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A New Era in Prenatal Diagnosis: The Use of Cell-Free Fetal DNA in Maternal Circulation for Detection of Chromosomal Aneuploidies

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Prenatal screening for chromosomal aneuploidies is a fundamental part of routine obstetric care in most countries. Typically, maternal age, weight, ethnicity, serum biomarkers (including pregnancy-associated plasma protein A, human chorionic gonadotropin, α-fetoprotein, inhibin A, and estriol), and sonographic features (i.e., nuchal translucency) are included in a risk algorithm to determine the probability of the fetus being affected. Pregnant women identified as at high risk according to the prenatal screen can then undergo invasive procedures, such as amniocentesis and chorionic villus sampling, to confirm the diagnosis. Current prenatal-screening methods are able to identify approximately 90% of pregnancies affected by trisomy 21 (Down syndrome) at a false-positive rate of approximately 5%. Given that the prevalence of chromosomal aneuploidies is generally quite low, a false-positive rate of 5% means that a large number of women with unaffected pregnancies undergo invasive procedures, putting the fetus at an unnecessary risk for miscarriage. The discovery of fetal cell-free DNA in the plasma of pregnant women 14 years ago opened up the possibility of identifying chromosomal abnormalities noninvasively, through a single blood sample. Approximately 10% of cell-free DNA in the maternal circulation is of fetal origin, and this property was initially exploited to determine rhesus D status and the sex of the unborn fetus. The advent of next-generation DNA sequencing, however, has allowed prenatal detection of chromosomal aneuploidies, including trisomy 21, from maternal blood. In brief, the proportion of chromosome 21 DNA molecules in maternal plasma is measured directly; an increase above a predetermined threshold is indicative of trisomy 21. The clinical performance of this noninvasive prenatal test has been promising, with recent clinical studies having shown a diagnostic sensitivity of 100% and a diagnostic specificity of 98%–99%, compared with full karyotyping by invasive means. When used as a second-tier screening procedure, this technology also has the potential to markedly reduce the number of women undergoing invasive diagnostic procedures, which produces considerable cost savings. In this article, 4 leaders in the field of noninvasive prenatal diagnosis provide their opinion on this exciting advancement.

Can you briefly describe how next-generation sequencing (NGS)[7] has been applied to the detection of chromosomal aneuploidies with cell-free fetal DNA? Are there any other laboratory techniques that have been explored for this purpose?

Barry Hoffman: NGS noninvasively detects chromosomal aneuploidy of the fetus prenatally by determining the relative proportion of DNA from the affected chromosome in the cell-free fragmented DNA circulating in the maternal blood. Many millions of sequencing reads at an appropriate depth of coverage are required to reliably quantify the miniscule increment or decrement in chromosomal dosage in the maternal circulation due to the fetal aneuploidy, and this has become feasible only with the advent of NGS technology. Either all of the circulating DNA fragments are sequenced without preselection by using massively parallel shotgun sequencing (MPSS) in a nontargeted approach, or DNA fragments from the target of interest, in this case the aneuploidic chromosome along with one other, are first selected before subsequent sequencing.

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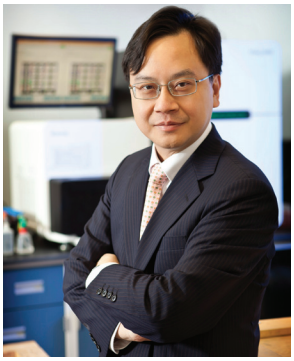
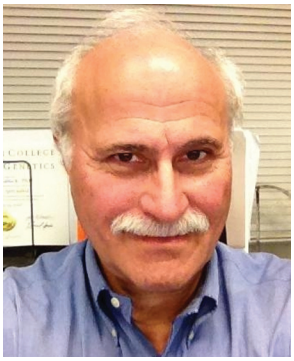
[7] Nonstandard abbreviations: NGS, next-generation sequencing; MPSS, massive parallel shotgun sequencing; NIPT, noninvasive prenatal testing; TAT, turnaround time; ISPD, International Society for Prenatal Diagnosis.

The latter requires considerably less sequencing, thereby improving throughput and decreasing reagent cost, but is inherently more complex by requiring a strategy to select DNA from targeted regions. Some of the methods correct for the fraction of cell-free DNA in the maternal circulation that originates from the fetus, as this influences the magnitude of aneuploidic chromosomal dosage observed against the backdrop of the much larger maternal disomic contribution. Typically, the fetal fraction is of the order of 10%, but results that are sufficiently reliable can be obtained when the fraction is as low as 3%–4%. Another approach is to enrich the fetal fraction, for example by exploiting methylation differences between fetal and maternal DNA, before sequencing to increase the fetal signal.



Dennis Lo: Most published studies on the use of NGS for detecting chromosomal aneuploidies are based on random, or shotgun, sequencing. When mapped back to the reference human genome, the sequencing data yield a proportional representation of each chromosome in maternal plasma. When used to detect a fetal trisomy, the aneuploid chromosome would show an increased representation in maternal plasma in the presence of a fetus suffering from the trisomy. Apart from random sequencing, a number of researchers have published on approaches based on targeted sequencing, in which sequences from the chromosomes of interest are selectively captured or amplified and then sequenced. Apart from NGS, a number of reports have studied the use of the allelic ratio of plasma RNA and DNA methylation markers for the noninvasive prenatal detection of fetal chromosomal aneuploidies. In my opinion, these methods are not as mature as strategies based on NGS, especially in terms of robustness and population coverage.



Jacob Canick: Professor Lo's hallmark finding in 1997, that DNA fragments of fetal as well as maternal origin are present in the circulation of pregnant women, provided the biological basis for developing an NGS strategy for fetal-aneuploidy detection. Simultaneous sequencing of millions of such DNA fragments allows for computerized identification of the chromosome of origin for each fragment. Therefore, the ability to discern a fetal trisomy on a background of cell-free DNA from a euploid mother is really an exercise in analytical imprecision. A fetus affected by a particular trisomy will have a 50% increase in that chromosome's contribution to the total fragments sequenced, but the increase is diluted according to the proportion of fetal DNA present in the maternal plasma sample. The actual increase caused by the trisomic fetus will be, on average, one half of whatever the percent fetal DNA contribution is in that sample. Other molecular methods that have been examined previously with some success involve epigenetic differences between fetal and maternal DNA (e.g., methylation) and RNA (e.g., fetal-specific vs maternal-specific gene transcription). However, the degree of epigenetic variation is different in different populations, so that so-called universal coverage by using such methods is difficult to attain.

Dirk van den Boom: The key questions after Dennis Lo's 1997 publication related to (A) choosing the most appropriate analyte (DNA, RNA) to enable commercially viable sample-collection procedures; (B) maximizing the yield of the chosen analyte in nucleic acid extraction procedures; and (C) identifying the most appropriate technology to reliably detect the fetal aneuploidy in the chosen analyte given that the majority of information in maternal blood/ plasma is maternally derived. A variety of methods were initially researched, among them RNA-based methods, methods relying on epigenetic differences between the maternal and fetal genome, and different detection technologies, including digital PCR, mass spectrometry, and sequencing. NGS is currently the favored and dominant detection technology, because it provides the necessary amount of data points that allows for accurate detection of a fetal trisomy. At the Sequenom Center of Molecular Medicine, we have chosen to implement a whole-genome approach towards fetal aneuploidy detection by using NGS. DNA is extracted from maternal plasma. Most of the extracted DNA is apoptotic in nature and highly fragmented into relatively short continuous stretches (around 150 bp). Then, a DNA library representative of the maternal and fetal genomes is prepared from these fragments. Following amplification, several million DNA fragments of the library are sequenced in a massively parallel fash-

ion. For each component of the library, the process generates 36 bases of continuous sequence information (sequence read), sufficient to uniquely identify the chromosomal origin of the sequence/fragment. After completion of the sequencing process, all sequence reads generated per patient sample are mapped back to their chromosomal location on the human genome, and the frequency of fragments that align with each chromosome is counted. With these data in hand, one can now calculate the relative representation of each chromosome vs a set of reference chromosomes. The value strongly correlates with the size of the chromosome and is a very precise and stable measure, because it is based on a large number of measurements of independent markers (millions of data points per sample, with nearly each sequence read being an independent marker). At this point, the detection of a fetal trisomy, such as trisomy 21, becomes rather simple. The cell-free DNA derived from the plasma of a pregnant women carrying a fetus with trisomy 21 will contain more fragments from chromosome 21, due to the extra copy of that chromosome in the fetal genome and hence in the cell-free fetal DNA present in maternal plasma. In the sequencing process this will lead to an overrepresentation of chromosome 21 sequences relative to a set of reference chromosomes. Thus, a trisomy 21 is identified as a significant increase in the chromosome 21 representation compared to plasma from pregnant women carrying a euploid fetus. This specific case for trisomy 21 can be generalized to the identification of significant changes (increases or decreases) in the chromosome representation of any chromosome. While the MaterniT21™ Plus laboratory test currently marketed by the Sequenom Center of Molecular Medicine currently focuses on chromosomes 21, 18, and 13, our chosen whole-genome approach is generically applicable, so that additional fetal aneuploidies can be detected and reported without changes to the test method as additional clinical validations are performed and demonstrate an acceptable level of performance. As the cost of NGS decreases, this technology could eventually lead to a noninvasive molecular karyotype with a similar content and performance to today's invasive cytogenetic test.

Have different NGS platforms been evaluated for the purpose of noninvasive prenatal diagnosis? Are there notable differences?

Barry Hoffman: A number of NGS platforms have been evaluated for noninvasive prenatal testing (NIPT), including those from Illumina, Ion Torrent, and Applied Biosystems. All 3 platforms clonally amplify the DNA fragments, either by emulsion-based PCR or solid-phase bridging PCR, before sequencing, which has the downside of introducing adenine–thymine (AT) guanine–cytosine (GC) bias that must be later corrected. The first 2 platforms use sequencing-by-synthesis mediated by DNA polymerase, while the latter employs synthesis-by-ligation mediated by DNA ligase. The nucleotides sequentially added to the single-stranded DNA template by the polymerase or ligase are identified by the analyzer to produce the sequence. In the case of the Ion Torrent analyzer, the 4 nucleotides are cyclically added one at a time, and the hydrogen ion that is released when one is incorporated is detected. The other 2 platforms incorporate fluorescently modified nucleotides into the DNA strand to determine the sequence. Despite these differences, published studies have shown that all 3 platforms are capable of accurately diagnosing trisomy 21 noninvasively. New technologies on the horizon can eliminate the need of amplifying the template and adding nucleotides to determine the sequence. These advances will substantially reduce reagent costs and the turnaround time (TAT) of the test, thereby facilitating the application of NGS to clinical medicine.

Dennis Lo: Thus far, most publications have reported the use of the sequencing-by-synthesis platform. There are also a couple of publications reporting the use of the sequencing-by-ligation platform. The results generated by using such platforms are generally very comparable. However, the details of the sequencing protocols and bioinformatics analyses do affect the no-call rate and whether the sequencing results would require additional clinical parameters (e.g., gestational age and maternal age) for interpretation. Since both platforms require the use of amplification, GC bias has been observed. For the detection of chromosomal aneuploidies involving chromosomes particularly prone to this bias (e.g., chromosomes 13 and 18), special bioinformatics algorithms correcting such effects are needed and have been shown to be very effective. Two recent papers reported the use of a single-molecule sequencer that does not require an amplification step for analyzing maternal plasma DNA. As a result, GC bias does not appear to be a problem anymore. It would be interesting to test a number of emerging single-molecule sequencing platforms (e.g., nanopore sequencing) for this application.

Jacob Canick: To my knowledge, 2 NGS platforms have been tested and found suitable for this application. The most extensive work has been done by using the sequencing-by-synthesis method on the Illumina Hi-Seq 2000 platform. One study in 2010, in which sequencing-by-ligation was done by using the SOLiD™ 3 System from Applied Biosystems, provided evidence that this method should also be effective.

Dirk van den Boom: While there are a few publications describing preliminary evaluations of other NGS platforms such as the SOLiD™ system or the Heliscope system (Helicos), we chose to implement the MaterniT21 Plus laboratory-developed test on the Illumina HiSeq 2000 platform. The main drivers for this decision were the high data quality and maturity of the technology at the time of our clinical-validation study. Suitability for the clinical laboratory work flow, sample capacity, and cost considerations were also key factors in our choice of instrumentation for initial commercialization.

What is the TAT for this method? Within what time period of gestation should this test be performed? How does this compare to current practices for maternal-serum screening?

Barry Hoffman: The TATs of commercially available NGS-based tests that detect trisomy are typically on the order of 1.5–2 weeks, somewhat longer than the 2- to 5-day reporting times routinely achieved by traditional maternal-serum screens in the first or second trimester of pregnancy. NGS throughput depends on how well the testing laboratory has automated, simplified, and efficiently meshed from "end to end" the discrete steps required to generate a clinical result (target selection, library construction, template preparation, sequencing, data processing, and interpretation). Other interrelated factors that bear on the throughput include the inherent speed of the core sequencing technology used, the number of multiplexed samples that can be combined in a single run, and the quality and length of the sequencing reads that in turn impact on the required depth of coverage and alignment with the genome. Although the absolute and relative amount of fetal DNA in the circulating cell-free DNA of the mother is lower earlier in gestation, published studies have shown that NIPT can reliably detect fetal trisomies as early as the 10th week of gestation. Such sampling in the late first trimester fits nicely with elements of current obstetrical practice and the emerging concept of a late first-trimester routine first hospital visit of the pregnancy, in which the fetus is assessed for anatomic anomalies, screened for trisomies, and assessed for the likelihood of early-onset preeclampsia.

Dennis Lo: The TAT is approximately 7–10 days. The test can be performed from 10 weeks of gestation onwards. This time frame is highly compatible with current practices for maternal serum. For example, pregnant women can first be screened by ultrasound for assessment of nuchal translucency of their fetuses and first-trimester maternal-serum testing. Women classified as high risk by such tests can then be referred to undergo the NGS test using maternal-plasma DNA. Similarly, the time frame is also compatible with second-trimester maternal-serum screening and the integrated test.

Jacob Canick: In a study in which the Sequenom Center for Molecular Medicine tested almost 2000 patient samples in 9 weeks, we arranged for all the samples to be tested in real time, and the TAT was under 10 days for 18 of the last 20 sets of 90 samples run. This is similar to the TAT typical for full karyotype analysis of amniotic fluid and chorionic villus samples. Most of that time is consumed by the current NGS and bioinformatics technology, which should be able to be reduced within the next few years. Maternal serum screening (with or without fetal ultrasound) is expected to have a TAT of no more than 2–3 working days, although in most cases once the sample is in the testing lab, running the multiple-marker assays and reporting out the screening results typically take 1 day.

Dirk van den Boom: In our center, we currently average a TAT of <7 business days upon accessioning of a sample. This TAT is based on the laboratory's experience with over 40 000 clinical samples. Through an independently designed and executed clinical study, we validated the utility and imprecision of the test encompassing the period of 10–22 weeks of gestation. Noninvasive detection of fetal aneuploidies through analysis of cell-free fetal DNA from maternal plasma is a direct test and does not use surrogate markers, which could be confounded by other clinical variables, such as ethnicity, smoking, gestational age, or insulin-dependent diabetes mellitus. As a result, this test technology enables diagnostic sensitivity and specificity that are significantly improved over the commonly employed serum biochemical-screening tests.

Is this test available commercially now? If so, how expensive is it?

Barry Hoffman: NIPT of fetal cell-free DNA in the maternal plasma to detect trisomy 21, 18, and 13 is currently offered commercially in the US by a number of vendors, including Sequenom Center for Molecular Medicine (MaterniT21 Plus®), Ariosa Diagnostics Inc. (Harmony Prenatal Test®), and Verinata Health (Verifi®). These tests are directed to pregnancies at high risk for trisomy 21 and are available upon request by a US physician. Related testing for sex chromosome aneuploidy (XO and, recently, XXY, XXX, and XYY) is available in some cases, as is gender assignment. The cost of trisomy testing ranges from $795 to $2760. In Asia and Europe, different commercialization agreements have been formulated to provide similar testing. In Canada, the Harmony Test was launched in January 2013.

Dennis Lo: The test is available commercially now. Costs have been quoted as ranging from approximately $1000 to $3000 per test.

Jacob Canick: Yes. As indicated above, in the US there are currently 3 commercial laboratories offering DNA-based maternal-plasma aneuploidy testing. LifeCodexx AG of Konstanz, Germany, is now offering testing in German-speaking European countries, and in China the Beijing Genomics Institute and Berry Genomics are accepting samples. Natera, Inc., of San Carlos, California, has announced its intention to offer testing by the end of 2012. The list prices for the tests offered in the US range from $795 to $2700, with some health insurance programs in place.

In your opinion, do you think this test will eventually become available in most clinical laboratories, or do you see it being offered by specialized centers only?

Barry Hoffman: Currently, NIPT for trisomy 21 in North America is available only from specialized national or regional laboratories operated by the commercial vendors, and this is likely to remain so in the near future. However, as molecular testing evolves and becomes more widespread, one can envision the development of turnkey packages of prenatal genetic tests, automated end to end and targeted to (ever expanding) sets of selected anomalies that would be suitably user-friendly to implement in clinical laboratories seeking to offer a prenatal service. Transitioning the testing from vendors to clinical laboratories would require an alternative business model driven by licensing fees and the sale of proprietary kits (reagents). It can also be argued that disseminating the testing to local or regional centers better fits the time-sensitive nature of the testing and the existing complex, specialist infrastructure, over and above the testing itself, required to deliver a quality prenatal-screening service. This infrastructure includes pre- and posttest counseling, follow-up invasive testing, extensive caregiver and patient communication, and statistical analysis of the database to determine a test's clinical performance against expected benchmarks. The requirement for such an infrastructure precludes the likelihood of noninvasive molecular prenatal testing being offered by most clinical-service laboratories.

Dennis Lo: As NGS still involves rather expensive instruments and requires extensive bioinformatics support, I believe that it might be more streamlined to be offered by specialized centers, each serving a particular geographic region.

Jacob Canick: Inherent in the new method is the need for costly advanced sequencing platforms, information collection and storage systems, and bioinformatics, as well as highly specialized technical and scientific input, with redundancy built in to assure no lapse in clinical result reporting. In addition, intellectual property issues have resulted in suits and countersuits between commercial entities. Although dependent on the outcome of these lawsuits, it seems unlikely that in the near future many clinical laboratories will validate and offer this test as a clinical service. Eventually, advances in bioengineering will no doubt simplify, speed up, and reduce cost issues, so that these and even more complex genomic methods will become possible for most laboratories.

Dirk van den Boom: At this point NGS-based tests still have a rather high complexity in work flow and the computational and bioinformatic infrastructure requirements, and they demand significant capital investment. Adequate analytical and clinical validation of NGS-based tests for use in prenatal diagnostics is not trivial and requires large sample collections. Therefore, I believe only specialized centers should offer this technology until such time that deployable, simple-to-use test systems that do not compromise accuracy could be made available.

A recent position statement published by the International Society for Prenatal Diagnosis (ISPD) in 2011 stated that the use of NGS for the advanced screening of trisomy 21 should be performed only in high-risk populations. Have any advances been made in the past year to support widespread screening in low-risk populations as well?

Barry Hoffman: When the ISPD issued its recommendation, it was being prudent, inasmuch as the clinical studies to date had validated the test only in pregnancies at high risk for Down syndrome. Since then, numerous studies in high-risk pregnancies have confirmed that NIPT of cell-free DNA in the maternal plasma can detect >99% of pregnancies with fetal trisomy 21, at a false-positive rate of <1%, but the performance of screening for trisomies 18 and 13 has been less robust. The ISPD also recognized that just because the test exhibits near-perfect performance in high-risk populations doesn't mean that the same performance would necessarily be realized in a routine-risk population undergoing screening. In late November 2012, the first study was published that assessed the performance of screening for trisomy 21 and trisomy 18 by using chromosome-selective sequencing in a routine population of 2049 women. The study concluded that NIPT of fetal cell-free DNA in the maternal plasma of a routine-risk population was as effective as previously reported in high-risk groups. A number of other stud-

ies assessing the performance of the test in a routine population of women undergoing screening for aneuploidy are currently under way, with results expected within the next 6–12 months. Validating the test for routinely screened populations will simplify the decision of women and caregivers to choose the NIPT of circulating cell-free fetal DNA in the maternal circulation as the "go-to" preferred approach of aneuploidy prenatal screening.

Dennis Lo: The main biologic determinant of the accuracy of fetal aneuploidy testing using maternal plasma DNA sequencing is the fractional concentration of fetal DNA in maternal plasma. As far as I know, there is no evidence that women classified as high risk by conventional screening tests have a different fractional fetal DNA concentration profile than that in low-risk women. Thus, I am confident that the NGS test should have the same analytic performance in low-risk populations. The question is more an economic one, as there are many more low-risk women than high-risk ones. Hopefully, with the future reduction in the cost of sequencing and associated processes, such economic consideration will become less of an issue. I am thus optimistic that in the medium to long term, maternal plasma DNA testing for aneuploidy screening will become a first-line test.

Jacob Canick: The issue of offering DNA-based screening to all pregnant women should be approached by asking 2 questions: Is there a biological/scientific basis to expect different performance in the general population compared to the high-risk population, and are there more pragmatic, nonscientific reasons to wait before implementing population-based screening? We have examined whether there are any covariates to this type of testing (indirect variables that impact results), a number of which are related to the question of differences in low- and high-risk pregnancies, and the answer has been consistently no. For example, the reason for being called high risk does not change either the fetal fraction or the test result (*z* score) that is assigned for either trisomy 21 or euploid patients. In addition, specific phenotypic markers that are currently used (i.e., the various serum markers and the fetal ultrasound marker, nuchal translucency) either are not or are weakly associated with fetal fraction or *z* score. Currently, the more important reasons for not using NGS to test the general population are practical: excessive cost and TAT, uncertainties about insurance reimbursement, and insufficient testing facilities.

Dirk van den Boom: The utility of NGS has been extensively validated in pregnant women at high risk for fetal aneuploidy. This includes women of advanced maternal age, family history of fetal aneuploidy, positive serum-screening results or ultrasound findings. In addition to the ISPD, the American College of Obstetricians and Gynecologists and the Society for Maternal Fetal Medicine issued a joint-committee opinion recommending that cell-free fetal DNA testing be offered to patients at increased risk of aneuploidy. The California Technology Assessment Forum also recently completed an independent evaluation and recommended that the use of cell-free fetal DNA as a prenatal advanced screening test for fetal aneuploidy for trisomy 21 and 18 in high-risk women meet all 5 criteria for safety, efficacy, and improvements in health outcomes. An initial study with a targeted approach combined with NGS for detection of fetal trisomy has recently been published. This study included a rather limited sample set of low-risk women, and I believe substantially more clinical validation will be required before such a test should be generally applied in a screening scenario. At this point, currently available serum screening–based approaches are still more cost-effective for the general/low-risk population—despite their poorer performance—and they may identify pregnancy complications currently not detectable by cell-free fetal DNA.

Has this technology been applied to other chromosomal aneuploidies aside from trisomy 21? What about diseases arising from single point mutations?

Barry Hoffman: Published studies have demonstrated the potential of NGS to detect a wide spectrum of molecular anomalies in fetal circulating cell-free DNA, including chromosomal aneuploidies, deletions, insertions, single-nucleotide polymorphisms, and copy number variants. The technology must be optimized to identify each type of anomaly, with issues such as length of sequenced transcripts and development of specialized bioinformatic algorithms bearing strongly on the quality and reliability of the outcome. It is conceivable that NGS will supplant chip-based arrays in the detection of fetal anomalies, and there is nothing to preclude the development of "multimodal" sequencing platforms capable of reliably detecting all manner of DNA molecular anomalies and variants in a single pass. Currently, the technology has been applied commercially to the detection of sex chromosome aneuploidy, such as Turner (monosomy X) and Klinefelter (XXY), and to the testing of Rh incompatibility.

Dennis Lo: In addition to trisomy 21, the technology has been applied to trisomies 13 and 18, as well as Turner syndrome. There are also data showing that the sequencing test can be used to detect Down syndrome caused by robertsonian translocation, as well as chro-

mosomal microdeletions. For the latter, the depth of sequencing would need to be increased. We have shown that the sequencing approach can be used for the noninvasive prenatal diagnosis of monogenic diseases (e.g., β-thalassemia), including cases caused by point mutations. For such applications, a targeted sequencing approach appears to be more cost-effective.

Jacob Canick: It is interesting to note that trisomy 21, the most common aneuploidy at birth, also appears to be the easiest of the clinically important aneuploidies to identify by NGS of maternal plasma DNA. Trisomies 18 and 13, and monosomy X, have been more difficult to identify with the same level of performance as for trisomy 21, but the performance for those aneuploidies is now sufficiently high to allow clinical use of NGS to test for them. However, positive test results for any of these aneuploidies, including trisomy 21, are not sufficient to make a definitive diagnosis. Invasive diagnostic testing is still recommended for all positive NGS results to make sure that false-positive euploid fetuses are identified.

Dirk van den Boom: The clinical-validation study for the MaterniT21 Plus test included cases with fetal trisomy 18 and 13, in addition to trisomy 21. Both were detected with very high diagnostic accuracy. Additionally, our technology for fetal aneuploidy detection uses an approach that in principle can assess the whole genome. As samples of fetal trisomies other than 21, 18, or 13 become available for clinical validation, the performance of the current test using NGS can be evaluated, and a decision can be made if these other trisomies should be reported. Early results for the detection of fetal sex aneuploidies by NGS, for example, have been published recently as well. Several research groups have published proof-of-concept studies for the targeted detection of single point mutations in cell-free fetal DNA, in particular for cases indicated by family history and/or carrier status of the parents. Dennis Lo and his team have recently pioneered work to assess detection of single point mutations in a more generic fashion by sequencing an entire fetal genome from cell-free fetal DNA. While this work is extremely exciting, potential commercial implementation will require further advances in reducing associated sequencing costs and, of course, analytical and clinical validation beyond what has been accomplished to date.

Can this technology be applied to twins? What about in vitro fertilization donor pregnancies?

Barry Hoffman: Multiple gestation is currently a contraindication for the NIPT of cell-free fetal DNA in the maternal plasma and is specifically ruled out by ultrasound examination before ordering the test. When twins are detected, an alternative is traditional screening using biochemical and imaging biomarkers, although it is well established that the performance of the traditional screen in multiple gestation is considerably poorer than in singleton pregnancies. There is no a priori reason, however, why the molecular analysis of circulating cell-free fetal DNA to detect an increment in the proportion of fetal trisomy 21 in the maternal plasma could not be applied to multiple gestations. Undoubtedly, validating the test in such pregnancies will be hampered by the rarity of affected fetuses and complexities related to mono- and dizygosity, but there should be more fetal DNA relative to the maternal background, thus increasing the fetal fraction and the reliability of the analysis, particularly in the case of monozygotic twins with their identical DNA and leaky placentas. In addition, the zygosity of the twins will be evident from such testing. In principle, the analysis of fetal cell-free DNA in the maternal circulation should perform as equally well in egg donor pregnancies as in spontaneous conceptions. Limited data now available from the recently reported MELISSA trial shows this to be true. All 36 assisted-reproduction pregnancies that were included in this prospective trial of the accuracy of the Verifi test in a "real world" mix of pregnancies were correctly assessed.

Dennis Lo: The technology can be applied to twins. We have shown that the technology can also allow one to determine the zygosity of twin pregnancies. Furthermore, for dizygotic twins, maternal-plasma DNA sequencing can allow one to measure the amount of DNA released by each twin, which might have interesting research and clinical applications. The technology can be applied to in vitro fertilization donor pregnancies.

Jacob Canick: A study from our group and a study from Diana Bianchi's group indicate that NGS testing for aneuploidies will identify twin pregnancies in which one or both of the fetuses are affected. While these results are based on fewer cases than the literature on singleton pregnancies, it appears that twin pregnancies have, on average, a higher fetal fraction than singleton pregnancies, contributing to better test performance. Bianchi and colleagues have briefly addressed the question of pregnancies achieved by in vitro fertilization and have found no differences in performance, although no data were provided.

Dirk van den Boom: Yes, the technology concept underlying the MaterniT21 Plus test is suitable for application in twin pregnancies. The ability to detect a fetal

trisomy in maternal plasma is mainly driven by the relative amount of fetal DNA vs maternal DNA. In the published validation studies, the fetal fraction averaged around 13%, with the major component almost always being of maternal nature. In twin pregnancies where only one of the gestations is trisomic, the overabundance of the respective chromosome is still detectable as long as the effective fetal fraction is above the detection threshold. The nontrisomic fetus adds only a small amount to the euploid maternal component. We have reported on over 700 twin gestations. As with twins, the MaterniT21 Plus test can be applied to in vitro fertilization donor pregnancies. The analysis of the chromosomal representation underlying the noninvasive detection of fetal trisomy in maternal plasma, in principle, is not impacted by donor eggs.

In your opinion, do you see this test eventually acting as a "stand alone" diagnostic test for prenatal screening and replacing the maternal serum screen altogether? Or will it simply be an add-on to current screening procedures?

Barry Hoffman: There is no question that NIPT of fetal cell-free DNA in maternal plasma is a far superior test than the traditional trisomy screen that uses biochemical and imaging biomarkers. However, the former is also considerably more expensive. It can be expected that individuals who can afford to pay for the molecular testing, either privately or through insurance, will seek it as their initial test. However, it is unlikely that government-funded prenatal-screening programs will have the funds to pay for the universal application of molecular testing to all women undergoing prenatal screening. A cost-neutral solution to this dilemma is contingent testing, whereby traditional screening is carried out first and molecular testing offered only to the screen positives. The performance of such screening approaches to that of molecular testing alone and the delay to a final result should be acceptable if due attention is paid to speeding the initial screen-positive samples to the second stage of testing. In Ontario, preliminary financial analysis has shown that contingent screening is cost neutral when the positive rate of the traditional initial screen is set to 5%. The positive rate cutpoint can be increased as the cost of molecular testing decreases.

Dennis Lo: For maternal serum screening for Down syndrome, I think that the sequencing test is definitely superior and would likely replace the conventional approach eventually. However, as discussed above, for the short term the expenses associated with the sequencing test would make it perhaps more cost-effective to perform conventional screening first and then refer the high-risk women classified by such screening to the sequencing test.

Jacob Canick: I expect that NGS will eventually become a first-line test offered to all pregnant women. It is clear that for the common trisomies the test already has superior performance to current prenatal-screening methods. The detection rate approaches 100%, and the false-positive rate is substantially less than 1%, with relatively low failure rates. As a first-line test, the positive predictive value for Down syndrome would be close to 50%. That is, one of every 2 invasive diagnostic procedures would result in identification of a fetus with Down syndrome. Predictive values would be lower for the other trisomies, since their prevalence in the population is lower. There remains the question of uninformative tests caused by samples that have, for example, too low a fetal fraction or by methodological failures, but such patients can be offered standard screening as an alternative. My expectation of first-line testing reasonably depends on continued improvements in throughput and cost being achieved during the next few years.

Dirk van den Boom: The use of cell-free fetal DNA affords diagnostic sensitivity and specificity that can outperform current serum screening for fetal aneuploidy. However, maternal-serum biochemical screens for indications other than fetal aneuploidy could not, in principle, replace maternal-serum screening. Additionally and equally importantly, there will still need to be an assessment for fetal neural tube defects that currently cannot or are not addressed by NGS.

A 2012 paper in Nature by Fan et al. and a 2012 paper in Science Translational Medicine by Kitzman et al. describe the noninvasive determination of the entire fetal genome from maternal blood. What implications do you foresee arising from this discovery? How will this build on current clinical practices?

Barry Hoffman: Targeting the molecular analysis of fetal cell-free DNA in the maternal circulation to a specific anomaly, or even a limited set of anomalies, is reasonably straightforward in terms of current clinical practice and ethical norms. As the number of anomalies included in the molecular testing increases, so does the difficulty of coherently presenting the findings to the ordering physician, the genetic counselor, the parent(s), the fetus (eventually), and the related kindred. But, noninvasively sequencing the entire fetal genome is the ultimate open-ended test, unquestionably an exciting technical tour de force, but utterly devastating, insofar as applying the information clinically will require an entirely new ethical, educational, technical,

and physician-delivery framework, none of which is currently in place. It will take time, resources, new insights, and much hard work before the potential of NGS at the level of the entire genome can be fully realized in the clinic.

Dennis Lo: Using an approach based on paternal genotyping, maternal haplotyping, and maternal plasma DNA sequencing, Kitzman and colleagues have confirmed our approach and shown that the method is scalable and robust, even when applied to a deeper level of sequencing. The paper by Fan and colleagues also confirmed the general concept but posed the additional question as to whether genotyping of the father's DNA could be spared and whether one could directly deduce the paternally inherited fetal alleles that were not present in the mother's genome from the maternal-plasma DNA-sequencing data. Taken together, these papers demonstrate the feasibility of noninvasive fetal genome sequencing. While this development is technologically and scientifically very exciting, for clinical applications it might be more cost-effective to perform targeted sequencing for selected genomic loci involved in monogenic diseases common in a particular population, as has been recently achieved. This would also make counseling easier to do and would reduce the ethical complexity on introducing such testing clinically.

Jacob Canick: Both publications, which show that fetal and maternal genomes can be fully sequenced from samples of maternal plasma, build on a proof-of-concept study by Dennis Lo and his colleagues in 2010 showing that the complete genomes of both mother and fetus are present in the DNA fragments present in the maternal circulation and that these fragments are in a constant relative proportion. The implication of the discoveries is clear. The potential here is to identify many of the single-gene disorders in the fetus, both inherited and newly formed, without having to use invasive and risky fetal-sampling techniques. The time frame for clinical implementation of these discoveries is unknown at present, but 10 years is perhaps not an unreasonable guess.

Dirk van den Boom: Common to other potentially disruptive technologies in medical care, the use and implementation have to be accompanied by adequate ethical considerations and appropriate analytical and clinical validation. While current tests are focusing on the detection of trisomy 21, 18, and 13, a noninvasive karyotype similar in information depth to comparative genomic hybridization arrays performed on amniotic fluid are within reach as sequencing technologies evolve. NIPT is extremely powerful and will allow for the diagnosis of many disorders, which are currently assessable only by obtaining samples from invasive procedures. It is important that this new technology gets applied to improve health outcomes. Of particular interest could be diseases that allow for treatment in utero as well as for prenatal identification of metabolic diseases, which could allow for dietary adjustments at birth, before symptoms develop.

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