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ORIGINAL RESEARCH ARTICLE

Non-invasive prenatal diagnosis for translocation carriers—YES please or NO go?

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Abstract

Introduction: The presence of an unbalanced familial translocation can be reliably assessed in the cytotrophoblast of chorionic villi. However, carriers of a balanced translocation often decline invasive testing. This study aimed to investigate whether an unbalanced translocation can also be diagnosed in cell free DNA by whole-genome non-invasive prenatal screening (NIPS).

Material and methods: Pregnant women carrying a fetus with an unbalanced familial translocation, for whom NIPS as well as microarray data were available, were included in this retrospective assessment. NIPS was performed in the course of the TRIDENT study.

Results: In 12 cases, both NIPS and microarray data were available. In 10 of 12 cases the unbalanced translocation was correctly identified by NIPS without prior knowledge on parental translocation. One was missed because the fetal fraction was too low. One was missed because of technical restrictions in calling 16p gains.

Conclusions: This study supports the hypothesis that routine NIPS may be used for prenatal diagnosis of unbalanced inheritance of familial translocations, especially with prior knowledge of the translocation allowing focused examination of the involved chromosomal regions. Our study showed that routine shallow sequencing designed for aneuploidy detection in cell free DNA may be sufficient for higher resolution NIPS, if specialized copy number software is used and if sufficient fetal fraction is present.

KEYWORDS

cell free DNA screening, copy number variant analysis, fetal fraction, non-invasive prenatal screening, unbalanced translocation

Abbreviations: BAM file, compressed binary version of a SAM file that is used to represent aligned sequences; cfDNA, cell free DNA; CNV, copy number variant; FF, fetal fraction; NIPD, non-invasive prenatal diagnosis; NIPS, non-invasive prenatal screening; TRIDENT, Trial by Dutch laboratories for evaluation of non-invasive prenatal testing study.

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1 | INTRODUCTION

The incidence of balanced reciprocal chromosome translocations ranges from 1/800 to 1/1100 in the general population to approximately 1/30 in couples with multiple miscarriages.¹ Carriers of a balanced chromosome aberration often experience multiple miscarriages of unbalanced products of conception and are therefore very anxious about taking any risk when their pregnancy advances beyond the gestation of those that miscarried. For this reason, some women tend to decline invasive testing. We have previously hypothesized whether non-invasive prenatal screening (NIPS) could be a second-best option for carriers of balanced aberrations, who are not willing to take the 0.1%–0.2% risk for miscarriage induced by invasive testing.² Carrier status of a balanced chromosome translocation or inversion is typically an indication for invasive prenatal diagnosis using chorionic villus sampling. Experience from cytogenetic testing of chorionic villi showed that the presence or absence of the (un)balanced translocation can reliably be diagnosed by cytogenetic analysis of the cytotrophoblast of chorionic villi.^{3–5} For this reason, we previously hypothesized that analysis of cell free (cf) DNA in maternal plasma—of which the fetal part originates from the cytotrophoblast—should also give a reliable diagnosis.² When NIPS shows the unbalanced product of the parental structural chromosome aberration such a result might be considered as a definitive diagnosis.

In the Netherlands, a known familial translocation/inversion is an exclusion criterion for prenatal screening with NIPS. However, pregnant women and their partners may not always be aware of being a carrier and because whole-genome NIPS may reveal subchromosomal aberrations, a familial translocation may be detected by chance through prenatal screening, as was shown before.^{6–13} In the first year of the TRIDENT-2 study, we found four such cases.¹⁴ Therefore, we have retrospectively analyzed all data of the Dutch laboratories involved in both TRIDENT studies (1 and 2). We have searched for fetuses with a familial unbalanced chromosome aberration, of which both cfDNA and microarray data were available. The aim of this study was to investigate whether the unbalanced fetal karyotype can be detected in cfDNA. We were particularly interested in whether shallow sequencing for common aneuploidy analysis is sufficient to reveal the unbalanced familial translocation and whether the analysis resolution can be increased by targeted (focused) analysis in a software dedicated to copy number variant (CNV) detection that is routinely used for microarray analysis.

2 | MATERIAL AND METHODS

We retrospectively re-evaluated data of prenatal screening and diagnostic testing of pregnancies in 2014–2019. We collected cases in which the fetus was shown to be affected with an unbalanced familial translocation and for which both NIPS and invasive diagnostic testing results were available. As carrier status of a balanced translocation is an exclusion criterion for the Dutch TRIDENT studies, the available cases were discovered without a priori knowledge on parental karyotypes (with the exception of case 8). Therefore,

Key message

Routine non-invasive prenatal screening may be used for prenatal diagnosis of unbalanced inheritance of familial translocations. Shallow sequencing designed for aneuploidy detection may be sufficient for higher resolution, if specialized copy number software is used and if sufficient fetal fraction is present.

the WISECONDOR results in this cohort represent initial blind analysis. Sequencing of cfDNA was performed according to various protocols (see Appendix S1) and the results were analyzed by using WISECONDOR, which has a resolution of ~15 Mb.^{15,16} It visualizes the results on chromosome plots in addition to the z-scores (if abnormal) and uses “within chromosome normalization”.

In a few cases ($n = 5$), the BAM files of the routine NIPS were analyzed in the dedicated CNV calling software NEXUS BioDISCOVERY COPY NUMBER v.10, which we routinely use for microarray analysis. We used matched reference sets, to assess whether the detection of the unbalanced fetal karyotype is feasible at a higher resolution than with WISECONDOR in routine NIPS data.

Fetal fraction (FF) percentage was calculated (where possible) by use of the SeqFF algorithm.¹⁷ SeqFF determines the FF based on a difference in autosomal regional read counts between fetal and maternal reads. Two statistical models trained with read counts over specific autosomal regions in a large sample set were used to predict the “fetal” fraction of the test samples. The FF calculation by the SeqFF method is therefore independent of fetal gender.¹⁷

2.1 | Ethical approval

Here, we describe the cytogenetic follow up of the additional findings (in fetuses with unbalanced translocations) found in the TRIDENT-1 study and in the first 2 years of the TRIDENT-2 study. Permission for the TRIDENT-1 study was granted by the Dutch Minister of Health, Welfare and Sport (350010-118701-PG) on March 28, 2014. A license for the TRIDENT-2 study was granted by the Dutch Minister of Health, Welfare and Sport in 2017 (1017420-153371-PG) on September 20, 2016. Additionally, the study in the general population was approved by the Medical Ethics Committee of the Amsterdam UMC, VU University Medical Center (VUMC No.2017.165) on March 27, 2017. All women consented to their data being used for research purposes.

3 | RESULTS

3.1 | Routine WISECONDOR analysis

Twelve prenatal cases of unbalanced familial translocations were collected in which both NIPS and genomic microarray data were available. Table 1 shows the results of routine NIPS, fetal

TABLE 1 Results of routine NIPS analysis, invasive testing and targeted CNV analysis in cfDNA with Nexus BioDiscovery Software for samples with imbalances <10 Mb

Case	Routine NIPS results (Wisecornder)	GA at NIPS	mln reads	FF	Fetal imbalance/karyotype (invasive testing results)	Chromosomal imbalance (Mb)	Targeted analysis in Nexus Copy NUMBER software
1	Chr11 18.2 Mb gain	12.0	15.1	n.a.	+der(22)t(11;22)(q23.3;q11.2)mat arr[GRCh37]11q23.3q25(116722111_134945165)x3, 22q11.1q11.21(16053473_20311988)x3	Chr11 18.2 Mb gain Chr22 3.7 Mb gain	n.a.
2	Chr12 35 Mb gain Chr18 5 Mb loss not called, but visually detectable	12.5	10.4	6%	der(18)t(12;18)(q23.1;q22.3)mat arr[GRCh37] 12q23.1q24.33(98750482_133777645)x3, 18q22.3q23(72777472_78004048)x1	Chr12 35 Mb gain Chr18 5 Mb loss	Chr12 35 Mb gain Chr18 5 Mb loss
3	Normal ^a	12.5	20.1	8%	der(6)t(6;16)(q27;p13.13)pat arr[GRCh37] 6q27(167805497_170919470)x1, 16p13.3p13.13(88366_10830753)x3	Chr6 3.1 Mb loss Chr16 10.7 Mb gain	Chr6 3.1 Mb loss Chr16 10.7 Mb gain
4	Chr4 12 Mb loss Chr12 8 Mb gain	13.1	14	13%	der(4)t(4;12)(q34.3;p13.31)mat arr[GRCh37] 4q34.3q35.2(178689242_190963766)x1, 12p13.33p13.31(191619_8003758)x3	Chr4 12 Mb loss Chr12 8 Mb gain	Chr4 12 Mb loss Chr12 8 Mb gain
5	Chr16 39.7 Mb gain	13.4	15.7	n.a.	der(10)t(10;16)(p15.3;q12.1)mat arr[GRCh37] 10p15.3(100026_1125755)x1, 16q12.1q24.3(50428067_90155062)x3	Chr10 1.1 Mb loss Chr16 39.7 Mb gain	n.a.
6	Chr10 ~48 Mb gain	14.6	23.9	n.a.	+der(10)t(5;10)(p15.3;q11.2)mat	Chr5 ~6 Mb gain Chr10 ~48 Mb gain	n.a.
7	Chr3 47 Mb gain Chr11 15 Mb loss	12.1	19.2	13%	der(11)t(3;11)(q25.2;q23.3)mat arr[GRCh37] 3q25.2q29(152973729_197851986)x3, 11q23.3q25(120391473_134933972)x1	Chr3 44.9 Mb gain Chr11 14.5 Mb loss	n.a.
8	visually detectable Chr1 8.9 Mb loss Chr10 11.7 Mb gain	13.5	17.6	8%	der(1)t(1;10)(q43;p14)pat arr[GRCh37] 1q43q44(240361422_249222527)x1, 10p15.3p14(95229_11780837)x3	Chr1 8.9 Mb loss Chr10 11.7 Mb gain	Chr1 8.9 Mb loss (called but noisy) Chr10 11.7 Mb gain
9	Chr6 44 Mb dup	13.6	12.7	n.a.	rec(6)dup(6q)inv(6)(p25.3;q22.2)mat arr[GRCh37] 6p25.3(156975_472826)x1, 6q22.2q27(118406846_170919483)x3	Chr6 0.3 Mb loss Chr6 44 Mb gain	n.a.
10	Normal ^b	11.2 (at NIPS sampling)	20.2	n.a.	der(6)t(6;9)(q27;p21.3)pat arr[GRCh37] 6q27(164768212_170919482)x1, 9p21.3(203,861-22937078)x3	Chr6 6 Mb loss Chr9 23 Mb gain	n.a.
11	Chr12 8 Mb gain not called, but visually detectable Chr18 11.3 Mb loss	12.0	18.6	n.a.	der(18)t(12;18)(q24.31;q22.1)mat arr[GRCh37]12q24.31q24.33(125760991_133777902) x3, 18q22.1q23(66746699_78014123)x1	Chr12 8 Mb gain Chr18 11.3 Mb loss	n.a.
12	Chr11 18.2 Mb gain	12.3	18.6	7%	+der(22)t(11;22)(q23.3;q11.2)mat arr[GRCh37] 11q23.3q25(116690712_134934063)x3, 22q11.1q11.21(16595552_20311646)x3	Chr11 18.2 Mb gain Chr22 3.7 Mb gain	Chr11 18.2 Mb gain Imbalance on chr22 not detectable

FF, fetal fraction estimated with SeqFF; GA, weeks of gestation at NIPS blood sampling; n.a., not analyzed; NIPS, non-invasive prenatal screening.

^aChromosomal imbalance missed by routine analysis due to technical noise in 16p in the experiment and no a priori knowledge on the parental translocation (many samples showed 16p gains in this experiment, which were all initially interpreted as technical noise). Invasive test done due to ultrasound anomalies at 22 weeks: bilateral mild ventriculomegaly, hypoplasia of cerebellum, edema, suspected sub-aortic ventricular septal defect, gall bladder agenesis, hypospadias, left hand syndactyly, right hand cleft and syndactyly.

^bChromosomal imbalance missed by routine analysis due to too low fetal fraction. Invasive test done due to ultrasound anomalies at 21.3 weeks.

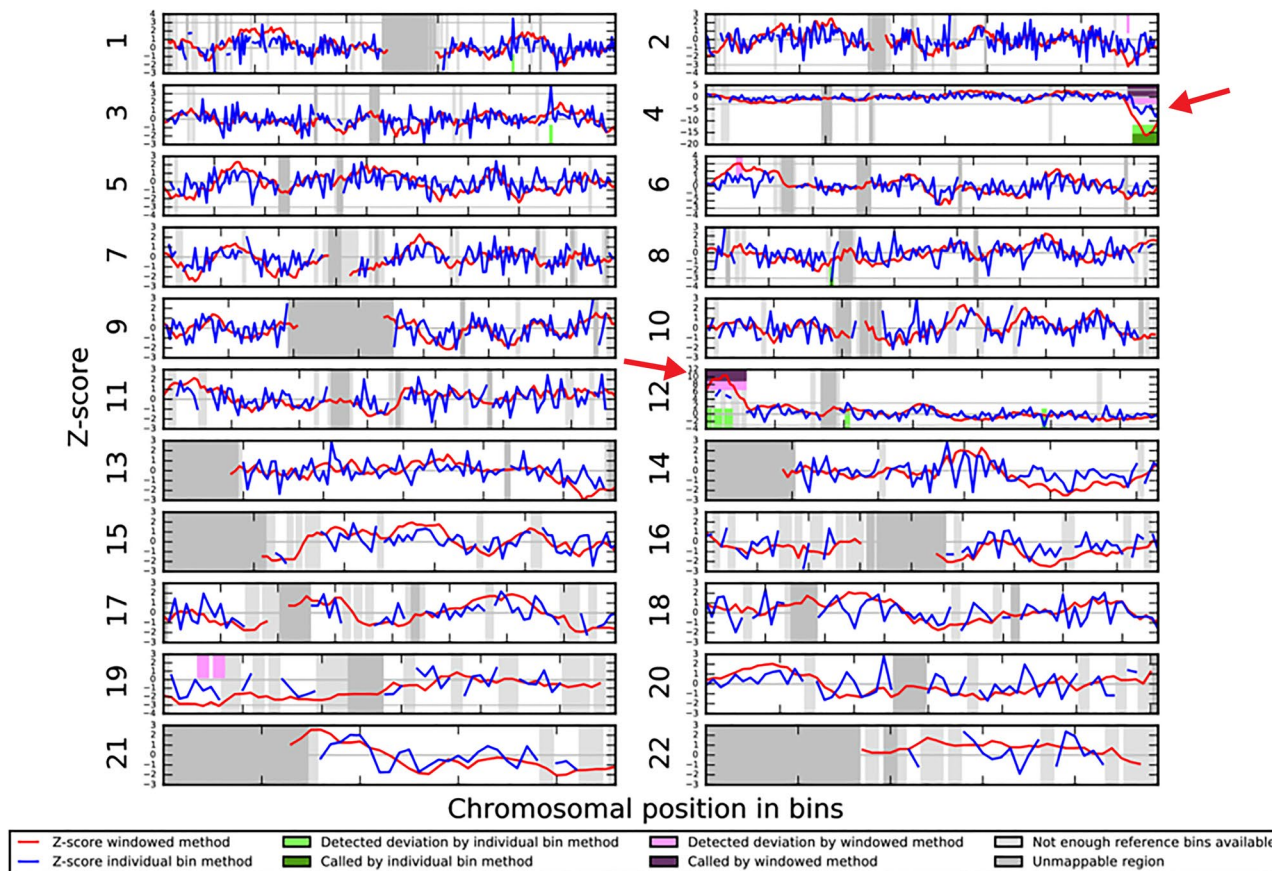


FIGURE 1 The results of WISECONDOR analysis in case 4 showing the presence of a 12-Mb loss at 4q and a 8-Mb gain at 12p. Invasive testing showed that the fetus was carrying a $\text{der}(4)t(4;12)(q34.3;p13.31)\text{mat}$

karyotype, and size of the chromosomal imbalances. All imbalances larger than 8 Mb could be detected with WISECONDOR despite the estimated resolution of ~15 Mb. It should be noted that the 8-Mb gain on chromosome 12 in fetus 4 was detected in a sample with a substantially higher FF when compared with the other fetuses (c.13% vs. 6%–8% most other fetuses) (see Table 1; Figure 1). In general, an FF of 6%–8% was sufficient to routinely detect at least one of the imbalances, with the number of usable sequence reads being 10–20 million. The aberrations smaller than 8 Mb were not called by WISECONDOR software, but in some cases were visible in the report graphics when knowledge on the parental aberration was taken into account.

In 9 of 12 cases, routine NIPS revealed the unbalanced translocation without prior knowledge on parental karyotype by showing at least one of the mal-segregated translocation segments. One aberrant case (3) showed a normal result in routine NIPS, because one of the imbalances (10.7 Mb) was located at a known problematic region (chromosome 16p), whereas the other imbalance on chromosome 6q was only 3 Mb and was not detectable with WISECONDOR. In our settings, chromosome 16p is often noisy, showing losses and gains, which are mostly technical false-positive calls. Therefore, this chromosomal region is often ignored, unless a very clear call is made. Retrospective analysis of the WISECONDOR

result after amniocentesis showed an unbalanced translocation, revealing the chromosome 16 aberration, but not the chromosome 6 aberration (see Figure 3).

Case 10 involving an unbalanced translocation $t(6;9)$ was missed during routine NIPS analysis because of too low FF in the first trimester sample. As we did not use a lower FF cut-off value for reporting results, a normal result was reported. However, retrospective analysis did not show the presence of Y chromosome material in the NIPS data, whereas the fetus was male. This suggests that the FF being too low had caused this false-negative NIPS result.

Case 8 involved an aberration that was almost missed during routine analysis. It showed subtle imbalances of ~9–12 Mb that were only visibly detectable on the report graphics, but that were not called by WISECONDOR, and at first were not considered as truly abnormal. The NIPS was requested without information on the balanced abnormal karyotype of the father, but it was noticed that a fetus from a previous pregnancy of this woman was already known in our database with an unbalanced translocation $t(1;10)$. Therefore, the chromosomes of interest were additionally inspected and the test was repeated so that data could be pooled to achieve deeper read depth and the aberrations became visually detectable (Figure 2).

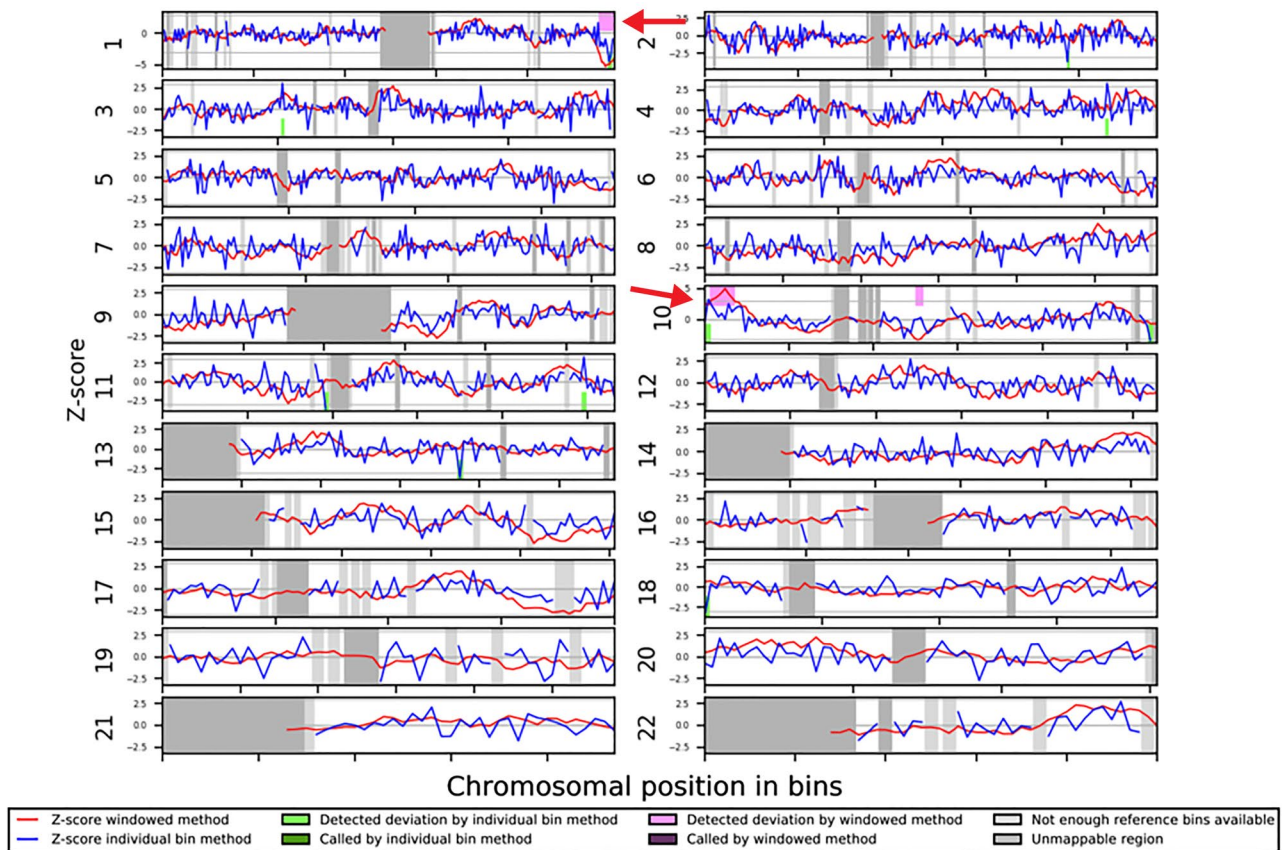


FIGURE 2 The results of pooled data in case 8 carrying $\text{der}(1)t(1;10)(q43;p14)\text{pat}$. After pooling the data from two runs, 36 mln reads were used for analysis by WISECONDOR and the imbalances on 1qter (8.9 Mb) and 10p (11.7 Mb) became detectable with WISECONDOR. The imbalances are not called by the windowed method, but the z-scores at 1qter and 10pter are noted by WISECONDOR as deviated, but too small to call, indicated by the light pink color. See also the change of scale on y-axis (z-score) indicating the presence of the paternal unbalanced translocation

3.2 | Additional analysis in NEXUS COPY NUMBER BioDISCOVERY software

Because the imbalances under 8 Mb were not visible with WISECONDOR, we were interested in whether the smaller imbalances could be detected by software that is dedicated for CNV calling in various microarray data. Therefore, we additionally analyzed the BAM files of the cases with small imbalances (cases 2, 3, 4, 8, and 12) with NEXUS COPY NUMBER BioDISCOVERY software. Interestingly, focused Nexus analysis revealed the 3–5 Mb aberrations that could not be detected with WISECONDOR analysis. Case 3 is especially interesting as NEXUS COPY NUMBER software was able to detect a 3-Mb deletion on chromosome 6q (Figure 3).

4 | DISCUSSION

The data presented in the current study further support the hypothesis that NIPS may be a useful tool for the diagnosis of unbalanced products of known familial balanced translocations.¹⁸ Although doubts have been expressed as to whether whole-genome NIPS is ready for clinical detection of unbalanced chromosomal aberrations smaller

than autosomal trisomies,^{19,20} various studies showed that detection of subchromosomal aberrations is feasible.^{7,8,13} Recently the Dutch TRIDENT study also showed that detection of fetal structural aberrations with our routine NIPS protocol is feasible.¹⁴ Flowers et al¹⁸ have demonstrated that whole-genome NIPS successfully detected all imbalances larger than 15 Mb in their cohort of 42 women at risk for a fetal unbalanced familial translocation. Our study specifically shows that a priori knowledge of a familial translocation allowing focused analysis of the involved chromosomes, further helps in the detection of unbalanced products of parental balanced aberrations, especially if segments are small and/or FF is low. It further demonstrates that with a priori knowledge on parental balanced aberration, 92% (11/12) of our cases with unbalanced translocations can be routinely detected; only the case with the FF that was too low would be missed (case 10). However if a lower cut-off for FF were to be used, such a result would not have been reported as “normal”. Based on the results of this study, we found three important factors to be crucial for implementation of non-invasive prenatal diagnosis (NIPD) for translocation carriers: (a) the size and the location of the involved chromosomal segments, (b) the software resolution, and (c) the FF in the total cfDNA pool.

The resolution of the software that is routinely used for aneuploidy detection with NIPS may limit genome-wide analysis, as it is

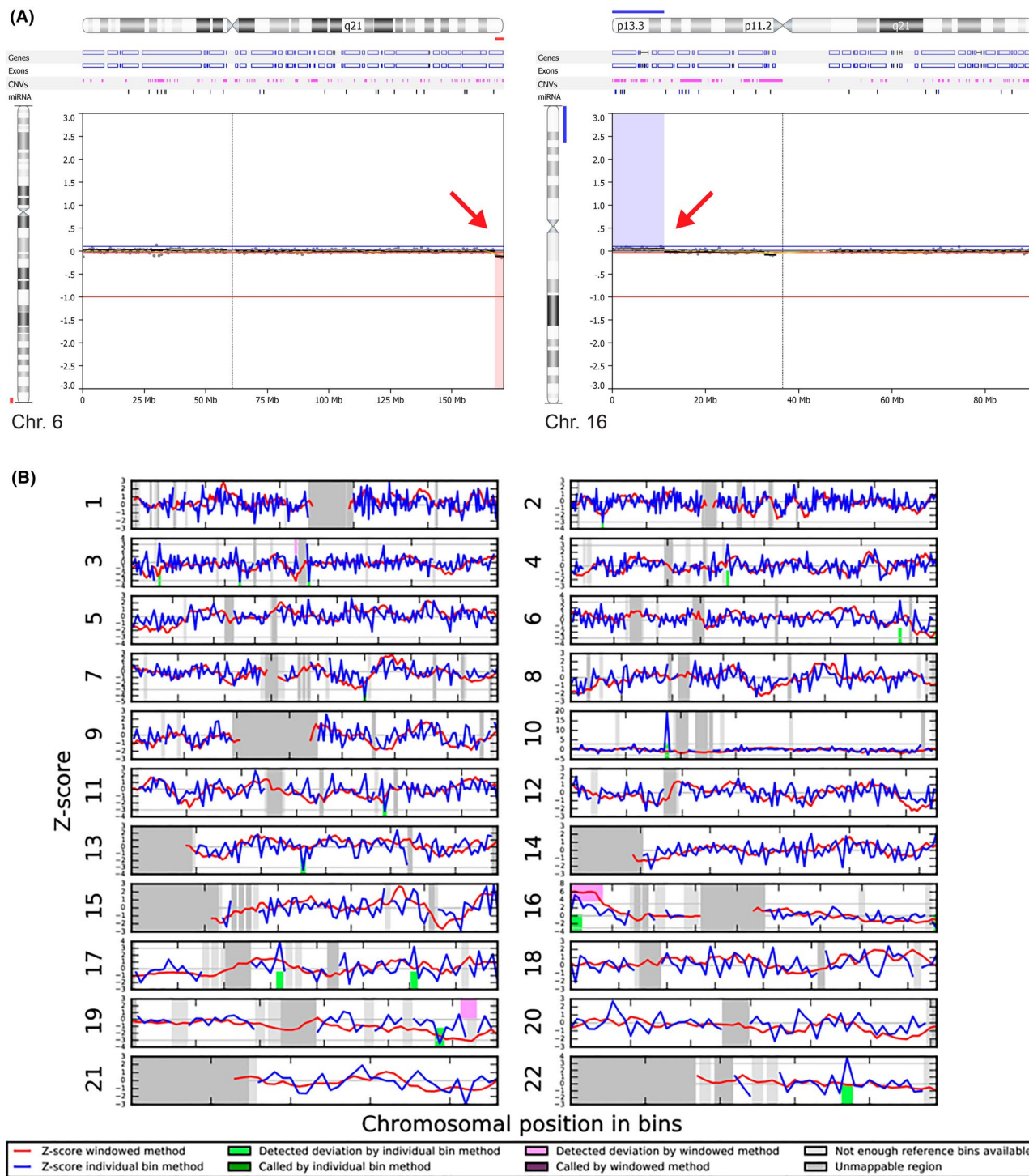


FIGURE 3 (A) Copy number analysis in NEXUS BioDISCOVERY SOFTWARE showing the small aberrations that are present in case 3 carrying a $\text{der}(6)t(6;16)(q27;p13.13)\text{pat}$. A 3.1-Mb terminal loss in the long arm of chromosome 6 as well as a 10.7-Mb terminal gain in the short arm of chromosome 16 were called by the dedicated CNV software. (B) Without prior knowledge on the carrier status, the 16p gain was recognized as technical noise because there were many samples with gains in 16p in several experiments. Retrospective analysis of the routine NIPS results in WISECONDOR only revealed the 16p gain (visual call—light pink event), but not the 6q loss

primarily designed for trisomy detection and needs to balance between resolution and the number of false-positive calls. As shown before, the resolution of our test is approximately 10–15 Mb,^{13,14} but smaller chromosome imbalances can be detected through focused

testing and higher sequence depth.²¹ This study shows that imbalances larger than 8 Mb could be detected, but smaller ones are missed by the routine NIPS without prior knowledge of the carrier status. However, instead of expensive deeper sequencing we show

that an increased analytical resolution can also be achieved by employing dedicated CNV software. A carefully selected reference set and focused (targeted) analysis in dedicated software can overcome the resolution limitations without changing the routine sequencing protocol. This approach allowed us to detect 3–5 Mb imbalanced products of chromosomal mal-segregation as early as 12–14 weeks of gestation (eg case 3). NEXUS software showed aberrations smaller than expected based on the number of reads and FF. Previously it was shown that for (highly sensitive) detection of a 10 Mb deletion, at least 10 million reads are needed in a sample with c.10% FF.²⁰ Although in case 2 with ~10 million reads even a 5-Mb deletion could be detected, it should be stressed that these small imbalances could only be identified with prior knowledge of the familial translocation allowing a focused analysis of the potential imbalances. Some of them will be visible in WISECONDOR (eg case 8), but some can only be detected by dedicated CNV software (eg 3-Mb deletion at 6qter in case 3).

Due to normal genomic variations and technical limitations, not all chromosomal regions produce reliable calls. The resolution is not only dependent on FF and sequencing depth, but on the involved chromosomal segments as well. For instance, 10qter is such a problematic region in that it may be deleted because of the presence of a fragile site in maternal DNA, which will complicate the detection of a fetal chromosome 10 aberration.²² Another example is that a 3-Mb terminal loss at 6qter (case 8) was reliably detected in NEXUS, but it was impossible to detect a 4-Mb gain of 22q11, which is known to be highly variable (case 12). It has to be noted that the algorithm that detects such small CNVs also produces significant noise, and therefore potentially produces many false-positive findings. Therefore, genome-wide high-resolution CNV analysis in cfDNA requires further optimization allowing sophisticated filtering of the background noise. Interpretation of smaller events is challenging because samples vary in FF and sequencing depth. Consequently, the same true fetal CNV can have a different mean probe log ratio or z score in different samples. That is why a future algorithm should be able to correct for FF and sequencing depth (such as Illumina VeriSeq 2, which provides additional statistical scores per event).

Because of the existence of problematic chromosomal regions, prior knowledge on the carrier status seems to be crucial for correct recognition of potentially unbalanced profiles. In our study a gain on chromosome 16p in case 4 was initially recognized as a technical false-positive event. However, with prior knowledge on the carrier status it would be reported as potentially abnormal. We currently analyze samples in WISECONDOR giving special attention to the involved chromosomal segments and if necessary we perform a targeted analysis in NEXUS BioDISCOVERY software, especially if one of the imbalances is beyond the resolution of routine WISECONDOR analysis. The WISECONDOR software viewer can also be used for specific region analysis and finally it is also possible to adjust WISECONDOR settings to improve the resolution. However, the latter was not done for the current cases, because the NIPS results were produced in the course of the Dutch TRIDENT studies and all laboratories are required to use the same settings.

Another crucial parameter is FF of the cfDNA. In our study, in which the test was performed between 12 and 14 weeks of gestation, it seems that an FF of approximately 6%–8%, as estimated by SeqFF, was sufficient to detect a loss as small as 3 Mb (case 4). These results show that early testing allowing early intervention in the case of an unbalanced result is feasible. However, the question is whether current FF measurements are reliable enough. It is known that SeqFF gives only an approximate assessed percentage of cfDNA fragments, which are assumed to be of placental origin, but in fact, it cannot distinguish true fetal cfDNA from other fragments of maternal origin. This may lead to overestimation of FF and therefore to false-negative results. The most reliable measurement of FF for both sexes is based on trio (index, mother, father) SNP analysis,²³ but this approach is not routinely used in most commercially available tests. FF measurement is especially important in samples with normal results. To ensure a sufficient amount of fetal fragments in the analyzed samples, Flowers et al suggested a minimal FF of 5% next to a deeper sequencing to achieve about 36 million reads.¹⁸ As the risks for an unbalanced live-born offspring of a couple carrying a balanced translocation is approximately 5%–10% (1%–20% depending on the unique breakpoints),¹ in prospective studies most test results will be normal. In all normal cases, sufficient FF should be measured with reliable methods, otherwise such a result has to be still recognized as screening and not a diagnosis. When FF is too low, resampling later in pregnancy might be considered, as suggested previously.^{2,18}

5 | CONCLUSION

This study supports the hypothesis that routine NIPS may be used for the prenatal diagnosis of unbalanced products of familial translocations, especially with prior knowledge of the translocation allowing a focused examination of the involved chromosomes. Our study also shows that routine shallow sequencing designed for aneuploidy detection in cfDNA may be sufficient for higher resolution NIPS if specialized copy number software is used and if sufficient FF is present.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

MIS and DVO performed the conceptualization and wrote the original draft. Data curation and formal analysis were by MIS, FSJ, MB, WGdV, RvdH, ES, EV, SB, MH, MM, MJ, NdH and DVO. Supervision was by DVO; and reviewing and editing were by MIS, FSJ, MB, WGdV, RvdH, ES, EV, SB, MH, MM and DVO.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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