ORIGINAL ARTICLE

Noninvasive prenatal aneuploidy testing of chromosomes 13, 18, 21, X, and Y, using targeted sequencing of polymorphic loci

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ABSTRACT

Objective This study aims to develop a noninvasive prenatal test on the basis of the analysis of cell-free DNA in maternal blood to detect fetal aneuploidy at chromosomes 13, 18, 21, X, and Y.

Methods A total of 166 samples from pregnant women, including 11 trisomy 21, three trisomy 18, two trisomy 13, two 45, X, and two 47,XXY samples, were analyzed using an informatics-based method. Cell-free DNA from maternal blood was isolated, amplified using a multiplex polymerase chain reaction (PCR) assay targeting 11 000 single nucleotide polymorphisms on chromosomes 13, 18, 21, X, and Y in a single reaction, and sequenced. A Bayesian-based maximum likelihood statistical method was applied to determine the chromosomal count of the five chromosomes interrogated in each sample, along with a sample-specific calculated accuracy for each test result.

Results The algorithm correctly reported the chromosome copy number at all five chromosomes in 145 samples that passed a DNA quality test, for a total of 725/725 correct calls. The average calculated accuracy for these samples was 99.92%. Twenty-one samples did not pass the DNA quality test.

Conclusions This informatics-based method noninvasively detected fetuses with trisomy 13, 18, and 21, 45,X, and 47, XXY with high sample-specific calculated accuracies for each individual chromosome and across all five chromosomes. © 2012 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

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INTRODUCTION

Until recently, pregnant women seeking information about the chromosomal health of their fetus had two options: (1) noninvasive screening by biochemical analysis of maternal serum and/or ultrasonography, or (2) invasive testing by chorionic villus sampling (CVS) or amniocentesis. Noninvasive screens are considered safe but have poor accuracy. Serum screens have false negative rates between 12% and 23% and false positive rates between 1.9% and 5.2%.[1–3] Detecting chromosomal abnormalities using ultrasonography screening depends on gestational age (GA); only 35% of fetal anatomic abnormalities were detected among 15 000 women screened by ultrasonography, and only 17% of these were detected prior to 24 weeks' gestation.[4] Invasive screens have considerably higher accuracy but carry a procedure-related miscarriage risk.[5,6] The diagnostic accuracy of karyotyping cultured cells is from 97.5% to 99.6% when obtained by CVS,[7–11] and from 99.4% to 99.8% when obtained by amniocentesis.[12]

Decades of research on noninvasive DNA-based prenatal testing are finally reaching fruition. In the short term, these tests offer a more accurate alternative or adjunct to serum screens. Ultimately, they may replace invasive testing provided they demonstrate accuracies at least as high and match their diagnostic scope.

Initial research efforts targeted the isolation and subsequent analysis of circulating fetal cells from maternal blood. Given the ~ 1 : 1 000 000 ratio between circulating fetal and maternal cells, these approaches struggled to reliably detect and isolate fetal cells and have largely been unsuccessful.[13–15] More recent efforts focused on analyzing cell-free DNA (cfDNA) in maternal plasma as it contains appreciable amounts of fetal DNA.

Fetal trisomy detection using cfDNA from maternal blood has been reported using massively parallel shotgun sequencing (MPSS).[16–24] MPSS detects higher relative amounts of DNA in maternal plasma from the fetal trisomic chromosome compared

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with reference chromosomes. The MPSS method shows good accuracy for detecting trisomy 21 (T21) and trisomy 18 (T18) given sufficient fetal cfDNA levels; however, detection of trisomy 13 (T13) and sex chromosome abnormalities is more limited[16,20–26] because some chromosomes are represented in sequencing data with high variability. This limits the scope of chromosomal abnormalities that can be accurately detected with these purely quantitative methods.[16,25–27] This limitation is exacerbated in samples drawn in the first trimester, as they tend to have lower fetal cfDNA fractions in maternal plasma. Additionally, only a few percent of sequencing reads are relevant for detecting aneuploidy on chromosomes of interest; thus, many more overall reads are required to reach a given level of accuracy.

A related quantitative approach termed Digital Analysis of Selected Regions (DANSR) selectively sequences loci only from chromosomes of interest by including a targeted amplification step. This method represents a significant increase in sequencing efficiency and has recently been shown to detect T21 and T18 in clinical samples.[28–30] However, as with all purely quantitative methods, the approach depends on low chromosomal amplification variation between target and reference chromosomes, thus limiting its diagnostic accuracy for some chromosomes.

Liao *et al.* recently described a method that selectively sequences single-nucleotide polymorphisms (SNPs) and determines copy number by comparing fetal to maternal SNP ratios between target and reference chromosomes.[31] The use of SNPs may mitigate chromosome-to-chromosome amplification variability; however, the need for a reference chromosome partly negates this advantage. Because the study only interrogated chromosome 21, this was not examined.

Although the sensitivity and specificity of these assays are considerably improved over serum screens and ultrasound, they currently do not achieve the same scope and accuracy as amniocentesis or CVS. To address some of these limitations, we introduce a method called Parental Support™ (PS), which determines fetal copy number from maternal blood samples at chromosomes 13, 18, 21, X, and Y with high accuracy at all chromosomes. A key novel feature of PS is that it calculates a per-test, per-chromosome accuracy for each sample, offering clinicians an individualized risk score for each patient. Here, we present the initial results of this methodology.

METHODS

Patients

Pregnant couples were enrolled at selected prenatal care centers under Institutional Review Board-approved protocols pursuant to local laws. Women were at least 18 years of age, had a GA of at least 9 weeks, singleton pregnancies, and signed an informed consent. A total of 166 maternal blood samples were drawn, and paternal genetic samples were collected (blood or buccal). The cohort included eleven T21 (Down syndrome), three T18 (Edwards syndrome), two T13 (Patau Syndrome), two 45,X (Turner syndrome), two 47,XXY (Klinefelter syndrome) samples, and 146 samples from women with putatively euploid pregnancies; normal fetal karyotype was confirmed by molecular karyotyping for 62 samples where post-birth child tissue was available.

Putative euploid samples were drawn prior to invasive tests from women without known risk indicators. Most aneuploid samples were drawn after invasive testing aneuploidy diagnosis with confirmatory fluorescence *in situ* hybridization and/or cytogenetic karyotype analysis at independent laboratories; individual sites sent Natera reports. PS detected Klinefelter syndrome in two putative euploid samples drawn from the same woman at different times; this was confirmed by molecular karyotyping of cord blood. Being a proof-of-principle study, laboratory researchers were not blinded to sample karyotype; however, the PS algorithm was blinded to sample karyotype, and the results of the algorithm were not informed by human oversight. No reports were released to physicians or patients.

Sample preparation and measurement are described in the Supporting Information.

Data analysis

Genome sequence alignment was performed using a proprietary algorithm adapted from the Novoalign (Novocraft, Selangor, Malaysia) commercial software package. A chromosome copy number classification algorithm was implemented in MATLAB (MathWorks, Natick, MA, USA) leveraging a proprietary statistical algorithm termed Parental Support™ (PS).[32–37] The technique uses parental genotypes, data from the Hapmap Database,[38] and the observed number of sequence reads associated with each of the relevant alleles at SNP loci. A simplified explanation of the PS method follows and is described in greater detail in the Supporting Information

The PS algorithm uses measured parental genotypes and crossover frequency data[35,38–40] to create, *in silico*, billions of possible monosomic, disomic, and trisomic fetal genotypes at measured loci, each considered as a separate hypothesis. PS then uses a data model that predicts what the sequencing data is expected to look like for a plasma sample containing different fetal cfDNA fractions for each hypothetical fetal genotype. Bayesian statistics are used to determine the relative likelihood of each hypothesis given the data, and likelihoods are summed for each copy number hypothesis family: monosomy, disomy, or trisomy. The hypothesis with the maximum likelihood is selected as the copy number and fetal fraction, and the absolute likelihood of the call is the calculated accuracy, analogous to a test-specific risk score.

Briefly, different probability distributions are expected for each of the two possible alleles at a set of SNPs on the target chromosome depending on the parental genotypes, the fetal fraction, and the fetal chromosome copy number. By comparing the observed allele distributions to the expected allele distributions for each of the possible scenarios, it is possible to determine the most likely scenario and precisely how likely that scenario is.

The PS algorithm uses a data quality test; samples must pass a DNA quality threshold for results to be reported. Any sample with $<4.0\%$ fetal fraction or with a DNA quality metric below the threshold was reported as a no call. The DNA quality metric was based on the quality of the plasma sequence data, noise levels, how well the data corresponded to the statistical model, and the calculated accuracies.

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Chromosomal copy number was reported: one, two, or three for chromosomes 13, 18, 21, and X; and zero or one for Y. The copy number was either reported for all or none of the five target chromosomes. The copy number determinations were considered to be correct for putative euploid samples where the copy number was two for chromosomes 13, 18, 21, and X + Y.

RESULTS

The 166 samples were analyzed using the PS method. Twenty-one samples did not meet the stringent DNA quality test used by the algorithm (20 putative euploid samples and one 45,X sample). For the remaining 145 samples (Table 1), the copy number was reported for chromosomes 13, 18, 21, X, and Y. All were correct (725/725), giving a sensitivity and specificity of 100%. Altogether, 706/706 euploid chromosomes and 19/19 aneuploid chromosomes were called correctly, including eleven trisomy 21, three trisomy 18, two trisomy 13, one 45,X, two 47, XXY, 57 46,XX, and 69 46,XY samples; confirmation was available on all aneuploid samples and 58 of the putative euploid samples that passed the DNA quality test. Each copy number determination included a sample-specific calculated accuracy; the mean calculated accuracy across all chromosomes called was 99.92%. The combined calculated accuracy (the accuracy at all five chromosomes conservatively treated as independent and multiplied together) exceeded 99.8% for 130 of 145 samples, including 17/19 of the aneuploid samples.

The mean measured fetal fraction for all samples was 12.0%, with a range of 2.0–30.8%. Regression analysis revealed a strong positive correlation between the fetal fraction and GA (p-value < 0.01, Figure 1). Figure 2 shows the distribution of correct and no-call samples as a function of fetal fraction. The samples that did not pass the DNA quality test cluster in the low fetal fraction region.

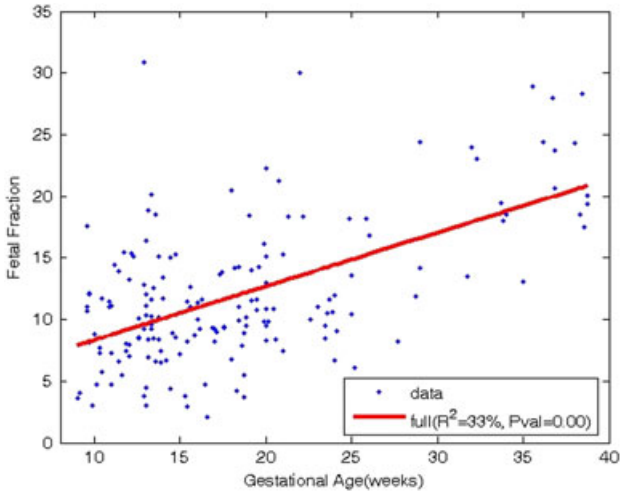

Figure 1 Fetal fraction as a function of gestational age. Fetal DNA was determined as described in the Supporting Information. Each spot represents a single sample, and fetal fraction (y axis) was plotted as a function of gestational age (x axis). Regression analysis reveals a positive correlation between fetal fraction and gestational age (red line: $R^2 = 0.33$, $p < 0.005$)

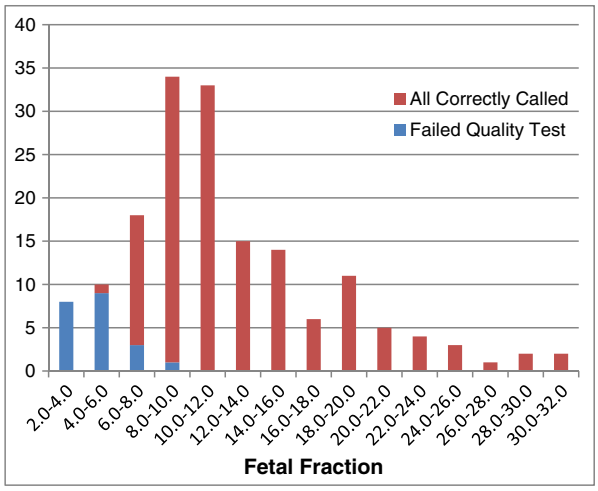

Figure 2 Histogram of samples stratified by fetal fraction

The median GA was 17.0 and 17.5 weeks for euploid and aneuploid samples, respectively. An average of 9.69 million reads were made for each sample; of those, 8.85 million reads (91.3%) mapped to the genome, and 6.47 million reads (66.7%) mapped to the targeted SNPs, were determined to be informative, and were used by the algorithm. We observed more than 95% of the targeted loci in sequencing results for the majority of the samples. The average depth of read was 344, and the median depth of read was 255 per SNP.

DISCUSSION

The data presented in this proof-of-principle study of Parental Support™ methodology demonstrate that PS enables accurate detection of fetal aneuploidy from maternal blood. The method measures cfDNA isolated from maternal plasma using targeted sequencing of 11 000 SNPs and Bayesian-based maximum likelihood informatics analysis. By focusing on polymorphic loci, multiple pieces of information – the number and identity of each allele – are measured in each sequence read. The use of advanced statistical methods allows PS to incorporate parental genotypic information and enhance the predictive power of data generated from high-throughput cfDNA sequencing. This approach offers numerous advantages, including and most importantly greater clinical coverage and sample-specific calculated accuracies.

The PS method increases clinical coverage of viable chromosomal abnormalities by approximately twofold, with comparable accuracies at each chromosome compared with previously reported methods (Figure 3).[16–25,28–31] In this cohort, PS detected trisomy 13, 18, 21, 47,XXY, and 45,X. On the basis of DNA mixing experiments and prior plasma samples analyzed with experimental assays, we expect that PS will also detect monosomy 21, 47,XXX and 47,XYY, uniparental disomy, and triploidy; corresponding samples have not yet been tested with the current molecular biology protocol. Whereas most published methods focus only on detecting autosomal trisomies, the combined at-birth prevalence of sex chromosome abnormalities is slightly higher than that of autosomal trisomies (Figure 3),[41,42] emphasizing the need for methods that detect sex chromosome abnormalities during pregnancy.

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Table 1 **List of 145 samples with PS copy number result**

GA (weeks)	Fetal fraction	PS called karyotype	Average confidence	Confirmed karyotype	GA (weeks)	Fetal fraction	PS called karyotype	Average confidence	Confirmed karyotype
18.1	14.19	47,XY +13	100	47,X?,+13	23.3	9.47	46,XY	99.998	46,XY
22.0	30.00	47,XY +13	100	47,XY,+13	36.1	24.44	46,XY	100	46,XY
20.5	8.41	47,XY +18	99.998	47,XY,+18	17.0	8.21	46,XY	100	46,XY
19.1	8.81	47,XX +18	100	47,XX,+18	27.5	8.26	46,XX	100	46,XX
20.1	12.98	47,XY +18	100	47,X?,+18	20.0	8.31	46,XX	99.036	N/A
20.0	22.30	47,XX +21	100	47,XX,+21	19.6	9.83	46,XX	100	N/A
14.1	6.70	47,XX +21	99.954	47,XX,+21	23.5	11.62	46,XY	99.874	N/A
13.1	6.93	47,XY +21	99.508	47,XY,+21	26.0	16.84	46,XY	100	N/A
17.3	9.22	47,XX +21	100	47,XX,+21	12.0	7.01	46,XY	99.616	N/A
12.2	15.13	47,XX +21	100	47,XX,+21	36.6	20.68	46,XY	100	N/A
13.0	16.38	47,XY +21	100	47,XY,+21	18.2	4.24	46,XY	97.604	N/A
13.0	11.12	47,XX +21	100	47,XX,+21	11.0	7.25	46,XY	100	N/A
22.1	18.35	47,XX +21	100	47,XX,+21	9.5	8.15	46,XX	99.988	N/A
34.0	18.51	47,XX +21	100	47,XX,+21	12.4	8.49	46,XX	100	N/A
19.0	18.43	47,XX +21	100	47,XX,+21	14.5	8.55	46,XY	99.998	N/A
18.0	20.51	47,XX +21	100	47,XX,+21	13.2	9.24	46,XY	100	N/A
10.6	11.49	47,XXY	100	47,XXY	12.4	10.11	46,XY	100	N/A
12.0	13.23	47,XXY	100	47,XXY	13.0	10.37	46,XX	100	N/A
21.0	15.30	45,X	100	45,X	9.4	10.70	46,XX	100	N/A
13.3	8.47	46,XY	99.992	46,XY	9.4	11.01	46,XX	100	N/A
16.0	11.39	46,XX	100	46,XX	13.2	11.60	46,XX	100	N/A
12.6	12.83	46,XY	100	46,XY	13.0	12.07	46,XY	100	N/A
15.3	8.19	46,XY	99.990	46,XY	9.5	12.11	46,XX	100	N/A
16.6	9.30	46,XX	100	46,XX	13.3	12.53	46,XY	100	N/A
16.0	10.01	46,XX	100	46,XX	14.3	14.99	46,XX	100	N/A
25.0	10.46	46,XX	100	46,XX	13.5	15.09	46,XX	100	N/A
15.4	11.06	46,XX	99.044	46,XX	14.5	15.30	46,XX	100	N/A
11.0	11.13	46,XY	100	46,XY	11.5	15.45	46,XY	100	N/A
38.4	17.50	46,XX	100	46,XX	12.6	30.83	46,XY	100	N/A
9.4	17.58	46,XY	100	46,XY	18.0	6.73	46,XY	99.968	N/A
38.5	20.05	46,XY	100	46,XY	15.0	7.19	46,XX	99.456	N/A
13.6	6.52	46,XX	98.120	46,XX	10.2	7.30	46,XX	99.906	N/A
13.4	6.59	46,XY	99.998	46,XY	13.6	7.47	46,XX	99.880	N/A
10.2	7.73	46,XY	100	46,XY	18.4	7.86	46,XY	99.992	N/A
12.0	7.92	46,XX	100	46,XX	11.6	8.02	46,XY	99.920	N/A
13.2	9.61	46,XX	100	46,XX	13.1	8.42	46,XY	97.290	N/A
10.3	11.68	46,XX	100	46,XX	12.4	8.58	46,XY	100	N/A
14.0	11.69	46,XY	100	46,XY	15.6	8.69	46,XX	100	N/A
11.3	13.89	46,XY	100	46,XY	14.4	8.86	46,XY	100	N/A
13.1	18.85	46,XY	100	46,XY	18.5	8.86	46,XY	99.972	N/A
13.2	20.11	46,XX	100	46,XX	17.1	8.98	46,XX	100	N/A
38.3	28.35	46,XY	100	46,XY	12.6	9.28	46,XY	100	N/A
21.0	7.43	46,XY	99.990	46,XY	17.4	9.32	46,XY	99.574	N/A
19.6	16.17	46,XY	100	46,XY	17.4	9.40	46,XX	100	N/A
20.5	21.22	46,XY	100	46,XY	18.6	9.53	46,XX	100	N/A

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Table 1 (Continued)

GA (weeks)	Fetal fraction	PS called karyotype	Average confidence	Confirmed karyotype	GA (weeks)	Fetal fraction	PS called karyotype	Average confidence	Confirmed karyotype
20.3	10.83	46,XY	100	46,XY	20.1	9.87	46,XY	100	N/A
35.0	13.07	46,XY	100	46,XY	18.3	9.93	46,XX	100	N/A
23.3	8.49	46,XY	100	46,XY	18.6	10.17	46,XX	100	N/A
33.6	17.99	46,XY	100	46,XY	13.5	10.18	46,XX	100	N/A
34.5	23.95	46,XX	100	46,XX	13.2	10.23	46,XY	99.998	N/A
33.5	19.49	46,XX	100	46,XX	19.3	10.80	46,XY	100	N/A
25.6	18.14	46,XY	100	46,XY	20.0	10.88	46,XX	99.998	N/A
23.6	10.59	46,XY	100	46,XY	18.3	10.99	46,XY	100	N/A
29.0	24.41	46,XX	100	46,XX	10.6	11.04	46,XX	100	N/A
24.1	9.07	46,XY	100	46,XY	15.3	11.46	46,XY	100	N/A
36.5	27.98	46,XY	100	46,XY	19.1	11.56	46,XY	100	N/A
28.5	11.90	46,XX	100	46,XX	16.2	11.60	46,XX	100	N/A
24.0	6.67	46,XY	100	46,XY	19.3	11.63	46,XX	100	N/A
29.0	14.21	46,XX	100	46,XX	9.5	12.01	46,XX	99.996	N/A
38.0	24.28	46,XY	100	46,XY	14.2	13.43	46,XY	100	N/A
36.6	23.74	46,XY	100	46,XY	17.2	13.64	46,XY	100	N/A
31.5	13.54	46,XY	100	46,XY	17.3	13.81	46,XX	100	N/A
23.0	11.05	46,XY	100	46,XY	19.1	14.03	46,XY	100	N/A
25.0	13.56	46,XX	100	46,XX	18.3	14.27	46,XY	100	N/A
35.4	28.88	46,XX	100	46,XX	19.4	14.30	46,XY	100	N/A
22.4	10.02	46,XY	100	46,XY	11.1	14.44	46,XX	100	N/A
32.2	23.06	46,XX	100	46,XX	20.0	15.09	46,XX	100	N/A
38.2	18.50	46,XY	100	46,XY	12.1	15.40	46,XX	100	N/A
38.5	19.37	46,XY	100	46,XY	13.4	18.51	46,XX	100	N/A
24.6	18.22	46,XX	100	46,XX	13.5	8.39	46,XX	99.936	N/A
21.2	18.38	46,XY	100	46,XY	16.1	9.24	46,XY	100	N/A
23.4	10.55	46,XX	100	46,XX	24.0	11.94	46,XX	100	N/A
15.4	12.68	46,XX	100	46,XX					

PS, Parental Support™; GA, gestational age.

Significantly, PS calculates a sample-specific accuracy for each chromosome copy number call, a feature that informs which individual calls are highly reliable and which ones may require follow-up. This approach represents a paradigm shift in testing. Traditional diagnostics typically utilize a single-hypothesis rejection test of a metric, such as *z*-score, to determine if the sample is positive or negative. Test accuracy is measured on a large cohort, and individual test accuracies are assumed to be the same as for the cohort. However, actual accuracies for samples whose parameters lie in the cohort distribution tail may differ dramatically from those in the test cohort bulk. Conversely, screening tests produce a risk score, but the risks typically are widely distributed and many samples receive an intermediate risk.

Parental Support™ uses a novel informatics approach to leverage the best of both methods: it models data distributions associated with both euploid and aneuploid hypotheses to optimize decision thresholds and produce sample-specific accuracy calculations. For example, a normal karyotype with a 99% calculated accuracy can be converted to a traditional risk score by simply combining 1/100 with the prior age-specific aneuploidy risk (calculated accuracies reported here do not leverage prior probabilities on euploidy and aneuploidy). Because the sample-specific calculated accuracy is based on the parameters of each sample, it is more accurate than tests quoting a single accuracy for all samples, especially for samples whose parameters lie in the tail of the cohort. Importantly, Figure 4 shows that these calculated accuracies are meaningful; the sample-specific calculated accuracies are grouped and compared with empirical accuracies, showing that they are aligned and that calculated accuracies are slightly conservative. Thus, a result with a high calculated accuracy can be treated differently to results with intermediate accuracies, allowing doctors to make better-informed decisions.

A benefit of using sample-specific versus cohort-based accuracy calculations is illustrated by comparing sensitivity rates for MPSS and PS for aneuploid samples with low fetal fraction. In one study, MPSS samples with fetal fraction <9% had a false negative rate of 3/21 (14.3%) when analyzing chromosome 21.[20] For the five analogous aneuploid samples in this data set, the PS method

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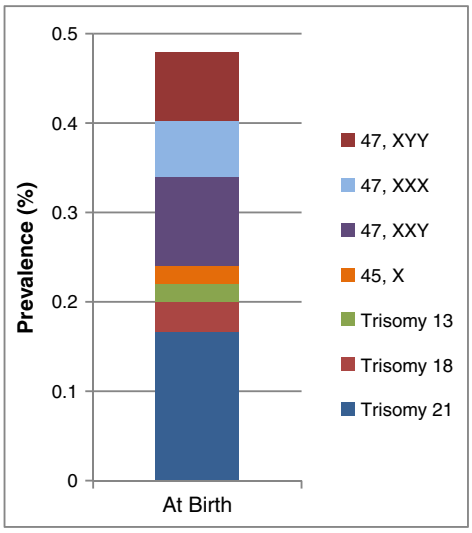

Figure 3 At-birth prevalence of aneuploidy

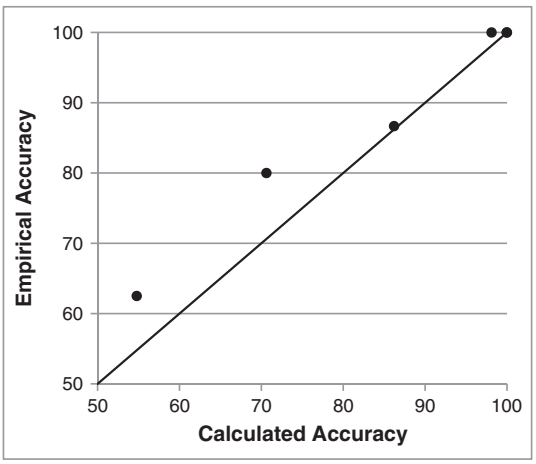

Figure 4 Relationship of calculated accuracy and empirical accuracy. The graph includes all copy number calls, including those that were reported by the algorithm as a no call, and excludes results for the eight samples with fetal fraction below 4%. Copy number calls were grouped by calculated accuracy (confidence), and for each group, the overall empirical accuracy was graphed against the average calculated accuracy in that group

reported a no call for one sample (20%), and the remaining four samples were reported with 100% accuracy on all five chromosomes, (the average calculated accuracy of the four reported samples was 99.87%). While the number of samples in this low fetal fraction cohort is small, this example demonstrates that at the similar lower detection limits of MPSS-based and PS-based methods,[20] MPSS tends to make incorrect calls on low quality samples, whereas the PS method tends to make no-calls. The presumption is that a no call is preferred to a false negative result, as a no call simply requires a redraw and retest, whereas a miscall can result in lifelong consequences.

Calculating accuracies is particularly beneficial in early GA pregnancies. Prenatal testing in early pregnancy is typically preferred as it facilitates earlier decision making; the drawback is typically lower fetal fractions, which correlate with an increased error rate. This is especially acute in single hypothesis rejection-based tests (e.g. MPSS and DANSR) that were validated using a cohort with a significantly higher average GA.[16,23,24,28,43] PS identifies samples for which incorrect results are likely, for example, due to low amounts or quality of fetal DNA, thus decreasing the chance of false negatives.

Parental Support™ also offers various other benefits over previous methods. Because PS relies on comparing the relative amounts of alleles at a set of loci, it obviates problems with chromosome-to-chromosome amplification variation that generate poor accuracies for chromosomes 13, X, and Y in previous methods.[17,25–27] In this study, average calculated accuracies of calls at chromosomes 13, 18, 21, X, and Y were statistically similar: 99.92%, 99.86%, 99.89%, 99.93%, and 99.99%, respectively. Utilizing allelic data obviates the requirement for a reference chromosome that is presumed to be euploid, and PS is therefore uniquely expected to detect triploidy. Moreover, incorporating parental data allows PS to detect abnormalities that preserve chromosome copy number, such as uniparental disomy.

Importantly, because PS informatics maximally utilizes available information in the data set, combining it with high-fidelity parental allelic information and HapMap data, it generates more powerful test statistics with narrower distributions, similar to a diagnostic. Indeed, 90.1% of these results (748 of 830 chromosome calls) return a calculated aneuploidy probability of either ≤0.1% or ≥99.9%.

Lastly, thermodynamic design of PCR probes dramatically reduces probe–probe interaction, allowing targeted enrichment of 11 000 loci in one reaction, considerably more than previous targeted methods.[28–31] Inclusion of more loci results in higher accuracy and is efficient: more than two-thirds of sequence reads map to informative loci. In contrast, the MPSS methods universally amplify all DNA indiscriminately even though chromosomes 13, 18, 21, X, and Y represent only ~14% of the genome. The average number of reads used per sample in this data set was 6.47 million (~1.3 million per chromosome). Additionally, Figure 5 indicates that performance does not degrade appreciably under 5 million reads; the average mapped read count for samples in this bin was 4.2 million, considerably lower than the MPSS methods, which require ~20–30 million reads. It is anticipated that similar levels of accuracy will ultimately be achieved using significantly fewer sequence reads, resulting in reductions in cost and increases in throughput.

Taken together, PS is an encouraging, novel method for detecting fetal chromosomal abnormalities noninvasively.

Study limitations

Although this study demonstrates the promise of targeted cfDNA sequence analysis, there are several caveats. The putative euploid samples were comprised of average-risk women whose fetal ploidy status was not independently confirmed. The likelihood of a chromosomal abnormality in this cohort was low, but one pregnancy with Klinefelter's syndrome was detected in the putative euploid cohort. The mid-term risk for whole chromosomal abnormality is ~0.7%,[44,45] and the risk of the putative euploid cohort having

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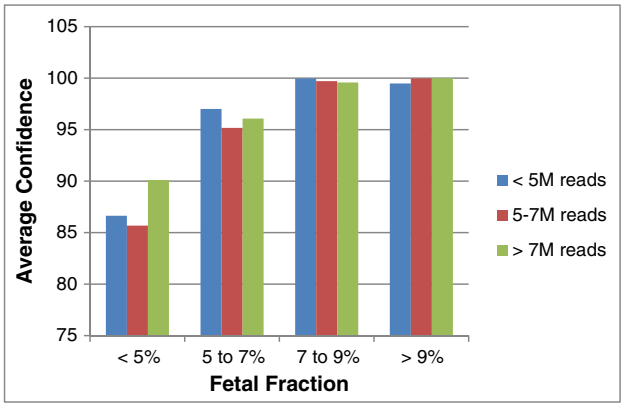

Figure 5 Calculated confidence as a function of number of sequence reads and fetal fraction

no aneuploid pregnancies is only 36%; therefore it is not surprising to find an aneuploid sample.

The prevalence of confined placental mosaicism and its impact on prenatal screening is unclear. Assuming that a significant portion of the fetal DNA present in maternal blood is derived from the placenta, the presence of placental mosaicism could undermine the significance of any algorithm-generated accuracies in each sample. Importantly, no method relying on cfDNA found in maternal plasma could overcome this limitation.

Our analysis included samples from women with GAs > 20 weeks, which do not represent the early stages of pregnancy for which this method is intended. However, because PS generates a sample-specific calculated accuracy that takes into account fetal fraction and GA, the method is expected to either accurately call copy number at low fetal fractions with high confidence or not return a call. As each accuracy calculation is generated independently, the inclusion of these women should have no effect on method validation or calculated accuracy for samples with early GA and/or low fetal fraction. Additionally, previous studies also included samples from patients with average GAs > 20 weeks.[28,43]

Aneuploid samples were confirmed prior to blood draw using invasive procedures, which increases fetal cfDNA in maternal blood minutes post-procedure.[46–50] We are not aware of any studies that measured fetal cfDNA levels more than a day post-procedure. Regardless, elevated fetal cfDNA levels would not be expected to affect results because PS calculates a sample-specific accuracy involving numerous parameters, including fetal fraction. Obtaining statistically significant results, however, requires a larger cohort; a large-scale clinical trial is underway (NCT01545674).

Twenty-one samples failed our stringent quality control test, resulting in a 12.6% no-call rate. Failures were typically due to low fetal fraction and poor DNA quality, although the PS method still made several calls at low fetal fractions with high calculated accuracies. Note that previous methods report accuracies for calls only on a subset of chromosomes, not on all five (13, 18, 21, X, and Y), and usually exclude the sex chromosomes.[20,23,24,30] In the single published study that reported detection of 45,X, the unclassified (analogous to a no call) rate was 10.2% for 45,X alone, and higher when autosomal trisomies were included.[24] In contrast, the PS method returned calls with high accuracies on all five chromosomes and included detection of 45,X and certain sex chromosome trisomies, with a comparable overall no-call rate.

A combination of high no-call rate and long turnaround time could prompt doctors to request invasive procedures in response to no calls; this would exacerbate rather than mitigate the problem of unnecessary invasive procedures. However, the PS method enables a fast turnaround time (<1 week), thus PS allows for redraws and reanalyses with sufficient time to avoid invasive procedures after a no call. Indeed, examination of no call, redraw, and reanalysis rates for a commercially available noninvasive paternity test utilizing the PS methodology that interrogates fetal cfDNA in maternal blood revealed that 126 of 2307 (5.5%) commercial cases collected between July 2011 and July 2012 generated inconclusive results. Ninety-seven of those 126 cases submitted a second sample, 93 of which returned a result (95.9%), resulting in an overall 0.23% no-call rate after a single redraw (manuscript in preparation). This indicates that redraws are not patient-specific.

Although the results were reported by PS informatics in a blinded fashion, sample collection was intentionally unblinded as this study was intended as a proof-of-principle report of the PS method.

Future directions

Preliminary data from an improved method involving an increase in the number of PCR assays to 19 500, an increase in reaction concentration, and an updated version of the PS algorithm, show a no-call rate of significantly below 10%, in line with other commercially available tests, without a change in the accuracy (manuscript in preparation).

Because PS uses a targeted amplification approach, future efforts could target panels for detection of sub-microscopic imbalances (microdeletions/microduplications).[51,52] Additionally, PS focuses on polymorphic loci, which allows for parental haplotype reconstruction, and thus detection of fetal inheritance of individual disease-linked loci. This is not possible for quantitative methods that utilize sequence counts of non-polymorphic loci.

CONCLUSION

Parental Support™ analysis of targeted regions of the genome represents a novel, promising method for prenatal aneuploidy testing. Here, chromosome copy number was determined at chromosomes 13, 18, 21, X, and Y with 100% sensitivity and 100% specificity for all samples passing the quality test. The PS method obviates issues with amplification variation and generates a more powerful sample-specific calculated accuracy for samples with low fetal fractions of cfDNA. Together, this holds promise for the development of a noninvasive screening test with accuracy and scope comparable to current invasive testing.

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WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Decades of research on DNA-based noninvasive prenatal aneuploidy testing are reaching fruition.

WHAT DOES THIS STUDY ADD?

- Presented here is a proof-of-principle study describing a novel informatics-based noninvasive prenatal method for detecting fetal aneuploidy of chromosomes 13, 18, 21, X, and Y with high calculated accuracy across all five chromosomes for the samples tested.
- This method was shown to detect trisomy 13, 18, 21, 45,X, and 47,XXY in a fetus with 100% accuracy.

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