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Performance of cell free DNA as a screening tool based on the results of first trimester screening

Mahtab Motevasselian[1], Mohammad Amin Omrani[2], Soraya Saleh Gargari[3*], Sarang Younesi[4], Mohammad Mahdi Taheri Amin[4], Pourandokht Saadati[4], Soudabeh Jamali[4], Mohammad-Hossein Modarresi[5], Shahram Savad[5], Majid Rahmani[4], Saloomeh Amidi[4], Saeed Delshad[4], Fariba Navidpour[4], Samira Chagheri[4], Yalda Mohammadi[4], Sheyda Khalilian[6], Solat Eslami[7,8] and Soudeh Ghafouri-Fard[6*]

Abstract

The advent of non-invasive prenatal testing (NIPT) in the screening of fetal abnormalities has optimized prenatal care and decreased the rate of invasive diagnostic tests. In this retrospective descriptive study, we began with 1874 singleton pregnancies. After exclusion of some cases, the study cohort ended up with 1674 cases. We analyzed the performance of NIPT based on the results of first trimester screening (FTS) using serum screening combined with NT. The cases were also compared to diagnostic testing/pregnancy outcomes. Notably, in the subgroup with FTS risk < 1000, NIPT was reported to be normal in all cases with no false negative results. In the risk group of 1/300-1/1000, NIPT could detect all trisomy 21 cases with one false positive result. Moreover, in the risk group of 1/11 – 1/300, NIPT could detect all cases of trisomy 21, 13 and 18 with low false positive rate. However, the false positive rate for sex chromosomal abnormalities was high. Taken together, the current study confirms the applicability of NIPT as a tool for detection of fetal trisomies with high sensitivity and specificity. Yet, the high rate of false positive results for sex chromosome abnormalities should be considered in the interpretation of the results.

Keywords Non-invasive prenatal testing, NIPT, Screening

*Correspondence:
Soraya Saleh Gargari
soraya_saleh2000@yahoo.co.uk
Soudeh Ghafouri-Fard
s.ghafourifard@sbmu.ac.ir
[1]Department of Obstetrics and Gynecology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[2]Urology and Nephrology Research Center (UNRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]Men's Health and Reproductive Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[4]Department of Nilou Laboratory, Tehran, Iran
[5]Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[6]Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[7]Department of Medical Biotechnology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran
[8]Non-communicable Diseases Research Center, Alborz University of Medical Sciences, Karaj, Islamic Republic of Iran

Introduction

Non-invasive prenatal testing (NIPT), alternatively named as cell-free DNA (cfDNA) testing is a method for detection of chromosomal abnormalities due to its high sensitivity and specificity [1–3]. In recent years, NIPT has been used as a screening test in the general obstetric population in many parts of the world [2, 4]. Congenital abnormalities such as trisomies 21, 13 and 18 have a great economic, social and cultural burden on families and society [5]. The aim of prenatal screening methods is to obtain correct information to optimize prenatal care for both mother and fetus [6]. Screening methods for these abnormalities are based on calculating the individual risk according to the age of the mother and measurement of nuchal translucency (NT) and/or maternal serum markers and/or other sonographic markers in the first trimester [7, 8].

Several factors might contribute to false positive or false negative results of the NIPT test, including the maternal copy number variations, maternal malignancies, confined placental mosaicism, vanishing twins, and human errors [9, 10]. NIPT has now been used as a screening test and has not completely substituted the diagnostic invasive methods such as chorionic villus sampling and amniocentesis [11]. However, it is an alternative to invasive tests in high-risk people after combined screening (>1:10) [12]. The application of NIPT in the clinical practice has been investigated by several groups [1, 2].

There is not a global consensus in the healthcare setting for the introduction of NIPT in the prenatal screening programs. Some offer NIPT only to high-risk pregnant women according to a previous increased-risk FCT result, advance maternal age or medical history that increases the risk of a trisomy [13]. However, ACOG recommends NIPT to all pregnant women after consultation and sonography of the first trimester, considering the available facilities [14], due to its high diagnostic sensitivity and specificity [3].

Based on these recommendations, we decided to analyze the results of the 1874 plasma NIPT data from low-risk pregnant women. We also assessed the demographic data available from samples referred for NIPT to the laboratory. Our study recommends the use of NIPT in maternal plasma as a screening test for trisomies 21, 18 and 13 in low-risk pregnancies.

Patients and methods

This retrospective descriptive study included 1874 singleton pregnancies assessed in Nilou Laboratory, Tehran, Iran during 2020–2022. All patients received genetic counseling during the process of deciding about aneuploidy screening. FTS/NIPT was performed as a part of routine clinical care. NIPT was performed after evaluation of gestational age based on the ultrasound data. Pregnancy outcomes were compared with the screening results using fetal karyotyping or neonate physical exam by pediatricians. The corresponding ethical committee confirmed the study protocol. All study participants signed informed consent forms.

Initial exclusion criteria were nuchal translucency (NT)≥3, history of abnormal pregnancies including children with trisomy 21 and intrauterine fetal death, abnormal ultrasound findings indicative of increased aneuploidy risk, any chromosomal abnormality in parents, maternal age≥35 years. After exclusion of some cases, the study cohort ended up with 1674 cases. Figure 1 demonstrates the details of patients' enrolment.

NIPT

Five mL of peripheral blood was used for NIPT. Blood samples were collected in the EDTA tubes. Double-centrifugation method was used for separation of plasma. For elimination of the remaining cells, samples were centrifuged twice at 1600 g for 10 min at 4 °C and 16,000 g for 10 min, respectively. The circulating DNA was extracted using QIAamp kit (QIAGEN, Hilden, Germany). Subsequent steps were performed according to the previous study [15]. Massively parallel sequencing was performed in Ion Torrent (Life Technology) genome analyzer. If the fetal fraction was below 3%, no result was reported.

First trimester screening (FTS)

FTS was based on the maternal serum levels of the pregnancy-associated plasma protein-A (PAPP-A) and free beta subunit of human chorionic gonadotropin (β-hCG) at 9–13^{+6} weeks gestation in addition to the ultrasound fetal NT at 11–13^{+6} gestation. Cases were classified to high and low risk groups based on the results of FTS with a cutoff risk of 1/300. FTS was performed using serum screening combined with NT. High risk group included those with FTS risk>1/300, but NT<3; and low risk group included those with FTS risk<1/300.

Diagnostic confirmation

Amniocentesis and fetal karyotyping was performed in cases with positive NIPT results. Cases were also followed up until labor and neonate physical exam was performed. Cases were classified as true positive/false positive or true negative/false negative based on the accordance of NIPT results with fetal karyotyping and neonate physical exam. Cases with missing follow-up data (diagnostics or birth outcomes) were excluded.

Statistical analysis

The sensitivity, specificity, positive likelihood ratio, and positive and negative predictive values of NIPT were computed in the whole cohort and within the risk

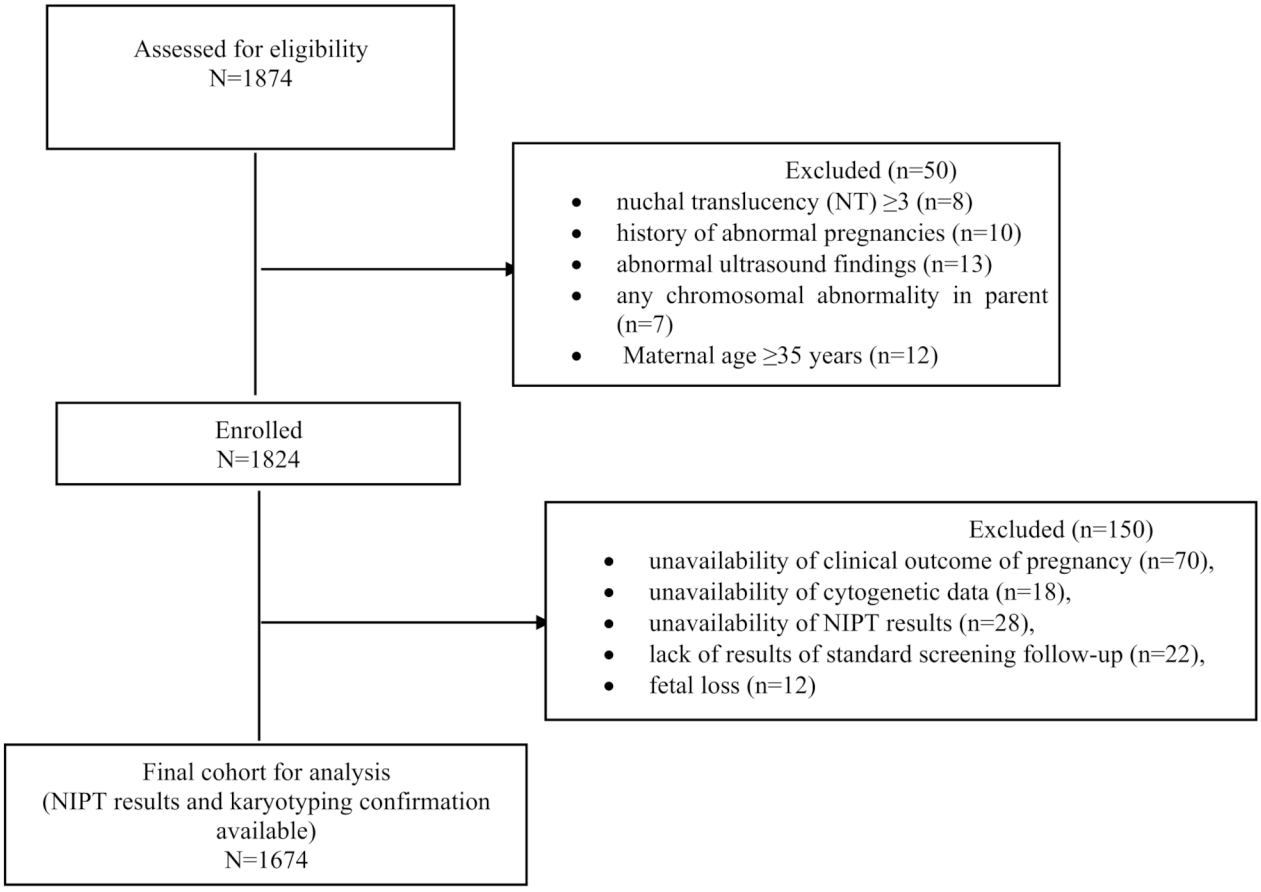

Fig. 1 Patients' enrollment flowchart

subgroups. When appropriate, exact (Cloppere-Pearson) 95% confidence intervals (CIs) were reported. Comparisons between low and high risk subgroups were performed using the Fisher's exact test.

Results

General data

Fifty cases among 1874 cases had at least one of the mentioned exclusion criteria. In addition, 150 cases were excluded from analysis because of the following reasons: unavailability of clinical outcome of pregnancy ($n=70$), unavailability of cytogenetic data ($n=18$), unavailability of NIPT results due to technical failure ($n=28$), lack of results of standard screening follow-up ($n=22$), and fetal loss ($n=12$). Consequently, a total of 1674 pregnancies were assessed.

FTS was performed using serum screening combined with NT. Cases were classified to high and low risk groups based on the results of FTS with a cutoff risk of 1/300. High risk group included those with FTS risk > 1/300, but NT < 3; and low risk group included those with FTS risk < 1/300.

Table 1 Demographic data of included cases

Parameters	Values
Maternal age (yr, mean ± SD)	28.4 ± 4.65
Body mass index (mean ± SD)	28.9 ± 6.45
Gestational age at time of testing	16w ± 8.6

Demographic characteristics

Demographic and pregnancy characteristics of the assessed cohort of pregnant women are shown in Table 1.

NIPT results

A total of 1543 cases were included in the low risk group (risk < 1/300). Among these cases, 1150 had risk < 1/1000. In this subgroup of pregnancies, NIPT was negative in all cases and postnatal results were normal. However, in the subgroup of pregnancies with risk of 1/300-1/1000 ($n=393$), four positive NIPT results for trisomy 21 were reported. Fetal karyotype following amniocentesis confirmed the presence of trisomy 21 in three cases out of four. Moreover, two cases of trisomy 13, two cases of trisomy 18, and 38 cases of sex chromosome abnormalities were reported in this subgroup all of them were shown to have normal karyotype after amniocentesis.

Table 2 Detailed information about subgroups

FTS Risk Group	Subgroup (Risk Score)	Number of Cases	NIPT Positive (T21)	NIPT False Positives (T21)	NIPT Positive (T18)	NIPT False Positives (T18)	NIPT Positive (T13)	NIPT False Positives (T13)	NIPT Positive (SCAs)	NIPT False Positives (SCAs)
Low Risk	risk < 1/1000	1150	0	0	0	0	0	0	0	0
	1/1000 < risk < 1/300	393	4 (1%)	1 (0.25%)	2 (0.5%)	2 (0.5%)	2 (0.5%)	2 (0.5%)	38 (9.6%)	38 (9.6%)
High Risk	1/300 < risk < 1/11	118	18 (15.2%)	2 (1.6%)	5 (4.2%)	3 (2.5%)	3 (2.5%)	1 (0.8%)	24 (20.3%)	18 (15.2%)
	1/10 < risk < 1/2	13	6 (46.1%)	0	0	0	0	0	0	0

Table 3 Performance of NIPT to screen trisomies 21, 18 and 13 and sex chromosomal abnormalities

Variable	T21	T18	T13	SCAs	T21/T18/T13/ SCAs
Sensitivity	25/25 100% (86.6–100)	2/2 100% (17–100)	2/2 100% (17–100)	6/6 100% (61–100)	35/35 100% (90–100)
Specificity	1646/1649 99.82% (99.5-99.95)	1667/1672 99.68% (99.2-99.86)	1669/1672 99.8% (99.4-99.95)	1612/1668 96.5% (95.5–97.3)	1639/1706 95.9% (94.8–96.7)
PPV	25/28 89% (72.8–96.3)	2/7 28% (5–64)	2/5 40% (7–77)	6/62 9.6% (4.5–19.5)	35/102 34% (25.8–43.9)
NPV	1649/1649 100% (99.76–100)	1672/1672 100% (99.7–100)	1672/1672 100% (99.7–100)	1668/1668 100% (99.7–100)	1639/1639 100% (99.7–100)
Likelihood ratio	525	315.4	525	29.07	24.46

SCAs, Sex chromosome abnormalities; NPV, negative predictive value; PPV, positive predictive value; T, trisomy

95% Confidence intervals are shown

In the high risk group (risk > 1/300 and NT < 3 mm), 13 cases had risk values between 1/2 and 1/10, while 118 cases had risk values between 1/11 and 1/300. A total of 6 and 18 cases were reported as NIPT positive for trisomy 21 in each subgroup, respectively. Amniocentesis confirmed the abnormality in 22 cases. Tow false positive cases were in the latter subgroup. Moreover, three and five cases were reported to have trisomy 13 and trisomy 18 in this subgroup, respectively. Amniocentesis and fetal karyotyping confirmed presence of trisomy 13 in two cases. Similarly, two cases of trisomy 18 were confirmed by amniocentesis. In addition, 24 cases of abnormal sex chromosomes were reported in NIPT. Amniocentesis and fetal karyotyping revealed the presence of 45X karyotype in four cases and 47XYY karyotype in two cases. Among false positive cases, a case of hypothyroidism and a case of hyperthyroidism were reported. Table 2 shows the detailed information.

In total, NIPT had 100% sensitivity for detection of trisomies and sex chromosomal abnormalities. However, positive predictive values (PPVs) for trisomies 21, 18 and 13 and sex chromosomal abnormalities were 89%, 28%, 40% and 9.6%, respectively (Table 3).

Finally, we compared performance of NIPT in different risk groups (Table 4). In total, PPV of NIPT was higher in high risk pregnancies compared with low risk pregnancies (P value < 0.0001). Such pattern was also observed for individual trisomies (P values = 0.002, < 0.0001 and < 0.001 for trisomies 21, 18, and 13, respectively.

Discussion

NIPT has an established situation in the screening of chromosomal abnormalities, with very low false positive rates in high-risk populations, thus reducing unnecessary worry and conduction of invasive prenatal procedures. However, it was not clear whether NIPT is useful for low risk pregnancies. In the current study, we analyzed NIPT performance in a group of Iranian pregnant women based on the results of FTS. It is worth mentioning that one of the major biases of using first trimester screening results as the separator is that the advanced maternal age will increase the priori risk for both FTS and NIPT, which is going to influence the test performance of both assays. To overcome this challenge, we excluded cases with maternal age ≥ 35 from the study. Additionally, SCAs may not be readily identified by neonatal exam. Therefore, all positive cases with SCAs had a karyotype. Finally, neonatal exam was used to determine concordance of the results of NIPT and fetal karyotyping for all of the false positive cases.

Notably, in the subgroup with FTS risk < 1000, NIPT was reported to be normal in all cases with no false negative results. In the risk group of 1/1000-1/300, NIPT could detect all trisomy 21 cases with one false positive result. Moreover, in the risk group of 1/300-1/11, NIPT could detect all cases of trisomy 21, 13 and 18 with low false positive rate. However, the false positive rate for sex chromosomal abnormalities was high. We also analyzed test performance within subgroups. We observed a higher prevalence of false positives in the low-risk subgroup (1/300-1/1000) compared to the very low-risk subgroup (< 1/1000). Similarly, within the high-risk groups,

Table 4 Comparison of cell-free DNA test performance for trisomies 21, 18, 13 and SCAs between women at low and high risks for aneuploidy[a]

Variable	High risk[a] (*n* = 131)	Low risk (*N* = 1543)	*P* value (High vs. low risk)
Trisomy 21 (*n* = 25)			
Sensitivity	22/22 100% (85–100)	3/3 100% (43.8–100)	0.38
Specificity	75/77 97.4% (91-99.5)	1497/1498 99.9% (99.6–100)	1.00
PPV	22/24 91.6% (74-98.5)	3/4 75% (30-98.7)	**0.002**
NPV	77/77 100% (95.1–100)	1498/1498 100% (99.7–100)	1.00
Prevalence[b]	16.79	0.19	
Likelihood ratio	38.5	1498	
Trisomy 18 (*n* = 2)			
Sensitivity	2/2 100 (17–100)	0/0 -	1.00
Specificity	75/80 96.1% (89.3–98.9)	1497/1499 99.8% (99.5–99.9)	1.00
PPV	2/7 40% (7.1–76.9)	0/2 0 (0–82)	**< 0.0001**
NPV	80/80 100% (95.1–100)	1499/1499 100% (99.7–100)	1
Prevalence	1.52	0%	
Likelihood ratio	26	-	
Trisomy 13 (*n* = 2)			
Sensitivity	2/2 100% (17–100)	0/0 -	0.4
Specificity	75/76 98.6% (92.9–99.9)	1497/1499 99.8% (99.5–99.9)	1.00
PPV	2/3 66.6% (11.8–98.3)	0/2 0 (0–82)	**< 0.001**
NPV	76/76 100% (95.1–100)	1499/1499 100% (99.7–100)	1.00
Prevalence	1.52		
Likelihood ratio	76		
SCAs (*n* = 6)			
Sensitivity	6/6 100% (61–100)	0/0 -	**0.002**
Specificity	75/93 80% (71–87)	1497/1535 97.5% (96.6–98.2)	1.00
PPV	6/24 25% (12–45)	0/38 0% (0-9.2)	**0.003**
NPV	93/93 100% (95.1–100)	1535/1535 100% (99.7–100)	1.00
Prevalence	4.58		
Likelihood ratio	5.16		
Trisomy 21,18, 13 & SCAs (*n* = 32)			
Sensitivity	32/32 100% (89.3–100)	3/3 100% (43.8–100)	**< 0.0001**
Specificity	75/99 75.7% (66.4–83.2)	1497/1540 97.2% (96.3–97.9)	1.00
PPV	32/56 57.1% (44.1–69.2)	3/46 6.5% (2.2–17.5)	**< 0.0001**
NPV	99/99 100% (95.1–100)	1540/1540 100% (99.7–100)	1.00
Prevalence	24.42	-	
Likelihood ratio	4.12	-	

SCAs, Sex chromosome abnormalities; NPV, negative predictive value; PPV, positive predictive value.; [a] Based on results of first trimester screening; [b] percent

the performance of NIPT was better in the very high-risk subgroup (1/2−1/10) with no false positives, compared to the high-risk subgroup (1/11−1/300) which had some false positive cases. These findings suggest that FTS results can help inform the likelihood of false positives in NIPT results. In our previous report of karyotype analysis of amniotic fluid cells in more than 15,000 cases, among 5131 cases of positive FTS results for trisomy 21, we found 315 cases of trisomy 21 and 118 cases of other chromosomal abnormalities, reaching a FPR of about 3% [16]. Thus, NIPT has a lower FPR compared with FTS in the same center.

In a retrospective study, Walter et al. have analyzed uptake of NIPT in three risk groups for trisomy 21 based on FTS (<1: 1000, 1:101–1:1000 and ≥1: 100, respectively) [17]. They reported a significant upsurge in the use of NIPT as part of FTS in all three-risk groups compared with those reported in the previous studies. Notably, the rate of invasive diagnostic test was lower in their cohort compared with the previous studies, which is explained by a significant decrease in the rate of invasive diagnostic test in the intermediate risk group. However, invasive diagnostic test rate in the high-risk group was reported to be stable over time. Cumulatively, they concluded that uptake of NIPT is becoming more common during recent years [17].

Another study in 831 samples has reported 100% sensitivity of NIPT in the detection of trisomies 21, 18 and 13. Moreover, specificity of this test has been reported to be more than 99% for each trisomy, suggesting this method as a method with greater accuracy and clinical utility compared to the conventional biochemical screening [18]. Similarly, prenatal testing with the use of NIPT has been shown to have considerably lower false positive rate and higher positive predictive value for detection of trisomies 21 and 18 compared with standard screening in a multicenter study [19]. A previous meta-analysis has also shown the high performance of NIPT performs as a screening tool for trisomy 21 in a general pregnant population [20]. Moreover, the false positive rate of NIPT has been lower than FTS [20]. Thus, this test can be used as an alternative or supplement to FTS, particularly in the group of patients with high risk pregnancies.

A prospective multicenter study to compare the performance of NIPT versus FTS for detection of trisomy 21 has shown that NIPT has higher sensitivity, a lower false positive rate, and higher PPV compared with FTS [21]. False positive rate and PPVs of NIPT have been 0.06% and 80.9% for detection of this trisomy [21]. Moreover, a nationwide implementation study on NIPT as a first-tier test in Netherlands has reported PPVs of 96%, 98% and 53% for trisomies 21, 18, and 13, respectively, which have been higher than expected [22]. The PPV values in the current study were comparable with the latter study, except for PPV of trisomy 18 which was lower in our study.

A meta-analysis of available literature has indicated that at a combined FPR of 0.13%, NIPT can detect >99%, 98% and 99% of cases of trisomy 21, 18 and 13, respectively. However, the number of reported SCA cases has been less than what is needed for precise valuation of performance of screening [1].

In the current study, the NPV was 100% for all mentioned trisomies in both high risk and low risk groups. Thus, NIPT can be recommended as a screening tool not only for high risk pregnancies, but also for low risk pregnancies. This suggestion is based on the observed higher accuracy and lower false positive rate of NIPT compared with FTS. In fact, the cumulative sensitivity and specificity values of NIPT for detection of chromosomal abnormalities were 100% and 95.9% in the current study. These values are higher than reported values for FTS [23–25].

The PPV of sex chromosome abnormalities screening by NIPT wan only 9.6%, much lower than the value reported in the literature. A former meta-analysis reported the pooled PPV of NIPT for sex chromosomal abnormalities to be 49.4% [26]. Another study reported a similar PPV and emphasized that this value was higher for sex chromosome abnormalities with a supernumerary Y chromosome and lower for monosomy X [27]. Similarly, the detection efficacy of NIPT for monosomy X was reported to be 25% in another study [28]. Thus, authors suggested conduction of an invasive examination when necessary to confirm the results of abnormal screening [28]. The lower PPV reported in the current study might be explained by the relative abundance of monosomy X cases in the assessed patients. A possible explanation for discordant NIPT result in these cases is monosomy X rescue that leads to uniparental isodisomy [29]. Thus, at least some discordant monosomy X results might be due to true mosaicism in the pregnancy. This issue is important in clinical outcomes and should be considered in patient counseling [29]. Moreover, a previous study showed association between maternal chromosome copy number variations (CNVs) with sizes of 1–1.6 Mb and false-positive NIPT results in sex chromosomal abnormalities [30]. These CNVs might be more prevalent among Iranian patients. This supposition is in accordance with the results of population-based investigations in Iran that revealed distinct genetic variations in this population and a number of high-frequency CNV regions in healthy persons [31]. Therefore, the observed low PPV in the current study might be explained by the presence of certain CNVs among Iranian subjects that result in high false-positive results. This necessitates design of population-specific kits for assessment of sex chromosomal abnormalities. The relative abundance of monosomy X cases in the mentioned population might

be due to higher parental age at pregnancy, reflecting a problem with population selection.

Conclusion

The advent of NIPT as a screening tool has optimized prenatal care and decreased the rate of invasive diagnostic tests. The current study has confirmed high sensitivity and specificity of this method in the detection of chromosomal trisomies. Of particular note, this method can be applied in the subgroups of patients with higher risk of trisomies based on the FTS results to decrease the rate of invasive tests. Moreover, we recommend this test for low-risk pregnancies as well. In brief, while combined FTS for all pregnancies and contingency testing with NIPT for those identified as high risk pregnancies is more effective at the population level, at the individual level, NIPT is suggested as a screening method with a higher detection rate and a lower false positive rate for trisomies. Yet, the high rate of false positive results for sex chromosome abnormalities should be considered in the interpretation of the results. Our study had some limitations. First, it was based on the results of a single center. Thus, additional multicenter studies are needed to confirm the results of current study. Second, this center is a referral lab for several complicated cases. Thus, it is possible that the referred cases are not true representative of the whole population of pregnant women, particularly in terms of risk stratification. Finally, not all of the patients in our study may truly be 'low-risk.' In fact, the prevalence of chromosome conditions in this study population is higher than expected for a 'low-risk' population.

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Author contributions
M.M, S.G., S.Y., M.A., M.M, S.S., designed and supervised the study. S.G.F., and S.K. wrote the draft and revised it. M.A.O., P.S., S.J., M.R analyzed the data. S.A., S.D., F.N., S.C., Y.M., and S.E. collected the data and performed the experiment. All the authors read and approved the submitted version.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Informed consent has been obtained from all patients. Ethical approval for this study has been obtained from the Ethical Committee of Nilou Lab. All methods were carried out in accordance with relevant guidelines and regulations.

Consent to publish
Informed consent has been obtained from all patients.

Competing interests
The authors declare no competing interests.

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