Performance evaluation of the NeoBona test, a new paired-end massive parallel shotgun sequencing approach for cfDNA based aneuploidy screening

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cfDNA testing by paired-end MPSS

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Abstract

Objective: To assess the performance of screening for fetal trisomies 21, 18 and 13 by cell-free DNA (cfDNA) analysis of maternal blood using a new method based on paired-end massive parallel shotgun sequencing (MPSS).

Methods: Blind study of 1000 plasma samples (1 mL) obtained from women undergoing screening for trisomies 21, 18 and 13 at 11-13 weeks' gestation. The study included 50 cases with confirmed fetal trisomy 21, 30 with trisomy 18, 10 with trisomy 13 and 910 unaffected pregnancies. Paired-end MPSS allowed simultaneous assessment of fetal fraction, cfDNA fragment size distribution and chromosome counting, these were integrated into a new analysis algorithm to calculate Trisomy likelihood ratios (t-score) for each chromosome of interest. Each sample was classified as trisomic or unaffected using chromosome specific cut-offs set at t-score values of 1.5 for trisomy 21 and 3.0 for trisomies 18 and 13.

Results: Valid results were provided in 988 (98.8%) cases; 12 samples (1.2%), 9 euploid and 3 trisomy 21 pregnancies, did not pass QC criteria and were excluded from further analysis. All 47 cases of trisomy 21, all 10 of trisomy 13, 29 of 30 with trisomy 18 and all 901 unaffected cases were correctly classified. The median fetal fraction was 10.2% (range 0.3, 33.8%) and both

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trisomic and unaffected cases were correctly identified even at low fetal fractions of less than 1%.

Conclusions: Our method for cfDNA analysis of maternal plasma utilizing paired-end MPSS can provide accurate prediction of fetal trisomies and the use of a new multicomponent *t*-score removes the need to reject samples with fetal fraction <4% which potentially extends the benefits of cfDNA analysis to a larger proportion of pregnancies.

Introduction

Screening for fetal aneuploidies by analysis of cell-free (cf) DNA in maternal blood was made possible by the advent of Massive Parallel Shotgun Sequencing (MPSS) which allows digital counting of cfDNA fragments, either using whole genome sequencing [1,2], or targeted approaches [3,4]. Detection of fetal trisomies using counting statistics, such as Z-score or normalized chromosome values (NCV), becomes easier and more robust when the proportion of fetal to total cfDNA in maternal blood is high because of better separation between normal and aneuploid cases [5,6]. The sensitivity of detecting fetal trisomies at low fetal fraction is mostly dependent on the amount of useful counts on the chromosome of interest or sequencing depth [6,7]. Inclusion of fetal fraction in analysis algorithms can improve specificity because in cases of low Z-scores or NCVs it helps distinguish between aneuploid cases with low fetal fraction from euploid cases with a higher fetal fraction [5].

Paired-end MPSS allows accurate digital counting while also determining the length of each cfDNA fragment by sequencing its both extremities [8]. As cfDNA fragments of fetal origin are slightly shorter than maternal ones, size differences can be used to determine fetal fraction [8]. Additionally, in case of fetal aneuploidy, counting differences detected from all cfDNA fragments would appear more evident if confirmed on shorter fragments only [8,9].

The NeoBona is the first cfDNA based screening test developed to exploit paired-end MPSS through a novel bioinformatics approach, which has the advantage of combining conventional counting statistics with the distribution of cfDNA size to provide this double check of chromosome counting data. Additionally, by integrating sequencing depth on each chromosome and fetal fraction it allows calculation of a unique trisomy score (*t*-score) thereby quantifying the likelihood of fetal trisomy. The objective of this study is to evaluate the performance of this new method on a large blinded set of archived maternal plasma samples tested without previous knowledge of their outcomes.

Methods

Study population

Samples were collected at King's College Hospital, London, U.K., from singleton pregnancies undergoing screening for trisomies 21, 18 and 13 by a combination of fetal nuchal translucency thickness and maternal serum free ß-hCG and PAPP-A at 11-13 weeks' gestation [10]. Gestational age was determined from the measurement of the fetal crown-rump length [11]. Women with a high-risk from the combined test had chorionic villus sampling for fetal karyotyping. Karyotype results obtained from genetic laboratories and details on pregnancy outcomes obtained from the maternity computerized records or the general medical practitioners of the women, were added into the database as soon as they became available. All patients gave written informed consent to provide samples for research, which was approved by the NHS Research Ethics Committee.

Blood samples were collected into EDTA BD vacutainer^{TM} tubes (Becton Dickinson UK limited, Oxfordshire, UK) and centrifuged at 2,000g for 10 min within 15 minutes of collection (plasma 1) followed by 10 min at 16,000g to further separate cell debris (plasma 2). Samples of Plasma 1 and 2 were divided into 0.5 mL aliquots in separate Eppendorf tubes, labelled with a unique patient identifier and stored at −80^{o}C for up to 9 years until analysis. A total of 50 trisomy 21 cases, 30 trisomy 18, 10 trisomy 13 and 910 normal controls were selected with 1 mL of

available plasma 1 or 2. Aneuploidies were selected at random and each case was matched to 11-12 controls that were sampled on the same or next day. Normal controls were uncomplicated pregnancies resulting in live birth after 38 weeks' gestation of phenotypically normal neonates assumed to be euploid. Maternal bloods were collected between April 2006 and February 2015, none of the samples were previously thawed and refrozen. Plasma samples (2 tubes of 0.5 mL per patient) were coded and sent on dry ice from London to the central laboratory of Labco Diagnostics in Barcelona, Spain, where blinded cfDNA analysis was performed using the NeoBona test.

Analysis of samples

The only information regarding each sample provided to the laboratory was patient-unique identifier, date of collection and plasma 1 or plasma 2. Each case was assessed for volume, adequacy of labelling and risk of contamination or sample mixing before evaluation of fetal trisomy. Although in 60 of the 1000 samples the volume was less than 1 mL (500 -950 µL) these were included in the analysis. Plasma samples from each patient were collected into 96 deep well plates. Plates of plasma 1 samples underwent a second centrifugation step at 16.000g before DNA extraction. Samples were processed in batches of 96 using VeriSeq NIPT v1.0 chemistry (Illumina Inc, San Diego, US) on a fully automated workstation (Hamilton Star, Hamilton, Reno, US) designed to handle plasma isolation, column based DNA extraction, set up of sequencing library, quantification, normalization and pooling. Sequencing libraries from each batch of 96 samples were collected in two separate pools of 48 double indexed samples which underwent paired-end MPSS for two sets of 36 cycles using NextSeq 500 and 550 sequencers with TG NextSeq 500/550 High Output Kit v1.2 (Illumina inc, San Diego, US). Sequencing outputs were analyzed using the VeriSeq NIPT software v1.0 (Illumina inc, San Diego, US).
After de-multiplexing and filtering, sequence alignment was performed against HG19 for data normalization and inter-chromosome comparisons [7]. Regions affected by poor alignment were filtered out and further normalization was applied based on a principal component decomposition as described in Zhao *et al.* [12]. Fetal fraction assessment based on molecular size distributions and differences in coverage between fetal and maternal cfDNA, was complemented with X and Y chromosomes data in case of male fetuses [8,9,13]. NCVs were calculated for chromosomes 13, 18 and 21 as previously described [6,14]. NCV counting statistics are similar in principle to the conventional Z-score, with fixed cut-off of around 3.0 to discriminate between trisomic and unaffected pregnancies, the main difference for NCVs being that each chromosome of interest is only normalised against a specific set of chromosomes, optimised for comparable sequencing coverage to minimise variations.
Trisomy likelihood ratios (*t*-score) for each chromosome of interest were calculated for each sample based on the estimated fetal fraction, counting statistics (NCVs) derived from both total and short DNA fragments, and sequencing depth. The likelihood ratio reflects the probability for a sample to be affected given the observed counting statistics and fetal fraction, versus the probability of a sample to be unaffected given the same counting data. Thus, using this analysis approach, trisomic samples with low fetal fraction can result in a higher *t*-score if they have, for instance, a higher depth of sequencing enabling efficient counting on short DNA fragments which are mostly of fetal origin.
Samples were classified as being compatible with the presence or absence of trisomy 21, 18 or 13 using predefined chromosome specific cut-offs at *t*-score values of 1.5 for trisomy 21 and 3.0 for trisomies 18 and 13.
Analysis QC were applied to monitor sequencing depth, the distribution of cfDNA fragments sizes, sequencing coverage for chromosome denominators and for the estimate of fetal fraction. Results were considered valid only for samples passing all QCs.

Results were provided to King's College Hospital where the classification for each case was compared to pregnancy outcome and detection and false positive rates were estimated.

Results

The characteristics of the study population are summarized in Table 1. In trisomy 21, compared to euploid pregnancies, the median maternal age, fetal NT, serum free ß-hCG were higher and serum PAPP-A was lower; in trisomies 18 and 13, the median maternal age and fetal NT were higher and serum free ß-hCG and PAPP-A were lower.

The cfDNA test provided results for 988 cases (98.8%). Twelve samples in total (1.2%), 9 euploid and 3 trisomy 21 pregnancies, failed to provide a result and were excluded from further analysis. The reasons for QC failure were first, size distribution of cfDNA fragments outside of the expected range (n=6), second, low sequencing depth for the observed fetal fraction (n=4), third, unusually high DNA concentration (n=1), and fourth, insufficient sequencing coverage for the determination of fetal fraction (n=1).

The cfDNA test correctly classified all 47 pregnancies with fetal trisomy 21, all 10 with trisomy 13, 29 of 30 (96.7%) with trisomy 18 and all 901 unaffected pregnancies (Table 2). In one case of trisomy 18 *t*-score and NCV values for chromosome 18 were compatible with normal chromosome copy number; in this case the fetal fraction was 11%. One trisomy 21 and one unaffected pregnancy had the same NCV of 3.5, while differing in t-scores for trisomy 21 which were 10 and -14, respectively. Therefore, using the predefined cut-offs at *t*-score values of 1.5 for trisomy 21 and 3.0 for both trisomy 18 and 13 resulted in detection rates of 100% for trisomies 21 and 13 and 96.7% for trisomy 18 with false positive rate of 0% for all trisomies.

The mean fetal fraction was 10.6% for euploid pregnancies, 11.1% for trisomy 21, 9.4% for trisomy 18 and 8.9% for trisomy 13 (Table 2). One case of trisomy 21, three of trisomy 18 and 58 unaffected pregnancies were correctly identified despite showing fetal fractions below 4%, including 1 trisomy 18 and 9 euploid cases with fetal fractions lower than 1% (Table 2 and Figure 1).

Discussion

The findings of this study demonstrate the feasibility of a new approach for cfDNA testing of maternal blood in screening for fetal trisomies 21, 18 and 13. Paired-end MPSS of cfDNA coupled with a novel analysis algorithm provided simultaneous assessment of fetal fraction, distribution of size of DNA fragments and chromosome counting. Trisomy likelihood ratios for each chromosome of interest could than be calculated for each sample based on the estimated fetal fraction, chromosome specific counting statistics on total and short fragments and sequencing depth. We used this novel approach to examine stored plasma samples and at preselected chromosome specific cut-offs at *t*-score values of 1.5 for trisomy 21 and 3.0 for trisomies 18 and 13, the test correctly classified all cases of trisomy 21, trisomy 13 and unaffected pregnancies and 29 of 30 cases of trisomy 18. Such high performance of screening is compatible with the results of the best of previous studies utilizing cfDNA testing to screen for trisomies 21, 18 and 13 [15].

In the only case of trisomy 18 misclassified the fetal fraction was 11%, it is therefore highly unlikely that this error was related to technical issues affecting test sensitivity. Unfortunately, no more sample was available to repeat the test and exclude errors due to laboratory mishandling.

Also trisomy rescue generating a normal cell line in the cytotrophoblast could not be definitely ruled out as the underlying cause of this discrepancy, as prenatal diagnosis was only performed on long term CVS culture by QF-PCR and karyotype but not on direct preparation.

The basis for cfDNA testing using counting methods is that in trisomic pregnancies the number of molecules derived from the extra fetal chromosome, as a proportion of all sequenced molecules in maternal plasma, is higher than in euploid pregnancies. The ability to detect the small increase in the amount of a given chromosome in maternal plasma in a trisomic compared to a disomic pregnancy is directly related to the fetal fraction and the depth of sequencing [3,16-19]. Trisomy cases with low fetal fractions used to be much more difficult to discriminate from normal samples by counting statistics only as they can produce NCVs having similar values to those occasionally observed in normal samples with higher FF [6], thus reducing test specificity. Also the sensitivity could be affected if, for the sequencing depth used, the proportion of fetal cfDNA is too low to allow discrimination of trisomies by counting statistics only [7, 18].
For these reasons, when the fetal fraction is below 4%, which occurs in 0.5-6.1% of pregnancies, the cfDNA test is usually presented as a failure and no result is reported [15].

Some of the problems due to low fetal fraction have now been overcome by the application of the multicomponent *t*-score, as the resolution in discriminating between trisomic and unaffected pregnancies is no longer dependent only on fetal fraction but also on the new possibility of performing additional counting statistics on short DNA fragments, which are mostly of fetal origin. Consequently, trisomic pregnancies with low fetal fraction could result in higher *t*-score than pregnancies with higher fetal fraction and lower total sequencing depth or less efficient counting statistics on short fragments. This approach proved to be highly efficient at low fetal cfDNA amounts, as all 4 aneuploid cases with FF between 0.8 and 3.5% could also be detected.
The effectiveness of the new multicomponent *t*-score to improve overall specificity was evident in one case of trisomy 21 and one unaffected sample which were correctly classified despite generating the same NCV, thus undistinguishable by conventional MPSS analysis algorithms.

Despite testing archived plasma samples and with suboptimal volumes in 6% of cases, failure to provide a result was only observed in 1.2% of samples. The most common reason for test failure was an abnormal distribution of size of cfDNA fragments, which affected size based counting and the measurement of fetal fraction. This artefact was likely to be caused by cfDNA shearing resulting from sample degradation, it is therefore expectable to be less frequent in routine clinical samples collected in dedicated tubes, designed to prevent cell lysis and stabilize cfDNA. Four more samples failed the QC which combines sequencing metrics and estimated fetal fraction, thus determining if the analysis output has statistical confidence in scoring a sample. Repeating the test on a second aliquot of the same plasma would probably have yielded a valid result. In clinical routine, when the test is usually performed within a few days from sampling and with enough volume to be repeated, also this technical failure is expected to decrease.

The novel approach presented in this study has the potential of extending the advantages of cfDNA based aneuploidy screening to a wider proportion of pregnancies. Complementing conventional counting statistics with size based chromosome counting and fetal fraction ensured that accurate prediction of trisomic status was provided in the 62 of our cases with fetal fraction <4%. Consequently, it is no longer necessary to exclude samples from analysis just because the fetal fraction is <4% if enough sequencing depth is reached for the correspondent amount of cfDNA and size based counting is performed at the same time.

References

1. Chiu RWK, Chan KCA, Gao Y, Lau VYM, Zheng W, LeungTY, Foo CHF, Xie B, Tsui NBY, Lun FMF, Zee BCY, LauTK, Cantor CR, Lo YMD. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. *Proc Natl Acad Sci USA* 2008; **105**: 20458–20463.

2. Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proc Natl Acad Sci USA* 2008; **105**: 16266–162271.

3. Sparks AB, Struble CA, Wang ET, *et al.* Noninvasive prenatal detection and selective analysis of cell-free DNA obtained from maternal blood: evaluation for trisomy 21 and trisomy 18. *Am J Obstet Gynecol* 2012; **206**: 319.e1–9.

4. Nicolaides KH, Syngelaki A, Ashoor G, Birdir C, Touzet G. Noninvasive prenatal testing for fetal trisomies in a routinely screened first-trimester population. *Am J Obstet Gynecol* 2012; **207**: 374.e1-6.

5. Yeang CH, Ma GC, Hsu HW, Lin YS, Chang SM, Cheng PJ, Chen CA, Ni YH, Chen M. Genome-wide normalized score: a novel algorithm to detect fetal trisomy 21 during non-invasive prenatal testing. *Ultrasound Obstet Gynecol* 2014; **44**: 25-30.

6. Rava RP, Srinivasan A, Sehnert AJ, Bianchi DW. Circulating fetal cell-free DNA fractions differ in autosomal aneuploidies and monosomy X. *Clin Chem* 2014; **60**: 243-250.

7. Fan CH, Quake SR. Sensitivity of Noninvasive Prenatal Detection of Fetal Aneuploidy from Maternal Plasma Using Shotgun Sequencing Is Limited Only by Counting Statistics. *PLoS ONE* 2010; **5**: e10439

8. Yu SC, Chan KC, Zheng YW, Jiang P, Liao GJ, Sun H, Akolekar R, Leung TY, Go AT, van Vugt JM, Minekawa R, Oudejans CB, Nicolaides KH, Chiu RW, Lo YM. Size-based molecular diagnostics using plasma DNA for noninvasive prenatal testing. *Proc Natl Acad Sci USA* 2014; **111**: 8583-8588.

9. Kim SK, Hannum G, Geis J, Tynan J, Hogg G, Zhao C, Jensen TJ, Mazloom AR, Oeth P, Ehrich M, van den Boom D, Deciu C. Determination of fetal DNA fraction from the plasma of pregnant women using sequence read counts. *Prenat Diagn* 2015; **35**: 810-815.

10. Nicolaides KH. Screening for fetal aneuploidies at 11 to 13 weeks. *Prenat Diagn* 2011; **31**: 7-15.

11. Robinson HP, Fleming JE. A critical evaluation of sonar crown rump length measurements. *Br J Obstet Gynaecol* 1975; **82**: 702-710.

12. Zhao C, Tynan J, Ehrich M, Hannum G, McCullough R, Saldivar JS, Oeth P, van den Boom D, Deciu C. Detection of fetal subchromosomal abnormalities by sequencing circulating cell-free DNA from maternal plasma. *Clin Chem* 2015; **61**: 608-616.

13. Fan CH, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Analysis of the size distributions of fetal and maternal cell-free DNA by paired-end sequencing. *Clin Chem* 2010; **56**: 1279-1286.

14. Sehnert AJ, Rhees B, Comstock D, de Feo E, Heilek G, Burke J, Rava RP. Optimal detection of fetal chromosomal abnormalities by massively parallel DNA sequencing of cell-free fetal DNA from maternal blood. *Clin Chem* 2011; **57**: 1042-1049.

15. Gil MM, Quezada MS, Revello R, Akolekar R, Nicolaides KH. Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: updated meta-analysis. *Ultrasound Obstet Gynecol* 2015; **45**: 249-266.

16. Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE, Neveux LM, Ehrich M, van den Boom D, Bombard AT, Deciu C, Grody WW, Nelson SF, Canick JA. DNA sequencing of maternal plasma to detect Down syndrome: An international clinical validation study. *Genet Med* 2011; **13** : 913-920.

17. Canick JA, Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE. The impact of maternal plasma DNA fetal fraction on next generation sequencing tests for common fetal aneuploidies. *Prenat Diagn* 2013; **33** : 667–674.

18. Benn P, Cuckle H. Theoretical performance of non-invasive prenatal testing for chromosome imbalances using counting of cell-free DNA fragments in maternal plasma. *Prenat Diagn* 2014; **34** : 778-783.

19. Wright D, Wright A, Nicolaides KH. A unified approach to risk assessment for fetal aneuploidies. *Ultrasound Obstet Gynecol* 2015; **45**: 48-54.

Table 1. Characteristics of the study population.

Characteristics	Euploid n=910	Trisomy 21 n=50	Trisomy 18 n=30	Trisomy 13 n=10
Maternal age in years, median (IQR)	31.9 (27.3-34.9)	37.9 (35.3-41.3)	35.4 (28.8-40.5)	33.5 (30.0-34.9)
Maternal weight in kg, median (IQR)	65.0 (59.0-75.0)	68.0 (60.7-75.0)	66.8 (60.4-75.5)	64.5 (60.6-64.9)
Maternal height in cm, median (IQR)	165 (160-169)	166 (161-172)	166 (160-171)	167 (164-170)
Racial origin				
Caucasian, n (%)	563 (61.9)	41 (82.0)	17 (56.7)	8 (80.0)
Afro-Caribbean, n (%)	244 (26.8)	6 (12.0)	6 (20.0)	1 (10.0)
South Asian, n (%)	32 (3.5)	1 (2.0)	3 (10.0)	0
East Asian, n (%)	26 (2.9)	2 (4.0)	2 (6.7)	0
Mixed, n (%)	45 (4.9)	0	2 (6.7)	1 (10.0)
Cigarette smoker, n (%)	55 (6.0)	3 (6.0)	1 (3.3)	1
Method of conception				
Spontaneous, n (%)	880 (96.7)	46 (92.0)	26 (86.7)	10 (100)
Ovulation drugs, n (%)	9 (1.0)	3 (6.0)	2 (6.7)	0
In vitro fertilization, n (%)	21 (2.3)	1 (2.0)	2 (6.7)	0
Crown rump length in mm, median (IQR)	61.8 (57.0-67.6)	66.1 (60.0-73.0)	56.1 (51.9-61.6)	59.0 (51.1-63.1)
Gestational age in weeks, median (IQR)	12.6 (12.2-13.0)	12.9 (12.5-13.4)	12.2 (11.8-12.6)	12.4 (11.8-12.7)
Fetal nuchal translucency, median (IQR)	1.7 (1.5-1.9)	4.4 (3.4-6.2)	6.5 (3.6-7.9)	5.2 (2.3-6.3)
PAPP-A MoM, median (IQR)	1.126 (0.766-1.563)	0.695 (0.441-0.869)	0.227 (0.135-0.327)	0.371 (0.282-0.570)
Free ß-hCG MoM, median (IQR)	0.995 (0.678-1.582)	2.259 (1.574-3.109)	0.293 (0.171-0.362)	0.314 (0.204-0.747)

IQR: Interquartile range; PAPP-A = pregnancy associated plasma protein-A; ß-hCG = ß-human chorionic gonadotropin; MoM = Multiple of the median.

Table 2. Results of cfDNA analysis.

Result	**Euploid n=901**	**Trisomy 21 n=47**	**Trisomy 18 n=30**	**Trisomy 13 n=10**
Normalized chromosome value (NCV) for chromosome 21, median (range)	-0.01 (-3.43, 3.55)	11.50 (3.59, 25.67)	0.32 (-1.88, 3.48)	-0.048 (-1.36, 1.25)
t-score for trisomy 21, median (range)	-23.2 (-1074.2, 0.6)	101.0 (7.2, 392.1)	-12.0 (-178.4, -1.1)	-20.5 (-121.8, -5.2)
Normalized chromosome value (NCV) for chromosome 18, median (range)	0.01 (-3.25, 6.17)	-0.24 (-2.42, 2.71)	12.24 (-1.22, 36.91)	0.72 (-1.49, 2.56)
t-score for trisomy 18, median (range)	-31.3 (-1960.6, 1.0)	-38.6 (-322.2, -3.6)	94.5 (-17.9, 765.2)	-21.9 (-247.2, -7.2)
Normalized chromosome value (NCV) for chromosome 13, median (range)	0.001 (-4.55, 4.44)	-0.09 (-1.91, 2.58)	0.42 (-2.30, 2.16)	14.76 (6.31, 28.50)
t-score for trisomy 13, median (range)	-37.6 (-2591.6, 0.1)	-48.6 (-449.6, -1.3)	-15.2 (-442.6, 2.0)	209.3 (23.9, 479.5)
Fetal fraction, median (range)	10.2 (0.3, 33.8)	10.7 (3.8, 19.8)	9.6 (0.8, 23.0)	7.9 (4.0, 15.3)
Trisomy 21 *t*-score > 1.5, n (%)	0	47 (100)	0	0
Trisomy 18 *t*-score > 3.0, n (%)	0	0	29 (96.7)	0
Trisomy 13 *t*-score > 3.0, n (%)	0	0	0	10 (100)

Figure legend

Normalized chromosome values (left) and *t*-score (right) for Chromosome 18 in 29 trisomy 18 (red circles) and 901 unaffected (open circles) pregnancies, plotted against fetal fraction. The plot on normalized chromosome values illustrates the limitation of this method because the values of two cases of trisomy 18 (arrows) at low fetal fraction <4% were similar to those of two unaffected cases (oval) at high fetal fraction, these would have been wrongly classified as positive being above the cut-off of 3.0 (interrupted horizontal line). The plot on *t*-scores demonstrates that all cases of trisomy 18 have a value well above the cut-off of 3.0 for trisomy 18 (interrupted horizontal line), including one case with FF<1%.

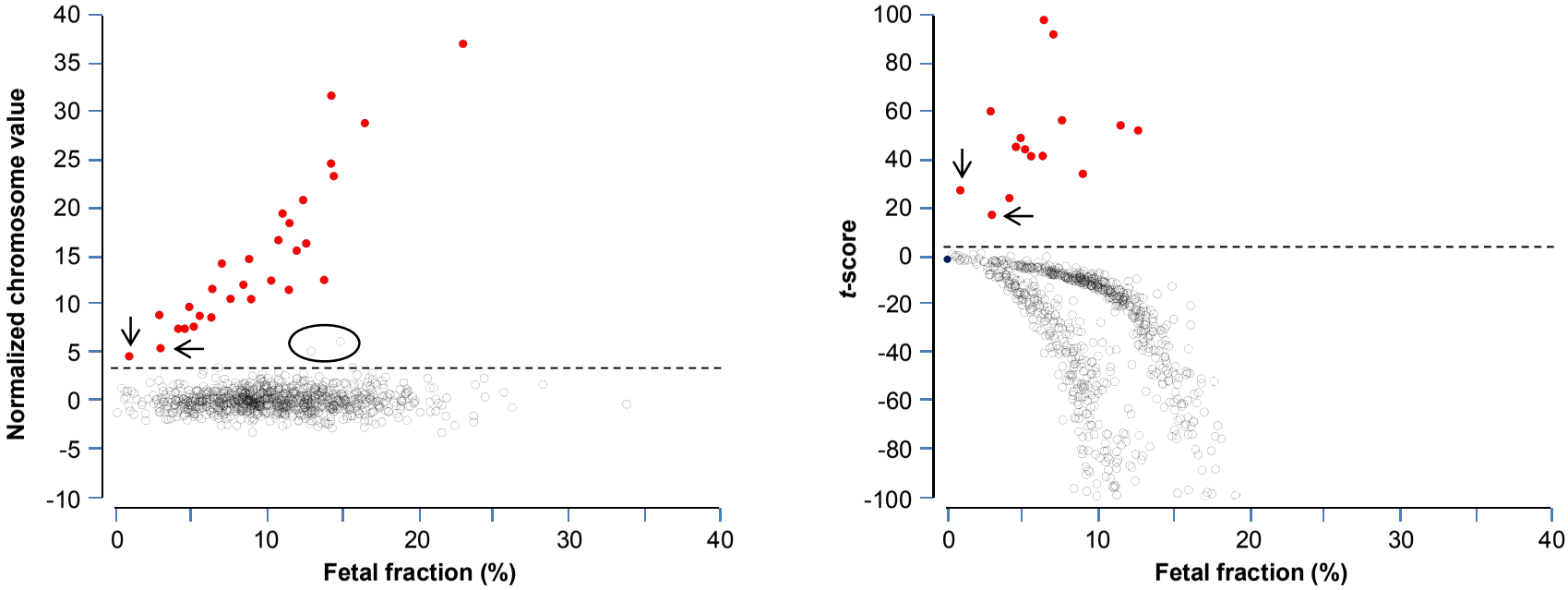

Figure 1