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GENOME-WIDE NIPT FOR RARE CHROMOSOMAL CONDITIONS:

TECHNICAL, BIOLOGICAL, AND CLINICAL CONSIDERATIONS

A **GenomeWeb/Roche** White Paper

This white paper is based on a webinar presentation by Dr. Francesca Romana Grati of TOMA Advanced Biomedical Assays and the Impact Lab Group, in which she reviewed the current state of noninvasive prenatal testing (NIPT) technologies as well as their expanding applications into rare and uncharacterized genomic disorders.

Grati discussed the technical considerations for labs that adopt cell-free DNA testing and focused on the specific differences between targeted tests and genome-wide tests.

Targeted tests, which can be based on a variety of technologies, provide a high analysis depth on a subset of chromosomes or critical regions of the genome – generally chromosomes 21, 18, 13, the sex chromosomes, and regions susceptible to microdeletions. Genome-wide tests, on the other hand, rely on massively parallel shotgun sequencing (MPSS), resulting in shallow analysis depth across all chromosomes. This approach can detect large fetal and maternal chromosomal imbalances (larger than five to seven megabases), but the detection of microdeletions and smaller duplications is not as reliable, she noted.

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Grati outlined three key factors that influence the statistical precision of NIPT testing with MPSS:

- Fetal fraction
- Size of the analyzed chromosomes
- Sequencing depth

Regarding fetal fraction, she noted that the "z-score" value for euploid samples is around zero with a standard deviation of one, and this does not increase with increasing fetal fraction. In trisomic samples, however, the z-score values are greater than zero and increase with increasing fetal fraction. As a result, "trisomic samples cannot reliably be discriminated from euploid ones at low fetal fraction levels, typically below 4 percent," she said.

Chromosome size is another factor with multiplexed MPSS tests because they use a limited amount of reagent to analyze all chromosomes in proportion to their size. "Essentially, the larger the chromosome size, the greater the number of circulating cell-free DNA fragments from that chromosome in the maternal plasma, and the greater the number of reads for that chromosome," she said.

This is a key point for trisomy testing, she noted, since chromosome 21 is the smallest chromosome and accounts for just 1.5 percent of the entire genome. "This means that with MPSS, chromosome 21, which is the most important chromosome, has the lowest analysis depth."

Sequencing depth is dependent on a range of technical parameters that can vary from lab to lab, and even from one session to another session in the same laboratory, Grati said. These parameters include the size of the NGS cartridge, the number of sample inputs during multiplexing, and the concentration of multiplexed samples.

For targeted approaches, key factors influencing statistical precision are:

- Fetal fraction
- Accuracy of cfDNA fragment amplification and enrichment
- Density of probes along the chromosome of interest

Of note, Grati said, "the most important difference" between genome-wide MPSS-based tests and targeted tests is the fact that "the statistical precision for … the targeted method depends not on the size of the chromosome under investigation but on the number of probes that are characterized to investigate the chromosome of interest." For most targeted methods, "the number of probes that are characterized to investigate, for example, chromosome 21, is the same as the number of probes that are used to analyze larger chromosomes such as chromosome 13."

In particular, Grati explained that her lab uses Roche's Harmony test with DANSR technology [illegible]es microarray to analyze the amplified fragments, with each [illegible]mple analyzed individually in each sub-array. This means that the analytical conditions are independent of the number of sample inputs and the reagent supply and can thus be standardized for each experiment and lab.

Grati also shared details of an internal study she conducted to compare the analysis depth of targeted tests versus genome-wide tests – particularly for small chromosomes. Based on that analysis, she estimated that with targeted methods, the number of reads for chromosome 21 is at least 3.5 times greater than with MPSS tests. For the 22q region, meanwhile, the number of reads using targeted methods is at least 70 times greater than with MPSS.

She stressed that labs must take these differences into account when considering which approach to adopt and noted that no single testing technology provides both breadth of chromosome targets and analysis depth.

Medical providers should consider these technical aspects when selecting which method to use, and in particular should ensure that "the appropriate technology-based pre- and post-counseling is provided to women," she noted.

Grati also addressed the biological, clinical, and counseling considerations of NIPT for rare chromosomal conditions, and in particular for rare autosomal trisomies (RATs).

A key consideration, she noted, is that the majority of cell-free fetal DNA analyzed in these tests is not derived directly from the fetus but rather from an external layer of the placenta called the cytotrophoblast.

For cfDNA tests, "the results reflect the genetic constitution of the cytotrophoblast, and therefore can provide false positive or negative results if the conceptus under investigation is affected by a feto-placental mosaicism in which the cytotrophoblast does not match the fetus," Grati noted.

In particular, cfDNA tests may provide a false positive result if the conceptus under investigation is involved in a confined placental mosaicism (CPM), with an abnormal cytotrophoblast but a normal fetus. Conversely, the test may provide a false negative result in the case of a true fetal mosaicism (TFM), which is a normal cytotrophoblast but an abnormal fetus.

"The risk that the abnormal cell line which is detected in the cytotrophoblast is also extended to fetal tissue is not the same for all chromosomes and it largely depends on the type of chromosome and on the type of abnormality involved in the abnormal cell line in the cytotrophoblast," Grati said.

Rare autosomal trisomies are particularly difficult to interpret, Grati said, due to the variable origins of a RAT call. Such a result may be due to a non-mosaic fetal RAT, a feto-placental mosaic RAT involving the cytotrophoblast, or a maternal mosaic constitutional or somatic RAT (including possible maternal malignancies). Grati noted that there is no way to reliably determine the biological origin of a RAT call from the sequencing data.

Even in cases where a positive result is due to a non-mosaic fetal RAT, this presents a challenge for counseling. Such cases usually lead to a first trimester spontaneous miscarriage, she said, but "releasing this information to the couple just increases anxiety because a miscarriage is neither preventable nor clinically actionable." In addition, "this information is of limited utility to calculate the recurrence risk because the recurrence risk of a miscarriage is mainly related to maternal age and the number of previous consecutive spontaneous abortions rather than the karyotype of the previous miscarriage."

In the case of mosaicism, whether it has clinical impact depends on the tissue in which the mosaicism resides, but this information is not known in prenatal diagnosis due to variability in the distribution of abnormal cell lines in the different fetal tissues and organs, Grati said. "Counselling in these cases should be conducted with extreme caution as a true fetal mosaicism does not necessarily mean that the individual will be abnormal or mildly abnormal," she said.

Grati shared results of study her lab conducted that reviewed the clinical outcomes of 153 RAT-positive results from six different published studies, in which a total of 71,893 women were screened. The most common outcome associated with a high-risk RAT result was a normal live birth in 40 percent of cases, she said, followed by miscarriage in 27 percent of cases. One conclusion from this analysis, she noted, is that genome-wide cell-free DNA testing is not an efficient screen for fetal anomalies or pregnancy complications. For example, based on this analysis, only one abnormal fetus was detected out of 6,500 women tested, while only one TFM case was detected out of every 5,000 women tested.

Grati said that "another layer of complexity" arises from the fact that there are no guidelines from professional societies regarding optimal patient management in cases where a RAT is detected. "In fact a woman with high-risk results for a rare autosomal trisomy should be carefully counselled about all the options she has, including the possibility to do nothing because of the negligible risk of some RATs to be confirmed as a true fetal mosaicism; to do [chorionic villus sampling] in order to confirm the presence of the abnormal cell line in the placenta and assess the degree of mosaicism for possible pregnancy complications; to do an amniocentesis in order to confirm or exclude the presence of a true fetal mosaicism; to do both CVS and amnio simultaneously in order to assess the presence and the distribution of the abnormal cell line in both the placental and fetal compartments; and also the possibility to do [cord blood sampling]."

Overall, Grati noted, genome-wide cfDNA testing is "fraught with counseling difficulties" due to the different possible biological reasons for a positive result, uncertainty in the performance of different cfDNA tests, uncertainties about genotype-phenotype correlations in true fetal mosaicism cases, and a lack of data on how to properly counsel women in the case of a positive result.

A key take-home for labs running cfDNA tests is that they should understand the limitations of the methodologies available, in both technical and clinical terms. MPSS-based genome-wide cfDNA analysis provides a lower depth of analysis than targeted cfDNA analysis and increases the complexity of clinical care by screening for conditions of unclear clinical significance.