal deletions/duplications were detected, including 30 cases of X chromosomal deletions/duplications and 7 cases of Y chromosomal deletions/duplications. Parent-of-origins of five cases of sex chromosomal deletions/duplications were determined. One case was with *de novo* X chromosomal variations, while the sex chromosomal deletions/duplications in other four cases were inherited from their parents. Overall, our results presented a detailed manifestation of sex chromosomal abnormalities of 186 fetuses in Fujian Province and suggested the important roles of single nucleotide polymorphism (SNP) array analysis in the prenatal diagnosis of sex chromosomal abnormalities. Also, determining the parent-of-origins of the deletions/duplications was critical for the prenatal diagnosis of sex chromosomal abnormalities.

KEYWORDS

sex chromosomal abnormalities, copy number variations, prenatal diagnosis, single nucleotide polymorphism array, Turner syndrome

Introduction

The integrity of sex chromosomes, including X and Y chromosomes, is critical for normal embryonic development. Sex chromosomal abnormalities are associated with multiple defects, including sexual organ dysplasia, low reproductive ability, and infertility (Leggett et al., 2010). Turner syndrome (45, X), triple X syndrome (47, XXX), Klinefelter syndrome (47, XXY), and XYY syndrome (47, XYY) are common aneuploidies of sex chromosomes (Nielsen and Wohlert, 1991). Moreover, large scales of sex chromosomal deletions/duplications are also detected in abnormal fetuses and associated with different phenotypes (Zhang et al., 2018). With the increase in the maternal age at conception, the incidence of sex chromosomal abnormalities during pregnancy is growing every year (Lei and Dong, 2019; Li et al., 2021). So, for pregnant women, prenatal diagnosis about sex chromosomal abnormalities is deeply needed.

Karyotype analysis and non-invasive prenatal testing or screening (NIPT or NIPS) (Deng et al., 2019; Deng et al., 2021; Shi et al., 2021) could be used to detect or screen the aneuploidies of sex chromosomes. However, in mosaic cases, sex chromosomal abnormal cells and normal cells exist together. Classic karyotype analysis and NIPT analysis are limited in determining mosaic sex chromosomal abnormalities (Ma et al., 2021). On the contrary, the single nucleotide polymorphism (SNP) array is validated in detecting chromosomal syndromes, mosaic chromosomal syndromes, and chromosomal deletions/duplications, with high accuracy and high resolution (Samango-Sprouse et al., 2013).

From 2019 to 2022, more than 10,000 cases of SNP arrays were conducted in Fujian Maternity and Child Health Hospital. In this study, we retrospectively analyzed the SNP arrays of 186 early fetuses with sex chromosomal copy number variations (CNVs). Among them, 72 cases of Turner syndrome, 24 cases of triple X syndrome, 37 cases of Klinefelter syndrome, and 15 cases of XYY syndrome were detected. Moreover, mosaic sex chromosomal CNVs were determined. Sex chromosomal deletions/duplications were found in 37 cases and parent-of-origins of the deletion/duplications were determined in five cases. Overall, our results presented a detailed manifestation of sex chromosomal abnormalities during pregnancy in Fujian Province and sex chromosomal abnormalities using parental samples.

Materials and methods

Clinical patients

This was a retrospective analysis of 186 fetuses with sex chromosomal abnormalities at the Medical Genetic Diagnosis and Therapy Center, Fujian Maternity and Child Health Hospital, including 74 male fetuses and 112 female fetuses. The mean maternal age at conception was 30.98 years old. The clinical conditions of the fetuses were examined by level 2/3D ultrasound. Genetic counseling and prenatal diagnosis were provided for all pregnant women. Informed consent for the SNP analysis from each patient was obtained. All analyses were approved by the Fujian Maternal and Child Health Hospital ethics committee (ID No. 2020KY113).

Chromosomal microarray analysis

In total, 119 amniotic fluid samples, 53 chorionic villi, 13 cord blood samples, and one skin tissue were collected by the nurse in accordance with the Declaration of Helsinki. Fetal DNA was extracted using a QIAGEN DNA Mini Kit, according to the manufacturer's instructions. SNP array experiments were carried out according to Affymetrix CytoScan 750K array standard protocols. The Affymetrix CytoScan 750K array includes 550,000 CNV probes and 200,000 SNP probes for the CNV analysis and is wildly used for prenatal diagnosis. The microarray was scanned using a GeneChip Scanner 3000 system and annotated using Chromosome Analysis Suite (ChAS) software based on the hg19 human reference sequence. The chromosomal abnormalities in each sample were assessed by the American College of Medical Genetics and Genomics (ACMG) guidelines by ChAS software.

Prenatal diagnosis

Based on the Database of Genomic Variants (DGV), Online Mendelian Inheritance in Man (OMIM), and Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (DECIPHER), the CNVs of sex chromosomes are classified into likely benign and benign, pathogenic variants, likely pathogenic variants, and variants of uncertain clinical significance (VOUS). Peripheral blood from the parents of the fetuses with sex chromosomal deletions/duplications was used to determine the parent-of-origins of the deletions/duplications. The types of CNVs were further determined by the results of the pedigree analysis and SNP arrays.

Results

General sex chromosomal abnormalities in our study

From 2019 to 2022, 186 fetuses were detected with sex chromosomal abnormalities at Fujian Maternity and Child

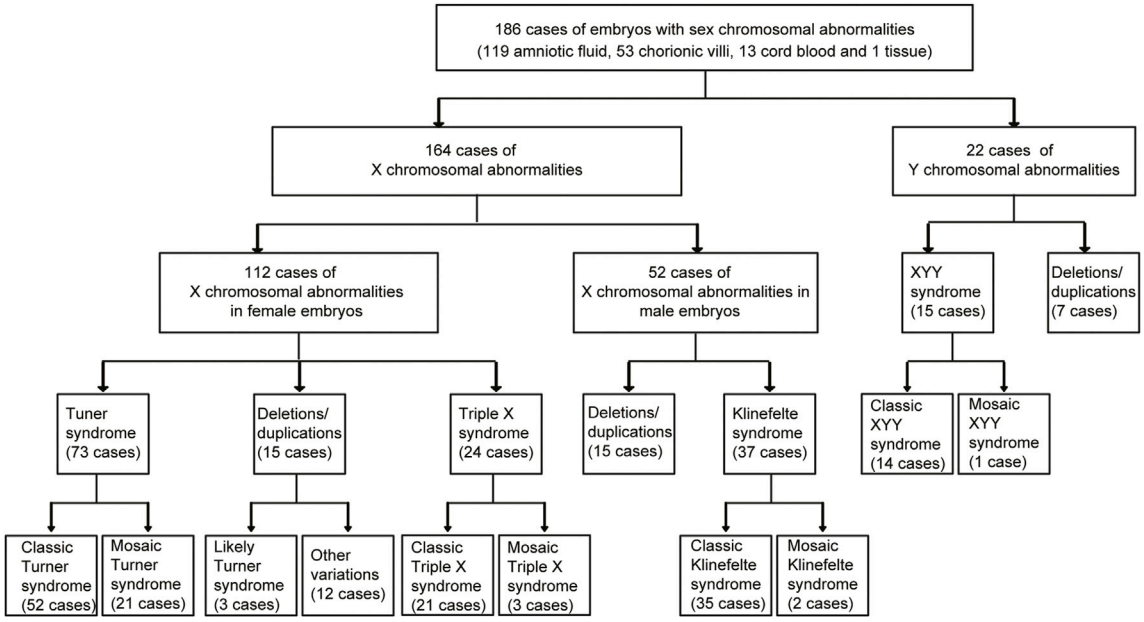

FIGURE 1
Retrospective analysis of 186 fetuses with sex chromosomal abnormalities.

Health Hospital. The sex chromosomal abnormalities of those 186 samples are shown in Figure 1, including 164 cases of X chromosomal abnormalities and 22 cases of Y chromosomal abnormalities. A total of 112 cases of X chromosomal abnormalities were detected in female fetuses, while 52 cases of X chromosomal abnormalities were detected in male fetuses. Turner syndrome (45, X) and triple X syndrome (47, XXX) are common abnormal manifestations of the X chromosome in female fetuses. We detected 73 cases of Turner syndrome and 24 cases of triple X syndrome (Figure 1). Klinefelter syndrome (47, XXY) was detected in 37 cases of female fetuses. XYY syndrome (47, XYY) is a common abnormal manifestation of the Y chromosome in male fetuses, and 15 cases of XYY syndrome were detected. Moreover, 37 cases of large scales of sex chromosomal deletions/duplications were detected, including 15 cases of X chromosomal deletions/duplications in female fetuses, 15 cases of X chromosomal deletions/duplications in male fetuses, and 7 cases of Y chromosomal deletions/duplications in male fetuses.

In mosaic cases, sex chromosome abnormal cells and normal cells exist together. We found 21 cases of mosaic Turner syndrome out of 73 cases of Turner syndrome. Moreover, three cases of mosaic triple X syndrome, two cases of mosaic Klinefelter syndrome, and one case of mosaic XYY syndrome were detected (Figure 1).

Clinical phenotypes of fetuses with sex chromosomal abnormalities

Turner syndrome, triple X syndrome, Klinefelter syndrome, and XYY syndrome have diverse phenotypes. The clinical phenotypes of fetuses with classic sex chromosomal aneuploidies are shown in Table 1. We found that 35 (67.3%) fetuses with classic Turner syndrome had their growth arrested. Two cases of classic Klinefelter syndrome also had their growth arrested. However, other sex chromosomal aneuploidies did not affect the early growth of the embryos. Moreover, five cases of classic Turner syndrome were with nuchal translucency (NT) thickening, three cases of classic Turner syndrome were with congenital heart disease, and three cases of classic Turner syndrome were with lymphocystoma. Also, 11 (52.4%) cases of classic triple X syndrome, 25 (71.4%) cases of classic Klinefelter syndrome, and 10 (71.4%) cases of classic XYY syndrome were previously detected with a high NIPT risk of sex chromosomal abnormalities.

In contrast with triple X syndrome, Klinefelter syndrome, or XYY syndrome, Turner syndrome had the most mosaic cases. The mosaic CNV values, mosaic percentage, and clinical phenotypes of early fetuses with mosaic Turner syndrome are shown in Table 2. The mosaic CNV values of Turner syndrome ranged from 1.3 to 1.9 and the percentage of the 45, X cell ranged from 10% to 80%. Some embryonic phenotypes of mosaic Turner

TABLE 1 Clinical phenotypes of fetuses with classic sex chromosomal aneuploidies.

Ultrasound	Classic Turner syndrome (N = 52)	Classic triple X syndrome (N = 21)	Classic Klinefelter syndrome (N = 35)	Classic XYY syndrome (N = 14)
Embryonic growth arrest	35	0	2	0
NT thickening	5	0	3	1
Congenital heart disease	3	1	1	1
Lymphocystoma	5	0	0	0
Fetal growth restriction	0	1	1	0
Choroid plexus cysts		1	1	0

TABLE 2 CNVs and percentage of mosaic Turner syndrome.

Case	CNV value	45, X cell (%)	Ultrasound/NIPT
1	Undetermined	10	Embryonic growth arrest
2	Undetermined	30	Embryonic growth arrest
3	1.3	70	Embryonic growth arrest
4	1.8	20	Embryonic growth arrest
5	1.2	80	NT thickening
6	1.3	70	Embryonic growth arrest
7	1.3	70	Embryonic growth arrest
8	1.9	30	Non-structural abnormalities
9	1.7	30	NIPT high Turner risk
10	1.7	30	Fetal echogenic bowel
11	1.5	50	NIPT high Turner risk
12	1.7	30	NIPT high Turner risk
13	1.8	20	Hydronephrosis
14	1.8	20	NIPT high Turner risk
15	1.4	60	NIPT high Turner risk
16	1.86	14	NIPT high Turner risk
17	1.82	18	NIPT high Turner risk
18	1.7	30	Non-structural abnormalities
19	1.77	23	NIPT high Turner risk
20	1.68	32	NIPT high Turner risk
21	1.5	50	Non-structural abnormalities

syndrome were similar to those of classic Turner syndrome, like embryonic growth arrest, NT thickening, and a high NIPT Turner risk (Table 2).

X chromosomal deletions/duplications and prenatal diagnosis

Except sex chromosomal aneuploidies, 30 cases of large scales of X chromosomal deletions/duplications were detected in our study, including 15 cases of X chromosomal deletions/duplications in female fetuses and 15 cases of X chromosomal deletions/duplications in male fetuses. Detailed information on the 15 cases of X chromosomal deletions/duplications in female fetuses is shown in Table 3. The X chromosomal deletions/duplications mainly occurred in Xp22.31, Xp22.33, and Xq28 regions. In the female fetuses, five (33.3%) cases were with Xp22.31 deletions, four (26.7%) cases were with Xp22.33 deletions, and two (13.3%) were with Xq28 deletions (Table 3).

The X chromosomal deletions/duplications in the Xp22.31 region were mostly associated with the STS OMIN gene. Loss of the STS gene was associated with multiple defects, including X-linked ichthyosis. X chromosomal deletions/duplications in the Xp22.33 region were mostly associated with the SHOX OMIN gene. SHOX gene deletion can lead to short stature or Leri–Weill dyschondrosteosis disorder. Deletions/duplications in Xp22.31 and Xp22.33 regions were considered pathogenic CNVs in female fetuses (Table 3).

Detailed information on 15 cases of X chromosomal deletions/duplications in male fetuses is shown in Table 4. Similar to female fetuses, the X chromosomal deletions/duplications in male fetuses also occurred in the Xp22.31 and Xp22.33 regions. We found that nine (60%) cases of male fetuses were with Xp22.31 deletions, three (20%) cases were with Xp22.33 deletions, and one was with Xp22.33 duplication (Table 4). Deletions/duplications in the Xp22.31 and Xp22.33 regions were also associated with STS and SHOX OMIN genes and were considered as pathogenic CNVs in male fetuses (Table 4).

Parent-of-origins of the X chromosomal deletions/duplications

Origins of the X chromosomal deletions/duplications in female or male fetuses were determined using parental samples. Xp22.2 duplications were found in case 1 female fetuses (Table 3). After chromosomal analysis of the parents of case 1, we found that her mother but not her father shared similar Xp22.2 duplications and suggested that the Xp22.2 duplications in case 1 female fetuses were inherited

TABLE 3 X chromosomal deletions/duplications in female fetuses.

Case	Regions (starts–ends)	CNV	Size (Mb)	Number and representative OMIM gene	Prenatal diagnosis
1	Xp22.2 (9,505,232–10,476,941)	3	0.972	5 (*MID1*)	Probably benign
2	Xp22.31 (6,449,836–8,143,509)	1	1.6	8 (*STS*)	VOUS
3	Xp22.31 (6,449,558–8,141,076)	1	1.69	6 (*STS*)	VOUS
4	Xp22.31 (6,449,558–8,141,076)	1	1.69	6 (*STS*)	VOUS
5	Xp22.31 (6,835,778–7834,078)	0	0.998	3 (*STS*)	VOUS
6	Xp22.31 (6,449,558–8,141,076)	1	1.69	5 (*STS*)	VOUS
7	Xp22.33 (168,551–2,958,480)	1	2.79	29 (*SHOX* and *ARSE*)	Pathogenic
8	Xp22.33p11.1 (168,551–58,527,155)	13	58.4	300 (*SHOX* and *ARSE*)	Pathogenic
	Xp11.1q28 (61,882,314–155,233,098)		93.4	411 (*MECP2* and *PLP1*)	
9	Xp22.33p21.3 (168,551–26,023,162)	1.87	25.8	118 (*SHOX* and *STS*)	Pathogenic (likely mosaic Turner)
	Xp21.3q28 (26,031,561–155,233,098)	1.40	129.2	628 (*ARX* and *DMD*)	
10	Xp22.33p22.31 (168,551–8,881,475)	1	8.7	40 (*SHOX*)	Pathogenic (likely mosaic Turner)
	Xp22.31q28 (8,931,445–155,233,098)	1.4	146.3	671 (*MID1* and *HCCS*)	
11	Xp22.33p22.32 (168,551–4,422,774)	1	4.2	31 (*SHOX*)	Pathogenic
12	Xq12 (67,210,899–67,626,475)	3	0.416	1 (*OPHN1*)	VOUS
13	Xq24q25 (118,395,148–125,416,121)	1	7	38 (*UBE2A*, *LAMP2*, and *UPF3B*)	VOUS
14	Xq28 (154,120,632–154,564,050)	1	0.443	6 (*F8*)	VOUS
15	Xq28 (147,550,751–155,233,098)	1	7.68	107 (*F8*)	Pathogenic

TABLE 4 X chromosomal deletions/duplications in male fetuses.

Case	Region (starts–ends)	CNV	Size (Mb)	Number and representative OMIM gene	Prenatal diagnosis
1	Xp21.1 (31,987,021–32,181,659)	0	0.195	1 (*DMD*)	VOUS
2	Xp22.12 (20,220,457–20718,134)	2	0.498	1 (*RPS6KA3*)	VOUS
3	Xp22.31 (6,455,361–8,135,568)	0	1.6	5 (*STS*)	Pathogenic
4	Xp22.31 (6,449,836–8,141,076)	0	1.69	6 (*STS*)	Pathogenic
5	Xp22.31 (6,449,836–8,141,076)	0	1.69	6 (*STS*)	Pathogenic
6	Xp22.31 (6,715,163–7,918,931)	0	1.2	4 (*STS*)	Pathogenic
7	Xp22.31 (6,449,836–8,141,076)	0	1.69	5 (*STS*)	Pathogenic
8	Xp22.31 (6,683,449–7,814,664)	0	1.13	3 (*STS*)	Pathogenic
9	Xp22.31 (6,455,151–8135,568)	0	1.68	4 (*STS*)	Pathogenic
10	Xp22.31 (6,455,151–8135,568)	0	1.68	4 (*STS*)	Pathogenic
11	Xp22.31 (6,455,151–8135,568)	0	1.68	4 (*STS*)	Pathogenic
12	Xp22.33 (168,552–1234,634)	0	1.1	5 (*SHOX*)	Pathogenic
13	Xp22.33 (168,551–629,999)	0	0.461	5 (*SHOX*)	Pathogenic
14	Xp22.33 (387,396–629,998	0	0.243	1 (*SHOX*)	Pathogenic
15	Xp22.33p22.32 (2,372,667–5,718,525)	2	3.3	11 (*ARSE*)	Probably benign

from her mother (Figure 2A). Moreover, her mother had normal phenotypes and the Xp22.2 duplications in case 1 were defined as probably benign CNVs.

Xp22.33p22.31 and Xp22.31q28 deletions were found in case 10 female fetuses (Table 3). We found that her parents had no Xp22.33p22.31 or Xp22.31q28 deletions, suggesting that the X

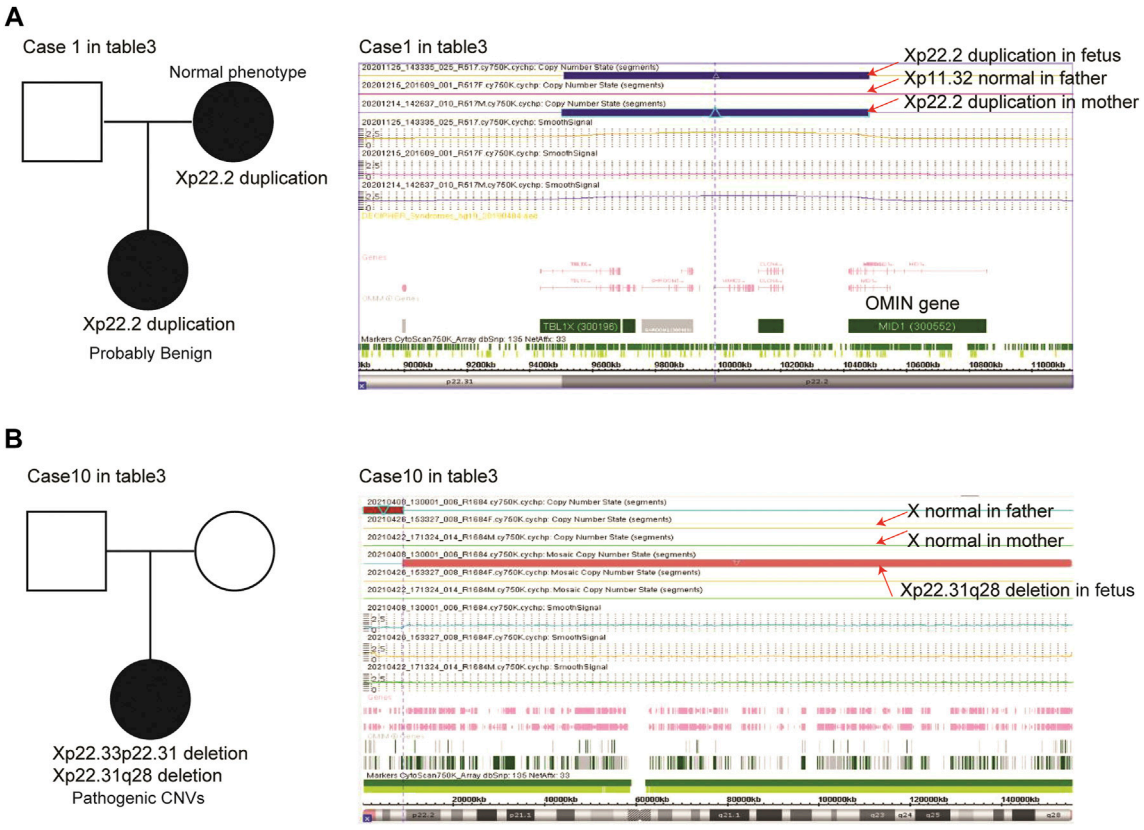

FIGURE 2
Parent-of-origins of the X chromosomal deletions/duplications in case 1 **(A)** and case 10 **(B)** female fetuses.

chromosomal variations in case 10 were *de novo* alterations (Figure 2B). Larger scales of Xp22.31q28 deletions influenced 671 OMIN genes, including *MID1* and *HCCS* genes, and this alteration was defined as pathogenic CNVs.

Parent-of-origins of the X chromosomal deletions/duplications were also determined in case 8 and case 15 male fetuses (Table 4). The Xp22.31 deletions in case 8 male fetuses were inherited from his mother (Figure 3A). The case 8 male fetus and his mother shared similar X chromosomal deletions (Figure 3B). Although his mother had normal phenotypes, the Xp22.31 deletions were known pathogenic CNVs. The Xp22.33 duplications in the case 15 male fetus were inherited from his father. His father had normal phenotypes, and the Xp22.33 duplications in case 15 were defined as probably benign CNVs (Figure 3A).

Y chromosomal deletions/duplication and prenatal diagnosis

We also detected seven cases of large scales of Y chromosomal deletions/duplications. Detailed information on Y chromosomal deletions/duplications is shown in Table 5, including two cases of Yp11 duplications and five cases of Yq11 deletions. Yq11 included AZFa, AZFb, and AZFc regions. Alterations of these regions were associated with male infertility, and Yq11 deletions/duplications were considered pathogenic CNVs. In the DGV, loss of Yq11.223q11.23 is defined as VOUS.

Parent-of-origins of the Y chromosomal deletions/duplications were also determined in case 2. The Yp11.32 deletions in the case 2 male fetus were inherited from his father (Figure 3C). The case 2 male fetus and his father shared similar Y chromosomal deletions (Figure 3D). His father had normal phenotypes, and Yp11.32 deletions in case 2 were defined as probably benign CNVs.

Discussion

Turner syndrome, triple X syndrome, Klinefelter syndrome, and XYY syndrome are the most common abnormal manifestations of sex chromosomes (Nielsen and Wohlert, 1991). In our study, 122 (65.6%) cases out of 186 cases with

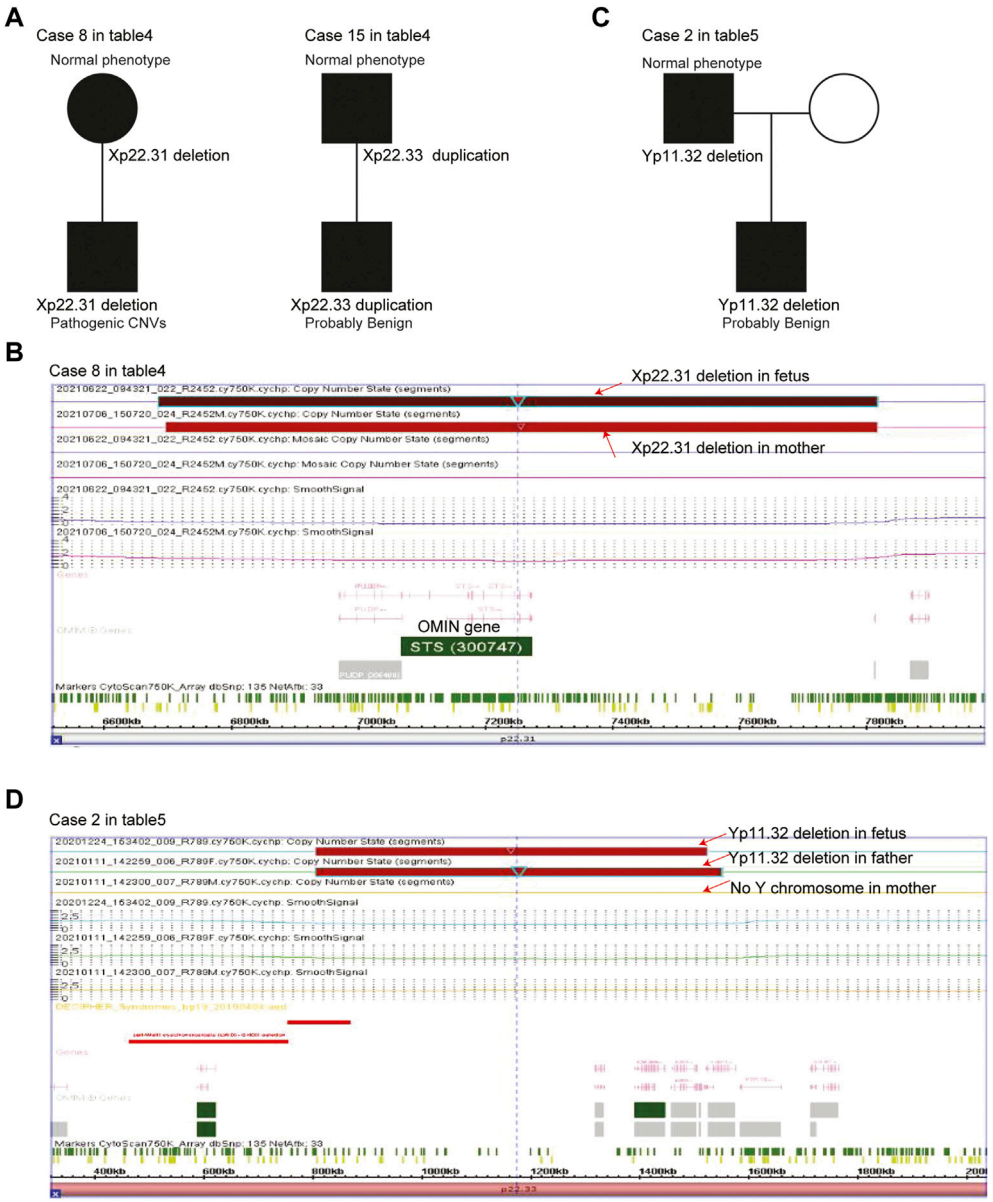

FIGURE 3
Parent-of-origins of the sex chromosomal deletions/duplications in male fetuses. **(A)** Pedigree of case 8 and case 15 female fetuses with X chromosomal deletions/duplications. **(B)** Xp22.31 deletions in the case 8 male fetus and his mother. **(C)** Pedigree of the case 1 female fetus with X chromosomal deletions/duplications. **(D)** Yp11.32 deletions in the case 1 male fetus and his father.

sex chromosomal abnormalities were classified into those four subtypes. One missing X chromosome in females (Turner syndrome) was associated with severe defects and with the absence of further fetal development in some cases. Also, 35 (67.3%) fetuses with Turner syndrome had their growth arrested. However, most girls with triple X syndrome grow up healthy and have normal sexual development. An extra X chromosome (Klinefelter syndrome) or Y chromosome (XYY syndrome) in male fetuses usually has no severe defects. Those and our results highlighted the different phenotypes of males or females with sex chromosomal abnormalities (Migeon, 2020).

In our study, more than 40% cases were associated with Turner syndrome. Turner syndrome was correlated with the deletion of one entire X chromosome in all embryonic cells (classic Turner syndrome) or in partial of embryonic cells (mosaic Turner syndrome). We identified 52 cases of classic and 21 cases of mosaic Turner syndrome. The mosaic percentage of Turner syndrome was from 10% to 80%.

TABLE 5 Y chromosomal deletions/duplications in male fetuses.

Case	Region (starts-ends)	CNV	Size (Mb)	Number and representative OMIM gene	Prenatal diagnosis
1	Yp11.31q11.221 (485,572–18,016,216)	2	17.4	36 (*SRY* and *AZFa*)	Pathogenic
2	Yp11.32 (803,294–1,519,822	0	0.717	8 (*CSF2RA*)	Probably benign
3	Yq11.1q11.23 (13,134,517–28,799,654)	0	15.6	29 (*AZFa*, *AZFb*, and *AZFc*)	Pathogenic
4	Yq11.221 (16,189,079–28,799,654)	2	16	32 (*AZFb* and *AZFc*)	Pathogenic
5	Yq11.222q11.2 (20,094,029–28,398,950)	0	8.3	21 (*AZFb* and *AZFc*)	Pathogenic
6	Yq11.223q11.23 (24,741,034–2,8,372,003)	0	3.6	10 (*AZFc*)	VOUS
7	Yq11.223q11.23 (25,863,808–27,609,692)	0	1.75	6 (*AZFc*)	VOUS

However, the SNP array could not detect the mosaicism as low as 5% (Zheng et al., 2019) and should be detected by whole-exome sequencing or other technologies (Murdock et al., 2017). Moreover, other X chromosomal abnormalities, like isochromosome Xq, isodicentric Xp, ring X chromosome, or large scales of X chromosomal deletions were also associated with Turner syndrome (Gravholt et al., 2017). Three cases with large scales of X chromosomal deletions were found in our study. However, isochromosome Xq, isodicentric Xp, and ring X chromosome were not determined. Those results highlighted the complexity of Turner syndrome and should be further studied.

In our study, 37 cases of large scales of X chromosomal deletions/duplications were detected, including 30 cases of X chromosomal deletions/duplications and 7 cases of Y chromosomal deletions/duplications. The prenatal diagnosis of those variations was difficult. Changes in STS (Zhang et al., 2020; Crane and Paller, 2022) and SHOX (Hirschfeldova et al., 2012; Ogushi et al., 2019) genes were associated with multiple genetic defects, and chromosomal alterations involved in those genes were defined as pathogenic CNVs. Determining the parent-of-origins of the deletions/duplications is critical for the prenatal diagnosis of sex chromosomal abnormalities (Chen et al., 2020). In our study, we detected three cases of X or Y chromosomal deletions/duplications which were inherited from their parents with normal phenotypes and were defined as probably benign CNVs. However, because of the economic pressure and other concerns, most parents refused further testing. Also, 12 cases of sex chromosomal deletions/duplications defined as VOUS could not be further classified.

Overall, using SNP arrays, our results showed a detailed manifestation of sex chromosomal abnormalities in Fujian Province and validated some sex deletions/duplications using parent samples. Our analysis suggested that Xp22.2 duplications, Xp22.33 duplications, and Yp11.32 deletions were probably benign CNVs. However, some cases with mosaic sex chromosomal abnormalities should be further studied using other technologies. Moreover, parent-of-origins of the sex chromosomal abnormalities were critical for prenatal diagnosis and should be used more widely.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at https://www.ncbi.nlm.nih.gov/, GSE208389.

Ethics statement

The studies involving human participants were reviewed and approved by the Fujian Maternal and Child Health Hospital ethics committee (ID: No. 2020KY113). The patients/participants provided their written informed consent to participate in this study.

Author contributions

HW designed the study, performed the data analysis, and wrote the manuscript. YW and BL collected the samples and performed the SNP array. HH revised the manuscript. NL and LX supervised the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Synthesis of positive plasmas with known chromosomal abnormalities for validation of non-invasive prenatal screening

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Non-invasive prenatal screening (NIPS) is a DNA sequencing-based screening test for fetal aneuploidies and possibly other pathogenic genomic abnormalities, such as large deletions and duplications. Validation and quality assurance (QA) of this clinical test using plasmas with and without targeted chromosomal abnormalities from pregnant women as negative and positive controls are required. However, the positive plasma controls may not be available for many laboratories that are planning to establish NIPS. Limited synthetic positive plasmas are commercially available, but the types of abnormalities and the number/quantity of synthetic plasmas for each abnormality are insufficient to meet the minimal requirements for the initial validation. We report here a method of making synthetic positive plasmas by adding cell-free DNA (cfDNA) isolated from culture media of prenatal cells with chromosomal abnormalities to the plasmas from non-pregnant women. Thirty-eight positive plasmas with various chromosomal abnormalities, including autosomal and sex chromosomal aneuploidies, large deletions and duplications, were synthesized. The synthetic plasmas were characterized side-by-side with real positive plasmas from pregnant women and commercially available synthetic positive plasmas using the Illumina VeriSeq NIPT v2 system. All chromosomal abnormalities in the synthetic plasmas were correctly identified with the same testing sensitivity and specificity as in the real and commercial synthetic plasmas. The findings demonstrate that the synthetic positive plasmas are excellent alternatives of real positive plasmas for validation and QA of NIPS. The method described here is simple and straightforward, and can be readily used in clinical genetics laboratories with accessibility to prenatal cultures.

KEYWORDS

non-invasive prenatal screening (NIPS), synthetic plasma, NIPS validation, cell free DNA, fetal fraction

1 Introduction

The discovery of cell-free DNA (cfDNA) of fetal origin in blood plasma of pregnant women paved a new way for non-invasive prenatal screening (NIPS) (Lo et al., 1997). With advances in next-generation sequencing (NGS) technology, tens of millions of short sequence tags can be generated from cfDNA in a single maternal plasma sample. By counting the number of sequence tags mapped to each chromosome, fetal aneuploidies can be correctly detected. This accurate and reliable genomic screening for common fetal aneuploidies clearly outperforms the traditional serum protein screening (Chiu et al., 2008; Fan and Quake, 2010; Norton et al., 2015). NIPS has transformed prenatal care in countries and regions where it is available (Norton, 2022).

As a screening test, NIPS is routinely offered to women at as early as 10 weeks' gestation. This test can be established in clinical genetics laboratories using commercially available platforms, for example the Illumina VeriSeq NIPT v2 system, or laboratory developed sequencing and bioinformatic pipelines. In either way, clinical validation and continuous monitoring of NIPS performance using both negative and positive plasma controls are required to ensure the test is performed appropriately. Negative plasmas can be obtained from female donors with normal pregnancies following appropriate protocols. However, positive plasmas that carry fetal cfDNA with targeted chromosomal abnormalities are usually very difficult to collect in a timely manner, in particular for laboratories new to this test, due to limited availability of such positive specimens. Although synthetic positive plasmas are commercially available, they are usually insufficient for the initial validation due to limited abnormality types and sample quantity. Therefore, development of reliable alternatives of the positive plasmas for NIPS validation and QA is needed to help and facilitate applications of NIPS. We describe here a simple method of making synthetic positive plasmas that are reliable and excellent alternatives of positive maternal plasmas for validation and monitoring NIPS performance.

2 Materials and equipment

2.1 Materials

Thirty-eight de-identified culture media were collected from backup cultures of chorionic villus cells or amniocytes that were submitted for prenatal diagnosis at the University of California San Francisco (UCSF) Clinical Cytogenetics Laboratory after reporting.

Twenty de-identified remaining plasmas of phenotypically normal non-pregnant females (age 20–42 years old) were collected after testing pathogens of infectious diseases at the UCSF Clinical Microbiology Laboratory. These samples that would be otherwise discarded were used as donor plasmas to make synthetic positive plasmas.

Two maternal blood samples from pregnancies with fetal aneuploidies were collected in Cell-Free DNA BCT tubes (Streck, Nebraska, United States) after obtaining the consent of each individual.

In addition, six synthetic positive plasmas, including two with trisomy 21, two with trisomy 18, and two with trisomy 13, were purchased from SeraCare Life Sciences (SeraCare Life Sciences, Massachusetts, United States).

Two hundred negative control plasmas with normal fetal cfDNA for NIPS system validation and training were provided by Illumina (Illumina, California, United States).

2.2 Reagents and kits

AmnioMAX-II complete media (ThermoFisher Scientific, Massachusetts, United States).

QIAamp MinElute ccfDNA kits (Qiagen, Hilden, Germany).

High sensitivity DNA kit (Agilent, California, United States).

VeriSeq NIPT Extraction and Library prep kit (Illumina, California, United States).

2.3 Equipment

Agilent 2100 Bioanalyzer (Agilent, California, United States).

Avanti J-15R centrifuge (Beckman Coulter, Indiana, United States).

Eppendorf MiniSpin plus centrifuge (Eppendorf, Connecticut, United States).

Corning 25 cm^2 rectangular canted neck cell culture flask with vent cap (T25) (Corning, New York, United States).

Corning sterile 15 mL plastic conical centrifuge tube, graduated polypropylene, RNase & DNase-free (Corning, New York, United States).

Eppendorf 1.5 mL safe-lock clear tube (Eppendorf, Connecticut, United States).

Illumina VeriSeq NIPT v2 system (Illumina, California, United States).

Microlab STAR liquid handling system (Hamilton, Nevada, United States).

3 Methods

3.1 Isolation of plasma

Approximately 10 mL blood sample collected in a Cell-Free DNA BCT tube was centrifuged at 1,000 g for 10 min with centrifuge break off (Avanti J-15R centrifuge). The supernatant was then transferred to four 1.5 mL centrifuge tubes (1.1 mL plasma/tube).

Each tube with 1.1 mL plasma was further centrifuged at 5,600 g for 10 min (Eppendorf MiniSpin plus centrifuge), and 1.0 mL supernatant was transferred to a new centrifuge tube.

Isolated plasma could be stored at 4°C for up to 10 days. They could also be stored at −80°C for up to 2 years.

3.2 Extraction of cfDNA from culture media and from donor plasmas

Chorionic villus cells or amniocytes were first cultured to about 90% confluence in a T25 flask following a standard protocol (Segeritz and Vallier, 2017). The culture was then fed with 5 mL fresh AmnioMAX complete medium.

Three to 5 days after feeding (depending on cell growth), 3.0 mL culture medium was transferred from the flask into a 15 mL centrifuge tube and centrifuged at 1,000 g for 10 min with centrifuge break off (Avanti J-15R centrifuge).

Approximately 2.2 mL supernatant was transferred to two 1.5 mL centrifuge tubes (1.1 mL plasma/tube) (Eppendorf) and then centrifuged at 5,600 g for 10 min (Eppendorf MiniSpin plus centrifuge).

Two mL supernatant (1.0 mL from each tube) was used for cfDNA extraction. CfDNA was extracted using QIAamp MinElute ccfDNA Kit following the manufacture's instruction. CfDNA was eluted into 25.0 µL nuclease-free water provided in the kit and was checked for fragment size and quantity on Bioanalyzer using Agilent high sensitivity DNA kit following the kit instruction.

CfDNA from six donor plasmas was also extracted and measured in the same way to estimate the average concentration of the background cfDNA in the donor plasmas.

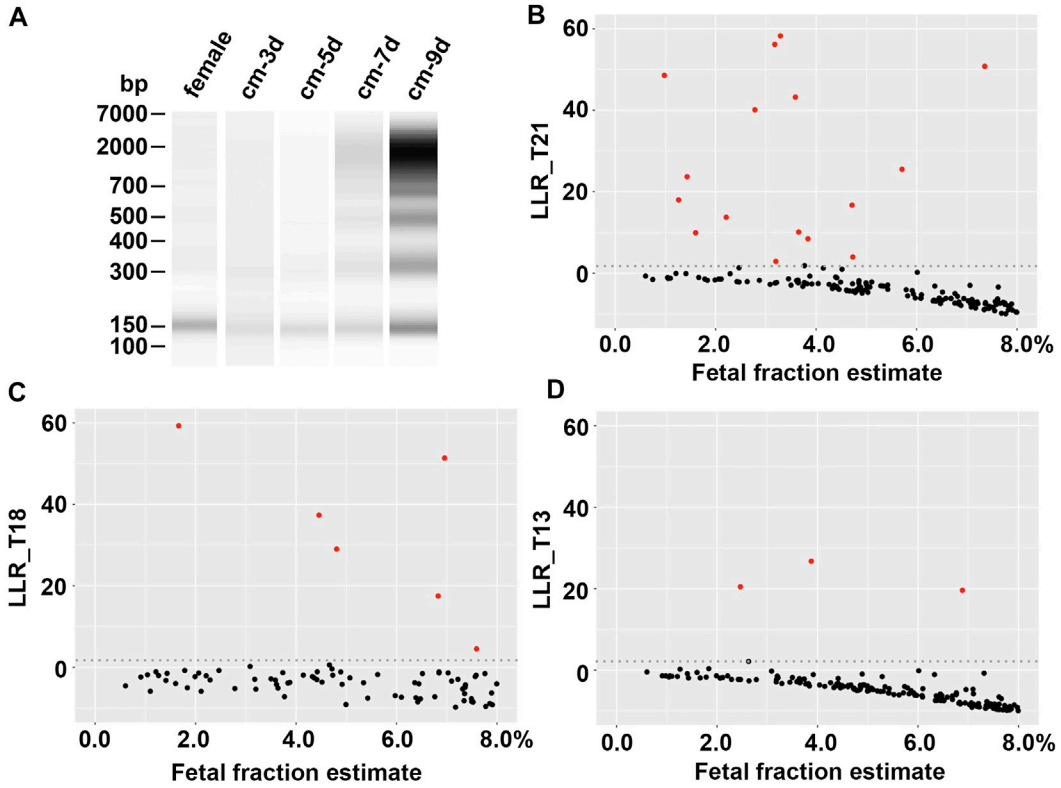

FIGURE 1
Evaluation of synthetic plasmas. **(A)** cfDNA size distribution. From left to right, cfDNA from a normal female plasma (female), culture media from a chorionic villus specimen with a 45,X karyotype collected at the day 3, 5, 7, and 9 of the culture, respectively (cm-3d, cm-5d, cm-7d, and cm-9d). **(B)**, **(C)**, and **(D)** Log likelihood ratios (LLRs) of synthetic plasmas (fetal fraction estimate ≤8%) with trisomy 21, trisomy 18, and trisomy 13, respectively. Red dot, synthetic positive plasma; black dot, negative maternal plasma; gray dotted line, LLR cutoff.

3.3 Synthesis of positive plasmas

Approximately 1.0 ng short cfDNA (130–190 bp) with targeted chromosomal abnormalities from a culture medium was added to 1.0 mL normal female donor plasma collected through step 3.1 to make a synthetic positive plasma. The expected average fraction of the cfDNAs from culture media in the synthetic positive plasmas is approximately 7%.

3.4 Characterization of synthetic positive plasmas for detecting targeted abnormalities

The synthetic positive plasmas were characterized using the Illumina VeriSeq NIPT v2 system according to the manufacturer's instruction. Briefly, cfDNA was extracted and the sequencing library was prepared using VeriSeq NIPT Extraction and Library Prep kits (Illumina) in Microlab STAR liquid handling system (Hamilton). The sample libraries were pooled and pair-end sequenced (36x2 cycles) on NextSeq550 (Illumina). The sequencing data were analyzed by VeriSeq NIPT software v2 (www.illumina.com/NIPTsoftware). This software aligned the sequencing reads to human reference genome GRCh37/hg19 and used a counting-based algorithm to generate the log-likelihood ratio (LLR) scores for chromosomes, as well as NCV_X and NCV_Y scores for sex classification. LLR thresholds for calling a sample high or low risk of specific chromosome abnormalities were internally validated. Data generated from fragment length and coverage analysis were used to estimate fetal fraction by the software.

3.5 NIPS data visualization

The LLRs of the synthetic positive plasmas with trisomy 21, trisomy 18, and trisomy 13, as well as the fetal fractions from the VeriSeq NIPT supplementary reports were plotted in RStudio (2021.09.2) using ggplot2 (3.3.6) for data visualization.

4 Results

A total of 38 cfDNA samples with targeted chromosomal abnormalities were extracted from cell culture media of chorionic villus cells or amniocytes. The quantity and size of the cfDNA were determined on Bioanalyzer using Agilent high sensitivity DNA kit, which showed a size range from 100 bp to >1 kb in discontinuous clusters, including a major cluster of

short sizes (130–190 bp) (Figure 1A). The average concentration of the cluster of short cfDNA is approximately 60 ng/mL in culture medium. This cluster of cfDNA was used to make synthetic plasmas, since its size range is most representable to the size range of fetal cfDNA in maternal plasmas (Kim et al., 2015; Jiang and Lo, 2016).

The average background cfDNA concentration measured by the same method in six donor plasmas was 13.7 ng/mL, ranging from 3.9 to 27.8 ng/mL. This range was in line with the findings of a broad survey of cfDNA from healthy donors (Raymond et al., 2017). Therefore, adding 1.0 ng abnormal cfDNA to 1.0 mL donor plasma resulted in an approximately 7% of average abnormal cfDNA fraction that would mimic the fetal fraction in the synthetic plasmas. This percentage was common in maternal plasmas based on the data reported in literatures. It was noteworthy that a wide range of fetal fraction (1%–15%) was estimated by the VeriSeq v2 system (Supplemental Table 1), most likely due to the various concentrations of the background cfDNA in the donor plasmas. In fact, this range of fetal fraction appeared to be consistent with a reported range (Canick et al., 2013; Artieri et al., 2017). We further analyzed the detectability of targeted chromosome abnormalities in synthetic positive plasmas with different fetal fractions to determine the sensitivity of the testing using the Illumina VeriSeq NIPT v2 system.

The abnormalities in the 38 synthetic positive plasmas included eighteen trisomy 21, six trisomy 18, four trisomy 13, four sex chromosomal aneuploidies (45,X and 47,XXY), one trisomy 7, one trisomy 16, two trisomy 20, one 10.5 Mb terminal deletion of chromosome 7p and 26.5 Mb terminal duplication of chromosome 9p, and one 26.3 Mb terminal duplication of chromosome 15q. All abnormalities in these synthetic positive plasmas were correctly detected by the Illumina VeriSeq NIPT v2 system (Supplementary Table S1). Figures 1B–D showed the LLRs of the synthetic plasmas with trisomy 21, trisomy 18, and trisomy 13, respectively, in comparison with that of the negative plasmas. Chromosomal abnormalities can be detected in the synthetic plasmas with the fetal fraction as low as 1% (Supplementary Table S1).

We also tested two real positive maternal plasmas with fetal trisomy 21 and trisomy 18, respectively, and six commercial positive plasmas, including two trisomy 21, two trisomy 18, and two trisomy 13 (SeraCare Life Sciences), in parallel with synthetic plasmas made in this study (Supplementary Table S1). There were no noticeable differences in sensitivity, specificity and other testing parameters between these samples and our synthetic plasmas.

5 Discussion

Short fetal cfDNAs in maternal plasmas were most likely derived from apoptosis (Jiang and Lo, 2016; Rostami et al., 2020). We noticed that cell culture media of prenatal specimens contain short cfDNA fragments that were probably derived from cell apoptosis during the culture. The sizes of such short cfDNA fragments are within the reported size range of fetal cfDNA in plasmas of pregnant women (Kim et al., 2015; Jiang and Lo, 2016). Therefore, it is possible to use this type of short cfDNA to make positive synthetic plasmas that could mimic maternal plasmas carrying fetal cfDNA with chromosomal abnormalities. Our study demonstrated that the synthetic positive plasmas can be readily and reliably used in clinical validation and QA of NIPS. The synthetic positive plasmas described in this study have been successfully used to validate and monitor the NIPS system in our laboratory, which are required by the national and state regulations. Negative synthetic plasma could also be synthesized using normal cfDNA as needed, although it may not be necessary since negative maternal plasmas are not difficult to collect.

Clinical laboratories that provide prenatal cytogenetic tests have unique advantages of making synthetic positive plasmas. It is required to maintain backup cultures for 2 weeks after reporting cytogenetic findings for all prenatal specimens in the United States. Other countries may also have similar requirements. Therefore, the laboratories can readily collect culture media of targeted abnormal cells from the backup cultures. The cfDNAs from the culture media can be directly used to make synthetic plasma after cfDNA extraction without further treatments. Synthetic positive plasmas may also be made using abnormal genomic DNA, but additional processes, such as fragmentation of long genomic DNA and isolation of short DNA, would be needed and those processes could be challenging.

The best time to collect short cfDNA from the culture medium of chorionic villus cell or amniocyte appears to be on day 3–5 after feeding the cells that grow at high confluency (~90%) with fresh culture medium (Figure 1A). Short culture time might not be able to collect enough cfDNA; long culture time might result in more background of large DNA, probably due to increased cell death and reduced apoptotic activities.

De-identified remaining plasmas after pathogen testing from phenotypic normal non-pregnant females, which would be otherwise discarded, are readily to collect from clinical microbiology or immunology laboratories with appropriate protocols. It is less likely that a phenotypically normal non-pregnant female donor would carry aneuploidies that are usually associated with abnormal phenotypes. To ensure aneuploidy-free in the donors, each non-pregnant plasma was used to synthesize two positive plasmas with different abnormalities if possible. An abnormality of donor origin would be indicated if an abnormality showed in both synthetic plasmas.

While the synthetic plasmas can be used as controls on the Illumina VeriSeq NIPT v2 system, they have not been tested on other NIPS systems for validation of different methodologies, such as single nucleotide polymorphism (SNP)-based NIPS, cfDNA size selection, and targeted sequencing. We did not test cfDNA from culture media of other cell types. In addition, abnormal prenatal cell cultures may not be accessible for every laboratory in needs to make synthetic positive plasmas.

In conclusion, we reported a practical strategy of making synthetic positive plasmas that could be used for NIPS validation and QA. This method could be especially helpful for clinical genetics laboratories that plan to implement NIPS testing.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of University of California San Francisco. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

ZQ and JY designed the study. ZQ analyzed the data and drafted the manuscript. JY edited and revised the manuscript. Both authors have read and approved the final version of this manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2023.971087/full#supplementary-material

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Patient attitudes and preferences about expanded noninvasive prenatal testing

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Introduction: Noninvasive prenatal testing (NIPT) using cell-free DNA (cfDNA) is typically carried out to screen for common fetal chromosomal anomalies, with the option to screen for a wider range of chromosomal changes (expanded NIPT) becoming increasingly available. However, little is known about pregnant patients' attitudes and preferences regarding expanded NIPT.

Methods: To address this gap, we surveyed general-risk patients having first-tier cfDNA screening at a private prenatal clinic on their expectations for expanded NIPT. Patients were asked questions regarding their current pregnancy and previous pregnancy history, their opinions on fetal DNA screenings during pregnancy and incidental findings, information and opinions on financial resources for NIPT, as well as socio-cultural questions to determine patient demographics.

Results: Of the 200 survey participants, the majority were educated, self-reported as white, had a higher than average income, and reported no aneuploidy risk factors. When asked what information they would like to receive from cfDNA screening, the vast majority of participants wanted all information available that could have an immediate impact on fetal health (88%) or an immediate impact on infant health from birth (82%). Many participants also wanted information that could have a future impact on the child's health or an immediate or future impact on the pregnant woman's own health. Most participants wanted information about the sex of fetus (86%) and common trisomies (71%), with almost half of participants desiring information about rare autosomal aneuploidies and/or all genetic information that may affect the baby. In addition, participants were found to be comfortable screening for conditions that are well-known, influence care during pregnancy, and are treatable. Finally, while most respondents either had insurance coverage for NIPT or were able to afford NIPT out of pocket, the majority of our participants felt that expanded NIPT should be either free for everyone or for those considered high risk.

Discussion: Our findings suggest that with appropriate pre-test counseling, pregnant patients may choose NIPT for an expanding list of conditions.

KEYWORDS

noninvasive prenatal testing, cell-free DNA, patient preference, surveys and questionnaires, aneuploidy, informed consent, incidental findings

Introduction

Noninvasive prenatal testing (NIPT) using cell-free DNA in maternal plasma to screen for fetal aneuploidy was first introduced clinically in 2011. NIPT typically consists of, at a minimum, screening for trisomies 21, 18, and 13, and it is primarily for these trisomies that most practice guidelines recommend screening for all pregnant people (Dondorp et al., 2015; Audibert et al., 2017; American College of Obstetricians and Gynecologists ACOG and Society for Maternal-Fetal Medicine SMFM, 2020; Dungan et al., 2022). A number of professional society guidelines have noted that cfDNA screening is more effective than traditional serum screens in screening for common aneuploidies, with higher sensitivities, specificities, and positive predictive values (PPVs) (Royal College of Obstetricians and Gynaecologists, 2014; Benn et al., 2015; Dondorp et al., 2015; Audibert et al., 2017; American College of Obstetricians and Gynecologists ACOG and Society for Maternal-Fetal Medicine SMFM, 2020; Dungan et al., 2022).

In addition to screening for common trisomies, NIPT for sex chromosome aneuploidies (SCAs) is optional and available in select countries. However, cfDNA screening for common trisomies and SCAs, even with the increased sensitivity of NIPT compared to traditional serum screening options, will miss ~17% of clinically relevant chromosomal anomalies (Wellesley et al., 2012). In the past few years, the use of NIPT has expanded both in volume and in the number and type of conditions for which screening is available (Ravitsky et al., 2021). The option to screen for additional chromosomal changes, such as rare autosomal aneuploidies (RAAs), select microdeletions, and copy number variants (CNVs) across the genome, collectively referred to as expanded NIPT, is becoming increasingly available through various laboratories.

Several recent publications have shown strong performance for the detection of RAAs and CNVs using expanded NIPT, with high sensitivities, specificities, and low no-call rates observed (Pescia et al., 2017; Pertile et al., 2021; Soster et al., 2021). Some studies have also shown the clinical impact that CNVs and RAAs can have on pregnancy and birth outcomes (Harasim et al., 2022; Mossfield et al., 2022), with the study by van Prooyen Schuurman et al. finding that most of the fetal chromosomal aberrations in their cohort were pathogenic and associated with severe clinical phenotypes (van Prooyen Schuurman et al., 2022). Because NIPT analyzes cfDNA from the placenta and not from the fetus, discordant results due to confined placental mosaicism (CPM) can occur. However, these CPM cases can also be associated with adverse perinatal outcomes (Eggenhuizen et al., 2021; Mossfield et al., 2022; van Prooyen Schuurman et al., 2022). To date, some professional medical societies have remained silent or have recommended against NIPT for RAAs or genome-wide CNVs, mainly citing the lack of large validation studies and the need for further research (Dondorp et al., 2015; Audibert et al., 2017; Kozlowski et al., 2019; American College of Obstetricians and Gynecologists ACOG and Society for Maternal-Fetal Medicine SMFM, 2020). The recent ACMG guidelines note that at this time there is insufficient evidence to either recommend or not recommend noninvasive prenatal screening for the identification of rare autosomal trisomies (Dungan et al., 2022).

Studies exploring patient preferences regarding prenatal screening, and NIPT in particular, have suggested that pregnant patients find NIPT for common aneuploidy screening to be a convenient and safe option that is preferable over conventional serum screening options because of its higher sensitivity and specificity (accuracy) (Farrell et al., 2014a; Lewis et al., 2014; Tiller et al., 2015; Lewis et al., 2016b; Sahlin et al., 2016; Abdo et al., 2018; Bowman-Smart et al., 2019b; Cornell et al., 2022). Fewer studies have explored patient preferences for expanded NIPT. To examine the attitudes and preferences of pregnant people regarding expanded NIPT, we surveyed general-risk patients having first-tier cfDNA screening at a private prenatal clinic in Canada on their expectations for expanded NIPT, including the factors they consider most important when making the decision to undergo expanded cfDNA screening.

Materials and methods

Pregnant patients presenting to a private prenatal clinic in Quebec City (Prenato Clinics Canada) for consideration of first-tier NIPT for common aneuploidy screening from April 2021 to September 2021 were approached for participation in this study. Inclusion criteria included pregnant patients 18 years of age or older, French-speaking, and ability to provide informed consent for research. We planned to enroll 200 participants. Patients were enrolled on a consecutive basis if they agreed to participate in the study and no advantages were given to participants that agreed to take part in the study. Once enrolled, participants were provided with an informational leaflet (Supplemental Appendix SA [English version]; Supplemental Appendix SB [French version]) describing NIPT, the various types of conditions that could potentially be screened by expanded NIPT, and possible effects of these conditions on the health of the fetus, the pregnancy, the mother, or the child after delivery. If necessary, the patients were free to ask additional questions to the medical team on site. Participants were asked to complete an anonymous electronic survey (Supplemental Appendix SC [English version]; Supplemental Appendix SD [French version]) exploring their attitudes and preferences about expanded NIPT. The survey consisted of a total of 28 questions, covering the following topics: Current pregnancy and pregnancy history; Fetal DNA screenings during pregnancy for the most common trisomies; Additional information that could be accessed through fetal DNA (incidental findings); Financial resources for fetal DNA screenings and incidental findings; and a Socio-cultural section.

Following completion of the survey, participants resumed routine clinical care with a consultation by a clinical nurse to obtain additional information, if needed, and to have blood drawn for NIPT. NIPT offered in this clinic included screening for trisomy 21, trisomy 18, trisomy 13, and sex chromosome aneuploidies (including expected fetal sex). Screening for microdeletions, RAAs, or CNVs was not available at this clinic at the time of the study.

The survey data were analyzed and responses were calculated as percentages. Responses to the Likert-scale questions related to comfort were collapsed into the following three categories: Comfortable (consisting of responses of comfortable and very comfortable), Neutral, and Not Comfortable (consisting of responses of not comfortable and not very comfortable).

TABLE 1 Characteristics of study participants.

Patient characteristics	N (%)
Age, years	
Range	19–40
Median	30
Gestational age, weeks completed	
Range	4–23
Median	12 (SD 2.3)
Previous children	
Yes	102 (51)
No	97 (49)
Previous miscarriage or loss of baby	
Yes	56 (28)
No	143 (72)
cfDNA screening in ≥1 previous pregnancy	
Yes	52 (26)
No or not applicable	147 (74)
Previous pregnancy with a genetic abnormality	
Yes	4 (2)
No	191 (96)
Family history of chromosomal abnormalities	
Yes	15 (8)
No	179 (90)
Method of conception	
Natural	184 (92)
IVF/Assisted reproduction/Other	15 (8)
Country of birth	
Canada	176 (88)
Other	19 (10)
Reported ethnicity	
White	149 (75)
Other	30 (15)
Highest level of education	
High school diploma	12 (6)
College degree	39 (20)
Professional training	20 (10)
Baccalaureate[a]	65 (33)
Master's degree	37 (19)
Doctorate	13 (7)
Other	8 (4)
Annual family income	
Less than $50,000	12 (6)
$50,001 to $100,000	57 (29)
$100,001 to $300,000	109 (55)
More than $300,000	9 (5)
Religion	
Catholic	108 (54)
Other	25 (13)
No religious affiliation	58 (29)
Considers religion (very) important	
Yes	12 (6)
Neutral	39 (20)
No	138 (69)

[a]A bachelor's degree from a university.
Numbers may not total 100% (200) as not all respondents answered every question.

Responses to Likert-scale questions related to importance were likewise collapsed into the following three categories: Important (consisting of responses of important and very important), Neutral, or Not Important (consisting of responses of not important and not very important). Because of the homogeneity of the data and the small sample size, comparison between response groups was not performed.

Results

Participant details

A total of 200 pregnant patients were included in the study cohort. Based on responses to questions 1–6 and 18–28 of the survey (Supplemental Appendix SC), the majority of participants were educated, self-reported as white, and reported no aneuploidy risk factors. Just over half of all participants had at least one child, and around one-quarter of participants had undergone cfDNA screening in a previous pregnancy. In addition, 60% of participants reported an annual family income of greater than $100,000 (see Table 1).

Desired information from cfDNA screening

Participants were asked a multi-part question regarding the sort of information that they would be interested in receiving with regard to incidental findings (Supplemental Appendix SC, question 9). The vast majority of participants wanted all information available that could have an immediate impact on fetal health (175; 87.5%) or that had an immediate impact on infant health from birth (163; 81.5%), as shown in Figure 1. Many participants also wanted information that could have a future impact on the child's health (138; 69%) or an immediate or future impact on the pregnant woman's own health (141; 70.5%). Only 35 participants (17.5%) did not want information from expanded NIPT and only wanted information about common trisomies. When asked what information they would like to receive through the fetal DNA test (Supplemental Appendix SC, question 7), most participants wanted information about the sex of fetus (172; 86%) and common trisomies (141; 70.5%), as shown in Figure 2. Fewer wanted information about other conditions such as rare trisomies (90; 45%) and CNVs (46; 23%).

Importance of factors in decision-making

Several questions in the survey also asked patients to indicate the importance of various factors in their decision-making process (Supplemental Appendix SC, questions 8, 10, 11). When asked about what factors were most important when making the decision to obtain information about common trisomies, participants responded that wanting a healthy child was important (198; 99%), with 88% of participants (n = 176) stating that wanting as much information as possible about their child's health or their own health was important (Figure 3). Other factors that were important to almost all participants included having a safe test with no risk of miscarriage (197; 98.5%), wanting the reliability of the results

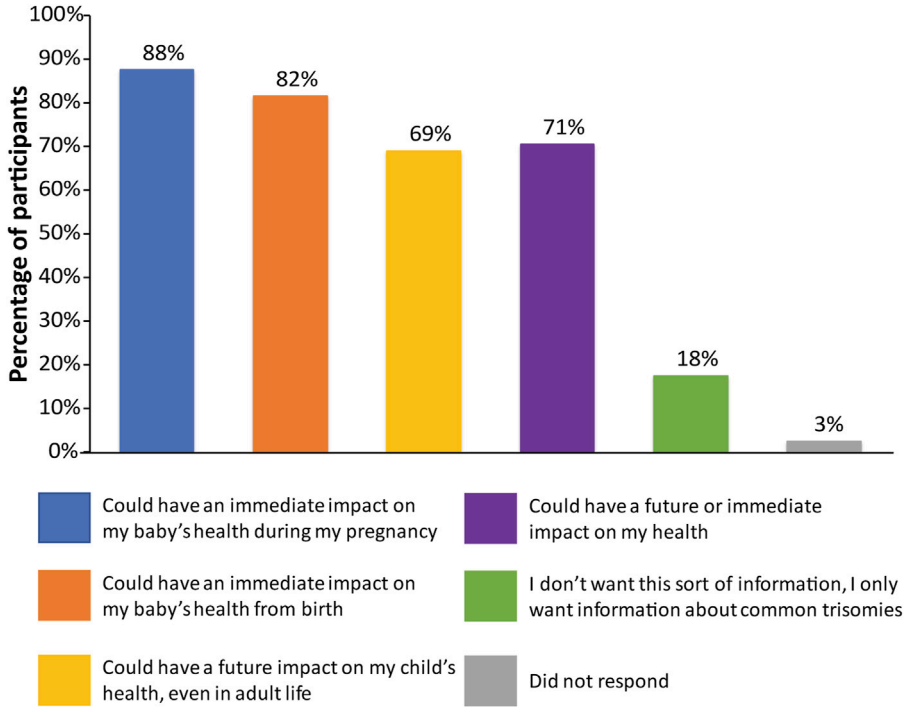

FIGURE 1
Patient preferences regarding information received from expanded NIPT. Patients were surveyed on the sort of information that they would like to receive with regards to incidental findings, with six options provided. Patients were allowed to select more than one option.

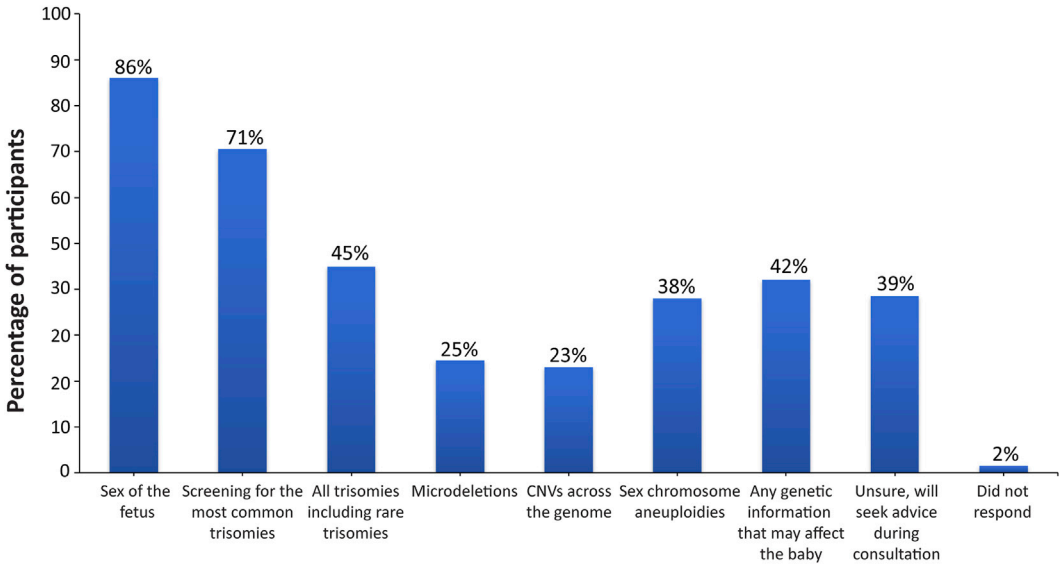

FIGURE 2
Desired information on the conditions screened for through the fetal DNA test. Patients were surveyed on the information that they would like to receive through the fetal DNA test, with eight options provided. Patients were allowed to select more than one option.

to be as high as possible (195; 97.5%), and that fetal DNA was the most effective test for finding any conditions (191; 95.5%). As can be seen from Figure 4, factors that were most important to participants when making the decision on whether to obtain information from expanded NIPT included wanting to know if their child has a genetic disorder (186; 93%), wanting as much

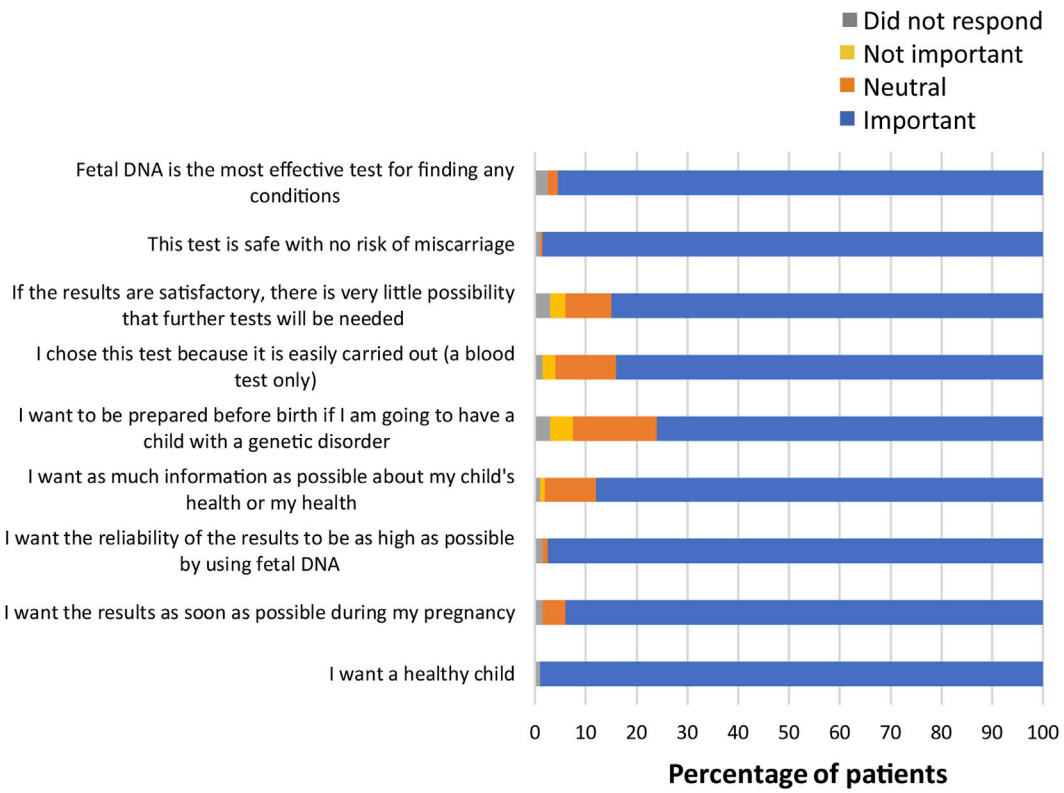

FIGURE 3
Importance of factors in decision-making for NIPT for common trisomies.

information as possible about their child's immediate health (183; 91.5%), and wanting as much information as possible about their child's future health (178; 89%). Wanting the result as soon as possible during the pregnancy was also viewed as important for most participants (181; 90.5%). Participants were then asked about the factors they considered to be important when making decisions about obtaining information regarding their own health (Figure 5). The vast majority responded that wanting as much information as possible about their own health was important (163; 81.5%). Almost half the participants (98; 49%) thought that regretting it later if they did not undergo the test was an important factor in the decision to get information on their own health. Sixty-four percent of participants (n = 128) noted that their religion beliefs were not an important factor in the decision.

Comfort of participants with information received by expanded NIPT

Participants were also surveyed on their comfort with incidental findings that may involve personal or family risk (Supplemental Appendix SC, question 12). As can be seen from Table 2, participants were found to be comfortable screening for conditions that are well-known (155; 77.5%), influence care during pregnancy (146; 73%), and are treatable (153; 76.5%). Although fewer, participants were still comfortable screening for conditions that are not well known (80; 40%), will not influence care during pregnancy (117; 58.5%), are not treatable (103; 51.5%), and do not appear until adulthood (105; 52.5%).

Attitudes of patients regarding coverage for expanded NIPT

Finally, all participants were surveyed on financial resources and reimbursement for fetal DNA screenings and incidental findings (Supplemental Appendix SC, questions 14–17). The majority of participants thought that these tests should be either free for everyone (123; 61.5%) or free for people who are high risk (51; 25.5%), as shown in Figure 6. When asked if cost of testing was a factor in their screening decision (Figure 7A), over half of participants replied "No", because either their insurance covers these tests (20; 10%); because they can afford the test they want (75; 37.5%); or because they do not want a test for additional genetic information, they are only interested in screening for common trisomies (19; 9.5%). Over a third of participants (71; 35.5%) had insurance that covered at least part of the cost of cfDNA screening. The participants were also asked how much they would be willing to pay for additional findings, with 43% of participants (n = 86) stating that they would be willing to pay at least $100 for it and 32% of participants (n = 64) stating the amount is not important (Figure 7B).

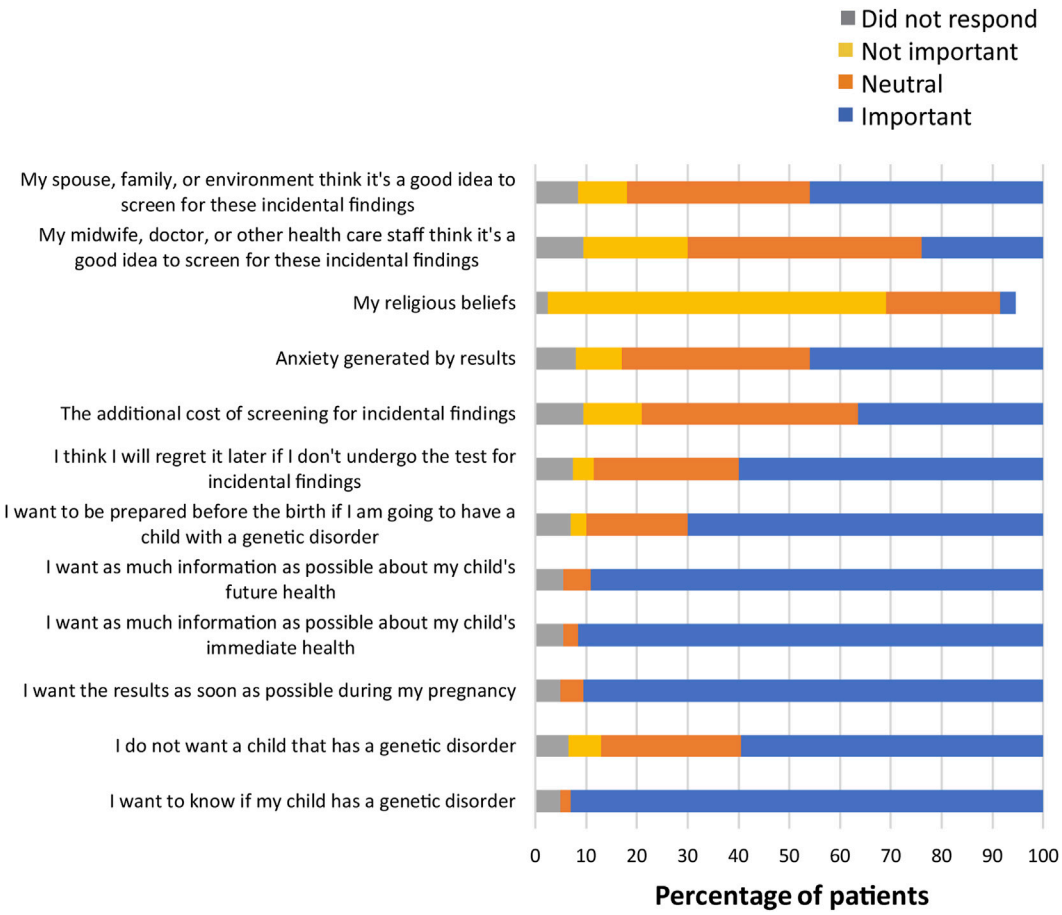

FIGURE 4
Importance of factors in decision-making for expanded NIPT.

Discussion

In this study we found that general-risk pregnant people that are undergoing first-tier NIPT, after reading a leaflet with detailed information on the advantages and disadvantages of expanded NIPT screening, are interested in information available through expanded cfDNA screening about both the current and future health of their fetuses and selves. A majority of the survey participants were also comfortable with screening for conditions that will not influence pregnancy care, do not appear until adulthood, or have no treatment. In addition, 42% of participants said they wanted to know any genetic information that could affect the baby.

Most major medical professional societies endorse the option of NIPT to screen for common autosomal aneuploidies (Benn et al., 2015; Dondorp et al., 2015; Audibert et al., 2017; BeSHG, 2017; Salomon et al., 2017; Kozlowski et al., 2019; American College of Obstetricians and Gynecologists ACOG and Society for Maternal-Fetal Medicine SMFM, 2020; BeSHG, 2020; Prieto et al., 2020; Dungan et al., 2022) with some also endorsing cfDNA screening for sex chromosome aneuploidies (Benn et al., 2015; American College of Obstetricians and Gynecologists ACOG and Society for Maternal-Fetal Medicine SMFM, 2020; Dungan et al., 2022). However, as noted above, the use of expanded NIPT has not been endorsed at this time. Our data clearly demonstrate that pregnant patients may be interested in receiving additional findings from expanded NIPT screening. Although the American College of Medical Genetics and Genomics (ACMG) notes that there should be personalized patient-centered counseling (Dungan et al., 2022), and the European Society of Human Genetics/American Society of Human Genetics (ESHG/ASHG) note that pregnant women's wishes regarding learning information beyond the common trisomies should be taken into account (Dondorp et al., 2015), most guidelines do not acknowledge the preferences of pregnant patients. Our data could thus contribute to a better understanding of patient preferences regarding expanded NIPT and could help to better adapt practice recommendations due to the rapid evolution of genomics, including in the prenatal field, in the near future. In the same way and supporting our results, the Netherlands prenatal screening program, which, since 2017, has offered NIPT with the option of genome-wide expanded options to all pregnant people, emphasizes patient's opinions and increasing reproductive choices of couples (Bilardo, 2021). A recent publication from this TRIDENT screening program in the Netherlands found that, following a pre-test counseling session with a certified obstetric counselor, 74.2% of patients chose to learn about additional

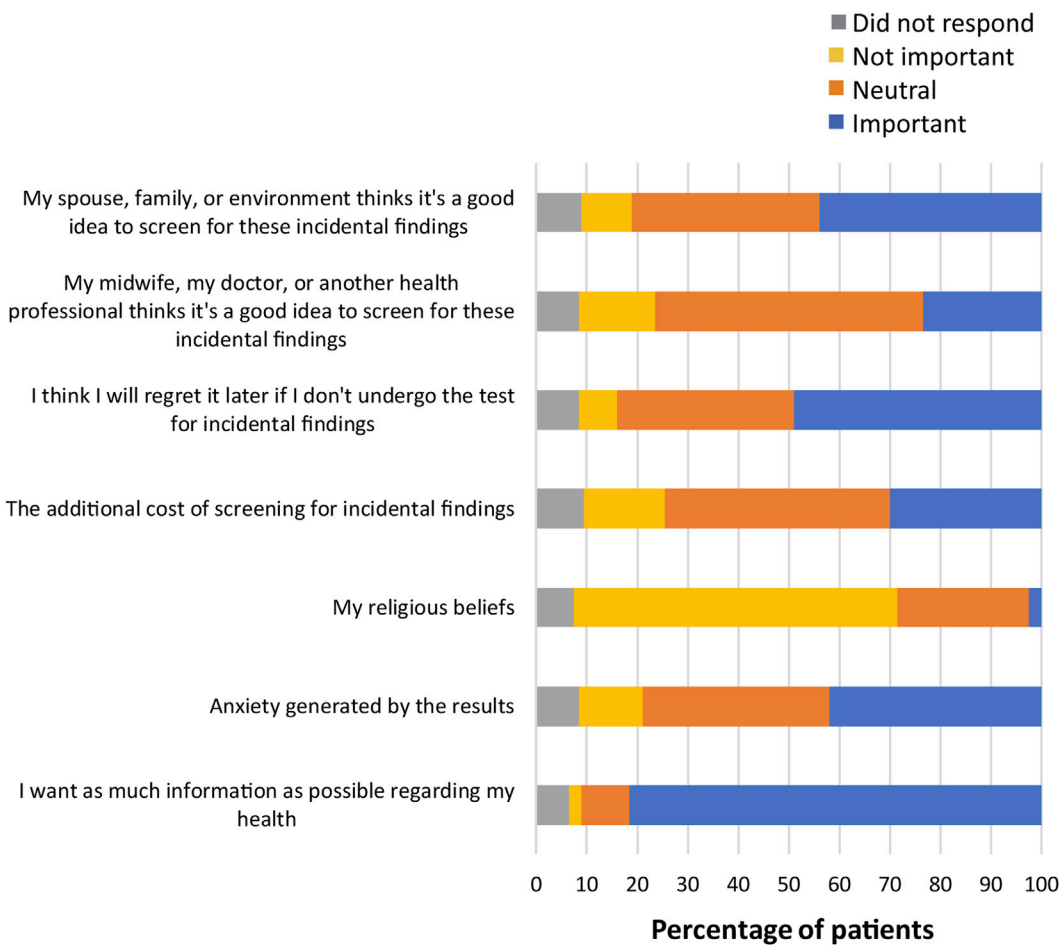

FIGURE 5
Importance of factors in decision-making for patient's own health.

TABLE 2 Participant's level of comfort with information from expanded NIPT.

	Did not respond, n (%)	Not comfortable, n (%)	Neutral, n (%)	Comfortable, n (%)
Get results that give an assessment of the risks, rather than just a "yes/no" answer	19 (9.5)	31 (15.5)	28 (14.0)	122 (61.0)
Screen for conditions that are well known	21 (10.5)	3 (1.5)	21 (10.5)	155 (77.5)
Screen for conditions that are not well known	25 (12.5)	56 (28.0)	39 (19.5)	80 (40.0)
Screen for conditions that will influence care during pregnancy	23 (11.5)	10 (5.0)	21 (10.5)	146 (73.0)
Screen for conditions that will not influence care during pregnancy	23 (11.5)	13 (6.5)	47 (23.5)	117 (58.5)
Screen for conditions that are treatable	25 (12.5)	2 (1.0)	20 (10.0)	153 (76.5)
Screen for conditions for which there is no treatment	24 (12.0)	37 (18.5)	36 (18.0)	103 (51.5)
If potential disorders do not appear until adulthood	23 (11.5)	24 (12.0)	48 (24.0)	105 (52.5)

findings other than common trisomies (van Prooyen Schuurman et al., 2022). Another study by the TRIDENT group on patient experiences found that 90.4% of respondents were glad to have been offered the choice between expanded and targeted NIPT, with 76.5% of the respondents choosing to undergo expanded NIPT (van der Meij et al., 2022). The authors concluded that the

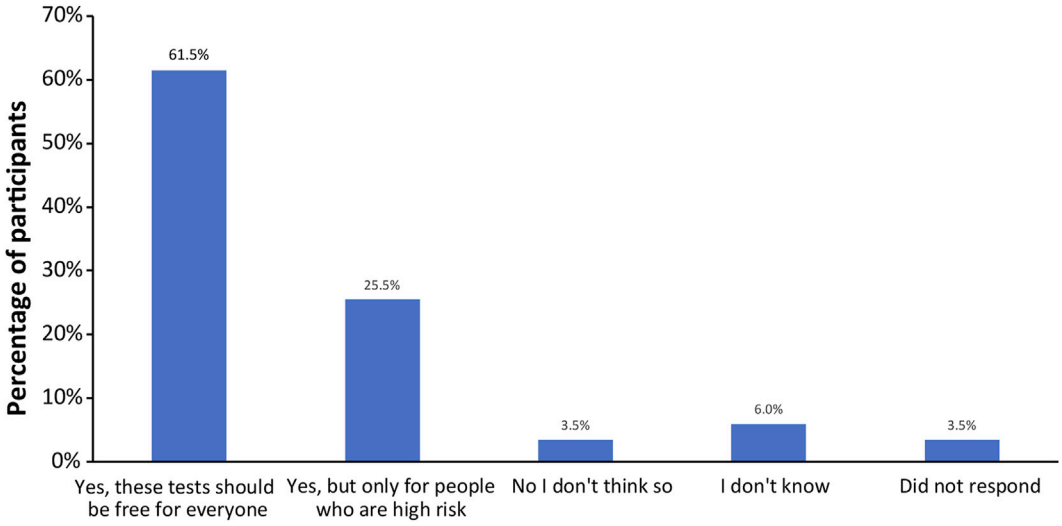

FIGURE 6
Opinions of participants on whether the public health plan should cover the costs for fetal DNA screening and incidental findings.

perspectives of pregnant patients should be included in the dialogue surrounding the expansion of NIPT.

A recent commentary by Bayefsky et al. suggested that criteria used for other health screening programs should be applied to genome-wide NIPT (Bayefsky et al., 2022). These include the condition being an important health problem, that there should be a recognizable latent stage as well as a valid and reliable test and accepted treatment for the condition, and the screening should be cost effective. The authors question whether NIPT should be applied for certain rare conditions if they are not a common cause of disease and disability in the general population. From our perspective, we believe that the prevalence of RAAs and CNVs is high enough to warrant screening for these additional fetal anomalies. In addition, recent publications have shown that the presence of RAAs and CNVs can impact both pregnancy and birth outcomes, and that measures taken during the pregnancy such as increased monitoring can be beneficial and should be considered (Mossfield et al., 2022; van Prooyen Schuurman et al., 2022). We therefore believe it is important that patients are offered the choice of having expanded noninvasive prenatal screening, provided they receive appropriate pretest counselling.

A number of previous studies have looked at patient preferences regarding conventional noninvasive prenatal screening. Here, when participants were asked about the conditions that they desired information on, the vast majority of participants (86%) wanted information on fetal sex, and 71% wanted information on common trisomies. This differs from a 2019 study (Bowman-Smart et al., 2019b) based on survey responses of 235 pregnant patients in Australia, which found that less than a third wanted to undergo NIPT for fetal sex, whilst 86% of respondents noted detection of chromosomal abnormalities as a reason for undergoing NIPT. A study by Farrell et al. (Farrell et al., 2014b) looking at the perspectives of 53 people that were either pregnant or had recently delivered found that accuracy, early timing, ease of testing, and fetal sex determination were the main advantages of NIPT. The recent study by van der Meij et al. noted the main reasons that participants chose expanded NIPT were 'wanting as much information as possible about the health of the child' and wanting 'to be prepared for everything' (van der Meij et al., 2022). Other studies that have looked at patient expectations and preferences from expanded NIPT often focus on conditions that are not currently available as part of routine cfDNA screening. Most of these studies have shown strong support for including predicted fetal sex (Bowman-Smart et al., 2019a; Haidar et al., 2021). A sizeable portion of pregnant patients are also interested in other expanded NIPT options, including sex chromosomal aneuploidy (Agatisa et al., 2015; Bowman-Smart et al., 2019a), microdeletions (Agatisa et al., 2015; Calonico et al., 2016; Farrell et al., 2016), childhood onset conditions (whether treatable or not) (Farrell et al., 2014a; Sullivan et al., 2019), and conditions of adult-onset (whether preventable or not) (Farrell et al., 2014a; Bowman-Smart et al., 2019a). However, most pregnant patients do not appear to be supportive of using NIPT for non-medical traits, other than fetal sex (Kooij et al., 2009; Bowman-Smart et al., 2019a; Haidar et al., 2021). A 2015 study (van Schendel et al., 2015) of 381 women who completed an online questionnaire on a Dutch website found that the vast majority of participants agreed with screening for a broad range of conditions including severe life-threatening disorders with no available treatment and disorders for which the child can already be treated during pregnancy such as heart disease. The study by van der Meij et al. also noted that most of the respondents were favorable toward a broader future screening offer such as screening for severe untreatable life-threatening disorders, disorders characterized by a mental disability, disorders that can be treated during pregnancy, and severe physical disabilities (van der Meij et al., 2022).

One disadvantage of a screening test is the low PPV that may be associated with it, which can lead to increased patient anxiety. PPV is the proportion of positive results that are truly positive and incorporates test sensitivity and specificity as well as the population prevalence of the condition. This can also lead to

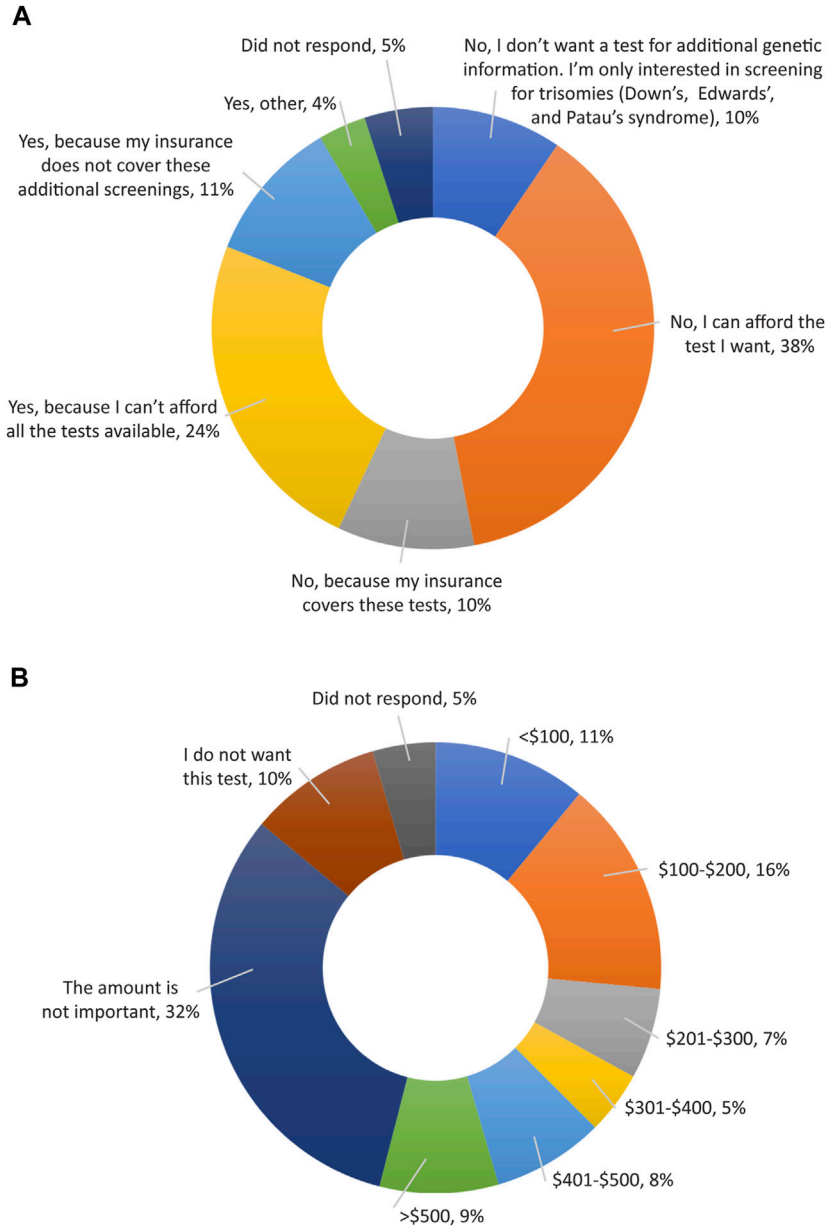

FIGURE 7
Opinions of participants on reimbursement for expanded cfDNA screening. **(A)** Cost of testing as a factor in a patient's screening decision. **(B)** Participant's views on how much they would be willing to pay to access genetic screening for additional information.

unnecessary invasive diagnostic procedures, which are associated with additional risks and costs. Studies looking at expanded NIPT have noted different PPVs for these additional findings; reasons for differing PPVs between studies may include differences in sequencing depth, the background risk profile of the population (e.g., proportion of advanced maternal age), differences in inclusion criteria for study participants, and whether maternal CNVs are included as true positives in the analysis. A recent study looking at test performance of genome-wide cfDNA screening in a real clinical population (Soster et al., 2021) found high sensitivity and specificity, with a PPV of 22.4% for rare autosomal trisomies and 72.6% for genome-wide CNVs. The study also found that 25% of the positive results would have been missed with traditional cfDNA screening. A recent study from the TRIDENT-2 group noted PPVs of 7.7% for rare autosomal trisomies and 44.1% for structural chromosomal aberrations (van Prooyen Schuurman et al., 2022). The 2017 Society of Obstetricians and Gynaecologists of Canada (SOGC) guidelines on prenatal screening for fetal aneuploidy in singleton pregnancies (Chitayat et al., 2017) note that any prenatal screen offered to Canadian women must have a detection rate of 75% with no more than 3%–5% false-positive rate, dependent on trimester of screening. With this scope, a long-time serum screening testing has been used as the preferred prenatal screening program while its PPV was as low as 3%–5%. Nevertheless, for 46% of our patients, the anxiety generated by the results is considered important in decision making when considering the use of an expanded screening test.

Regarding financial resources for NIPT, over 62% of our study participants thought that NIPT should be free for everyone and another 26% thought that is should be free for people with a high-risk pregnancy. Another study (Birko et al., 2019) carried out in Canada a few years ago that also looked at patient attitudes toward NIPT coverage noted similar results, with 67% of pregnant women responding that all patients should have access to NIPT free of charge and 30% saying that only patients with a high-risk pregnancy should be eligible. Studies of healthcare providers in Europe (Benachi et al., 2020), as well as the Lebanon and Quebec (Haidar et al., 2020), found that one of the primary barriers to uptake of NIPT was the cost and lack of reimbursement. Cost and insurance coverage were also noted as disadvantages of NIPT in a study by Farrell et al.(Farrell et al., 2014b) of patients in a clinic in the United States. According to the SOGC, as of January 2021, the cfDNA test is not publicly funded for all pregnant patients in Canada as first-line prenatal screening and is a self-paid or insurance-covered option for most pregnant people if they are not detected at risk by a first-step serum screen. In some Canadian provinces, there is funding for people who meet certain high-risk criteria (Wou et al., 2021). Our results show that a large proportion of patients in a financially well-off population are willing to cover the additional costs related to obtaining additional findings from expanded cfDNA screening.

With increasing use of NIPT, concerns over the potential for 'routinization' of prenatal screening have arisen (Lewis et al., 2016a; Cernat et al., 2019). While this concern lacks empirical confirmation in practice (Kater-Kuipers et al., 2018), the importance for patients to have sufficient understanding of prenatal screening options to allow for an informed choice consistent with one's values is well-recognized. Global medical societies emphasize the need for appropriate pre-test counseling (Benn et al., 2015; Dondorp et al., 2015; Rose et al., 2020; Dungan et al., 2022). Patients, however, have expressed feeling dissatisfied with both the quality and type of information available about NIPT, citing lack of provider knowledge and time constraints (Cernat et al., 2019). As the menu options for NIPT grow and include the potential for conditions with less well-defined phenotypes, reduced penetrance, variable expressivity, and/or later onset, pretest counseling will become more complex and difficult. In particular, understanding the positive predictive value of results for conditions of varying prevalence will be crucial. Pregnant patients, with their partners, can have different preferences for whether NIPT should consist of a fixed set of conditions or whether they should be able to decide which specific condition(s) to screen for in their pregnancy (van Schendel et al., 2014). ACMG recommends discussing the types of conditions that can and cannot be screened using NIPT as part of pretest counselling (Dungan et al., 2022). In addition, it is important to note that genomic abnormalities detected during NIPT may be of parental origin and may indicate a maternal health condition such as maternal malignancy (Turriff et al., 2022; van Prooyen Schuurman et al., 2022). It is important that patients are counselled on the possibility of these additional findings; in our study, most women indicated that they were interested in receiving information that had either an immediate or future impact on their own health.

A limitation of our study is the lack of diversity amongst participants. The majority of patients that carried out the survey self-identified as white, were intermediate to highly educated, were born in Canada, and had an annual family income of greater than $100,000. All participants also had to be French-speaking as the survey was carried out in French. This population largely reflects the population of Quebec City in eastern Canada, which uses the services of a private clinic for pregnancy monitoring, while free prenatal screening programs exist in the province of Quebec. It is therefore possible that the findings from our study may not translate into other more heterogeneous populations such as patients from a lower socio-economic background and different cultural groups. Future studies that are carried out in different regions and that include participants from different educational backgrounds and different socioeconomic backgrounds would be useful for comparison with the results observed in our study population. Another limitation is that the survey includes the opinions of a relatively small number of pregnant patients and did not include opinions of their partners or healthcare providers. However, in our study cohort, only a quarter of participants thought that the opinion of their healthcare provider was an important factor in their decision to screen for incidental findings.

Another limitation of the study was that patients were not fully informed on all of the conditions that can be screened for with genome-wide NIPT, the sensitivity and specificity of NIPT for these conditions, or the limitations of genome-wide NIPT, which may have impacted their responses. The informational leaflet did not contain any information regarding the limitations of expanded NIPT or provide details on the expected PPVs for the different fetal anomalies that are screened for by both traditional and expanded NIPT. It also did not give details on potential reasons for discordant results such as CPM, and the need for additional confirmatory follow-up testing that should be carried out following a positive NIPT result before any decisions regarding the pregnancy are taken. The participants took the survey before their routine consultation with a clinical nurse, and so their responses were based on the information provided in the leaflet. However, the participants were informed that they were also free to ask questions to the medical staff following reading of the brochure if they needed further information. It is possible that this was not sufficient information for them to make a truly informed choice when it came to the different questions in the survey. For example, some patients may not have been aware that presence of a rare autosomal trisomy or CNV could have an immediate impact on fetal health. This may help explain some of the contradictory responses from the participants, such as the fact that while most participants wanted all information available that could have an immediate impact on fetal health, 45% and 23% of women did not want information on rare trisomies and CNVs, respectively. In addition, the multiple-choice answering format of the survey could have contributed to these seemingly contradictory results. A knowledge evaluation of the patients could be carried out in the future to assess the patient knowledge of expanded NIPT following completion of the survey. In addition, the survey did not include any questions relating to patient anxiety associated with a false-positive result or the false reassurance associated with a false-negative result.

In summary, our findings suggest that with appropriate pre-test counseling, pregnant patients may choose NIPT for an expanding list of conditions. However, patients should be made aware of both the benefits and limitations of expanded NIPT and the potential for discordant results. It is very important that appropriate post-test counselling is provided in cases of a high-risk screening result before any decisions on the pregnancy are undertaken. Our results indicate that women can provide their perspective on their preferences on expanded NIPT screening. This study adds to the growing body of research looking at

the attitudes, experiences, and opinions of pregnant patients on cfDNA screening, which can be used to inform future policies around the implementation, availability, and scope of this screening technology.

Data availability statement

All of the data supporting the findings in this study are available upon reasonable request from the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Prenato clinic. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors contributed to the study conception and design. M-LD, PW, and JG developed the study tools and performed the data analysis. The manuscript was drafted by M-LD and PW. All authors revised the manuscript and approved it for publication.

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Conflict of interest

Author PW is an employee of and owns equity in Illumina, Inc. This study received funding from Illumina, Inc. The funder had the following involvement with the study: study design, data analysis, and preparation of the manuscript. Study funding was also used to support a research associate position to implement the study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2023.976051/full#supplementary-material

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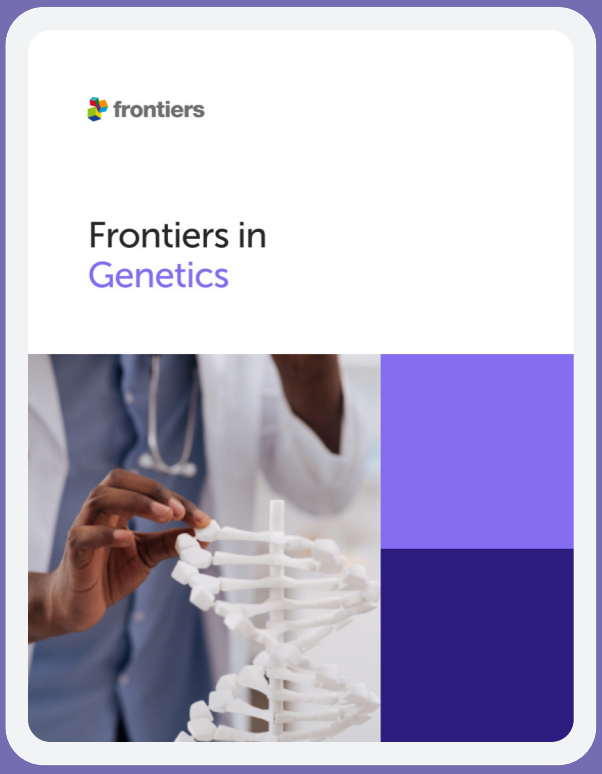

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