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Opinion Paper

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Circulating fetal cell-free DNA and prenatal molecular diagnostics: are we ready for consensus?

Abstract: Circulating fetal cell-free DNA (cffDNA) is emerging as the most reliable known target for prenatal molecular diagnostics. Different points of view are expressed in literature regarding the safe use of cffDNA for all types of molecular tests, above all those used to detect maternal DNA rather than fetal mutation. The aim of the present study was therefore to achieve consensus on guidelines conducive to standardizing current procedures, which differ between laboratories, and to design stringent technical protocols for the analysis of cffDNA.

Keywords: circulating fetal cell-free DNA; guidelines; prenatal diagnosis.

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In recent months numerous papers have appeared in the literature on the use of circulating fetal cell-free DNA (cffDNA) for prenatal testing, based on massively parallel sequencing. This approach is considered a useful tool for making a prenatal diagnosis of some Mendelian disorders and for detecting chromosomal alterations (e.g., aneuploidies). DNA gain associated with trisomies 21, 18 and 13 can be detected on fetal DNA and used for clinical diagnostics [1–3]. Although this technique is gaining ground in different fields of laboratory medicine, concern has been expressed regarding the routine use of cffDNA in laboratory clinical diagnostics. Recently Wu et al. [4] highlighted the risk of fetal DNA contaminating whole blood DNA samples, and some questions regarding the safety of using cffDNA, especially when performing molecular diagnostic tests, remain unanswered. In particular, the quantity of cffDNA, which can originate in different fetal tissues, generally increases with gestational age; therefore, for the purposes of clinical diagnostics, there is a need to standardize the best possible timing to obtain maternal blood samples when performing the test to screen maternal rather than fetal DNA mutations. Yet few general recommendations, guidelines and standardized operational procedures have been defined in the literature concerning the timing for maternal blood sampling. This aspect calls for consensually developed guidelines conducive to harmonizing the procedures currently used by different laboratories. Nor do all the techniques used for detecting mutation or aneuploidy have the same sensitivity: classical OLA CFTR-sequencing (in particular when a positive F508del homozygosity finding is made) based on the detection of genetic variants through allele-specific methods does not ensure the best possible performance and, in terms of quality of analysis, is error-prone in detecting of variants [5]. Nor, therefore, can qualitative PCR-based platforms be reliably used to detect low or high frequency alleles, such as those carried by fetal circulating DNA segments (cffDNA) contained in lots of maternal genomic DNA fragments (cfDNA), above all when the copy amount of these molecules is lower than 10%, as occurs in the early gestational weeks. In this case, if testing is fetus-oriented, some mutations may be found to be missing. However, classical Sanger sequencing or real-time PCR-based methods detect circulating fetal DNA alleles, not only in early gestation, but also between the 10th and 20th weeks; in such cases, the risk of contamination from circulating fetal DNA should certainly be taken into account [1–3]. This issue may become critical when the recently designed next generation sequencing platforms are used for molecular testing, as they have high sensitivity, which also depends on the depth of coverage, chemistry, pipeline and methods (counting or genotyping) employed. Here it is important to stress that the ratio between cffDNA and cfDNA may be reduced if maternal weight is increased (e.g., pregnancy in overweight or obese women). In this

setting, cffDNA may be considered diluted, its relative concentration often being below 4%; in such cases the performance of molecular methods employed to discover, e.g., the more common fetal aneuploidies, may be compromised. Therefore, for clinical molecular diagnostics in a prenatal setting, maternal saliva samples (in particular when obtained to assess the risk of Mendelian disorders) should be chosen as a valid alternative to blood DNA, as the latter seems to be prone to fetal DNA contamination, unlike saliva. A preliminary "non-invasive" genetic analysis made on DNA extracted from saliva is therefore a suitable diagnostic tool for use in pregnant women. It is important to bear in mind that DNA from saliva samples is currently used for molecular testing also in non-prenatal diagnostics [6].

Chiu et al. have recently reported that since circulating fetal DNA accounts for about 15% of total DNA, being cleared quickly from maternal plasma following delivery, the persistence of fetal DNA "*from one pregnancy into the next one*" can effectively be ruled out [7]; therefore, maternal cffDNA should also be used to confirm results obtained on saliva in the early gestational weeks.

A further issue is the need to optimize procedures for blood collection, transport and storage, as the amount of cell-free DNA significantly increases when samples are maintained at 23 °C and above, due to maternal cell degradation [8]. Few studies have demonstrated the possible contribution of circulating fetal pregnancy-associated progenitor cells, which may cause fetal cell microchimerism (FCM). During pregnancy, these cells, albeit in small numbers, may enter the maternal circulation thus causing maternal blood contamination; the DNA from these cells is probably detected only after cell enrichment before any molecular analysis, although DNA may be released following apoptosis, thus contributing to a relative increase in the amount of cffDNA [9]. Nevertheless, contamination from fetal DNA released from these stem cells should always be considered a possible additional confounding factor when high sensitivity molecular prenatal methods such as NGS are used for diagnostic purposes, above all due to the longer half-life of these cells in the bloodstream. However, Coata et al. [10] also showed that fetal microchimerism due to fetal hematopoietic CD34(+) cells persisting from previous pregnancies did not affect the outcome of genetic analysis.

The lack of sufficient data on the safety of using cffDNA or cfDNA for prenatal non-invasive molecular diagnostics, should prompt researchers and clinical laboratory professionals to define criteria and platforms that ensure the: 1) use of appropriate procedures for the enrichment of cffDNA when an analysis is made to rapidly discover fetal mutation; 2) harmonization and standardization of timing and procedures for blood drawing so as to obviate maternal cffDNA contamination of; 3) identification of the more sensitive and specific platforms to be used for molecular diagnostic analysis; 4) influence on sensitivity and specificity of full automated platforms, as compared to manual procedures followed for testing cffDNA samples.

The model proposed by Wu et al. [4] cannot guarantee the elimination of either contamination or confounding factors, for the following reasons: 1) the spiking of fetal mutant DNA alleles (obtained for example from peripheral blood leukoctyes) in normal DNA samples does not perfectly represent the situation of cffDNA fragments mixed with maternal cfDNA fragments. For routine diagnostics, DNA is obtained through whole blood extraction, yielding a mix of cffDNA and cfDNA; 2) the methods used for CFTR and F5 and F2 factors are not so sensitive as NGS and quantitative PCR methods, which can be strongly affected by contamination from fetal DNA. The different behaviors, in terms of sensitivity and specificity in the large panel of commercially available molecular platforms should therefore be carefully evaluated before introducing a layout in routine diagnostics, above all when cffDNA is used. Furthermore, information on the sensitivity and specificity of methods employed should be included in the clinical laboratory report.

In conclusion, as we are very close to introducing massive parallel sequencing in routine molecular prenatal diagnostics [2, 11], it is of the utmost importance to standardize bioinformatic tools in order to ensure the greatest possible accuracy in prenatal non-invasive diagnostics [11]. A careful consideration of the ethical impact of this new approach must also be made, and an increased awareness gained concerning the potential impact of non-invasive prenatal testing for single gene disorders on clinical practice, since this will have important implications for future policy and guidelines in prenatal care [12].

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