Review

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Non-invasive prenatal diagnostics of aneuploidy using next-generation DNA sequencing technologies, and clinical considerations

Abstract

Rapidly developing next-generation sequencing (NGS) technologies produce a large amount of data across the whole human genome and allow a large number of DNA samples to be analyzed simultaneously. Screening cell-free fetal DNA (cffDNA) obtained from maternal blood using NGS technologies has provided new opportunities for non-invasive prenatal diagnosis (NIPD) of fetal aneuploidies. One of the major challenges to the analysis of fetal abnormalities is the development of accurate and reliable algorithms capable of analyzing large numbers of short sequence reads. Several such algorithms have recently been developed. Here, we provide a review of recent NGS-based NIPD methods as well as the available algorithms for short-read sequence analysis. We furthermore introduce the practical application of these algorithms for the detection of different types of fetal aneuploidies, and compare the performance, cost and complexity of each approach for clinical deployment. Our review identifies several main technologies and trends in NGS-based NIPD. The main considerations for clinical development for NIPD and screening tests using DNA sequencing are: accuracy, intellectual property, cost and the ability to screen for a wide range of chromosomal abnormalities and genetic defects. The cost of the diagnostic test depends on the sequencing method, diagnostic algorithm and volume of the tests. If the cost of sequencing equipment and reagents remains at or around current levels, targeted approaches for sequencing-based aneuploidy testing and SNP-based methods are preferred.

Keywords: aneuploidy; cell-free fetal DNA; Down syndrome; fetal aneuploidies; next-generation sequencing; non-invasive prenatal diagnosis; prenatal screening.

Introduction

To date, invasive procedures, such as amniocentesis and chorionic villus sampling (CVS) have been used successfully for the detection of fetal aneuploidy in high-risk pregnancies. However, invasive methods are associated with significant risks of the fetal loss – a major adverse consequence of prenatal diagnosis in obstetric practices [1]. In 1997, a research paper on the discovery of circulating cffDNA from the Y chromosome of male fetuses in the maternal blood stream during pregnancy opened up new horizons for NIPD [2]. Since then there have been numerous studies describing the use of cffDNA for NIPD of chromosomal aneuploidy; in particular, trisomies 21 (Down syndrome), 18 (Edward syndrome) and 13 (Patau syndrome), all of which used digital PCR analysis [3–5].

The appearance of cffDNA in the maternal circulation occurs when normal placental cell death causes the chromosomes to break into short fragments, most of which are under 300 bp in length [6–8]. The proportion of cffDNA in maternal blood increases as pregnancy progresses. cffDNA comprises around 5%–10% of the total cell-free DNA during the first and second trimesters and can be detected reliably as early as the seventh week...
of gestation [2, 9]. Circulating cffDNA is rapidly cleared from maternal blood after delivery, except for cases where small amounts remain, including cells from previous pregnancies [10]. It has recently been found that the entire fetal genome, in the form of cffDNA, is present in maternal blood [11].

New NGS technologies permit the simultaneous, or ‘massively parallel’, sequencing of extremely large quantities of DNA molecules. NGS produces billions of short sequence reads per instrument run [12]. Massively parallel sequencing of fetal DNA from maternal blood has enormous potential, not only for increasing our understanding of the causes of prenatal genetic disorders in the fetus but also for designing non-invasive clinical diagnostic tests [11]. At the moment, non-invasive methods do not detect other genetic abnormalities, such as SNP and indel variations causing Mendelian disorders, and invasive procedures are thus still required. Non-invasive methods of fetal genotyping are currently in development, and accurate screening of both dominant and recessive single gene disorders may be possible in clinical practice in the near future [13]. The possibility of using massively parallel shotgun sequencing to detect non-invasive fetal trisomies from maternal blood by analyzing the relevant chromosomes in locus-independent assays has been demonstrated [14, 15], and recent studies have confirmed this finding [16, 17]. An alternative approach to sequencing whole genomes for non-invasive detection of fetal abnormalities would be to enrich for the region of interest using array capture prior to sequencing [18–20].

NGS technologies have already begun to show their remarkable potential for detecting the most common aneuploidies in live births, including Down, Edward, and Patau syndromes. These research discoveries have been translated into clinical tests, resulting in major benefits for NIPD. In this review, we describe different approaches to non-invasive detection of prenatal aneuploidy which, for their clinical application, use the NGS technologies of four innovative companies. The companies Sequenom, Inc (San Diego, CA, USA) and Verinata Health, Inc (Redwood City, CA, USA) offer methods based on collecting and analyzing information across the entire genome, while the approach of Ariosa Diagnostics, Inc (San Jose, CA, USA) and Natera, Inc (San Carlos, CA, USA) is based on the selection of only the chromosomes of interest. Both approaches are capable of detecting the most common fetal aneuploidies in the population. To date of submission of this manuscript, three of the aforementioned companies – Sequenom, Verinata Health, and Ariosa Diagnostics – have published clinical validation studies. These companies have furthermore launched cffDNA-based non-invasive prenatal laboratory developed tests (LDT) using next-generation sequencing through the Clinical Laboratory Improvement Amendments (CLIA) laboratories. Several clinical diagnostics laboratories in other countries have also launched NIPD screening tests using NGS, including the Beijing Genomics Institute in China. Although every company has sponsored clinical validation studies and published their results in peer-reviewed, high-impact industry journals, none of the tests are approved by the US Food and Drug Administration (FDA). Although tests and clinical validation studies are still ongoing, it is expected that the diagnostic accuracy, sensitivity, and specificity of these techniques will be very high.

**Limitations of current methods of prenatal diagnostics**

Many prenatal screening and diagnostic tests have been developed and introduced into clinical practice. While standard methods of prenatal care vary, combination screening using biochemical markers in serum, including pregnancy-associated plasma protein A (PAPP-A), free β-subunit of human chorionic gonadotropin (free β-hCG), α-fetoprotein (AFP) and unconjugated estriol (uE3), in conjunction with a sonographic measurement of nuchal translucency and detection of the presence or absence of the nasal bone, are commonly used. The detection rate of non-invasive combination screening has improved greatly over the past decade to over 90% in the first trimester of pregnancy [21]. However, achieving such a high detection rate requires exceptionally skilled physicians and strict adherence to protocols. Invasive diagnostic methods are still recommended for high-risk pregnancies.

Currently, diagnostic testing requires that the fetal cells that are to be tested be removed directly from the uterus using either CVS between 11 and 14 weeks of gestation, or amniocentesis between 15 and 20 weeks. While using direct fetal material provides over 99% accuracy, both of these procedures are invasive and are associated with an increased risk of transplacental hemorrhage and spontaneous abortion [22]. The risk of miscarriage following these procedures can be conservatively estimated at around 1% [23], but this varies greatly depending on a number of human and environmental factors including the country, the hospital or clinic, and the physician.
Whole-genome sequencing approaches for NIPD of fetal aneuploidy

In cases where a woman is carrying a fetus with aneuploidy – trisomy 21, e.g., the amount of copies of chromosome 21 is expected to be slightly higher in comparison with other autosomes. Rapidly developing NGS technologies, which provide a vast amount of data across the entire genome, appear to be suitable for counting genome representation and determining the over-represented chromosomes of interest in the affected fetus. Two scientific groups have independently shown that, from cffDNA obtained from maternal blood, NGS can clearly identify plasma samples from women carrying aneuploid fetuses by comparing them with samples taken from women with euploid fetuses [14, 15]. A short region at one end of each DNA molecule of maternal plasma was sequenced using synthesis technology on an Illumina platform, and mapped against the reference human genome to determine the chromosomal origin of each sequence. The density of sequenced tags from the chromosome of interest from an aneuploid fetus was compared with cases of trisomy and euploid pregnancies (Figure 1).

Fan et al. [14] used from 1.3 to 3.2 mL of plasma taken from pregnant women at risk for fetal aneuploidy. Their study examined a total of 18 normal and aneuploid pregnancies, which included nine cases of trisomy 21, two of trisomy 18 and one of trisomy 13. Amniocentesis or CVS was conducted to analyze and confirm the fetal karyotype. Cell-free plasma DNA was sequenced on the 1G Genome Analyzer platform (Solexa/Illumina, Inc, San Diego, CA, USA). Each of the 25 bp reads were mapped against the non-repeat-masked reference human genome build 36 (hg18) using ELAND from the Solexa data analysis pipeline. Sequencing resulted in approximately five million unique sequence reads per patient sample with, at most, one mismatch against the human genome. Each set of patient sequence reads covered approximately 4% of the entire human genome. The average sequence read density for 50-kb windows across the chromosome of interest was normalized using the median value obtained from all autosomes in the euploid cases. A 99% confidence interval in the distribution sequence read density of the chromosome of interest from an aneuploid fetus was compared with cases of trisomy and euploid pregnancies (Figure 1).

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The GC content of the sequenced reads of all the samples was not uniform. In addition, the coverage of chromosome 21 for trisomy 21 pregnancies was on average ~11% higher than that of the disomy 21 cases. The method described by Fan et al. reveals that the minimum fraction of fetal DNA that could be detected by NGS is approximately 2%.

The distribution of sequence reads across each chromosome for all samples was not uniform. In addition, the GC content of the sequenced reads of all the samples was ~10% higher than the value of the sequenced human genome [24]. The mean read density of chromosomes, which was reported to correlate with the GC content of the chromosomes, was observed to have a positive bias for chromosomes with high GC content. Interestingly – and differing from this report – Chiu et al. [25] observed a negative correlation between chromosome representation and GC content in their analysis employing massively parallel sequencing of fetal DNA from maternal plasma using ligation technology on the SOLiD™ 3 platform (Applied Biosystems/Life Technologies, Carlsbad, CA, USA). In both sets of data, a strong GC bias and the non-uniform representation of read density between chromosomes are likely to be explained by analytical rather than biological factors stemming from the sequencing process, which would be eliminated by the use of appropriate algorithms [26–28].

Importantly, blood samples were collected from 15 to 30 min after amniocentesis or CVS, and the average gestational period of the aneuploidy pregnancies (20.6 weeks) was longer than that of the euploid group (13.8 weeks). Since the proportion of fetal DNA in maternal blood was reported to increase with the length of gestation age [29, 30], the above-mentioned factors should be taken into consideration in future analyses. Nonetheless, this pioneering work has shown that NGS could be used as a non-invasive prenatal diagnostic tool for the quantitative measurement of a number of chromosomes.

Chiu et al. analyzed cell-free DNA from 5 to 10 mL of plasma taken from 14 trisomy 21 and 14 euploid pregnancies in women at risk for fetal aneuploidy [15]. In the majority of cases, maternal blood samples were collected before invasive obstetrics procedures had been carried out, with comparable median lengths of gestation for both the euploid and the trisomy case groups. One end of the clonally expanded copies of each plasma DNA fragment was sequenced (36 bp reads) and processed by standard post-sequencing bioinformatic alignment analysis with the Illumina Genome Analyzer, which uses ELAND software. The obtained mean of approximately 2 million unique reads per sample resulted in no mismatches with the repeat-masked reference human genome (NCBI Build 36, version 48). The number of reads originating from chromosomes of interest was normalized by the total number of reads generated by the sequencing run. Z-scores representing the number of standard deviations from the mean proportion of chromosomes-of-interest reads in a reference set of euploid cases were calculated for each case. A statistically significant difference between the parameter estimated in the test case and that in the reference group was 99% under z-score >3. Z-scores for chromosome 21 were above +5 (range 5.03–25.11) for all 14 trisomy 21 cases, i.e., at three standard deviations above the reference established from the male euploid fetuses. In the study, the method accurately detected all trisomy 21 cases and produced no false-positive results.

One caveat of using this approach is that the distribution of reads on each chromosome can vary from sequencing run to sequencing run; hence, these intra- and inter-run sequencing variations can increase the overall variation in the aneuploidy detection metric.

Both studies demonstrated that massively parallel sequencing can both identify and count DNA fragments in maternal plasma to detect small quantitative alterations in the genomic distribution of plasma DNA. Methods that do not require the differentiation between fetal and maternal DNA are currently being developed, and can be applied to arbitrarily small concentrations of fetal DNA in maternal plasma. Since fetal DNA is natively fragmented, no further fragmentation step for the library preparation is needed, substantially simplifying subsequent analysis.

Non-invasive NGS detection of the variation in the number of copied fetal chromosomes in maternal plasma is already in use on pregnant women in proof-of-concept studies, and has been brought into clinical application. A number of subsequent large-scale clinical studies were carried out by academic research institutions and biotechnology companies to validate various approaches to NIPD using massively parallel genomic sequencing for use in clinical practice [16, 17, 31–33]. Table 1 presents a summary of these studies for detection of Down syndrome.

### Targeted approaches to sequencing-based NIPD of fetal aneuploidy

The two methods described above rely on the massively parallel sequencing of all the DNA in a maternal plasma
sample without targeting specific chromosomes. However, several targeted approaches were recently developed based on the a priori selection of DNA regions for analysis. Compared to sequencing and counting all reads from all chromosomes, limiting the number of DNA regions to quantify greatly reduces the effort required to assess the dosage of a chromosome. Moreover, careful selection of the regions to quantify can potentially reduce the confounding variation in the number of reads per locus by taking into account only the loci with similar properties, e.g., GC content or the number of repeats of a particular sequence [19, 20].

Different strategies can be used to select and enrich for the genomic regions of interest. The enrichment step should meet two requirements: the method should minimize introductory bias (i.e., the output quantity of a fragment should depend only on its input quantity and not on other factors, such as the features of its sequence) to allow further quantitative analysis, and the method should be capable of dealing with small amounts of sampled DNA [34]. Using the SureSelect System (Agilent Technologies, Santa Clara, CA, USA), Liao et al. successfully performed a 213-fold enrichment of the selected loci of cfDNA from the blood samples of pregnant women [35] as an example of an in-solution, separation-based enrichment technique [34]. The enrichment did not introduce any bias in the ratio of maternal and fetal DNA.

Sparks et al. [19] describe a method for detecting chromosome aneuploidy using NGS-sequencing of DNA enriched by a so-called Digital ANalysis of Selected Regions amplification-based enrichment assay. The assay comprises three oligos per analyzed locus. Ligating oligos, “Left”, “Middle”, and “Right” can complementary hybridize to the respective DNA loci, thus forming a contiguous chain with two nicks, which can subsequently be ligated. Besides the fragment, complimentary oligos contain constitutive fragments on their ends, which are then used for PCR amplification with universal primers. The authors obtained and sequenced DNA products. Of the 298 samples analyzed (including 39 trisomy 21 and 7 trisomy 18 samples), all the aneuploid samples were correctly distinguished from the controls, the authors concluded to 100% sensitivity and specificity. The level of sequencing, covering only 420,000 reads per sample, was nevertheless sufficient to detect trisomy 21 and trisomy 18 reliably (z statistics exceeded 3.6 in all samples). This level corresponds to <5% of the level required by non-targeted approaches, and enables multiplexing [the study claims that 96 samples were processed simultaneously in one HiSeq2000 sample]. The level of sequencing, covering only 420,000 reads per sample, was neverthel
run (Illumina, Inc); thus greatly reducing the cost of analysis.

A method for detecting aneuploidy based on the assessment of heterozygosity for various polymorphisms is described by Natera in the US patent application US20110288780A1 [18]. The key feature of this method is that it takes the mixture of maternal and fetal DNA obtained from blood plasma into account separately from the DNA from one or both parents. As fetal DNA is represented exclusively in the cell-free fraction, purely maternal DNA can be extracted from the blood cells – white blood cells in particular. Paternal DNA can be additionally included in the analysis to improve the accuracy of the test, although the test can still be performed based solely on the samples taken from the pregnant mother. This method implies that the sequencing of DNA regions is targeted. One of the patent’s claims suggests the enrichment of the explored polymorphic loci with an assay of pre-circularized probes, which have been previously been described [36, 37]. Collected sequencing data contains information on the counts of sequences having each allele at each of the selected SNP loci. A statistical model estimates a likelihood ratio for the total number of reads containing one or another allele for every possible combination of parental genotypes and for each number of maternal \( n_m \) and paternal \( n_f \) chromosomes inherited by the fetus. This model takes the fraction of fetal DNA \( \sigma \) as a parameter. First, a maximum likelihood estimation is performed for the fetal DNA fraction \( \sigma \), then \( n_m \) and \( n_f \) are determined for the chromosomes of interest using the value of \( \sigma \) estimated in the previous step. As the approach described builds statistical models of the euploid state as well as all the other abnormal ploidy states, it not only finds the most likely ploidy state of the fetus for each chromosome, but also permits an estimation of the significance of this decision. This allows a statistically grounded identification of the samples where no reliable decision can be made without choosing arbitrary thresholds or estimating them from a training set. Unfortunately, the patent does not describe the results of the method’s performance tests, thus it is difficult to estimate its clinical efficiency. The sensitivity and specificity of the method depend on the fetal DNA fraction and the availability of the paternal genotype. The availability of paternal DNA allows for samples of the maternal peripheral blood with lower cffDNA content to be accepted for diagnosis. Methods using paternal content are likely to enable NIPD at earlier stages of the pregnancy when the concentration of fetal material is below the minimal threshold for methods utilizing only maternal samples. Separate sequencing of purely maternal cellular DNA may further improve performance. Along with trisomy 21, trisomy 18 and trisomy 13, sex chromosome aneuploidies (e.g., X0, XXY, XXX, XYY) can also be detected (Natera, personal communication), which is an important advantage of this method in light of the high occurrence of these abnormalities. However, the detection performance of the method is still to be published. The clinical trial of the Prenatal Noninvasive Aneuploidy Testing Using SNPs supported by the National Institutes of Health is underway [38]. As it is SNP-based, the method may need to be tested on patients from different populations.

The recent SNP-based targeted NIPD methods of Sparks et al. [20] and Rabinowitz et al. [18] seem to be highly efficient. As they can be performed on a sequencing machine with a lower price per run and lower throughput [e.g., PGM (Ion Torrent/Life Technologies, Carlsbad, USA) or MiSeq (Illumina, Inc)], these methods are preferred, especially for average-sized clinics. In addition to modeling disomy and trisomy, both methods model other ploidy states, thus enhancing statistical performance. The Natera method can potentially perform better because it extracts both cell-free DNA and cellular DNA from the same blood sample of purely maternal origin. It is also claimed that the Natera method can detect aneuploidy in sex chromosomes. Nevertheless, performance tests for the Natera method have not yet been published.

### Counting statistics for improvements in the sensitivity of the NIPD of fetal aneuploidy

Early reports suggest that inaccuracy in measuring genomic representation is variable [14, 15]. Although the algorithms used in recently published studies successfully classify fetal trisomy 21, they appear to be unable to effectively detect other aneuploidies, such as trisomy 18 and 13, which would inevitably occur in the population. However, it has been reported that high throughput massively parallel sequencing assays using specific bioinformatic algorithms, may enable the non-invasive detection of any type of fetal aneuploidy [17, 26–28].

The existence of a substantial GC bias in Illumina/Solexa and ABI/SOLiD sequencing has recently been shown. This issue limits the sensitivity of measuring genomic representation in chromosomes [14, 39–41]. Fan and Quake [26] have analyzed sequencing data collected in a previous study [14], as described earlier. In this study, a bioinformatic algorithm was developed to remove
GC-content-dependent artifacts in shotgun sequencing data by applying weight to each sequenced read based on the local genomic GC content of large regions of the human genome with relatively homogeneous GC content [42]. After calculating the standard z-statistic, z-scores for chromosomes 18 and 13 were increased in two of the two trisomy 18 and one of the one trisomy 13 cases, respectively. Consequently, all trisomy 18 and 13 cases were correctly classified. The algorithms for removal of the effect of GC bias used in these studies appear to be able to effectively detect cases of trisomy 18 and 13 as well as trisomy 21. Although the classification accuracy for trisomy 18 and 13 improved, there were not enough positive samples to measure a representative distribution.

Chen et al. [27] have demonstrated the successful use of two-plex massively parallel plasma DNA sequencing for NIPD of trisomy 18 and 13 on the Genome Analyzer Ix platform (Illumina) for a large sample set. Cell-free DNA from 5 to 10 mL of plasma from 25 trisomy 13, 37 trisomy 18, 86 trisomy 21, one sex chromosome mosaic case and 140 euploid pregnancies was used in the analysis. A total of 392 cases were analyzed, including 103 cases of women pregnant with euploid fetuses, which were used as normal controls for z-score calculation. As previously described, standard z-scores representing the number of standard deviations away from the mean proportion of chromosome 18 and 13 reads in a reference set of euploid cases were determined for each case [15, 32]. Based on the previous findings that the statistical power of the molecular counting approach increases with the number of molecules counted, a mean of approximately 4.6 million unique reads per sample, without mismatches to the non-repeat-masked reference human genome, was obtained. As a result, the classification accuracies for trisomy 18 and 13 were improved. For trisomy 18 detection, 31 of 37 trisomy 18 cases and 247 of 252 non-trisomy 18 cases were identified correctly, corresponding to sensitivity and specificity of 83.8% (95% CI 67.3%–93.2%) and 98.0% (95% CI 95.2%–99.3%), respectively. For trisomy 13 detection, 11 of 25 trisomy 13 cases and 247 of 264 non-trisomy 13 cases were identified correctly, corresponding to improved sensitivity and specificity of 44.0% (95% CI 25.0%–64.7%) and 93.6% (95% CI 89.7%–96.1%), respectively. As the imprecision of measuring the genomic representation of chromosomes was shown to be variable and dependent on the GC content of each chromosome, a statistical approach based on z-score calculation, but with an additional GC correction algorithm, has been developed in order to improve diagnosis of trisomy 18 and 13 [14, 15]. Specifically, all chromosomes from each sample were first divided into 50 kb bins using bioinformatics. Chen et al. determined the number of sequenced reads and GC content in each bin and applied the locally weighted scatter plot smoothing (LOESS) regression to calculate the predicted (P) value for each bin. Using the raw read counts (RCraw) the GC-corrected read counts (RCgc) of each bin were calculated with the correlation factor (F), which was derived from the median counts of all the bins (M) and the LOESS fit predicted value by the following equations:

\[ F = \frac{M}{P} \]  

and

\[ RC_{gc} = RC_{raw} \times F \]  

The standard z-score was calculated using the genomic representations of chromosomes 18 and 13, and all of the trisomy 13 cases (25 out of 25) were clearly identified. Two hundred and sixty-one of 264 non-trisomy 13 cases were correctly determined under z-score >3, indicating 100% (95% CI 83.4%–100%) sensitivity and 98.9% (95% CI 96.4%–99.7%) specificity of the GC correction approach. Thirty-four out of 37 trisomy 18 samples and 247 out of 252 non-trisomy 18 cases were classified providing 91.9% (95% CI 77.0%–97.9%) sensitivity and 98.0% (95% CI 95.2%–99.3%) specificity. This study has shown that the use of both the mapping of sequence reads against the non-repeat-masked genome and the GC correction approaches have improved the accuracy of trisomy 18 and 13 diagnosis. Essentially, correct aneuploidy detection was achieved by increasing the number of aligned reads in general and hence performing deeper sequencing. This bioinformatic method will be able to lower the cost of NGS in the future. Current algorithms for removing GC bias improve the precision of measuring the genomic representation of chromosomes as well as allow the effective classification of aneuploidies, most notably trisomy 13. Larger sample collections are required to further examine the algorithm for trisomy 18 detection.

An alternative for improving the accuracy of the detection of chromosomal abnormalities is to develop an optimized algorithm using a normalized chromosome value (NCV) from the sequencing data of the reference group (training set) of 71 samples with 26 abnormal karyotypes. This would minimize the intra- and inter-run sequencing variation as previously described [28]. Short, single-end reads of each plasma sample were sequenced on the Genome Analyzer Ix platform and unambiguously mapped to the non-repeat-masked reference human genome, allowing for up to two base mismatches during alignment. In the test set, the number of unique sequence tags varied from approximately 13×10^6–26×10^6. To determine cases of fetal aneuploidy, the NCVs for the
To return to the recent study by Sparks et al. [19], in the current report the authors introduced Fetal-fraction Optimized Risk of Trisomy Evaluation – an improved statistical algorithm for trisomy detection [20]. It estimates the risk of aneuploidy by computing an odds ratio that compares the probability of observing the outcome according to a model representing a disomic fetal chromosome and a model representing a trisomic fetal chromosome. Modeling the case of trisomy represents a major improvement in the study. Both disomic and trisomic models are normal distributions of the number of reads from the chromosome of interest scaled by the number of reads from a different chromosome. The mean value for the disomy model was taken as a mean proportion over the reference disomy samples (possibly with bootstrapping of samples). For the trisomy model, adjustment of the mean proportion was based on the estimated fetal DNA fraction. In order to assess the fetal DNA fraction for a sample, a set polymorphic loci was quantified together with the constitutive loci in the assay. A maximum likelihood estimation of the fetal DNA fraction was performed for every sample, based on loci where fetal and maternal genotypes differ. The standard deviation for both the proportion of reads mapped on the chromosome of interest and of the fraction of fetal DNA was estimated by bootstrapping the reference samples and taking into account polymorphic loci. As a result, of the total of 192 polymorphic and 576 non-polymorphic loci quantified in the samples from the training set, the polymorphic regions and 384 non-polymorphic loci showing the highest residual difference between normal and trisomic samples were selected. Thirty-six samples of trisomy 21 and 8 trisomy 18 from 167 samples in the test set were identified correctly, showing 100% for trisomy 21 (95% CI 88.0%–100%) and for trisomy 18 (95% CI 59.8%–100%) detection rate with a false-positive rate of 0% for trisomy 21 (95% CI 0%–3.6%) and for trisomy 18 (95% CI 0%–3.0%). The method is promising in terms of the small amount of sequenced reads required and the potential for screening for subchromosomal abnormalities. Nevertheless, a study on a larger cohort is required. Moreover, as the approach is SNP-based and the frequency of SNP genotypes can vary in different populations, further studies should be conducted to verify whether extending the cohort would require the number of screened polymorphic loci to be extended.

**Clinical considerations**

To date three diagnostic companies – Sequenom, Verinata Health, and Ariosa Diagnostics – have published
the results of sponsored clinical studies validating their methods and started offering NGS-based NIPD services commercially as LDTs [44–46].

Sequenom was the first to launch the NIPD screening test in November 2011. Presently, this non-invasive, LDT detects the increased number of reads from chromosomes 21, 18 and 13 resulting from whole-genome sequencing. In particular, the reports by Palomaki et al. [17, 33] provide a large scale international investigation into the determination of fetal trisomy 21, 18 and 13 involving around two thousand validation samples. The sensitivity for detecting cases of trisomy 21 was 98.6% (95% CI 95.9%–99.7%) with a false-positive rate 0.2% (95% CI <0.1–0.6). Failure to obtain results occurred in 0.8% of cases. Trisomy 18 and 13 detection rates were 100% (95% CI 93.9%–100%) and 91.7% (95% CI 61%–99%), respectively with false-positive rates of 0.3% (95% CI 0.1%–0.7%) for chromosome 18 and 0.9% (95% CI 0.5%–1.5%) for chromosome 13. The average gestation period at the time the maternal blood was sampled was 15 weeks and 3 days. Although these studies are large scale, 63.6% samples were excluded from analysis due to poor sample quality, volume or long processing time. The developed test has a high accuracy for determination of the most common trisomies in the population and can be offered in combination with other non-invasive methods for diagnosis aneuploidy as a pre-invasive procedure for high-risk pregnancies. However, since only four to eight samples can be analyzed in one sequencing run, and the processing and analysis of whole-genome sequencing is required, the test cost is high and thus is a limitation factor for widespread use of this test for diagnosis in a clinical setting.

Verinata Health launched the verifi™ prenatal test for diagnosis of trisomy 21, 18 and 13 as early as 10 weeks gestational age, based on a clinical study which was conducted by Bianchi et al. [16]. Bianchi et al. carried out a study examining 532 maternal blood samples where every sample was analyzed for six independent categories in order to define test performance and determine the presence of trisomy 21, 18, 13 aneuploidy male, female or monosomy X. It is the first published study which is able to detect sex chromosome aneuploidy, including monosomy X. The sensitivity for detection of trisomy 21, 18 and 13 was 100% (95% CI 95.9–100), 97.2% (95% CI 85.5–99.9) and 78.6% (95% CI 49.2–99.9), respectively, with a specificity of 100% (95% CI more than 98.5–100) for all trisomy cases. Also, all chromosomes of the human genome were analyzed using this approach. Current test determines of the presence of trisomy 21 with a 100% detection rate and may be utilized for trisomy 21 diagnosis along with other NIPD methods. Bianchi et al. [16] additionally provide the first report in which the non-invasive diagnosis of trisomy 13 occurs during the first trimester of gestation. However, because the detection rate of trisomy 13 is low, this test cannot be used as the sole screening method for Patau syndrome, and negative results should be confirmed with further testing.

Ariosa Diagnostics launched the Harmony Prenatal Test for NIPD of trisomy 21, 18 and 13 using direct sequencing of selective chromosome regions of interest, which was developed by Sparks et al. [20]. From a blinded validation set of 167 individuals, all trisomy 21 and 18 cases were correctly determined with 100% sensitivity (95% CI 88.0%–100% for chromosome 21 and 95% CI 59.8%–100% for chromosome 18) and 100% specificity (95% CI 96.4%–100% for chromosome 21 and 95% CI 97.0%–100% for chromosome 18). Data for the study of trisomy 13 is not shown. If one relies exclusively on published data, this test appears to be the most effective in the detection of fetal aneuploidy. However, only a small sample of positive trisomy cases were examined in comparison with the general number of samples examined in the study. One relevant advantage of this approach is the possibility of analyzing a huge amount of samples in one sequencing run, which thus reduces the cost of the test.

CffDNA-based NIPD of aneuploidy using NGS technologies is the first NIPD method that both appears in clinical practice and detects trisomy with high accuracy. Through the continuous development and improvement of algorithms for data sequencing and analysis, it has been possible to raise the accuracy, sensitivity and specificity of aneuploidy detection using this approach to 100%. Today, successfully used non-invasive integrated screening during the first and second trimesters for fetal aneuploidy is both safe for the fetus and accurate for diagnosis, which is reflected in a high sensitivity of between 72% and 95% (5% false-positive rate) reported during the last 10 years. However, in some cases, the patient does not pass all screening tests, necessitating successive tests, which require a lot of time [47–49]. The independent conduction of serum-based integrated screening during the first and second trimesters and the interpretation of results lead to unnecessary invasive procedures for normal pregnancies in 11%–17% of cases because the current false-positive rate is high. Although the combination of ultrasonographic detection, serum markers and maternal age at first trimester detect trisomy 21 at a rate of 90%, the false-positive rate is high – approximately 20% [50, 51]. One relevant advantage of NGS-based tests for the diagnosis of aneuploidy is the ability to obtain information about the ploidy of the fetus in early pregnancy. The combination of data obtained from NGS-based testing
with ultrasonographic detection, serum markers and maternal age at first trimester may increase the detection of aneuploidy with a low false-positive rate. After such screening, women at high risk of fetal aneuploidy can choose CVS during the first trimester or amniocentesis during the second trimester. This approach would decrease the likelihood that a woman with a normal fetus would undergo an invasive procedure.

Since the current NIPD tests are designed to identify a limited number of aneuploidy, only invasive procedures are defining multiple fetal chromosome abnormalities with high accuracy. Although the methods NGS for analyzing cfDNA have huge diagnostic potential for detecting all possible aneuploidy in a clinical practice setting, the screening for other multifactorial birth defects remains a big challenge. For instance, neural tube defects are one of the most common defects in the general population, with both genetic and environmental factors contributing to their development. Neural tube defects are successfully detected by measuring the level of an AFP in the amniotic fluid between 13 and 22 weeks’ gestation [52, 53].

NGS methods usually have a fixed price per run, thus a serious reduction of in cost can be achieved by analyzing multiple samples in one sequencing run, while bar-coding DNA samples in order to determine the origin of every read obtained. In theory, maximal multiplexing can dramatically decrease the price. Unfortunately, the time in which the test can be performed is naturally limited by the growth of the fetus. That is why it is not always possible for a clinic to collect hundreds of samples for each run if runs start approximately every 10–20 days (rough estimation, 11 days is the run time for the Illumina HiSeq2000 sequencer). We also believe that the test will be of no use if it takes more than a month to perform.

We compared some of the prenatal diagnostic methods based on the equipment they use (Table 2). According to rough estimates of minimal sequencing depth, equipment required, and maximal multiplexing, the Ariosa Diagnostics method appears to be the most cost-effective. Targeted approaches can be the only choice if performed on sequencing machines with lower run price and lower throughput, like PGM or MiSeq, which seems to be a more practical choice for a clinic with tens rather than hundreds of pregnancies to analyze every month. For targeted approaches, the sample-enrichment step of particular DNA regions should also be taken into account, both in terms of the cost of the equipment and reagents and in terms of personnel trained to perform it.

Despite providing high accuracy and sensitivity early in the pregnancy without risk to the fetus or the mother, NGS-based NIPD of aneuploidy has several disadvantages slowing down the propagation into the mainstream clinical use. The long test turnaround time, high reagent and equipment costs and high percentage of cases, where the diagnosis cannot be made due to insufficient cfDNA content or other factors impede mass adoption. When deciding on the NGS-based NIPD strategy these additional factors should be considered in addition to the diagnostic sensitivity, specificity and scalability. The concept of clinical utility may include elements of whether the clinical outcomes are effective and whether its implementation offers an economically efficient solution compared to alternative methods [54, 55].

**Conclusions**

Since the discovery of the cell-free fetal nucleic acid sequences in maternal peripheral blood, several methods for highly accurate and highly sensitive aneuploidy testing using NGS technology either for full genome sequencing or sequencing of targeted areas of the genome were developed. Prenatal tests utilizing these methods are already offered as screening tests for trisomy 21, 18 and 13, reducing the need for risky invasive procedures. Additional clinical trials are underway to validate these methods for use as diagnostic tests for both high-risk pregnancies and screening of the general population. The final decision on the implementation of a NGS-based test for NIPD of aneuploidy in clinical practice should be based on the criteria of high diagnostic accuracy,

<table>
<thead>
<tr>
<th>Company*</th>
<th>Study</th>
<th>Sequencing depth, reads</th>
<th>Equipment used</th>
<th>Multiplexing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequenom</td>
<td>Palomaki et al., 2011 [33]</td>
<td>NA</td>
<td>Illumina HiSeq 2000</td>
<td>4*8=32 (1)</td>
</tr>
<tr>
<td>Verinata health</td>
<td>Fan et al., 2010 [26]</td>
<td>~10 M</td>
<td>Solexa/Illumina</td>
<td>3×10⁹/10×10⁶=300</td>
</tr>
</tbody>
</table>

*Clinical study sponsor.

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clinical and cost-effectiveness and the ability to make a
diagnosis even in cases where the content of cffDNA is
low. Furthermore, large-scale validation studies should
be carried out independent from the tests’ manufactur-
ing companies. Tests implemented in a clinical setting
should not be time consuming, which is very important
in prenatal diagnosis. It is also important to take into
account the nationality of the patients in order to imple-
ment the test in clinics around the world. Tests should
also require a minimal cost of equipment and infrastruc-
ture in order to be available to small laboratories around
the world. Today NGS-based tests for diagnosis of tri-
somies 21, 18 and 13 may be combined with ultrasono-
graphic detection and serum markers for more accurate
diagnosis of fetal aneuploidy, in order to avoid invasive
procedures. Methods utilizing full genome sequencing
allow for accurate detection of other autosomal and sex
aneuploidies, but are limited by the high cost of sequenc-
ing. Sequencing of targeted areas of the genome allows
one to significantly lower the cost of sequencing while
providing high accuracy and sensitivity in diagnosing
common aneuploidies. Methods utilizing parental geno-
types, where DNA from one or both parents is available,
in addition to common trisomy detection, provide for
highly accurate counts of autosomes and sex chromo-
somes and can be performed using significantly cheaper
and easier to operate sequencing equipment. Our review
demonstrated that NGS-based NIPD is a rapidly evolving
field with many research teams developing and commer-
cializing tests using new technologies and performing
large scale clinical trials. As the new NGS technologies
become available, new methods for NIPD will be de-veloped that allow the analysis of a broader spectrum of
chromosomal abnormalities and genetic diseases, and
cost will be reduced. Several commercial NIPD provid-
ers developed proprietary fetal quantifiers and proto-
cols for increasing diagnostic accuracy of the tests and
these may not be publicly available. All of the reviewed
methods bear equipment, technology, cost, intellectual
property and performance risk; thus, careful consid-
eration should be given to each of these aspects when
deploying or developing NGS-based NIPD in a clinical
setting.

Data sources and method of study selection

We searched the PubMed, PubMed Central, Bookshelf,
FreePatentsOnline and ClinicalTrials.gov databases for
reports published after 1997 using the key words – ‘pre-
natal diagnostics aneuploidy’ and ‘fetal next-generation
DNA sequencing’. Both thorough and theoretical reviews
of relevant full-text articles were performed. An extensive
analysis of references and literature sources was con-
ducted for the most relevant publications.

Conflict of interest statement

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