Original Research

Performance and Diagnostic Value of Genome-Wide Noninvasive Prenatal Testing in Multiple Gestations

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OBJECTIVE: To evaluate the accuracy and diagnostic value of genome-wide noninvasive prenatal testing (NIPT) for the detection of fetal aneuploidies in multiple gestations, with a focus on dichorionic–diamniotic twin pregnancies.

METHODS: We performed a retrospective cohort study including data from pregnant women with a twin or higher-order gestation who underwent genome-wide NIPT at one of the eight Belgian genetic centers between November 1, 2013, and March 1, 2020. Chorionicity and amnionicity were determined by ultrasonography. Follow-up invasive testing was carried out in the event of positive NIPT results. Sensitivity and specificity were calculated for the detection of trisomy 21, 18, and 13 in the dichorionic–diamniotic twin cohort.

RESULTS: Unique NIPT analyses were performed for 4,150 pregnant women with a multiple gestation and an additional 767 with vanishing gestations. The failure rate in multiple gestations excluding vanishing gestations ranged from 0% to 11.7% among the different genetic centers. Overall, the failure rate was 4.8%, which could be reduced to 1.2% after single resampling. There were no common fetal trisomies detected among the 86 monochorionic–monoamniotic and 25 triplet cases. Two monochorionic–diamniotic twins had an NIPT result indicative of a trisomy 21, which was confirmed in both fetuses. Among 2,716 dichorionic–diamniotic twin gestations, a sensitivity of 100% (95% CI 74.12–100%) and a specificity of 100% (95% CI 99.86–100%) was reached for trisomy 21 (n=12). For trisomy 18 (n=3), the respective values were 75% (95% CI 30.06–95.44%) sensitivity and 100% (95% CI 99.86–100%) specificity, and for trisomy 13 (n=2), 100% (95% CI 20.65–100%) sensitivity and 99.96% (95% CI 99.79–99.99%) specificity. In the vanishing gestation group, 28 NIPT results were positive for trisomy 21, 18, or 13, with only five confirmed trisomies.

CONCLUSION: Genome-wide NIPT performed accurately for detection of aneuploidy in dichorionic–diamniotic twin gestations.

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Noninvasive prenatal testing (NIPT) is widely implemented into clinical care as a first-tier screening test. A meta-analysis on the performance of cell-free DNA screening in singleton pregnancies reported a weighted pooled sensitivity of 99.7% and a false-positive rate of 0.04% for the detection of fetal trisomy 21.[1]

In twin pregnancies, however, detailed information on the performance of NIPT is limited and there are several factors that make it more challenging. Although the total fetal fraction is higher in twin pregnancies, the contribution of cell-free DNA from each twin is lower.[2] Moreover, the contribution per fetus can be different, potentially varying by as much as twofold.[3] In addition, chorionicity has important implications for the accuracy of NIPT in twin pregnancies.[4] In dizygotic twins, each twin has its own placenta contributing to the circulating cell-free DNA, resulting in a discordant aneuploidy risk for both fetuses.[5]

Given the implementation challenges, and the lack of sufficient validation data related to the accuracy of NIPT in twin pregnancies, uniform recommendations are not available.[6–9] Nevertheless, an accurate NIPT for twin pregnancies is highly desired, as the accuracy of combined nuchal translucency and first trimester serum screening is limited in twin gestations, with a pooled sensitivity of 89.3% and a pooled specificity of 94.6% for the detection of trisomy 21.[10]

Our objective was to evaluate the accuracy and diagnostic value of genome-wide NIPT for the detection of fetal aneuploidies in multiple gestations, with a specific focus on dichorionic–diamniotic twins. We hypothesized that NIPT in multiple gestations, including dichorionic–diamniotic twins, would show high sensitivity and specificity for detection of aneuploidy.

METHODS

This was a retrospective cohort study of pregnant women with a multiple gestation having undergone NIPT at the clinical laboratories of one of the eight Belgian genetic centers (Katholieke Universiteit Leuven, Universitair Ziekenhuis Antwerpen, Universitair Ziekenhuis Gent, Vrije Universiteit Brussel, Université Libre de Bruxelles, Centre Hospitalier Universitaire de Liège, Université Catholique de Louvain, and l'Institut de Pathologie et de Génétique). These genetic centers perform all invasive prenatal and neonatal genetic testing in Belgium. Genetic counseling was provided by obstetricians before blood sampling for NIPT and consent for clinical testing was obtained. Contraindications for NIPT testing were ultrasonogram abnormalities (including a nuchal translucency measurement exceeding 3.5 mm), or a history of stem cell, organ, or tissue transplant. Peripheral blood samples were collected from pregnant women from 10 weeks of gestation onward in Cell-Free DNA BCT tubes, Cell-Free DNA collection tubes, or PAXgene Blood ccfDNA tubes. Plasma isolation was carried out using standard centrifugation techniques and methods for cell-free DNA extraction, library preparation, whole genome sequencing, and analysis were carried out by the different genetic centers as described in Appendix 1, available online at http://links.lww.com/AOG/C291.

Twin chorionicity and amnionicity were evaluated by ultrasonography to classify twin pregnancies as dichorionic–diamniotic, monochorionic–diamniotic, or monochorionic–monoamniotic. A *vanishing twin* was defined as a spontaneous reduction of a fetus in utero. In cases of a dichorionic–diamniotic twin pregnancy, parents were informed that the test accuracy for aneuploidy could be lower as compared with monochorionic twin pregnancies. Both common fetal aneuploidies (aneuploidies of chromosome 13, 18, or 21) and rare autosomal trisomies detected by NIPT were reported to the parents for further follow-up. Fetal sex chromosome aneuploidies were not reported per clinical protocol.

If NIPT was positive for a chromosomal abnormality, pregnant women were offered follow-up by standard invasive prenatal diagnosis based on DNA preferably extracted from amniotic fluid. Analysis was carried out using the Agilent ISCA 60K or 44K array, the CytoSure v3 microarray, the Cytoscan 750K, the HumanCytoSNP-12 v2.1 BeadChip, or by shallow whole genome sequencing.[11] Testing for the presence of a uniparental disomy was carried out when applicable (nonverified trisomy of chromosome 6, 7, 11, 14, 15, or 20) by polymorphic short tandem repeat analysis, by use of the HumanCytoSNP-12 v2.1 BeadChip, or by methylation-specific multiplex ligation-dependent probe amplification using the SALSA MLPA Probemix ME034-A1. In the event of a technical failure or an inconclusive result, blood sampling and NIPT analysis were repeated.

Pregnancy outcomes, including false-negative cases, were retrieved from the clinical databases available in each genetic center. Because all prenatal and neonatal cytogenetic testing in Belgium is performed in one of the genetic centers involved in this study, it is very unlikely that a false-negative case was missed. Data were analyzed using R studio 1.3.959 and Excel 16.41. Test performance was expressed as

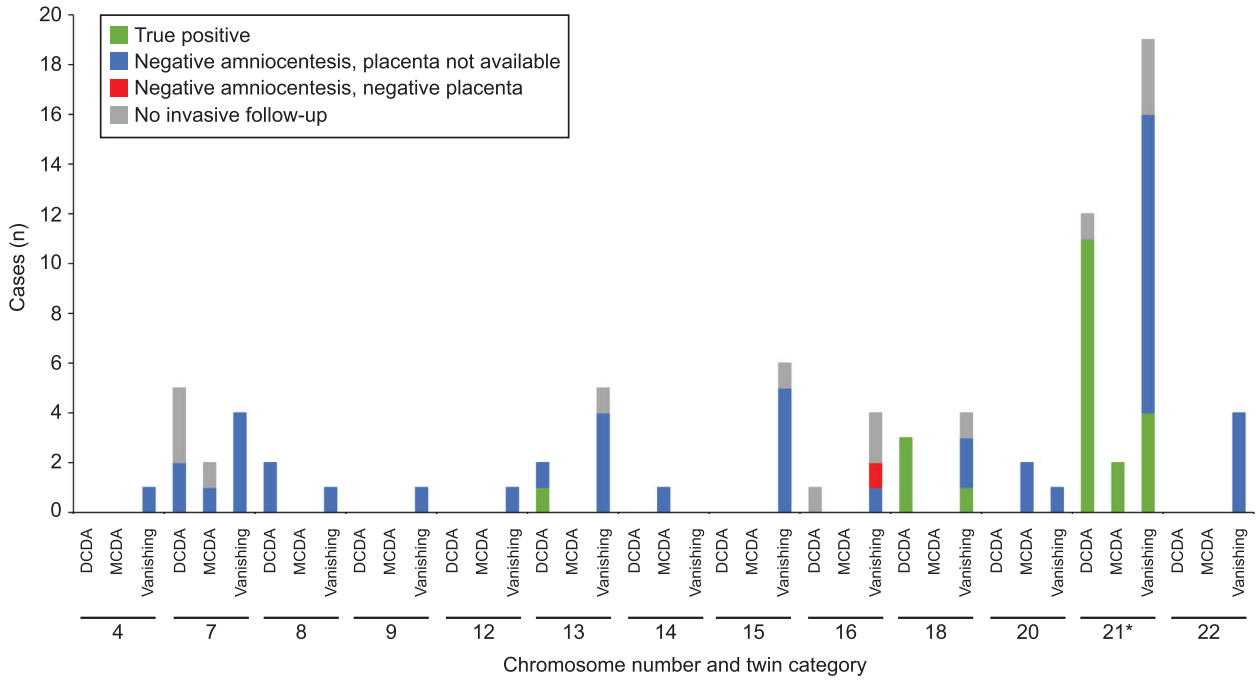

Fig. 1. Reported trisomies subdivided by twin category and by outcome of invasive follow-up. Chromosomes with zero trisomy cases were left out. *In one twin with unspecified amnionicity or chorionicity, trisomy 21 was detected by noninvasive prenatal testing and confirmed by amniocentesis in both twins. DCDA, dichorionic–diamniotic; MCDA, monochorionic–diamniotic; vanishing, vanishing twin.

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sensitivity, specificity, and positive and negative likelihood ratios. CIs were calculated using the Newcombe[12]-Wilson method without continuity correction. Chi-squared tests were used to compare percentages, with $P<.05$ considered statistically significant.

This study was approved by the Ethics Committee of the University Hospital of Antwerp.

RESULTS

We included 4,150 unique NIPT analyses in women with a multiple gestation and 767 in women with a vanishing gestation between November 1, 2013, and March 1, 2020. Among the multiple gestations, 2,716 (65.4%) were dichorionic–diamniotic twins, 790 (19.0%) monochorionic–diamniotic twins, 86 (2.1%) monochorionic–monoamniotic twins, eight (0.2%) monochorionic unspecified, and 25 (0.6%) triplets (Appendix 2, available online at http://links.lww.com/AOG/C291). For 525 (12.7%) twins, information on chorionicity, amnionicity, or both was unavailable. Of the 767 vanishing gestations, 13 were dichorionic–diamniotic twins originating from a triplet with one vanishing gestation, and seven were triplets with double vanishing gestations. Also, four quadruplets with double (n=3) or triple (n=1) vanishing gestations were included.

In this multicenter study, a large variation in failure rate was detected across centers, ranging from 0% to 11.7% in multiple gestations, excluding vanishing gestations. Overall, for 4.8% (n=198) of all multiple gestation samples excluding vanishing gestations, no result was obtained on the first analysis. After a single resampling, a conclusive result could be obtained for 4,101 out of 4,150 cases, resulting in an overall success rate of 98.8% for multiple gestations. Reasons for failure included low fetal fraction, poor quality, or a combination of both. In vanishing gestations, the failure rate was higher with 48 test failures out of 767 analyses (6.3%) after first sampling. After a second sampling, the success rate increased to 99.4%.

Noninvasive prenatal testing was positive for one of the common fetal trisomies in 17 (0.63%) of the dichorionic–diamniotic twins (Fig. 1). Twelve (0.44%) dichorionic–diamniotic cases had an NIPT result positive for trisomy 21, three (0.11%) for trisomy 18, and two (0.07%) for trisomy 13. Confirmatory invasive genetic testing results were available for all but one trisomy 21 case. Amniocentesis confirmed the trisomy in 14 cases; for one trisomy 21 case no amniocentesis

Table 1. Diagnostic Performance of Noninvasive Prenatal Testing in Dichorionic–Diamniotic Twin Pregnancies

				Likelihood Ratio			
	Incidence	Sensitivity	Specificity	Positive	95% CI	Negative	95% CI
Trisomy 21	0.44 (12/2,716)	100 (11/11) (74.12–100)	100 (2,704/2,704) (99.86–100)	∞		0	
Trisomy 18	0.11 (3/2,716)	75.00 (3/4) (30.06–95.44)	100 (2,712/2,712) (99.86–100)	∞		0.25	0.046–1.365
Trisomy 13	0.07 (2/2,716)	100 (1/1) (20.65–100)	99.96 (2,714/2,715) (99.79–99.99)	2,715	382.58–19,267.02	0	

Data are % (n/N) or % (n/N) (95% CI) unless otherwise specified.

was performed, but chorionic villous sampling confirmed the trisomy, and an increased nuchal translucency was detected by ultrasonography on which selective feticide was performed. The remaining case, with a positive NIPT for trisomy 13 that was not confirmed by amniocentesis, had no placental follow-up to evaluate for confined placental mosaicism. In all but one of the cases where the trisomy was confirmed by amniocentesis, the trisomy was restricted to one twin; in one trisomy 18 case, both twins were affected. In one dichorionic–diamniotic twin with trisomy 21, the other twin was triploid.

Two of 790 monochorionic–diamniotic twins had a NIPT result positive for trisomy 21. In both cases, trisomy 21 was confirmed in the two fetuses. In one twin with unspecified amnionicity or chorionicity, trisomy 21 was detected by NIPT and confirmed by amniocentesis in both fetuses. There were no common fetal trisomies detected among the 86 monochorionic–monoamniotic and 25 triplet cases.

In 767 vanishing gestation pregnancies, NIPT detected a common trisomy in 28 cases (3.65%). Nineteen cases were positive for trisomy 21, of which three were confirmed in the remaining fetus and one in the deceased fetus. There were four trisomy 18 cases, of which one was confirmed in the deceased fetus. In five cases, NIPT was positive for trisomy 13, including one case that had a positive NIPT result for trisomy 7 as well. One trisomy 13, one trisomy 18, and three trisomy 21 cases were lost to follow-up. The other 18 cases (12 with trisomy 21, two with trisomy 18, and four with trisomy 13) were not confirmed, but in all these cases only the remaining fetus was tested.

Overall, 36 rare autosomal trisomies were detected by the genome-wide NIPT analyses (Fig. 1). The majority of rare autosomal trisomies were detected in vanishing gestations (n=23), including one triplet with a vanishing gestation, leading to an monochorionic–monoamniotic twin gestation. In vanishing gestations, single cases of trisomy 4, 8, 9, 12, and 20 were detected. Furthermore, four cases with trisomy 7 were detected, including one case that was positive for both trisomy 7 and trisomy 13, as mentioned earlier. Additionally, four cases were positive for trisomy 16, four for trisomy 22, and six cases were positive for trisomy 15. Confirmatory invasive testing was available for 20 of the 23 vanishing gestation cases and showed that none of the rare autosomal trisomies could be confirmed in fetal tissue. Additionally, eight dichorionic–diamniotic twins were detected to have a rare autosomal trisomy. Five cases were positive for trisomy 7, two for trisomy 8, and one for trisomy 16. Invasive testing by amniocentesis failed to confirm an aneuploidy in four of these, and the other four were lost to follow-up. Lastly, NIPT detected a rare autosomal trisomy in five monochorionic–diamniotic twins. Two monochorionic–diamniotic twins were positive for trisomy 7 with one not confirmed by amniocentesis and one lost to follow-up. One monochorionic–diamniotic twin had an NIPT positive for trisomy 14, which was not confirmed by amniocentesis, and in two other monochorionic–diamniotic twins a trisomy 20 was detected, which were both not confirmed by amniocentesis. No rare autosomal trisomies were detected in monochorionic–monoamniotic twins or in triplet pregnancies.

According to Belgian guidelines, rare autosomal trisomies are communicated to the pregnant woman, stating the possibility of confined placental mosaicism, and ultrasonography and invasive follow-up are recommended.[13] In cases of confined placental mosaicism, analysis of uniparental disomy on amniotic fluid is warranted for chromosomes 6, 7, 11, 14, 15, and 20 because of their association with developmental disorders.[13,14] In our cohort, results of uniparental disomy analysis were available for eight cases; all were uniparental disomy negative.

The rate of both common fetal trisomies (3.7% vs 0.6%) and rare autosomal trisomies (3.0% vs 0.3%) was higher in vanishing gestations as compared with the dichorionic–diamniotic cohort ($P<.001$ for both comparisons).

Only one false-negative NIPT result was reported in a dichorionic–diamniotic twin gestation, in which fetal ultrasonography later in pregnancy detected structural anomalies. Amniocentesis was performed to confirm a diagnosis of trisomy 18 in one of the fetuses.

In five vanishing gestation cases, as well as in one quadruplet pregnancy with two vanishing gestations, the fetal sex detected with NIPT did not match the sex observed on ultrasonogram of the remaining fetus. In all six of these cases, NIPT detected a male fetus but the surviving twin was female.

Test characteristics for monochorionic–diamniotic, monochorionic–monoamniotic, and triplet pregnancies could not be calculated owing to low frequency of aneuploidies in those groups. Sensitivity and specificity for the detection of common aneuploidies in dichorionic–diamniotic twin gestations were both high (Table 1).

DISCUSSION

Studies on the accuracy of NIPT in twin pregnancies are limited and include relatively small numbers.[2,15–23] This study reports on one of the largest cohorts of women with a twin gestation undergoing screening for aneuploidy with NIPT, and demonstrates high sensitivity and specificity for detection of trisomy 21 in dichorionic–diamniotic twins.

For the common trisomies, there was only one false-positive dichorionic–diamniotic case. Confined placental mosaicism, previously demonstrated as a main cause of false-positive NIPT results in the general population,[24–26] could be the explanation for this false-positive result, but placental analysis was not performed for confirmation.

Noninvasive prenatal testing detected 12 (0.44%) trisomy 21 cases, three (0.11%) trisomy 18 cases, and two (0.07%) trisomy 13 cases in our dichorionic–diamniotic group. These incidences are comparable with those in the general population.[27,28] Twin pregnancies are historically believed to have a higher aneuploidy risk than singleton pregnancies because of a higher mean maternal age and because in a dizygotic pregnancy, each fetus has an individual aneuploidy risk. However, risks were mainly calculated based on statistical models rather than real data sets and observational data now suggest a lower birth prevalence of trisomy 21 than expected[29,30]; this is confirmed in our data set.

In this study, which includes NIPT results of one of the largest vanishing gestation cohorts described to date,[31,32] NIPT detected a higher percentage of both common and rare autosomal trisomies when compared with the dichorionic–diamniotic cohort. Chromosomal abnormalities are a major cause for spontaneous abortion as they are found in about half of evaluated products of conception.[33] Several cases of common fetal trisomies and all rare autosomal trisomies remained unconfirmed in our vanishing gestation cohort. In most cases, only the remaining fetus could be tested, and the trisomy result was suspected to originate from the deceased fetus. For one trisomy 18 case and one trisomy 21 case, the trisomy could indeed be traced to the deceased twin. False-positive results and sex discrepancies in vanishing twin pregnancies are a well-known problem due to skewing of the NIPT profiles by the cell-free DNA of the deceased twin[15,16,31,32] and should be discussed during genetic counseling. In case of a normal NIPT result, follow-up is the same as in singleton gestations. When NIPT demonstrates an increased risk of aneuploidy, invasive testing is offered to exclude the presence of the aneuploidy in the remaining twin. In our opinion, NIPT is useful in case of a vanishing gestation because it might reveal the cause of death of the vanishing gestation and indicate the need for ultrasonography follow-up of the remaining fetus.

Ninety-seven percent of all rare autosomal trisomies detected in chorionic villus samples can be explained by confined placental mosaicism[24–26]; a similar percentage is expected for NIPT samples. In our dichorionic–diamniotic cohort, none of the rare autosomal trisomies were confirmed, but the suspected association between a rare autosomal trisomy and an adverse pregnancy outcome warrants further close surveillance of the pregnancy.[34] Moreover, confined placental mosaicism poses a (small) risk for uniparental disomy, and testing is currently advised for chromosomes carrying imprinted regions.[13] Uniparental disomy data were available from two dichorionic–diamniotic twins where NIPT detected a trisomy 7; both were negative.

Large differences in the failure rate of NIPT in twin pregnancies have been reported, ranging from 0.5% to 13.2% after first sampling.[17,35–37] Likewise, this multicenter study shows a wide range in failure rate (0–11.7%). We obtained a result in all but 4.8% of multiple gestation samples excluding vanishing twins; this could be reduced to 1.2% after resampling. The

large differences in failure rate might be explained by variability in the threshold for fetal fraction set by the different genetic centers.

Few labs have performed NIPT in triplets and higher-order multiples, as large data sets are difficult to collect. Correct interpretation of NIPT and accurate determination of fetal sex seem achievable in triplet pregnancies,[2,21] as demonstrated in our cohort of 25 triplets, but more studies with larger cohorts and positive cases are required. In our opinion, NIPT would be a valuable screening option for higher-order multiples, because maternal serum markers cannot be applied and current screening is based on ultrasonography and maternal age only.[5,21]

Our results indicate excellent test performance of NIPT for detection of aneuploidy in one of the largest twin cohorts. However, we acknowledge the importance of ultrasonogram examinations to determine chorionicity and detect structural defects. Also, a positive NIPT result should always be confirmed by invasive testing, preferably by amniocentesis to rule out confined placental mosaicism. Given that the Belgian genetic centers involved in this study perform all invasive prenatal genetic testing and all neonatal testing in Belgium, complete follow-up of all positive NIPT results is ensured, allowing us to determine the false-positive cases, and optimal tracing of false-negative cases. Limitations of this study include the relatively low number of trisomy 13 and trisomy 18 cases. In addition, the different whole genome analysis methods used varied among centers, including the presence of different cutoffs for fetal fraction. However, this may increase generalizability of the results to other centers.

In conclusion, we demonstrate that genome-wide NIPT analysis is an accurate screening tool for common fetal trisomies in dichorionic–diamniotic twin gestations, and improves pregnancy management as current screening options, combined nuchal translucency measurement and first trimester serum screening, have limited sensitivity and specificity in multiple gestations.

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