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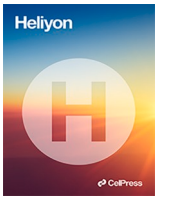

Research article

Performance analysis of non-invasive prenatal testing for trisomy 13, 18, and 21: A large-scale retrospective study (2018–2021)

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ABSTRACT

Background: Non-invasive prenatal tests (NIPT) are used to screen for trisomy 21, 18, and 13. This study investigated NIPT performance and the clinical significance of its results.
Methods: Pregnant women (n = 282,911) participating in a free NIPT (April 2018–December 2021) were screened for common trisomies, and the results were retrospectively analyzed. NIPT performance was evaluated by its positive predictive value (PPV), sensitivity, and specificity. Results were analyzed using number, percentage, and chi-squared/t-test analyses.
Results: After NIPT screening, patients with common trisomies (n = 746) included 457 with T21, 160 with T18, and 129 with T13. Seven false negative cases were identified. High PPV (86.81 %, 56.81 %, 18.18 %), sensitivity (99.25 %, 98.33 %, 100.00 %), and specificity (99.98 %, 99.98 %, 99.97 %) values were detected for trisomy 21, 18, and 13, respectively. The PPVs of common trisomies were significantly different between pregnant women older than 35 (85.53 %, 136/159) and those aged 35 or younger (58.90 %, 311/528) ($\chi 2$ = 125.02, P = 2.20e-16). As the NIPT uptake increased from 2018 to 2021, live-born birth defect incidence decreased.
Conclusion: NIPT performed well in screening for T21, T18, and T13. Our discoveries offer an important and useful guideline in laboratory and clinical genetic counseling.

1. Introduction

In China, the birth defect incidence rate is approximately 5.6 % [1], ranging from 1/200–1/150 in newborns with chromosome abnormalities [2]. These birth defects include chromosomal abnormalities, such as aneuploidy, deletion, and duplication [3]. The fetal chromosomal aneuploidies, trisomy (T) 21, 18, and 13, are the most common autosomal trisomies among humans. They cause serious deformities, disabilities, and even death, with no effective treatment to date [1]. Traditional detection methods include ultrasonic examination, serological prenatal screening, and chromosome karyotyping. Ultrasonic examination is mainly used to identify

Abbreviations: cell-free fetal DNA, cffDNA; confidence intervals, CI; confined placental mosaicism, CPM; false negatives, FN; false positives, FP; massively parallel sequencing, MPS; non-invasive prenatal testing, NIPT; polymerase chain reaction, nuchal translucency; NT, PCR; positive predictive value, PPV; positivity rate, PR; trisomy, T; true negatives, TN; true positives, TP.

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structural anomalies, such as fetal neural tube malformations. Serological screening can identify 60–95 % of fetus abnormalities with a false-positive rate of 5 %. Chromosome karyotyping is the gold diagnosis standard of anormalies in chromosome number and structural anomalies; however, it involves obtaining fetal samples through invasive methods, including amniocentesis or cordocentesis, which can cause infection, bleeding, and abortion [4,5].

In 1997, Lo et al. identified cell-free fetal DNA (cffDNA) in maternal peripheral blood. Subsequently, next-generation sequencing of cffDNA rapidly developed [6]. Since 2011, non-invasive prenatal tests (NIPT) based on cffDNA had been widely used to screen for T21, T18, and T13 [7], and some researches had indicated that NIPT exhibits high sensitivity and specificity [5,8–10]. However, uncertainties on the optimal application of NIPT and the nature and extent of its limitations still exist, necessitating further substantiating its accuracy in detecting common trisomies during pregnancy. In April 2018, a public health decision in Changsha city in China (Changsha Health and People's Livelihood Project) had executed the free NIPT for pregnant women with residency right [11–13]. By December 2021, 282,911 pregnant women were enrolled in the NIPT program. This NIPT implementation had provided the numerous samples acquired for the present research.

Our current work intended to retrospectively investigate the performance of NIPT for screening common trisomies and explore the clinical significance of these results.

We used an established NIPT protocol based on massively parallel sequencing (MPS) (BGI-500/2000 and semiconductor sequencing platform) for screening the acquired samples, as a first-tier screening tool. This research enriched the references for laboratory and clinical genetic counseling.

2. Materials and methods

2.1. Participants

The retrospective research covered 282,911 pregnant women (≥12 weeks of gestation) for a free NIPT (April 2018–December 2021), and 746 women had received the results of common autosomal trisomies (457 with T21, 160 with T18, and 129 with T13). Women were screened using semiconductor sequencing platform [n = 19,167] and BGI sequencing platform [n = 263,744]. The exclusion criteria included multiple pregnancies (three or more fetuses), transplantation, allogeneic blood transfusion, known chromosomal anomalies in any parent, allogeneic cell gene therapy, and immunotherapy within one year of the NIPT. After genetic counseling, pregnant women involved in the NIPT signed written informed consent forms (consenting participation in the scientific research). The research complied with the policies of the Helsinki Declaration. The basic characteristics of the pregnant women involved in the NIPT are stated in Table 1.

Table 1
Basic characteristics of 282,911 pregnant women uptaking NIPT. NIPT: non-invasive prenatal testing, Min:minimum, Max:maximum, IVF:in vitro fertilization.

Characteristic parameter	Number(n)	Percentage (%)
Maternal age (years)		
Mean	29.8	–
Min-Max	15–55	–
< 20	2876	1.02
>20-25	39674	14.02
>25-30	118357	41.84
>30-35	98375	34.77
>35-40	20967	7.41
>40	2662	0.94
Gestational age (Weeks + days)		
Mean	16^{+6}	–
Min-Max	12–30	–
Early pregnancy (0–12^{+6})	2747	0.97
Mid pregnancy (13–27^{+6})	279750	98.88
Late pregnancy (28–41^{+6})	343	0.12
Unknown	71	0.03
Pregnancy mode		
Naturally conceived	268569	94.93
IVF	12299	4.35
Unknown	2043	0.72
Number of fetus		
Singleton	274995	97.2
Twin	5483	1.94
Disappearance or reduction of one of the twins	66	0.02
Unknown	2367	0.84
NIPT sequencing platform		
Semiconductor sequencing	19167	6.77
BGI-500/2000	263744	93.23

2.2. NIPT study (part of the health and people's livelihood project in changsha)

In 2017, a free midterm serological screening of all pregnant women was conducted in the Hunan Province. In 2018, a public screening plan was formulated for pregnant women in Changsha; either spouse who held a Changsha residence or temporary residence permit could apply directly for a free NIPT e-Voucher. The application and use of the free NIPT e-voucher are shown in Fig. S1.

2.3. Experimental methods

2.3.1. BGI sequencing method

Peripheral blood samples were collected, the cffDNA was extracted and used for library construction [Registration permit No.20150250] [Registration permit No. 2017340059/20160193], sequencing and result analysis, according to the previous method (Lu. et al., the journal of Expert Rev Mol Diagn, 2022) [1] with slight modification. Briefly, the plasma (1.8 mL) was then collected equally in three 2 mL nuclease-free centrifuge tubes for further use. The plasma separation process required quality control (QC), including low-temperature treatment, plasma hemolysis determination, and plasma volume.

This library construction mainly included end repair and adaptor ligation. The reaction system for the end repair of a single sample was 50 μL, including 40 μL of extracted cffDNA, 9.4 μL of the end repair buffer, and 0.6 μL of the end repair enzyme. The reaction procedure was as follows: 10 min at 37 °C, followed by 15 min at 65 °C, and for ever at 4 °C. The reaction procedure for the ligation of a single sample was 80 μL, including 50 μL of a DNA solution, 24 μL of ligation buffer, 1 μL of ligase, and 5 μL of barcode. The reaction proceeded as follows: 20 min at 23 °C, followed by for ever at 4 °C. After purification, the reaction system for polymerase chain reaction (PCR) analysis of a single sample was 50 μL, including 21 μL of the DNA solution, 25 μL of the PCR reaction solution, 4 μL of primers. The reaction proceeded as follows: 2 min (1 cycle) at 98 °C, 15 s at 98 °C, 15 s at 56 °C, 30 min (12 cycles) at 72 °C, 5 min (1 cycle) at 72 °C, and for ever at 4 °C.

To generate a library pool, the concentration of the library was quantified using Qubit fluorescence quantitative analyzer (Life Tech, Invitrogen, USA), with the following criteria: blank control <0.6 ng/μL, negative and positive controls >2 ng/μL, and clinical samples >2 ng/μL. Quality control (QC) of sequencing met the following criteria: DNB concentration ≥8 ng/μL and Lane's QC, including the average value of original data volume ≥10 M, Q30 ≥ 85 %, and total quantity ≥400 M. QC of information analysis met the following criteria: effective data volume ≥3.5 M, GC mean (38 %, 42 %), Q30 ≥ 85 %, original data volume ≥5.2 M, comparison rate (70,100), repetition rate (0,5), abnormal chromosome number (0,3), fetal concentration (3.5,50). During the experimental process, the temperature and humidity of experimental areas were controlled at 19–25 °C and 20–80 %, respectively. Sequencing results were compared with the reference genomes (hg19, NCBI build 36), and z-scores were calculated for each chromosome.The results were interpreted using the software Halos-NIFTY (BGI-Shenzhen, Shenzhen, China). Z ≥ 3 indicated a high risk of aneuploidy, z < 3 indicated low risk of aneuploidy, 1.96< z < 4 indicated that the z value of the target chromosome was within the gray area and needed to be reconstructed library. The above steps were performed according to the BGI instructions.

2.3.2. Semiconductor sequencing method

The cffDNA extraction, library construction, library quantification, pooling, and QC were implemented following the rules of the detection kit (reagents were obtained from CapitalBio Genomics, Beijing, China) [Product code: S10020/S10010, Registration permit No. 20170019/20,170,021]. Semiconductor sequencing was implemented applying a Jingxin BioelectronSeq 4000 gene sequencer (CFDA registration permit No. 20203400708), and for DNA sequencing, the average length of the fragments used for library construction was around 135–145 bp and the sequencing read length was ~200 bp. Sequencing results were compared with the reference

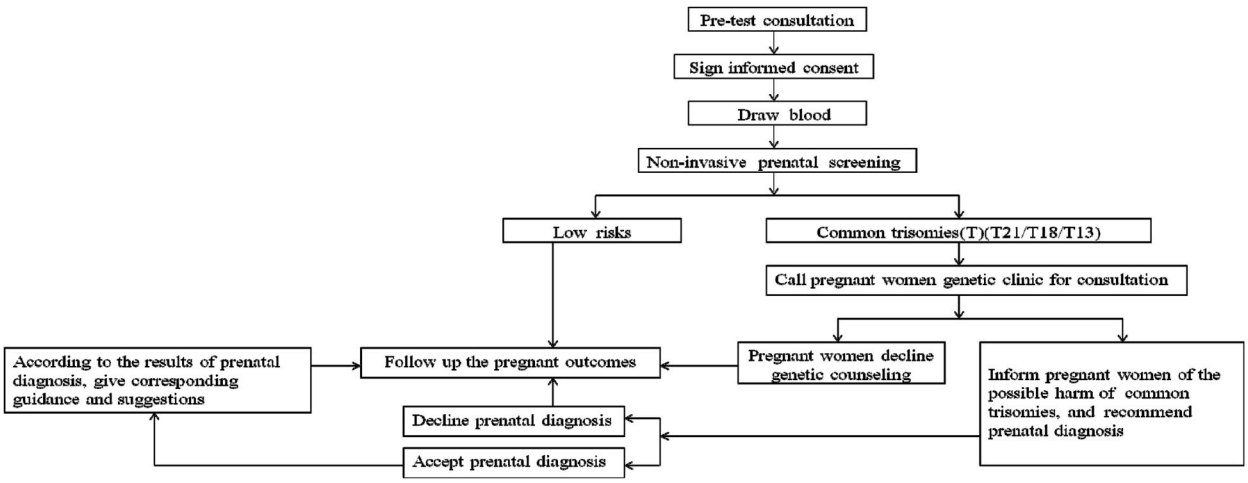

Fig. 1. The workflow of NIPT for detecting of common trisomies results. NIPT: non-invasive prenatal testing, T:trisomy.

genomes (hg19, NCBI build 36), and z-scores were calculated for each chromosome. $Z \leq 1.96$ indicated low risk of aneuploidy, $1.96 < z \leq 3$ indicated that the z value of the target chromosome was within the gray area. If the fetal DNA concentration was ≥4 %, it was considered negative. If the fetal concentration was <4 %, it was recommended to take blood samples again and retest to reduce the occurrence of false negative cases. If this was still the case after retesting, it was recommended to combine clinical information for further examination These steps were performed according to the manufacturer's instructions (CapitalBio Genomics, Product code: S30030). For details, please refer to the methods section (cffDNA preparation and sequencing, Bioinformatic analysis) reported by Wang et al. [14].

2.3.3. Chromosome karyotyping and/or chromosomal microarray analysis (CMA)

Pregnant women with common trisomies were immediately informed by telephone, received genetic counseling, and invasive prenatal diagnostic tests were recommended. Fetal chromosome karyotyping was carried by amniocentesis or cordocentesis and CMA was performed using a CytoScan 750 K array (Affymetrix, Santa Clara, CA, USA). The study protocol for the detection of NIPT trisomies is shown in Fig. 1.

2.4. Data collection and statistical methods

Basic clinical information about the pregnant women (for example, maternal age, gestational age, pregnancy mode, and fetuses number), the NIPT method and results, and pregnancy outcomes (received from postnatal questionnaires and/or telephonic follow-ups) were obtained from the Changsha Health and People's Livelihood Project System, prenatal care, and child health care systems. According to the Children's Health Manual formulated by China in 2009, newborns were required to be examined one week and 30 days after birth; thus, all live births underwent a detailed pediatric physical examination. All obstetric and pediatric electronic medical records and follow-up information in the prenatal care and child healthcare systems were completed by professional clinicians. Follow-up information, focused on health and livelihood systems, was received through online questionnaires completed by pregnant women and via telephone interviews held by professional medical personnel. The NIPT results were obtained from the local laboratory, BGI Changsha/Wuhan/Shenzhen, and CapitalBio Genomics (China). Pregnancy outcomes were followed up between 42 days and 3 months postpartum. The number of cases with true positives (TP), false positives (FP), true negatives (TN), and false negatives (FN) were collected and calculated. The positivity rate (PR), positive predictive value (PPV), sensitivity, and specificity were calculated (equations (1)–(4)).

$$PR = \text{number of positive NIPT} / \text{number of total NIPT} \times 100\% \quad [1]$$

$$PPV = TP / (TP + FP) \times 100\% \quad [2]$$

$$\text{Sensitivity} = TP / (TP + FN) \times 100\% \quad [3]$$

$$\text{Specificity} = TN / (TN + FP) \times 100\% \quad [4]$$

R software [R 3.6.1 GUI 1.70 EI Capitan build (7684)] was used to statistically analyze data. All data were presented as numbers or percentages. Confidence intervals (CI) of 95 % were calculated. The chi-squared test and *t*-test were performed to evaluate statistical significance, and a p-value of <0.05 was considered statistically significant.

3. Results

3.1. Annual uptake and implementation of NIPT

In April 2018, NIPT had been introduced as part of a free-livelihood project to screen fetuses for common trisomies in Changsha, Hunan, China. The specific implementations are listed in Table 2. As the NIPT uptake increased during 2018–2021, the incidence of birth defects decreased (Fig. S2).

Table 2
Analysis on the number of pregnant women, perinatal children, free NIPT coverage and the incidence of birth defects in Changsha. NIPT: non-invasive prenatal testing.

Time	Puerpera	NIPT	Positive T21	Positive T18	Positive T18	NIPT coverage rate	Perinatal baby	Liveborn baby	Birth defect	Incidence of perinatal defects
(year)	(n)	(n)	(n/‰)	(n/‰)	(n/‰)	(%)	(n)	(n)	(n)	(1/10,000)
2018	113002	61981	115/1.86	43/0.69	25/0.40	54.85	115129	114314	2322	201.69
2019	106955	86758	146/1.68	50/0.58	32/0.37	81.12	109106	108370	1914	175.43
2020	88716	67566	103/1.52	40/0.59	36/0.53	76.16	90401	89823	1591	175.99
2021	80505	66606	93/1.40	27/0.41	36/0.54	82.74	82263	81768	1440	175.05
Total	389178	282911	457/1.62	160/0.57	129/0.46	294.87	396899	394275	7267	183.09

3.2. Overall NIPT screen-positive and -negative results

Throughout the study, 282,911 pregnant women participated in the free NIPT in Changsha, which screened 746 cases of common trisomies, including 457 with T21, 160 with T18, and 129 with T13. The total screen positive rate was 0.26 % (746/282911). The overall PPVs were 86.81 %, 56.81 %, and 18.18 %, for T21, T18, and T13, respectively. The PPVs with common trisomies were significantly different between pregnant women older than 35 (85.53 %,136/159) and pregnant women aged 35 or younger (58.90 %, 311/528) ($\chi 2 = 125.02$, P = 2.20e-16). If cases were identified as positive after NIPT screening, further prenatal diagnostic tests were recommended. In total, 8.31 % (62/746) of the screen-positive cases were not diagnosed further using alternative diagnostic tests, and 52 cases of directly induced abortion, including 37 cases with fetal ultrasound anomalies, and only one aborted fetus that underwent karyotyping as 47, XN, +18. Fourteen special screen-positive cases were identified. In total, 253,622 follow-up results were obtained. The overall effective follow-up rate of pregnancy outcomes was 89.65 %, 99.46 % for screen-positive cases, and 89.62 % for screen-negative cases. The z-score distribution of screen-positive cases is shown in Fig. 2, and the z values of T21, T18 and T13 were approximately concentrated at 15 [Fig. 2(A)], 4 [Fig. 2(B)] and 5 [Fig. 2(C)], respectively. Some cases of spontaneous abortion and stillbirth also occurred in screen-negative cases, and pregnant women chose termination of pregnancy (TOP) for health reasons (gestational hypertension, eclampsia, and tumors) or because of fetal anomalies, such as the heart, kidney, cleft lip, and palate. The common trisomies screened by NIPT and their information regarding clinical follow-ups are shown in Tables 3 and 4.

3.3. Summary of false negative NIPT results

There were seven cases with singleton and spontaneous pregnancy of FN NIPT results, including five with T21 and two with T18. After rechecking the FN samples and retesting the NIPT results, the results remained negative. The clinical outcomes and results from further diagnostic tests are presented in Table 5.

3.4. Cases of NIPT common trisomies with ultrasound anomalies

Sixteen T21 pregnant patients, who were screened using NIPT, had ultrasound anomalies. These patients directly chose TOP without invasive prenatal diagnostic tests. Twelve T18 pregnant women had ultrasound anomalies, ten of whom directly chose TOP, whereas the remaining two underwent invasive prenatal diagnostic tests to identify the cause (one woman carrying a fetus with multiple malformations, and another carrying a fetus with cerebellar and spinal deformities). Karyotyping confirmed the presence of the 47, XN,+18, and the women subsequently chose TOP. Nine T13 cases were accompanied by ultrasound anomalies, including one fetus with multiple malformations resulting in spontaneous abortion, and the remaining women with direct TOP. These specific cases are illustrated in Fig. 3.

3.5. Special cases

In this research, fourteen unexpected special cases are shown in Figs. 4 and 5. Fig. 4 illustrated the results of NIPT screening for T21 in various cases. Cases 1, 2, 3, 6, 7, and 8 exhibited low z values for T21, with subsequent amniotic fluid diagnosis revealing a low proportion of mosaicism. Case 4 and 5 involved twins, with NIPT showing a high z-value for T21. The amniotic fluid diagnosis

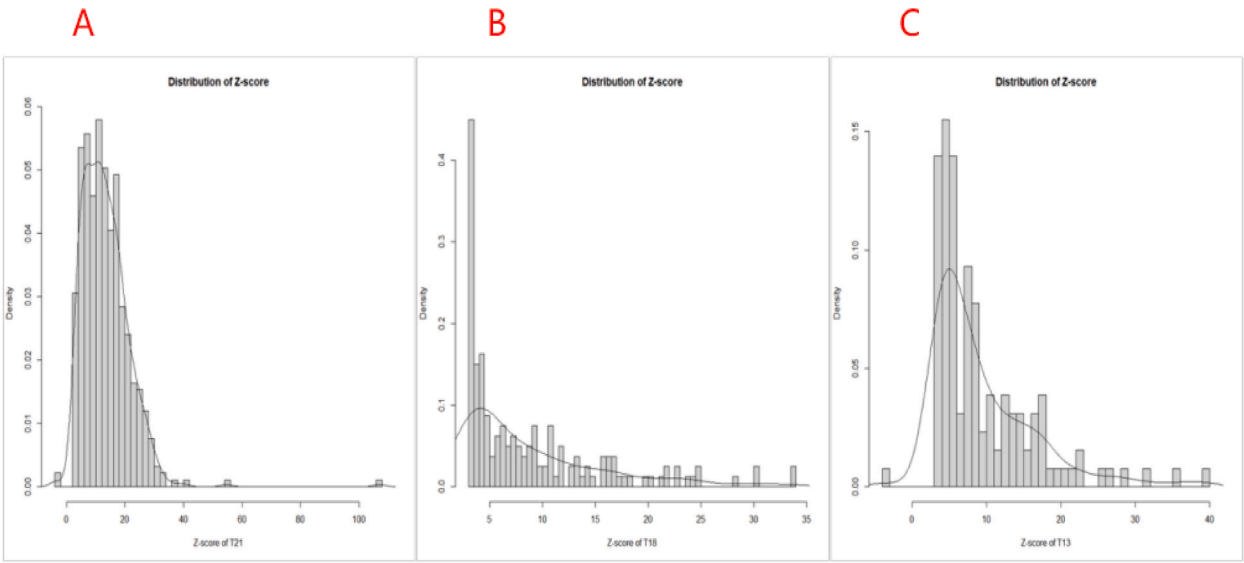

Fig. 2. The distribution of z-score of T21, T18 and T13. T:trisomy.

Table 3

Analysis of NIPT for screening T21, T18 and T13 with massively parallel sequencing at different maternal ages. NIPT:non-invasive prenatal testing, T:trisomy, PR:positive rate, CMA:chromosomal microarray, PPV:positive predictive value, NP:not participating, FN:false negative, UA:ultrasound anomalies, TOP: termination of pregnancy, IA:induced abortion, OA:other anomalies, CI:confidence intervals. The one asterisk (*) represents one fetus in twins.

Aberration	Maternal age	Pregnant	PR	Karyotyping/CMA(n)			FN	PPV	UA	TOP/abortion(n)				Livebirth(n)		Loss to	Sensitivity	Specificity
type(n)	(years)	women (n)	(‰)	Accor-dant	Discor-dant	NP	(n)	(%)	(n)	Aneuploidy	UA	IA	OA	Normal	Abnormal	follow-up(n)	(% (95%CI))	(% (95%CI))
T21 (418)	< 20	2	0.01	1	1	0	0	50.00	0	1	0	0	0	1	0	0	100.000 (2.500–100.000)	100.000 (99.998–100.000)
	>20-25	34	0.13	28	6	0	1	82.35	0	28	0	0	0	6	0	0	96.552 (82.236–99.913)	99.997 (99.995–99.999)
	>25-30	122	0.46	89	28	5	2	76.07	3	86	3	2	0	28	2	1	97.802 (92.285–99.733)	99.988 (99.981–99.991)
	>30-35	136	0.52	99*	19*	9	1	83.90	7	98*	7	2	1	28*	1	0	99.000 (94.554–99.975)	99.992 (99.979–99.989)
	>35-40	87	0.33	79**	7**	3	1	91.86	2	76**	2	0	0	7**	3	1	98.750 (93.231–99.968)	99.997 (99.994–99.999)
	>40	37	0.14	33	1	3	0	97.06	1	33	1	2	0	1	0	0	100.000 (89.424–100.000)	100.000 (99.996–100.000)
Total		418	1.58	329	62	20	5	84.14	13	322	13	6	1	71	6	2	98.503 (96.541–99.512)	99.974 (99.969–99.982)
T18 (149)	>20-25	22	0.08	7	14	1	0	33.33	1	7	1	0	1	12	1	0	100.000 (59.038–100.000)	99.994 (99.989–99.996)
	>25-30	41	0.16	12	23	6	1	34.29	3	12	1	1	0	25	2	0	92.308 (63.970–99.805)	99.991 (99.983–99.992)
	>30-35	57	0.22	26	29	2	0	47.27	2	25	2	1	0	28	1	0	100.000 (86.773–100.000)	99.988 (99.981–99.991)
	>35-40	23	0.09	9	8	6	1	52.94	5	9	5	1	1	7	0	0	90.000 (55.498–99.747)	99.997 (99.991–99.997)
	>40	6	0.02	4	1	1	0	80.00	1	4	1	0	0	1	0	0	100.000 (39.764–100.000)	100.000 (99.997–100.000)
Total		149	0.56	58	75	16	2	43.61	12	57	10	3	2	73	4	0	96.667 (88.472–99.594)	99.968 (99.961–99.976)
T13 (121)	>20-25	18	0.07	1	15	2	0	6.25	1	1	1	1	0	15	0	0	100.000 (2.500–100.000)	99.994 (99.988–99.996)
	>25-30	44	0.17	6	38	0	0	13.64	0	6	0	0	1	36	1	0	100.000 (54.074–100.000)	99.984 (99.978–99.989)
	>30-35	48	0.18	7	35	6	0	16.67	2	7	2	2	0	36	1	0	100.000 (59.038–100.000)	99.985 (99.976–99.988)
	>35-40	9	0.03	3	3	3	0	50.00	3	2	3	0	0	3	0	1	100.000 (29.240–100.000)	99.999 (99.994–99.999)
	>40	2	0.01	1	1	0	0	50.00	0	1	0	0	0	1	0	0	100.000 (2.500–100.000)	100.000 (99.998–100.000)
Total		121	0.46	18	92	11	0	16.36	6	17	6	3	1	91	2	1	100.000 (81.470–100.000)	99.961 (99.952–99.969)

Table 4
Analysis of NIPT for screening T21, T18 and T13 with semiconductor sequencing. NIPT:non-invasive prenatal testing, T:trisomy, CMA:chromosomal microarray, TOP:termination of pregnancy, PPV:positive predictive value, CI: confidence intervals.

Aberration type (n)	Karyotyping/CMA(n)			TOP/ abortion (n)	pregnancy outcome(n)	Loss to follow-up (n)	PPV (%)	Sensitivity (% (95%CI))	Specificity (% (95%CI))
	Accordant	Discordant	Not performed						
T21 (39)	34	4	1	34	5 normal livebirths	0	89.47	100.000 (89.718–100.000)	99.978 (99.936–99.991)
T18 (11)	7	3	1	8	3 normal livebirths	0	70.00	100.000 (59.038–100.000)	99.984 (99.944–99.994)
T13 (8)	1	4	3	3	4 normal livebirths	1	20.00	100.000 (2.500–100.000)	99.978 (99.921–99.985)

confirmed one fetus with T21 and one fetus with normal karyotyping, leading to the selective removal of the abnormal fetus. In Case 9, NIPT indicated a z-value of 10.13 for T21, which was not considered low. The final diagnosis from amniotic fluid analysis revealed a low proportion of mosaicism for T21 through CMA and FISH testing, and the child was followed up and everything was normal. Fig. 5 illustrated the results of NIPT screening for T18 and T13 in various cases. It could be seen that the z values of T18 of case1/2/3 were not high, but the final outcomes were one with normal karyotyping (case1), one with T18 (case2), and one with a microduplication from mother (case3). The z values of T13 of case1/2 were not high, but the final outcomes were one with a high proportion mosaicism of T13 (case1), and one with a microduplication from mother (case2).

3.6. Summary of the results

Investigating NIPT performance and the clinical significance had been verified. The biggest contribution of this study was the successful screening of T21/T18/T13 approximately 2 ‰ (457/289211), 1 ‰ (160/289211), 1 ‰ (129/289211), respectively. NIPT led to 8.98/10000 (356/396899) fewer live-born cases with T21, 1.64/10000 (65/396899) cases with T18, and 0.05/10000 (20/396899) cases with T13. These detailed information is shown in Table 2.

4. Discussion

In this research, correlation analysis indicated that after the introduction of the NIPT as a public welfare project (funded by the Changsha government in Hunan Province, China, NIPT uptake showed an upward trend and significantly led to significantly fewer aneuploid live-births. Concurrently, the total incidence of common trisomies screened by NIPT was 0.26 % (746/282911), and it was lower than the similar researches reported [5,9,17]. PPV refers to the proportion of true positive cases screened by NIPT. It is a successful test indicator for genetic counseling and may affect a pregnant woman's choice of NIPT and her subsequent decision after a positive NIPT result. Notably, the PPVs were similar to other reported researches [10,18–21]. Moreover, there were significant differences between pregnant women older than 35 (85.53 %) and those aged 35 or younger (58.90 %) ($\chi 2 = 125.02$, $P = 2.20e\text{-}16$). PPV increases as maternal age increases because the incidence rate of fetal chromosomal aneuploidy, which occurs during mitosis or meiosis, increases as maternal age increases. For older women, NIPT can reduce unnecessary prenatal diagnoses. In our study, there was a correlation between PPV and the z-score of positive samples, which is consistent with previous studies [22,23]. The decrease in PPV was not only related to the sensitivity of NIPT but also to the decrease in the incidence rates of T21, T18, and T13.

For NIPT, there are inevitably FP and FN cases. In the present study, 240 FP cases were identified, with an FPR of 0.09 %, and some screen-positive cases directly chose TOP due to ultrasonic anomalies, medical and personal reasons, and FP, indicating the possibility of unnecessary induced abortions. The main cause of inconsistency in karyotyping results is that circulating cffDNA originates from placental trophoblast cells and a few fetal cells, which were not representative of the fetus [16,24]. Additionally, assisted reproductive technology [25] and data analysis and annotation [26] are possible reasons for FPs. The high incidence of FP could have relations with the "trisomy rescue mechanism" in confined placental mosaicism (CPM), embryogenesis, maternal chromosomal anomalies, maternal cell contamination, maternal tumors, maternal solid organ transfer, fetal genetic anomalies, vanishing twin, and other factors that may lead to uniparental disomy [5,27,28].

Prenatal diagnosis is the gold standard for chromosomal diseases. Some pregnant women believe that if the z-score is low when aneuploidy is detected using NIPT, further examination is not required. However, in the present study, some special cases with lower z-scores were diagnosed as positive. Therefore, clinicians should recommend prenatal diagnostic test screening for pregnant women. In our study, some screen-positive cases did not undergo diagnostic tests, and in cases of anomalies such as fetal death, abortion, nuchal translucency (NT) thickening, cardiac dysplasia, umbilical hernia, and other abnormal ultrasonic results, the fetus was highly suspected to be T21, T18, and T13. Moreover, the prenatal diagnostic test rate was 92.09 % (687/746), indicating that the pregnant women had a high compliance rate with the NIPT guidelines [12]. This rate may be affected by the improvement in social and economic conditions, extensive publicity and education of medical personnel on birth defect knowledge and screening programs, and improvements in maternal education and cognitive levels. For example, some countries only provide women with high-risk pregnancies the NIPT. In contrast, in European, only Belgium and Netherlands provide all pregnant women access to the NIPT, with Belgium having a NIPT detection rate exceeding 75 % [19,29].

Table 5

Analysis of NIPT for false nagative results with massively parallel sequencing. NIPT:non-invasive prenatal testing, MA: maternal ages, GA:gestational ages, BMI:body mass index, CMA:chromosomal microarray, dup:duplication, Mb:megabases, TOP:termination of pregnancy.

Case	MA	GA	BMI	cff DNA concentration	Z-score of NIPT			The results of serum biochemistry screening	Other related abnormal findings	Diagnostic test (amniotic fluid)	Further investigation results	Pregnancy outcome
	(years)	(weeks $^{+}$ $_{days}$)	(kg/ m^2)	(%)	T21	T18	T13			(Yes or No)		
C.1	26	16^{+2}	19.56	14.25	−0.46	−0.55	−1.58	Low risk	Nill	47,XN,+21	WBC sequencing, failed due to poor sample quality. placental tissue unavailable.	Live birth
C.2	32	16^{+1}	20.82	13.87	−0.30	−0.23	0.64	Low risk	Nill	47,XN,t (7; 8) (p15.3; q24.3)pat,+21	WBC sequencing, normal, placental tissue available (result unavailable).	TOP
C.3	21	20^{+5}	24.36	12.53	−0.73	1.63	−0.16	Low risk	Premature amniotic fluid rupture cesarean section	No	47,XY,+21 [15]/46, XY [6] (newborn)	Live birth
C.4	30	16^{+1}	20.78	13.33	−1.35	−1.08	−0.62	Low risk	NT thickening (3.0 mm), amniotic fluid increased (85 mm), single umbilical artery	47,XN,+18		TOP
C.5	36	16^{+5}	25.85	8.25	1.35	1.49	0.94	Low risk	Fetal endocardial cushion defect (complete type)	FISH: 47,XN,+21 [75]/ 48,XN,+21,+21 [3]/ 46, XN [16]	WBC sequencing, normal	Stillbirth
C.6	29	16^{+5}	19.1	7.99	−2.77	−1.08	0.23	Low risk	Nill	No	47,XY,+21 (newborn)	Live birth
C.7	38	16^{+4}	25.24	5.47	−0.14	0.70	1.65	HCG-MOM 0.27; AFP-MOM 0.2; uE3-MOM0.27; risk of T21/T181: 1/51, 1/5	Fetal interventricular septum defect, single umbilical artery	karyotyping: 47,XN,+18, CMA: arr [hg19] 18p11.32q23 (136,227–78,013,728)*3, dup77.9 Mb	WBC sequencing, normal	TOP

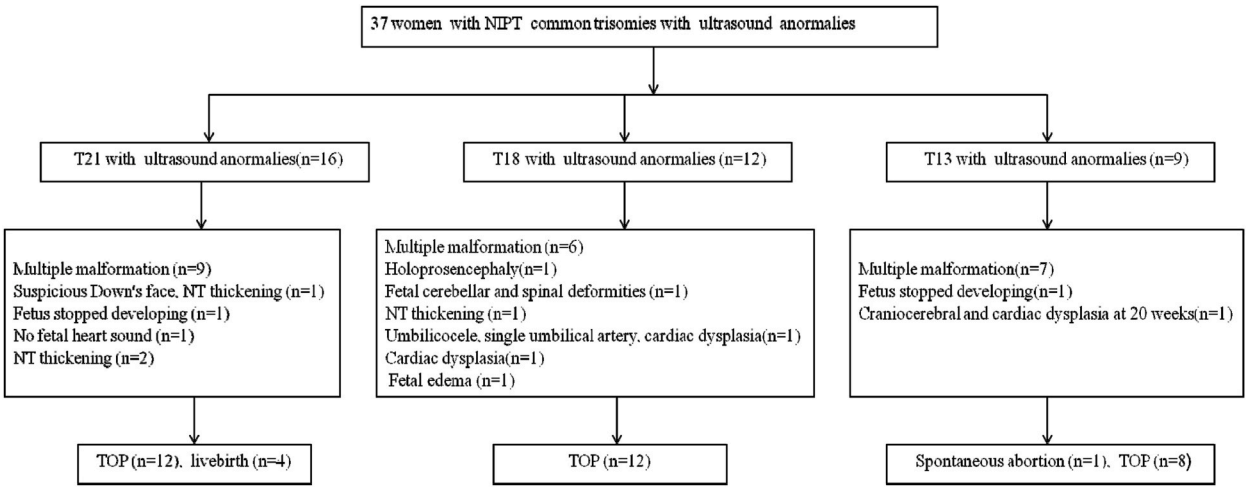

Fig. 3. NIPT common trisomies with ultrasound anomalies. NIPT: non-invasive prenatal testing, T:trisomy, NT: nuchal translucency, TOP: termination of pregnancy.

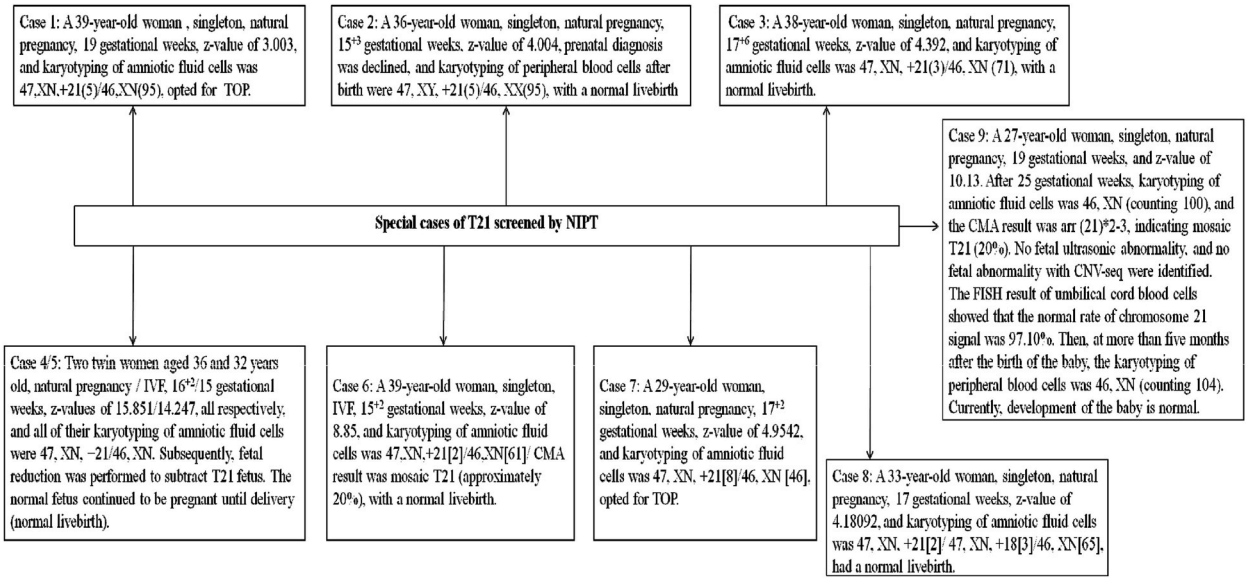

Fig. 4. Special cases of T21 screened by NIPT. NIPT: non-invasive prenatal testing, T:trisomy, IVF: in vitro fertilization, CMA: chromosomal microarray analysis, FISH: fluorescence in situ hybridization, CNV-seq: copy number variation sequencing, TOP: termination of pregnancy.

With the implementation of China's two- and three-child policies, the global incidence rate of infertility at 15–20 %, the increasing age of pregnant women, and the advancement of assisted reproduction techniques, the probability of having twins has increased [30]. Wei et al. demonstrated that the probability of chromosomal anomalies in twins were higher than that in singleton [31]. However, in the present study, only seven screen-positive cases of twin pregnancies were observed in our study, indicating a low-efficiency screening for twin pregnancy anomalies. According to the American Society of Medical Genetics and Genomics and other research reports, NIPT can be used as an effective screening program for aneuploidy in different age groups and twin pregnancies [32]. For FP cases there may be caused by vanishing twins, the vanishing fetus could still release DNA fragments to the maternal plasma for 7–8 weeks (<12–14 weeks generally) [33,34]. Therefore, we recommend that pregnant women wait eight weeks after the loss of one of the twins before undergoing NIPT (according to the protocol). Moreover, five live births with aneuploidies were documented in this study, of which one twin did not undergo diagnostic tests and the other (male) had non-mosaic T21. The other four live births were diagnosed with low-proportion mosaic T21. Their growth and intellectual development were documented as normal. Notably, the fifth case of 47, XN, +21 [2]/47, XN, +18 [3]/46, XN [65] reported in the literature was also identified. Autosomal trisomic mosaicism is rare in newborns. The possible mechanisms for its occurrence include two independent nondisjunction events in normal zygotes, two independent anaphase lag events in non-mosaic double aneuploidies zygotes, and independent trisomic rescue of different trisomies in

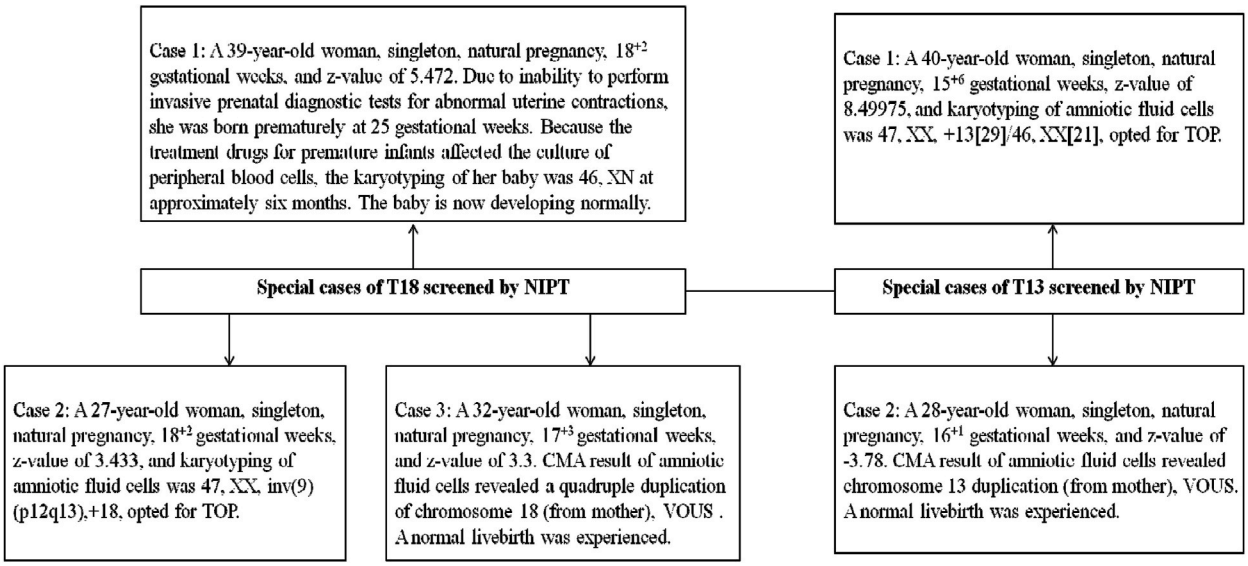

Fig. 5. Special cases of T18 and T13 screened by NIPT. NIPT: noninvasive prenatal testing, CMA:chromosomal microarray analysis, VOUS: variants of uncertain significance, TOP: termination of pregnancy.

different cell lines [15]. If prenatal diagnostic results revealed that one fetus of the twin was a chromosomal anomaly, the pregnant woman could opt for selective fetal reduction to prevent the birth of the fetus with the anomaly (two twins in this study were successfully reduced). In our study, there were few positive cases of T18 and T13, which may be due to the initiation of a self-rescue mechanism during meiosis to form abnormally fertilized eggs and CPM [35,36]. During the process of embryonic development, chromosomal variation might occur during the differentiation of different germ layers; therefore, karyotyping of the villus might be inconsistent with that of the amniotic fluid and cord blood. Generally, the clinical symptoms of mosaic trisomy depend on the proportion and location of occurrence. The earlier the occurrence, the higher the ratio of abnormal cells and the greater the impact of clinical consequences. However, because the mosaic location could not be accurately detected and the proportion of trisomy cells in important organs, such as the brain, which could not be obtained, it was necessary to exercise caution when judging the severity and prognosis of the fetus' phenotype based on the proportion of trisomy cells. This needed to be comprehensively judged in combination with obstetric examinations, such as ultrasound [35]. Moreover, the clinical phenotype of mosaic trisomy did not always correlate with the mosaic proportion, making it challenging to predict accurately. Prenatal detection was difficult to identify them and only possible through ultrasound to check for structural abnormalities. Functional abnormalities could not be assessed prenatally, causing confusion and anxiety for pregnant women and their families. This study followed eight cases of mosaic trisomy pregnancies to monitor the outcomes and development of the babies, which had certain clinical reference significance.

In the present research, in the FN cases, the cffDNA concentrations of cases 5, 6, and 7 were slightly lower than those of the other cases. However, FNs still occurred even in cases 1, 2, 3, and 4, displaying high cffDNA concentrations. Therefore, other unknown reasons are likely to be causing FNs. The serological screening of the other cases revealed a low risk; only case 7 was at high risk, and ultrasound anomalies were identified. The diagnostic results were T18, and the pregnancy women chose TOP. We investigated the causes of FN using various methods, such as sample mixed screening, backup sample retesting, maternal background screening, and placental mosaicism screening. However, we could not determine the underlying cause of the FN results for cases 1, 2, 5, and 6. According to previous studies, low DNA concentration, fetal cell and chromosome abnormalities, mosaicism, maternal CNVs, normal placental chromosomes, and statistical fluctuations in the detection z-score may be possible causes of FNs [37–39]. Therefore, screen-negative pregnant women still require regular ultrasound tracking and genetic counseling. Amniotic fluid or umbilical blood puncture is recommended for the diagnosis of ultrasound anomalies.

This research had several strengths. This public welfare project was supported by the Changsha Municipal Government. The uptake of NIPT was exceptionally large, and abnormal data resources were considerably rich. Through the efforts of many parties, the follow-up of pregnancy outcomes reached a high record. Moreover, valuable clinical information was accumulated and collected owing to the immense cooperation and support received from fetal parents and medical staff. Nevertheless, this study had some limitations. Our hospital housed the management unit of the Changsha Free NIPT People's Livelihood Project and was responsible for collecting blood samples and transferring them to our local laboratory and/or BGI/CapitalBio Genomics for testing. However, it was difficult to obtain the placenta, and it was not possible to perform karyotyping or further verification analysis of the placenta. It was challenging to analyze the true underlying cause of the FP and FN NIPT results and determine their correlation with the clinical occurrence. Furthermore, not all the positive cases were involved in the diagnostic tests. Many fetuses with fetal arrest, spontaneous abortion, ultrasound anomalies, and direct-induced abortion in this study did not undergo karyotyping, which affected the performance of the NIPT.

This research demonstrated that NIPT could effectively detect fetal common trisomies (T21, T18, and T13) and that the main

influencing factors were physical, chemical, and biological factors, and maternal age. Physical factors was referring tomicrowave, ultrasound, radiation, chemical factors including sulfur dioxide, hair dyes, paint, and heavy metals, and biological factors including parasites, bacteria, viruses, mycoplasma, and so on. The main clinical manifestations of chromosomal diseases are common and include multiple congenital malformations, mental and growth retardation, fetal abortion, and stillbirth. The data and clinical information on the NIPT in this study will greatly contribute to prevent birth defects. The innovation of this study is reflected in the specific visualization of abnormal cases.

5. Conclusion

This research aimed to clarify the clinical results of fetuses affected by common trisomies and provide valuable clinical information to assist in the decision-making process of pregnant women and their husbands and clinical genetic counselors. According to the recommendations of domestic and international guidelines, we implemented a free NIPT as a first-tier screening test for common fetal aneuploidy (chromosomes 21, 18, and 13) in a large clinical sample of general obstetrical population, which was representative of women seen in contemporary clinical practice in China. Our data confirmed that NIPT had high PPV, sensitivity, and specificity in screening for T21, T18, and T13 and, therefore, greatly contributed to the prevention and control of birth defects. The biggest contribution of this study was the successful screening of T21/T18/T13 approximately 2 ‰, 1 ‰, 1 ‰, respectively. And NIPT led to 8.98/10000 (356/396899) fewer live-born cases with T21, 1.64/10000 (65/396899) cases with T18, and 0.05/10000 (20/396899) cases with T13. Pregnant women should be fully informed, before and after clinical genetic counseling, as to the strengths and limitations of NIPT and interpretation the results. Invasive prenatal testing is recommended in women with positive screening results. In future studies, for the cases with FP and FN NIPT results and special cases, we should obtain the placenta and other tissues to thoroughly analyze the underlying causes.

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Ethics statement

All participants/patients (or their proxies/legal guardians) provided informed consent to participate in the study. This study was approved by the Ethics Committee of Changsha Maternity and Child Health Hospital (November 2, 2022, 20,221,102, No. EC-20221102-08).

Data availability statement

The data associated with the present study has not been deposited into a publicly available repository, and because the special information about pregnant women needs to be kept confidential. The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors or the first author.

CRediT authorship contribution statement

Yu-shan Lu: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ying-ying Chen:** Validation, Formal analysis. **Si-yi Ding:** Resources. **Li Zeng:** Resources. **Liang-cheng Shi:** Resources. **Yu-jiao Li:** Resources. **Jing-jing Zhang:** Resources. **Jin Fu:** Resources. **Shi-hao Zhou:** Writing – review & editing, Writing – original draft, Conceptualization. **Jun He:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33437.

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