Genome-Wide Sequencing for Prenatal Detection of Fetal Single-Gene Disorders

Ignatia B. Van den Veyver^{1,2} and Christine M. Eng²

¹Department of Obstetrics and Gynecology, Baylor College of Medicine, The Jan and Dan Duncan Neurological Research Institute at Texas Children's Hospital, Houston, Texas 77030

²Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030

Correspondence: iveyver@bcm.edu

New sequencing methods capable of rapidly analyzing the genome at increasing resolution have transformed diagnosis of single-gene or oligogenic genetic disorders in pediatric and adult medicine. Targeted tests, consisting of disease-focused multigene panels and diagnostic exome sequencing to interrogate the sequence of the coding regions of nearly all genes, are now clinically offered when there is suspicion for an undiagnosed genetic disorder or cancer in children and adults. Implementation of diagnostic exome and genome sequencing tests on invasively and noninvasively obtained fetal DNA samples for prenatal genetic diagnosis is also being explored. We predict that they will become more widely integrated into prenatal care in the near future. Providers must prepare for the practical, ethical, and societal dilemmas that accompany the capacity to generate and analyze large amounts of genetic information about the fetus during pregnancy.

To assess for fetal genetic risks and defects, pregnant women now have access to very sensitive and specific noninvasive screening methods for the common autosomal-trisomies. More recently, noninvasive screening for a few selected deletion syndromes is being investigated (Lau et al. 2013, 2014; Srinivasan et al. 2013) and has been added by some providers (Bianchi and Wilkins-Haug 2014), but the performance of noninvasive detection of such microdeletions is still being evaluated. Diagnostic procedures, such as amniocentesis and chorionic villus sampling (CVS), followed by karyotype analysis, are available for detection of all trisomies and large

chromosomal rearrangements. The addition of cytogenomic technologies such as chromosomal microarray analysis (CMA), allows detection of clinically significant unbalanced chromosomal abnormalities in 1%–1.7% of pregnancies of women undergoing amniocentesis or CVS for common risk factors and in 6%–7% when structural birth defects detected by prenatal imaging (Wapner et al. 2012; Hillman et al. 2013). If single-nucleotide polymorphism (SNP) arrays are used, regions of absence of heterozygosity can be found that may uncover uniparental disomy or a close relationship of parents that may predispose them to transmit

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ting rare recessive alleles (Schaaf et al. 2011). Although the higher detection rate of CMA tests is an important advance in prenatal genetic diagnosis (ACOG 2013), it still means that in the majority of cases a distinct genetic etiology for birth defects seen on prenatal ultrasound examination cannot be found (Hillman et al. 2014). Furthermore, none of these techniques can detect other types of mutations, such as point mutations and small insertion–deletion (indels) mutations, that cause the now more than 4600 known single-gene disorders and others yet to be characterized (Xue et al. 2014). Formerly, to find such mutations, targeted analysis of candidate genes was needed, requiring prior knowledge of clinical phenotypes caused by mutations in specific genes. This is challenging because some phenotypes or genetic disorders can be caused by mutations in different genes, while the genetic causes of other phenotypes are not yet defined. Furthermore, in prenatal diagnosis, certain phenotypic features, such as intellectual disability or minor birth defects and dysmorphic features, cannot be ascertained in the fetus before birth because of the limitations of prenatal imaging and the developmental stage at which they become recognizable. For other conditions with a distinct postnatal presentation, the prenatal phenotype may be incompletely defined (Filges and Friedman 2014).

The development of next-generation sequencing (NGS) technologies has revolutionized Mendelian disease gene identification and genetic diagnosis in pediatric and adult medicine because it has the ability to interrogate multiple genes at once, identifying deleterious variants that can then be correlated with the clinical presentation to make a molecular diagnosis (Berg et al. 2011; Gilissen et al. 2011; Gonzaga-Jauregui et al. 2012; Biesecker and Green 2014). Several clinical laboratories are now offering diagnostic whole-exome sequencing (WES) to search for mutations in the coding sequence of the ~20,000 human genes (de Ligt et al. 2012; Yang et al. 2013, 2014; Eng et al. 2014; Xue et al. 2014), providing results that are relevant for Mendelian disease diagnosis and risks for adult-onset conditions, including cancer, and pharmacogenetic information that can

guide therapeutic decisions (Korf 2013; Biesecker and Green 2014). Although NGS is not yet used extensively for prenatal diagnosis of Mendelian disorders (Hillman et al. 2014), it is slowly being introduced into this field for noninvasive detection of fetal aneuploidies, for carrier screening, and for research applications into the etiologies of fetal and maternal disorders (Hui and Bianchi 2013). While the NGS technology and its clinical applications, such as diagnostic WES, are advancing at an unprecedented pace, there are many practical challenges and ethical considerations that complicate its introduction into prenatal care (Berg et al. 2011; Gilissen et al. 2011; Gonzaga-Jauregui et al. 2012; Korf 2013; Biesecker and Green 2014).

NEXT-GENERATION SEQUENCING (NGS) TECHNOLOGY AND PITFALLS

NGS technology has completely revolutionized genetic sequencing because of unprecedented increases in speed and decreases in cost. Although it took years to sequence the first draft of the human reference genome, it is now possible to sequence an entire genome in a few days for a small fraction of that cost (Gonzaga-Jauregui et al. 2012). NGS is accomplished by massively parallel sequencing (MPS) of multiple sites through synthesis of millions of randomly distributed and overlapping small fragments of DNA of known sequence (Fig. 1) (Shendure and Ji 2008). The sequences from these fragments are then aligned to the human reference genome sequence to determine their location and compare their sequence content to that of the reference sequence. Multiple copies of each region are covered by overlapping fragments.

There are two basic strategies for NGS of genomic DNA (Fig. 1), a whole-genome approach and a more targeted approach (Bamshad et al. 2011; Biesecker and Green 2014). In the first, whole-genome sequencing (WGS), genomic DNA is fractionated into random small fragments. A sequencing library of all the fragments is then constructed by adding linkers and used as the template for sequencing by synthesis with fluorescent light-emitting nucleotides.

Genome sequencing for fetal single-gene disorders

Figure 1. Workflow for next-generation sequencing (NGS). (A) Library preparation: Genomic DNA is prepared from a prenatal sample (amniotic fluid or chorionic villi) and fragmented. This is followed by adapter ligation and preparation of the sequencing library. When WGS is performed, the library is directly denatured for use as sequencing templates (blue lines and arrows). For targeted approaches such as whole-exome sequencing (purple lines and arrows), an additional step to capture and enrich for fragments of interest (such as coding exons) for sequencing by hybridizing them to a library of baits with known sequence, followed by purification and bait release is required. (B) Sequencing procedure: Fragments are immobilized by hybridization to linkers (here, represented on a solid surface, but other methods exist), followed by multiple rounds of replication and clonal amplification. Differently fluorescently labeled nucleotides are then added to the single-stranded templates and emitting light is used to identify the added nucleotide that is complementary to the template. The sequenced fragments are then aligned to the reference genome sequence in multiple copies (sequencing reads). In WGS, the alignment covers all regions for which sequencing was successfully performed. In targeted sequencing, such as WES, only those sequences represented in the baits are covered, typically with more reads for each.

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The sequenced material originates from coding and noncoding regions of genes, from intergenic sequences and includes mitochondrial DNA. In the second approach, an additional step is added after the sequencing library is generated to enrich for and “capture” specific regions of interest by hybridizing the library of fragments to a collection of baits that represent these regions. After purification, only the purified captured fragments are sequenced, allowing increased sequencing depth and thereby accuracy at a lower cost. This is the basis for the development of diagnostic disease-focused multi-gene panels (Jones et al. 2013; Brett et al. 2014; Lepri et al. 2014; Onoufriadis et al. 2014; Xue et al. 2014), containing tens to up to hundreds of genes, as well as for WES (Lemke et al. 2012; Korf 2013; Xue et al. 2014). For WES, the baits are designed to capture the coding exons of nearly all genes, which represent ~1%–2% of the total genomic DNA but contain ~85% of known disease causing mutations (Bick and Dimmock 2011; Gilissen et al. 2012; Gonzaga-Jauregui et al. 2012; Korf and Rehm 2013; Xue et al. 2014).

Because NGS technology is more error-prone than Sanger sequencing, each region is sequenced multiple times and the sequencing depth is defined as the number of copies of each region represented in the pool of fragments (Korf and Rehm 2013). The Laboratory Quality Assurance Committee of the American College of Medical Genetics and Genomics (ACMG) has published standards for minimal coverage when NGS is used for diagnostic purposes. A minimum of 30-fold coverage is considered adequate for diagnostic WGS, and a minimum of 10- to 20-fold coverage of all bases is needed to make accurate diagnostic calls with targeted panels. Required coverage also depends on which individuals are sequenced. The ACMG recommends that a laboratory might ensure that diagnostic WES has a minimum mean coverage of 100-fold for the proband and that 90%–95% of bases in the defined WES targets reach at least 10-fold coverage, but that 70-fold might be used when trios are sequenced (Rehm et al. 2013). As technology is improving, deeper sequencing at reasonable cost is becoming available in a more routine manner (Yang et al. 2014).

APPLICATIONS OF DIAGNOSTIC SEQUENCING IN THE ADULT AND PEDIATRIC POPULATION

Gene Panels and Targeted Sequencing

NGS has resulted in a paradigm shift in how genetic diagnostic testing is approached, especially when a single phenotype or a phenotypic spectrum can result from disease-causing mutations in one of a variable number of genes or for conditions with highly variable phenotypes (Hennekam and Biesecker 2012). Rather than a lengthy stepwise approach with sequential mutation analysis of the most obvious candidate genes first, followed by analysis of less-likely candidate genes, disease-focused NGS panels permit analysis of up to several hundreds of genes at once for defined categories of phenotypes, such as congenital heart defects, skeletal dysplasias, mitochondrial disorders, disorders of glycosylation, ciliopathies, cardiac defects, epilepsies, Noonan syndrome, etc. (Jones et al. 2013; Korf 2013; Valencia et al. 2013; Brett et al. 2014; Chen et al. 2014; Lepri et al. 2014; Onoufriadis et al. 2014; Xue et al. 2014).

With phenotype or disease-focused NGS panels, sequencing typically provides a deeper and more comprehensive coverage of included genes at lower cost compared with WES or WGS, because the total amount of DNA sequence that needs to be covered is significantly less (Xue et al. 2014). NGS panels are also often complemented with other methods, such as Sanger sequencing for genes and regions that are difficult to sequence by NGS, or with array-based analysis for detection of microdeletions, microduplications and absence of heterozygosity.

WGS and WES for Mendelian Disease Gene Discovery and Clinical Genetic Diagnosis

WES, WGS, or targeted NGS sequencing of mapped candidate regions have quickly become the most widely used approaches for disease gene discovery, for which they have been highly successful (Bamshad et al. 2011; Gilissen et al. 2012, 2014; Beaulieu et al. 2014; Dymment et al. 2014; Makrythanasis et al. 2014; Rios and Delgado 2014). This has resulted in a sharp increase

in recent years in the number of Mendelian disorders for which the genetic basis is now known, from about 3000 a few years ago to a currently estimated approximately 4600 (Xue et al. 2014), and this number continues to rise.

A growing number of commercial and academic genetic diagnostic laboratories are now offering WES (de Ligt et al. 2012; Yang et al. 2013, 2014; Atwal et al. 2014; Eng et al. 2014; Fahiminiya et al. 2014; Iglesias et al. 2014; Lee et al. 2014). In some cases, WGS and clinical diagnostic WES are becoming near routine tests in pediatric and adult genetic clinics. Early published work from the Baylor Whole Genome Laboratory (WGL) on 250 clinical cases showed that WES provides up to a 25% increase in successful genetic diagnosis after all other genetic work-up is negative (Yang et al. 2013, 2014). More recently, results on 2000 additional cases, of which 88% were from pediatric patients, have confirmed the molecular diagnosis rate of 25%. Of the identified pathogenic mutations, 58% had not been previously reported (Yang et al. 2014). Other laboratories have shown similar successes with diagnostic WES for intellectual disability (16% diagnostic rate) (de Ligt et al. 2012) and for autosomal recessive conditions (25% diagnostic rate) (Fahiminiya et al. 2014; Iglesias et al. 2014). In one report, diagnostic success was enhanced by trio-sequencing of probands and their parents compared with proband-sequencing only (41% vs. 9%; Lee et al. 2014). Although the incremental diagnostic benefit of clinical diagnostic WES and WGS is significant, there are a number of caveats that need to be considered when evaluating its potential (Table 1).

Coverage of coding exons and mutations that are not well detected by NGS technology

Diagnostic WES is based on capturing as many coding exons as possible and typically 85%–95% of the targeted sequence yields sequencing data that are interpretable (Biesecker and Green 2014). Diagnostic laboratories can develop their own “exon capture” kits or use commercially available ones, leading to variation in the expect-

ed content of a sequenced exome. Some genes have coding exons that are difficult to sequence because of high GC-content or the presence of repeated sequences (including triplet repeat amplification mutations), resulting in absent or lower coverage of some exons. For other regions, alignment to the reference sequence cannot be resolved. These include breakpoints of balanced translocations and inversions, which can evade detection, unless paired-end sequencing is performed, wherein both sides of a fragment are linked together and sequenced in a way that the sequence is part of a single read (Talkowski et al. 2012). It has been estimated

Table 1. General points to consider in diagnostic application of WES/WGS

Technological challenges

Not all regions covered equally, some regions not covered (e.g., high GC-content; pseudogenes and highly homologous exons)
Fold coverage (sequencing depth or number of reads) determines accuracy

Sequencing assay design

Genome-wide (WGS): coding and noncoding; lower coverage of sequenced regions
Exome: most coding genes (some ncRNAs); variable design of different exome assays; higher coverage of sequenced regions than WGS
Targeted panels: selected regions and genes; disease- or phenotype-specific; variable design; higher coverage of sequenced regions

Poorly detected or undetectable mutation types

Mutations in genes with pseudogenes
Repeat amplifications
Large deletions and duplications; unbalanced translocations
Balanced translocations
Aneuploidy
Mosaic mutations (low-level mosaicism)

Result interpretation

Automated filtering for most relevant variants
Manual curation and annotation
Mutations relevant to proband's phenotype/indication
Incidental findings unrelated to indication for WES/WGS
Variants of uncertain significance (VUS)

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that at least one exon of up to 3000 genes has a highly homologous pseudoexon and some genes have entire pseudogenes (Xue et al. 2014). This makes interpretation of mutations in these genes challenging or unachievable by NGS or can be at the root of false-positive findings. Because NGS can have inaccuracies, multiple overlapping sequence reads are compared, and filtering is applied to the results before a “nucleotide call” is made. For example, if for a particular position, 90% of the aligned nucleotides are “A” but 10% are a mixture of other calls (“C,” “G,” and “T”), those other calls are rejected and “A” is chosen as the correct sequence (Biesecker and Green 2014). This approach limits the detection of low-level somatic mosaic mutations, unless special attention is paid to these filtering parameters. The practical consequences for using NGS for research (disease gene discovery) or diagnosis (mutation detection) are that “whole exome” and “whole genome” are essentially misnomers, as not all exons or mutation types are equally represented (Gilissen et al. 2012).

Result interpretation

Accurate interpretation and classification of sequencing results is the most complex and time-consuming component of diagnostic WES or WGS. Each individual genome contains millions of sequence variants, which is reduced by exome sequencing to between 20,000 and 50,000 (Gilissen et al. 2012; Gonzaga-Jauregui et al. 2012; Biesecker and Green 2014; Xue et al. 2014). These need to be filtered down to a much smaller manageable number that are more likely to be deleterious using automated bioinformatics tools with predefined parameters (Gilissen et al. 2012; Reid et al. 2014). This process is informed by the functional consequences of the variant itself, combined with information about the known or predicted function of the affected gene, including its known or predicted roles in human disease. One of the key steps in filtering variants for pathogenicity is comparison to variants catalogued in a variety of existing public data repositories and in-house databases accumulated by diagnostic laboratories, both of

which are continuously updated. However, the rigors of the clinical test reporting process require that manual curation and review of the most recent literature be performed for many variants. After variants are interpreted as potentially pathogenic, they need to be reported according to their relevance to the patient’s phenotype. Nonsense and frameshift mutations, or missense mutations already known to cause disease in a gene relevant to the phenotype, are typically reported as pathogenic and related to the indication for diagnostic exome sequencing.

In addition to the mutations related to the clinical phenotype, WES can incidentally reveal mutations that are clinically important but unrelated to the phenotype for which the diagnostic WES was performed, such as those predisposing to cancer or adult-onset conditions, or pharmacogenetic variants that affect response to and toxicity of medications (Berg et al. 2011; Gonzaga-Jauregui et al. 2012; Biesecker and Green 2014; Xue et al. 2014). How and when such changes, as well as variants of uncertain significance (VUS) are reported back to patients is a matter of active ongoing debate and an evolving process in the field that we will elaborate on more below (McGuire and Lupski 2010; Berg et al. 2013b; Green et al. 2013a,b; Krier and Green 2013; McGuire et al. 2013a,c; Holm et al. 2014).

PRENATAL GENE PANEL AND EXOME SEQUENCING ON CHORIONIC VILLUS AND AMNIOTIC FLUID SAMPLES

Prenatal Gene Panels

NGS-based diagnostic panels can be applied to prenatal cases in which the phenotype matches that of conditions discoverable by these panels. Most of these panels are not specifically designed for prenatal diagnosis and not all diagnostic laboratories accept prenatal samples for gene-panel tests, but they have been used in selected cases. The best examples are panels containing genes for Noonan syndrome, which are available for prenatal diagnosis and can be useful when an increased nuchal translucency measurement is found by first trimester sonog-

raphy or when there are cardiac defects in the fetus, for example, pulmonic stenosis, that are consistent with this diagnosis (Alamillo et al. 2012; Chen et al. 2014; Lepri et al. 2014). Skeletal dysplasia panels can be useful to differentiate between prenatally detected skeletal dysplasias, which can be important for counseling about neonatal survival prognosis and recurrence risk (Korf 2013).

Exome Sequencing

There is currently still very limited experience with the use of exome sequencing for prenatal diagnosis in ongoing pregnancies. In 2012, Talkowski et al. reported a case of a pregnancy with multiple fetal anomalies identified by ultrasound examination that included congenital heart defects (tricuspid atresia and hypoplastic right ventricle), micrognathia, abnormalities of the extremities, and evolving severe polyhydramnios with suspected tracheal atresia. An amnioreduction was performed in the third trimester that revealed a de novo balanced translocation 46,XY,t(6:8)(q13;q13)dn with no significant gains or losses detected at the translocation breakpoints. Genomic sequencing was performed, which revealed disruption of *CHD7*, the gene involved in CHARGE syndrome. This diagnosis was achieved in 13 d by paired-end sequencing of large-insert (2-kb) fragments of a whole-genome “jumping library” (Talkowski et al. 2012). Filges et al. also sequenced the exomes of a trio (affected fetus and both parents) for a family with one healthy child and two affected fetuses with recurrent intrauterine growth restriction, brain abnormalities (including microcephaly), renal and genitourinary abnormalities. They covered 67% of each genome at greater than 40-fold and 90% at greater than 10-fold to identify 34 genes that had nonsynonymous compound heterozygous sequence variants. From these, *KIF14*, encoding kinesin family member 14, emerged as the most likely causative gene, because it contained two truncating mutations, and has a cellular function and similarity to other kinesin genes causing ciliopathies with overlapping phenotypes (Filges et al. 2014). DNA from the unaffected

child and the first affected fetus was used for confirmation. The interpretation of pathogenicity of the mutations was supported by an existing animal model with *Kif14* mutations, the *Laggard* mouse, which has growth restriction, early death, and brain abnormalities that recapitulated the human findings in the family (Fujikura et al. 2013). This case illustrates the complexity and need to integrate data from multiple sources in determining the causal role of mutations in WES data.

In the first paper from our institution on 250 cases of clinical WES experience, there were four DNA samples from fetuses in non-continuing pregnancies, for one of which a diagnosis of Cornelia De Lange was revealed by identification of *NIPBL* gene mutation (Yang et al. 2013). Our more recent observational study on 2000 cases included DNA from 11 fetuses (0.6%) from terminated pregnancies. In this group, a diagnosis was made in 6/11 (54.5%), with the highest yield when a specific neurologic phenotype (1/1) or a neurological phenotype combined with a phenotype in other organ systems (5/7; 71%) was present (Yang et al. 2014). Although the number of fetal cases was limited, these data suggest that the diagnostic yield in prenatal samples may be comparatively high, especially when there are neurological findings present. Shamseldin et al. (2013) reported identification of *CHRNA1* mutations as the cause of recurrent fetal loss with fetal akinesia, and Filges and Friedman (2014) suggested that exome sequencing will be particularly beneficial for genetic diagnosis in lethal fetal disorders. Finally, Carss et al. (2014) reported exome sequencing results on a cohort of 30 euploid fetuses and neonates with structural fetal abnormalities that were identified by prenatal ultrasound examination and for which the previous work-up, including karyotyping and microarray analysis, had not yielded a molecular diagnosis. Nineteen (63%) of the analyzed samples originated from placenta, cord blood, chorionic villus samples or other fetal tissues of miscarried or terminated pregnancies. Eleven (37%) were from chorionic villus samples, postnatally obtained placentas, cultured amniocytes or postnatally obtained venous

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blood of live-born neonates. This supports that these various sources can yield DNA of sufficient quantity and quality to perform diagnostic WES. In that study, sequencing was performed at a mean coverage depth of 103-fold, with 92.7% of coding nucleotides covered by at least 10 reads. Thirty-five de novo mutations were identified (1.13 per fetal genome), three of which (10%) were interpreted as causative for the fetal phenotype, which included missense mutations in *FGFR3* and *COL2A1* and a 16.8-kb deletion in *OFD1*. Another five (17%) were VUS that needed further clarification. There were also 269 (5.3 per fetus) inherited autosomal recessive or X-linked rare functional variants (Carss et al. 2014). Finally, at least one large study has been initiated to evaluate the use of diagnostic exome sequencing on invasively obtained samples after a fetal structural anomaly is identified by prenatal ultrasound. The Prenatal Assessment of Genomes and Exomes (PAGE) study in the United Kingdom has a planned enrollment of 1000 cases (Hillman et al. 2014).

Technical Issues and Challenges Specific to Prenatal Exome Sequencing

The results of prenatal diagnosis of any modality in ongoing pregnancies are used to predict outcomes and to inform pregnancy management and reproductive choices. Obtaining results in a timely manner is therefore of critical importance (Table 2). If elective termination of pregnancy is contemplated, social and emotional burden for parents need to be considered together with legal limitations on timing; if pregnancies are continued, obtaining timely and accurate results of well-defined clinical significance is important to guide prenatal, perinatal and neonatal management. Time to results depends on different factors. When diagnostic WES is ordered because of fetal anomalies, it will likely only be initiated when other tests, such as karyotype and CMA, are normal. If prenatal sample volumes are limited, prior culture to obtain sufficient DNA of adequate quality may be required. The throughput of diagnostic WES in clinical laboratories will need

Table 2. Specific points to consider in prenatal application of WGS/WES

Clinical and technological challenges

- Limited sample size for fetal samples (amniotic fluid, CVS)
- Timing constraints from sample retrieval to results
- WES/WGS not clinically available on noninvasively obtained fetal DNA
- Incompletely defined clinical phenotype or “apparently healthy” fetus

Counseling and ethical challenges

- Cost and financial burden to patients and insurers
- Sometimes unrealistically high expectations of test performance
- Limitations in genetic counseling availability for complex test
- True meaning of informed consent, limitations if generalized consent
- Management of fetal incidental findings unrelated to indication (including adult-onset disorders)
- Discovery of inherited mutations relevant to other family members
- Trio sequencing can reveal incidental findings in parents
- Variants of uncertain significance and prenatal/perinatal decision making process
- Genetic privacy and confidentiality: fetal (and potentially paternal) results in maternal medical record, parental data in infants record after birth
- Result migration from maternal to infant’s medical record
- Data storage location: medical record versus laboratory
- Reclassification and reanalysis of data based on new disease gene information: when, how frequently, how to report

optimization to yield results faster than what is currently deemed acceptable for diagnosis in pediatric and adult patients, in which turnaround times vary and can be up to 3 mo and sometimes longer. Optimization of the procedures for DNA preparation, library construction, sequencing, data annotation, result interpretation and validation will be needed. This is technically achievable with newer faster sequencing equipment and bioinformatics tools, but will require an optimized and streamlined pipeline for data analysis and interpretation.

Importantly, clear guidelines for reporting in the prenatal context that are accepted by clinicians and families, e.g., the reporting of deleterious variants only, will also reduce interpretation time.

NONINVASIVE PRENATAL GENOME-WIDE DIAGNOSTIC SEQUENCING

Recently, extensive progress has been made in the analysis of cell-free fetal DNA from maternal plasma for diagnostic purposes. Lo et al. showed in 1997 that maternal plasma contains cell-free fetal DNA that could be assayed to noninvasively determine fetal gender in pregnancies with male fetuses (Lo et al. 1997), and subsequently, to determine the fetal RhD-genotype in Rh-negative women (Lo et al. 1998). The latter is now clinically used with high sensitivity and specificity for determining the fetal Rhesus genotype in pregnancies at risk for fetal Rh-alloimmunization and to guide the administration of anti-D immunoglobulin (Geifman-Holtzman et al. 2006; Tiblad et al. 2013; Soothill et al. 2014). Subsequently, cell-free fetal DNA has also been assayed to determine the presence of paternally inherited mutations, or to detect de novo mutations in the fetus responsible for single-gene disorders (Lench et al. 2013; Lewis et al. 2014) and for aneuploidy screening (Hui and Bianchi 2013).

In 2010, Lo et al. determined, by very deep sequencing of cell-free DNA from maternal plasma at 65-fold coverage, that the entire fetal genome is represented as a constant proportion of all the plasma cell-free DNA in fragments that are slightly larger (166 bp) than the maternal cell-free DNA fragments (143 bp). They showed that construction of a genome-wide fetal genetic map was possible and thereby provided proof-of-concept that noninvasive sequencing of the fetal genome can technically be achieved (Lo et al. 2010). They then used relative haplotype dosage (RHDO) analysis, a method they developed, to show that a fetus at risk for beta-thalassemia had inherited the paternal mutation but not the maternal haplotype that carried the mutant maternal allele. However, they still had to rely on sequence information from a

concurrently analyzed CVS sample to infer the parental haplotypes.

Subsequently, two groups published that ultra-deep sequencing of cell-free DNA in maternal plasma could be used to resolve the fetal genome sequence (Fan et al. 2012; Kitzman et al. 2012). Kitzman et al. (2012) combined shotgun sequencing of genomic DNA from maternal leukocyte-extracted DNA and paternal saliva-extracted DNA at 32- and 39-fold coverage respectively, with deep sequencing (78-fold coverage) of maternal plasma cell-free DNA in a pregnancy at 18.5 wk gestation. They could predict the inheritance of 2.8×10^6 parental heterozygous sites with 98.1% accuracy and also identified 2.5×10^7 de novo inherited sites, including 39 of 44 de novo inherited mutations with 88.6% sensitivity but a second trio sequenced at 8.5 wk gestation did not yield the same depth and accuracy (Kitzman et al. 2012). Fan et al. noninvasively sequenced the prenatal genome by molecular counting of parental haplotypes in maternal plasma by shotgun sequencing of two pregnant individuals and could identify fetal inheritance of a maternal 2.85 Mb deletion of the DiGeorge critical region. They also applied an exome capture approach on maternal plasma, allowing detection of fetal paternally inherited or de novo germline mutations. The basic principle underlying their approach is that transmitted haplotypes are overrepresented in maternal plasma, compared with the untransmitted haplotype (Fan et al. 2012). Although these are exciting new developments, both the cost and amount of labor needed to analyze the fetal genome noninvasively, currently preclude its clinical application.

COUNSELING AND ETHICAL CHALLENGES WITH FETAL GENOME-WIDE SEQUENCING

The issues related to filtering of sequencing variants to identify pathogenic ones, to discovery of incidental findings and variants of unknown significance that complicate postnatal diagnostic genome-wide sequencing, also complicate fetal exome sequencing, but with additional unique challenges and ethical considerations (Table 2).

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Cost and Financial Burden

Because of the high cost of WES/WGS, the financial burden to society and individual patients that comes with access to this important new diagnostic tool is an important limitation. However, this needs to be considered in perspective of the cost to patients and society of not knowing a diagnosis (Atwal et al. 2014). There is a need for research to learn better who will have the most benefit from the test and when in a diagnostic work-up it should be pursued. This should be paired with the development of technical and clinical guidelines to assure test quality and consistency among various providers.

Expectations of Test Performance

Although diagnostic WES or WGS have far greater capacity than other genetic tests to find disease-causing mutations, they still have significant limitations, as exemplified by currently published data of only ~25% incremental mutation detection rate when offered in pediatric and adult care (de Ligt et al. 2012; Yang et al. 2013, 2014; Fahiminiya et al. 2014). However, when a new test is offered at high cost after other standard genetic testing has failed to provide answers, patients and their healthcare providers may have unreasonable expectations about its potential benefit. Thus when no causative mutations are uncovered, patients may be disappointed or conversely, falsely reassured. The latter is especially concerning in prenatal testing, consequent to limitations of fetal imaging and because the prenatal presentation of many clinical phenotypes and syndromes is not fully understood. In addition, a benefit of exome or genome sequencing in the pediatric or adult population is the ability to review the results of prior sequencing in light of new disease gene discoveries. This can result in a diagnosis being made several years after the original testing. The risk and burden of delayed diagnosis may have special considerations in the prenatal setting.

Limitations in Genetic Counseling Availability

Fully informing individuals undergoing WES/WGS about the different categories of results

and their consequences, and offering them choices about which results they wish to have information about, requires lengthy and complex pretest counseling. In one case, it has been estimated that it could take up to 6 h to provide complete pretest counseling for clinical or research WES (Berg et al. 2011). There are not enough genetic health professionals (genetic counselors or medical geneticists) to fill this emerging counseling need, and it has been argued that the overall approach to genetic counseling has to be modified. Genetic counseling is currently primarily an individualized process in which both provision of general information and personalized risk assessment are addressed in a single session. In the future, virtual and interactive genetic counseling will need to be developed to achieve access to genetic counseling for larger numbers of patients, irrespective of their location, and to standardize the information that is provided during pretest counseling for complex genetic tests such as WES/WGS. This can then be complemented as needed with individualized genetic counseling to address specific risks and needs of patients based on their personal and family history.

The Meaning of Informed Consent

Because of limitations on genetic counseling time and personnel, paired with the complexity of the information and the multiple potential disease genes that are tested for, the meaning of informed consent in genomic testing is evolving. It is practically impossible to provide detailed information on all possible results and genetic conditions that a diagnostic WES or WGS test can yield, and patients can only be informed about the broad categories of results that can be obtained from diagnostic exome or genome sequencing (McGuire and Lupski 2010; Ross et al. 2013; Scollon et al. 2014).

Management of Incidental Findings

Clinical WES or WGS may reveal mutations in genes that are relevant for human health and disease, but that were unrelated to the initial reason for the genome-wide sequencing test in

~1%–6.5% of the time (Ding et al. 2014; Yang et al. 2014). Incidental findings, by some referred to as secondary findings (Christenhusz et al. 2013), occur in all healthcare fields (Borgelt et al. 2013; Yeh et al. 2013), including standard genetic testing, but their clinical and ethical complexity and frequency are much higher with genome wide-sequencing. How to handle these findings is widely debated (McGuire and Lupski 2010; Green et al. 2013b; McGuire et al. 2013a; Ross et al. 2013; Wolf et al. 2013; Eng et al. 2014) and their categorization with respect to pathogenicity is constantly evolving (MacArthur et al. 2012; Xue et al. 2012; Cassa et al. 2013; Dorschner et al. 2013). A study on 500 exomes from European and 500 exomes from African-American adults from the National Heart, Lung and Blood Institute (NHLBI) Exome Sequencing project estimated a frequency of 3.4% and 1.2%, respectively, in individuals of European and African descent, of high-penetrance actionable pathogenic or likely pathogenic variants (Dorschner et al. 2013). Furthermore, studies in asymptomatic individuals and analysis of the “1000 Genomes” project data have indicated that healthy individuals carry many variants that have been classified as pathogenic in mutation databases, raising the issue that such classification is not always accurate (MacArthur et al. 2012; Xue et al. 2012; Cassa et al. 2013). One study has used data from the NHLBI project to question pathogenicity of previously reported X-linked intellectual disability genes (Piton et al. 2013). Tabor et al. found that individual carrier burden for severe autosomal recessive conditions is 0.57% (Tabor et al. 2014).

Automated binning strategies to place incidental findings in specific categories relevant to the indication for testing or their general health risk have been proposed (Berg et al. 2013a,b) and the ACMG has recommended that laboratories offering diagnostic WES, interpret and report on specific mutations in 57 genes (subsequently modified to 56 genes) for which treatment or preventive measures are known to improve health outcome (Green et al. 2013a). These recommendations pertain to result reporting by laboratories but do not extend to the responsibility of clinicians to communicate

these findings to patients who can also opt out of receiving information on those genes during pretest counseling. These guidelines apply to genomic findings in children and adults and therefore deviate from earlier professional guidelines against testing for adult-onset disorders in minors (Borry et al. 2006; ESHG 2009). Whether testing for adult-onset disorders in children, and by deduction prenatally, should be limited is a matter of debate on its own (Mand et al. 2012; Anderson et al. 2014), but existing guidelines were directed toward actively seeking testing for adult-onset conditions and predated the era of genome-wide sequencing with its potential to uncover such findings incidentally and unintentionally. Recent survey studies have indicated that probands and parents of children undergoing WES are interested in having incidental findings disclosed (Townsend et al. 2012; Fernandez et al. 2014; Shahmirzadi et al. 2014).

Importantly, whereas issues surrounding the ethics of presymptomatic testing of children for adult-onset conditions extend prenatally, the recent ACMG guidelines on incidental findings specifically exclude prenatally obtained WES results (Green et al. 2013a), leaving a gap in guidance on how these—and any other incidental findings—should be handled if detected prenatally in fetal samples or in simultaneously sequenced parental genomes (Bui et al. 2014). It is imperative that a clear statement from the laboratory concerning the policy for reporting such results be communicated to the family.

Variants of Uncertain Significance (VUS)

The health consequences of deleterious mutations in genes that have not previously been associated with human disease, or of mutations with an uncertain functional consequence in known disease genes can be classified and reported as variants of VUS. VUS are seen at relatively high frequency in genome-wide sequencing and constitute the most complex counseling challenges for prenatal WES. It will be difficult to determine their influence on pregnancy outcome and postnatal prognosis for the fetus and to convey the reproductive risk of such findings when observed prenatally.

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Furthermore, at our institution, we are already seeing an increase in requests for preconception or prenatal genetic counseling to discuss options for preimplantation or prenatal genetic testing for a finding on diagnostic WES on a prior child or other family member. This is especially challenging when VUS are included in the WES report and it will be important that laboratories offering diagnostic WES consider this reproductive genetic counseling potential scenario.

Inherited Sequence Variants or Mutations

As with all genetic testing, any discovered mutation or VUS can be inherited or de novo. Inherited mutations are likely present in other relatives besides the sequenced trio, and these relatives may be at risk to pass the mutation to their offspring, or to develop an inherited condition themselves. When conditions are detected for which preventive measures can improve outcomes, providers may believe that they are ethically obliged to warn individuals at risk, which can be in conflict with the proband's (or parents') desire for confidentiality. The optimal time to address this possibility is during pretest counseling, to be repeated if such results are available. Parents can be encouraged to share information with their relatives and be provided tools to do so optimally, such as a counseling letter with information about available genetic services. However, this can be difficult because of the concern for stigmatization and for genetic discrimination and insurability. In the United States, genetic nondiscrimination legislature should protect individuals against health insurance discrimination, but it does not protect against life insurance discrimination (Korf 2013).

Sequence Data from Parental Samples

Considering the need for accuracy and rapid turnaround time (Lee et al. 2014), trio sequencing will be very attractive for diagnostic exome sequencing in ongoing pregnancies. If parental DNA is sequenced simultaneously with the fetal DNA to guide variant interpretation in a timely fashion, genetic defects could incidentally be

uncovered in a parent. When the genetic abnormality is inherited by the fetus and relevant to the condition affecting the fetus, it will be important information that is part of the diagnostic work-up. However, when an incidental finding is uncovered that is not relevant to the condition in the fetus, the situation becomes more complex. Certain mutations that predispose to adult-onset disorders (such as *BRCA1* and *BRCA2* mutations) could be important for parents because increased surveillance can improve the likelihood of early diagnosis, but not all parents will welcome such information. It is even more complex if the same finding is also found in the fetus, as it would be considered presymptomatic testing.

Genetic Privacy and Confidentiality

The privacy and confidentiality of stored data and future access to it are important considerations from a resource allocation and patient privacy perspective. Genomic sequencing data take up large amounts of server space. There is debate regarding where such data are best stored, for how long, and who should get access to it. Keeping the original raw sequence data in the diagnostic laboratories is the more straightforward solution, but some argue that this should be accessible within the electronic medical record. In prenatal diagnosis, there is additional complexity, because the fetal sequence will be obtained as part of prenatal care, and the results will be linked to the mother's demographic information and medical record in the diagnostic laboratory. Systems will need to be put in place for transfer of this information to the child's medical record after birth, although accounting for the fact that through trio sequencing, parental sequence information is also available.

Reanalysis and Reclassification of Data

Novel disease genes are uncovered at unprecedented pace and variants that were not previously considered to be clinically relevant or of uncertain clinical significance may require reinterpretation based on advancing knowledge. A systematic approach to identify impacted indi-

viduals and to recontact them (or their parents or guardians) to explore interest in reanalysis of sequence data may need to be developed. However, this will require significant coordination and infrastructure, in particular with prenatal sequence data. As sequencing costs continue to decline and technology improves, in the future it could become more practical and desirable to resequence individuals than to reanalyze existing sequences. Nevertheless, this issue highlights that with diagnostic WES or WGS, the concept of a diagnostic test result is evolving from a one-time single dataset in somebody's medical care to a life-long continuum.

CONCLUDING REMARKS

WES and WGS have rapidly increased the pace of Mendelian disease gene discoveries and have solidly entered the field of genetic diagnosis. It has improved our ability to diagnose the underlying causes of genetic disorders and birth defects in newborns, children, and adults. These new technologies are now entering the field of obstetrics and gynecology, most prominently for prenatal genetic diagnosis. Because incidental findings and VUS are more commonly detected in genome-wide sequencing than in any other genetic testing or screening modality, the counseling and informed consent challenges are complex (McGuire and Lupski 2010; McGuire et al. 2013b; Appelbaum et al. 2014; Holm et al. 2014; Scollon et al. 2014). In addition, the relatively high cost of NGS requires careful cost-benefit analysis and guidance on when preferentially to use diagnostic WES or WGS so that the diagnostic benefit is highest for a reasonable financial burden to patients and society.

There is therefore urgency for professional societies overseeing medical genetics, obstetrics and gynecological practice, and prenatal diagnosis to educate practitioners (Manolio and Murray 2014). Guidelines need to be developed for the prenatal use of these new diagnostic modalities to address the circumstances in which they are best offered, how pre- and post-test counseling should proceed, what the most optimal ways are to obtain informed consent, which categories of results should be reported,

and who decides on this within the informed consent process (Lohn et al. 2013; Appelbaum et al. 2014; Bui et al. 2014; Holm et al. 2014).

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