PEARLS OF LABORATORY MEDICINE

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TITLE: Cell Free DNA - General Principles and Clinical Applications

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Hello, my name is Deepika Sirohi. I am an Assistant Professor of Pathology at ARUP Laboratories and University of Utah. Welcome to this Pearl of Laboratory Medicine on “Cell free DNA- General Principles and Clinical Applications”.

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Cell free DNA (cfDNA) was first identified in the peripheral blood in 1948. With the development of sensitive technologies there has been a resurgence of interest in cfDNA over the past decade as a biomarker in clinical care. The source of cfDNA in the peripheral blood is from cells undergoing apoptosis or necrosis where it exists in a free form and a protein bound form. The former undergoes rapid degradation while the latter is preserved. The size distribution of cfDNA depends to a large extent on whether it is bound to single or multiple nucleoprotein complexes and is typically in the range of 160 to 200 base pairs.

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At the outset, there are certain terminologies used in this field that need to be defined. While cfDNA refers to any free DNA in the serum, cffDNA refers to the cell free fetal DNA fraction in the maternal serum and ctDNA refers to circulating tumor DNA.

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As with all clinical assays, pre-analytical variables also affect cfDNA. Fixative-containing blood collection tubes are preferred that prevent lysis of nucleated cells. Blood can be stored at ambient room temperature up to 7 days without compromising the yield of cfDNA, but these collection tubes are expensive. Alternatively, EDTA can be used as an anticoagulant with recommended sample processing within 6 hours. Prolonged storage in EDTA can cause lysis of normal cells with release of cfDNA and diluting of cfDNA of interest.

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In recent years, cfDNA has found increasing clinical applications, the foremost being in prenatal screening. In this setting, cfDNA has been used for fetal RhD genotyping in RhD negative mothers, fetal sex determination for sex linked disorders and detecting chromosome aneuploidies and other monogenic disorders in appropriate clinical settings. Emerging clinical applications include cancer diagnosis and monitoring, and other potential applications include transplant monitoring and autoimmune diseases. Of these, only selected applications in prenatal testing and oncology will be discussed in this pearl.

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cffDNA is by far most frequently used for prenatal screening also called non-invasive prenatal screening or NIPS, wherein fetal cfDNA in maternal peripheral blood is utilized to assess constitutional genetic disorders at an early stage during pregnancy which provides opportunities to make timely and informed decisions related to the pregnancy. The source of cffDNA in maternal peripheral blood is from apoptosis of placental cells and it exists in a highly fragmented form, comprising 6-20% of circulating DNA in maternal blood. Factors that affect levels of cffDNA include maternal body mass index (BMI) with lower fractions seen with increasing maternal BMI; gestational age with higher fractions seen with advanced gestational age; and placental health. Typically, a minimal fetal fraction of 4% is desirable for reliable testing.

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cffDNA is detectable as early as 5 weeks after conception with an incremental increase between 10 and 20 weeks and a more rapid increase after week 21. Levels decrease rapidly after delivery, disappearing within a few hours. Most laboratories offer cffDNA testing starting at gestational age of 9-10 weeks or later.

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Analysis of cffDNA can be SNP–based or shotgun sequencing of the whole genome. Accuracy of SNP-based assays depends on number of SNPs analyzed in the assay and can distinguish between maternal and fetal sources based on SNP differences. However, off-target abnormalities are not detected. The whole genome shotgun sequencing assays typically involve counting statistics of large number of maternal and fetal cfDNA to identify chromosomal origin and determine fetal aneuploidies.

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The next step in cfDNA assays is separating out cffDNA of interest. In the peripheral blood, cfDNA includes an admixture of germline/constitutional DNA from the mother and cffDNA from the fetus. These can be differentiated by various methods such as in case of a male fetus by the amount of Y- chromosome specific sequences. Bioinformatically, reads from X or Y chromosomes and aneuploidy chromosomes; benign or pathogenic copy number variations (CNVs) and single nucleotide polymorphisms (SNPs) can also be used to separate out cffDNA of interest. Moreover, the cffDNA are typically shorter in length than germline cfDNA and this property is also frequently utilized to isolate cfDNA of interest.

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Fetal disorders commonly amenable to prenatal testing by cffDNA include aneuploidies, most frequent of which are trisomies for chromosomes 21, 13, 18 and sex chromosomes; microdeletions and duplications such as those listed and triploidies.

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NIPS has excellent sensitivity, for the common aneuploidies and most sex chromosome aneuploidies. Average false positive rate for common aneuploidies is < 1-2/1000 with a specificity of 99.8-99.9%. However, the positive and negative predictive values are affected by disease prevalence in the population. Lower disease prevalence results in higher negative predictive value and a lower positive predictive value and vice versa and therefore the prevalence of disease must be taken into consideration while interpreting results.

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Microdeletions and duplications are individually rare occurrences, but collectively are more common than classic trisomies. Individual risk of having a fetus affected with a microdeletion is more than that of classic trisomies especially in women less than 35 years. As would be logical, larger deletions are more effectively detected.

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Like most laboratory tests, NIPS is also subject to false positive results. Common causes for false positive results include confined placental mosaicism, vanishing twins, maternal neoplasm, maternally derived genetic aberrations and maternal mosaicism. Due to these and other reasons, discordant results between NIPS and true fetal genotype are not entirely avoidable.

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Some other key concepts in understanding results from prenatal screening are to remember that prenatal disorders being tested are rare and as such a positive result does not always translate into an affected pregnancy. When NIPS is used as a secondary test for positive combined or sequential prenatal screens there is still a greater than 2% residual risk of having a fetus with a chromosome abnormity following a negative cell free test. In women more than 35 years, NIPS will detect only approximately 80% of chromosomal aneuploidies.

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Another significant clinical application of cfDNA is in oncology wherein it is referred to as circulating tumor DNA (ctDNA). ctDNA testing in cancers has several potential applications. It can

provide diagnostic information by identifying tumor specific changes, aid in early tumor detection, monitor for minimal residual disease and relapse/recurrence, and be informative of tissue specific epigenetic signatures. Quantitation of ctDNA also correlates with tumor burden. During the disease course it can be also used to identify clinically actionable variants; monitor treatment response; emergence of resistant mutation patterns; evaluate molecular heterogeneity across the tumor and tumor dynamics.

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Tissue sequencing is often limited by tumor heterogeneity both between different tissue and within a given tissue type, a need for invasive procedures to collect tissue, and contamination by normal cells. While cfDNA is an emerging technology and technically challenging, assaying for ctDNA which comprises a small fraction of cfDNA offers high specificity; significantly eliminates tumor heterogeneity; is very sensitive for early detection of cancers and has a rapid turnaround time.

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Assays used for detection of ctDNA depend on the underlying molecular alteration being tested. Some commonly used assays include comparative genomic hybridization (CGH) SNP arrays for copy number changes, next generation sequencing (NGS) that can detect multiple alterations, digital droplet PCR and Beads, Emulsification, Amplification, and Magnetics (BEAMing) to target specific genetic alterations. The analysis performed on the DNA can be targeted towards specific genes or alterations or be genome-wide to identify tumor specific alterations unique to the individual patient; de novo genetic alterations arising secondary to therapy and new actionable targets. The kinetics and variables affecting ctDNA are largely unknown; but the levels correlate with the rate of cellular turnover and in tumors can vary from 0.01 to 90%.

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In US, currently FDA approved ctDNA testing in cancers includes *EGFR* mutation testing for exon 19 deletion and L858R mutation at diagnosis for assessing suitability for *EGFR* directed therapies and T790M mutation at relapse for emergence of resistance. Colon cancer screening by testing for methylation based markers of *SEPT9, NDRG4* and *BMP3* gene promoter regions is also

available as an FDA approved test for screening individuals who have been offered conventional screening modalities for colorectal cancer but have not completed it.

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Another potential application of cfDNA is for management of patients undergoing organ transplants. Donor derived DNA was identified in plasma of kidney and liver transplants in 1998. In post-transplant individuals, donor derived cfDNA follows an L-shaped curve with highest level in the immediate post-engraftment phase followed by a rapid decline to baseline. The kinetics however differ across different transplants necessitating the need to establish normal dynamics for each transplant type. The donor cfDNA can be then quantitated by utilizing HLA DNA and copy number polymorphisms or SNP distribution in donor and the recipient. Monitoring donor cfDNA in transplant recipients can be used to guide therapeutic decisions and screen for opportunistic infections. The abundance of donor derived mitochondrial DNA can also aid in assessing organ damage associated pattern and the epigenetic markers can provide information about the cellular source of DNA.

Slide 20: References

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Slide 15: Disclosures

Slide 16: Thank You from www.TraineeCouncil.org

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