

Cell Free DNA Analysis for Chromosomal Abnormalities among Pregnant Females of Pakistan

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Authors' contributions

This work was carried out in collaboration among all authors. Author AIS proposed the topic of the review, did the literature search and edited the final draft of the manuscript. Authors SA and TS wrote the first draft of the review. Author SF managed the literature searches as well as contributed to figures. All authors read and approved the final manuscript.

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ABSTRACT

The advancement of modern molecular biology techniques has made it possible to detect fetal anomalies beforehand in order to tackle the upcoming situation. However, the idea is to devise the most sensitive screening tools with fewer chances of errors as well as noninvasive methods to diagnose fetal abnormalities. Previously used methods amniocentesis and chorionic villus sampling possess risks for the fetus on the other hand cell free fetal DNA (cffDNA) method is less invasive and reduces the risk to fetus. However, currently most cffDNA screening tests routinely evaluate fetal sex and sex chromosomal aneuploidies while in developed countries analysis of cffDNA is incorporated in high-risk pregnancies to detect the defects and mutations. In Pakistan where the prevalence of birth defect is reported approximately 7% as well as increased consanguineous marriages increase the chance of such defects. Centers in Pakistan offer cffDNA testing but with a hefty cost on the pocket. This review highlights the importance and prospects of exploring the maternal plasma Cell-free DNA (cfDNA) screening in high risk mothers in Pakistan as well as the limitations and strengths of the technique. Since the cffDNA sequencing is a major advancement in genomic medicine that has reduced the invasive procedures in clinical medicine.

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1. INTRODUCTION

Cell-free DNA (cfDNA) screening also designated as non-invasive prenatal testing (NIPT), a screening test accomplished by bioinformatics tooling and sequencing of fetal fragments of DNA in maternal plasma to rule out the chance of certain chromosomal abnormalities during pregnancy. Bioinformatics analysis classically consists of structural variation detection and computing the percentage of fetal fraction in sequenced genomic [1]. Simply, this testing evaluates circulating small fragments of DNA in a pregnant woman's blood. Unlike most DNA, cfDNA are free-floating fragments

(approximately 200 base pairs) released into bloodstream by dying or sloughed cells. Normally in human blood stream, their own cfDNA circulates but during pregnancy, cfDNA from the placental trophoblast cells also mixes with maternal cfDNA, which presents the genetic makeup of fetus. NIPT is utilized for direct DNA screening of cfDNA fragments numbers. This aids in identifying fetal RhD blood group genotyping, fetal trisomy (21, 18, 13), sex chromosome aneuploidy (45, X; 47, XXX; 47, XXY; 47, XYY) and specific microdeletions 22q; 15q; 11q; 8q; 5p; 4p; 1p36) (Fig. 1). Though, with the limitation that this technique cannot screen all genetic and chromosomal abnormalities [2,3].

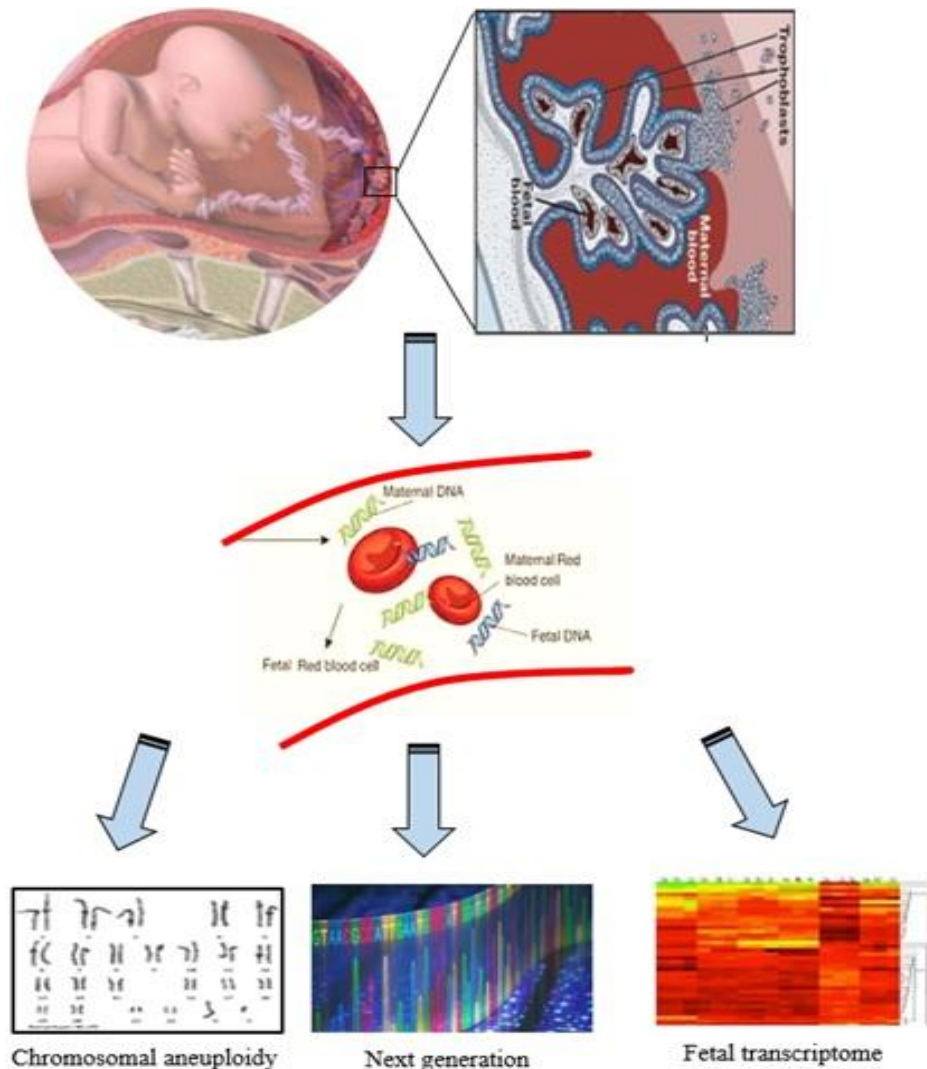


Fig. 1. Noninvasive prenatal cell free DNA screening during pregnancy

Cell free fetal DNA (cffDNA) is usually detectable in maternal blood after five to seven weeks of gestation. It comprises of approximately 11 to 13.4 percent in blood and increases as the pregnancy progresses. However, it is undetectable two hours after of delivery in maternal blood. Since last decade, antenatal cfDNA testing has gained worldwide recognition from being a less approved off-standard diagnostic examination (ultrasound or physical examination) to more authentic tool for diagnosing fetal genetic mutations (routine cytogenetic or microarray) [4]. The need to perform these tests is more in consanguineous marriages and in *in-vitro* fertilization. The reported rate of consanguineous marriages in Pakistan is 60-70% i.e. first cousin and second cousin marriages. On the other hand, the infertility is 22% prevalent in Pakistan and *in vitro* fertilization poses threat of high risk of chromosomal abnormalities in the unborn [5,6].

Cell free DNA (cfDNA) have multiple roles in the detection of different diseases. However, the purpose of this review is to highlight the importance of utilizing cfDNA as a screening tool for detecting fetal anomalies beforehand in high-risk pregnancies.

2. WHAT IS CELL FREE FETAL DNA?

The cell free fetal DNA (cffDNA) arises from the cytotrophoblasts and syncytiotrophoblast that undergo physiologic cycles of fusion and apoptosis during gestation, releasing DNA fragments from the placenta into the circulation of the mother [7]. Plasma sample is used for investigation of circulating DNA segments, which contains both maternal and placental cfDNA, as opposed to the seclusion of entire fetal cells from maternal blood. Fetal fraction which is the amount of placental to total (comprising of maternal and placental) cfDNA, increases as pregnancy progresses [8,9]. cfDNA testing is usually recommended to be carried out from the tenth week of gestation, since the fetal fraction in the maternal circulation reaches the ample amount required for an informative test result. Fetal fraction is considered as one of the several factors that influence the sensitivity. Other parameters include the number of sequenced cfDNA molecules, the proportion of bases of guanine and cytosine in a particular chromosome, and the presence of variants of the maternal and fetal copy number. Analyzing the circulating cfDNA, utilizes two basic sequencing approaches: random (whole-genome) and targeted [10].

3. ANTENATAL SCREENING IMPLEMENTATION GUIDELINES

Professional guidelines globally suggest cfDNA testing for trisomy 21, 18 and 13 as an option for women who are pregnant with a high risk of fetal aneuploidy [11]. Some guidelines also support all women's cfDNA testing, as it is the most sensitive test for these aneuploidies [12,13]. However, analyzing cfDNA is costlier than screening multiple markers. The efforts and resources required for pretest counseling related to maternal plasma cfDNA testing are greater than those required for standard screen counseling. Some women may not be fully aware of the limitations of the test if they consent to a blood test that does not pose a fetal risk, or they may not take sufficient account of the impact of a predictive positive result. It is also important to acknowledge that only one third of chromosomal anomalies can be identified by a diagnostic karyotype or microarray study in common trisomies [14,15]. Most cfDNA screening tests routinely evaluate fetal sex and sex chromosomal aneuploidies. Some laboratories report subchromosomal aneuploidy findings, including microdeletion. For both biological and analytical reasons, the positive predictive values for the detection of these conditions were lower than those of common autosomal trisomies, which lead to more false positive cases requiring invasive confirmatory tests to determine the true fetal karyotype [16,17,18].

4. EVALUATION OF FETAL FRACTION AND ITS IMPORTANCE

The proposed gold standard for validating different methods of estimating fetal fraction (FF) in singleton pregnancies should be useful in prenatal screening based on cell-free DNA methods. The gold standard overcomes a limitation in previous attempts to validate different methods of FF estimation that were restricted to comparing one method of estimation against another. In addition, experimental results indicate that the sequencing fetal fraction (SeqFF) method is an accurate method of estimating [10,19]. In early gestation and assisted conception, a low fetal fraction happens more frequently. Increased inflammation and apoptosis that occur in obese pregnant women's adipose tissue is negatively correlated with fetal fraction, resulting in increased release of maternal cfDNA into circulation [20]. Maternal thromboembolic disorders, heparin use and

vitamin B12 deficiency are correlated with low fetal fractions. As a consequence of maternal blood clotting and intramedullary hemolysis, or possibly to a direct impact on trophoblasts, maternal cfDNA is increased [21]. However, even with a sufficient fetal fraction, there may be false negative results due to true fetal mosaicism or an aneuploid fetus with an euploid placenta [10,22].

5. FACTORS CAUSING FALSE POSITIVE RESULTS IN cfDNA SCREENING

The usual standards for clinical practice advise confirmation of positive outcomes for cfDNA screening with an additional diagnostic karyotype or microarray study [23,24]. There may be disparity between karyotype result and cfDNA results due to chromosome reference ratio. Another biological reason for false positive outcomes is the expiration of a twin in utero ("vanishing twin syndrome") [25]. Another reason for sex-discordant outcomes, which is extremely rare, is that a male donor has previously given the mother a bone marrow or organ donation [26, 27]. Since sequencing is carried out on a sample containing both maternal and placental DNA, a lot of false positive results are of maternal origin [21]. Both constitutional and somatic, maternal mosaic sex chromosome aneuploidies (45, X and 47,XXX), are common causes for the low positive predictive values detected in sex chromosome cfDNA testing. Apoptotic cell-free DNA can be released into circulation when a pregnant woman has a malignant tumor. If this happens, entire genome sequencing techniques can detect a genome-wide imbalance that can be misinterpreted as fetal aneuploidy [28,29].

6. NONINVASIVE DIAGNOSIS OF FETAL SINGLE-GENE DISORDERS By cfDNA

cfDNA analysis is also used to detect fetal single gene disorders non-invasively in couples at high risk based on their personal or family history [30]. This test is based on the direct detection of DNA sequences in maternal plasma inherited from paternal or de novo mutants. In order to assess a maternally inherited condition or an autosomal recessive disorder, cfDNA techniques are used to evaluate whether the maternal mutant allele or haplotype is proportionally more or less compared to its nonmutant counterpart. The allele or haplotype which is present in larger amount is the one acquired from the developing fetus. In blood disorders such as beta thalassemia, sickle cell anemia and hemophilia, the evaluation of relative mutation dosages in

maternal plasma by means of a digital polymerase chain (ddPCR) reaction has been shown [31]. To recreate the haplotype structure enveloping the potentially mutated locus, sequence data from cfDNA analyses are used. A comparison is made between the combined quantity of cfDNA from the mutant haplotype and that from the opposite haplotype. The haplotype represented more abundantly by cfDNA is inherited by the developing fetus. This approach has been demonstrated for detecting congenital adrenal hyperplasia, Hunter's syndrome, hemophilia A [32] and beta thalassemia [33].

7. BIOINFORMATICS

Bioinformatics (Fig. 2) has become an important component in clinical laboratories generating, analyzing, maintaining, and interpreting data from molecular genetics testing [1]. Huge genomic database is formed by frequent use of NGS in the screening process. Such a great information has also become challenge for bioinformatics, which has become the important part of modern clinical laboratory. Genetic data acquirement from NIPT can be done by two sequencing approaches: sequencing of the whole genome by shotgun sequencing and targeted sequencing for sequencing of specific genomic regions of interest. Analysis of genomic mixture, called fetal fraction consists of detecting structural variation and calculating the proportion of fetal fragments in sequenced genome as already mentioned.

Conventionally DNA fragments from the affected chromosome was used for sequencing followed by eradication of long affected fragments, and also for quality control rectification of any laboratory induced error from the observed sequence matching to reported correct database [34]. Additionally, scores on the basis of count and length of sequence may be united to attain better identification of normal and affected DNA [35,36]. Routine testing which includes counting methods showed higher accuracy but only chromosomal counts are not enough to determine fetal fraction particularly in female babies as they similar karyotypes with their mothers [1]. In such cases the focus on deviation in DNA packaging nucleosomes in specific areas as well as fragment length distribution [35]. However, the length-sequencing method showed lower precision due to additional patient aspects involved such as body mass index, gestational age and other epigenetic factors. Recent researches into single-nucleotide

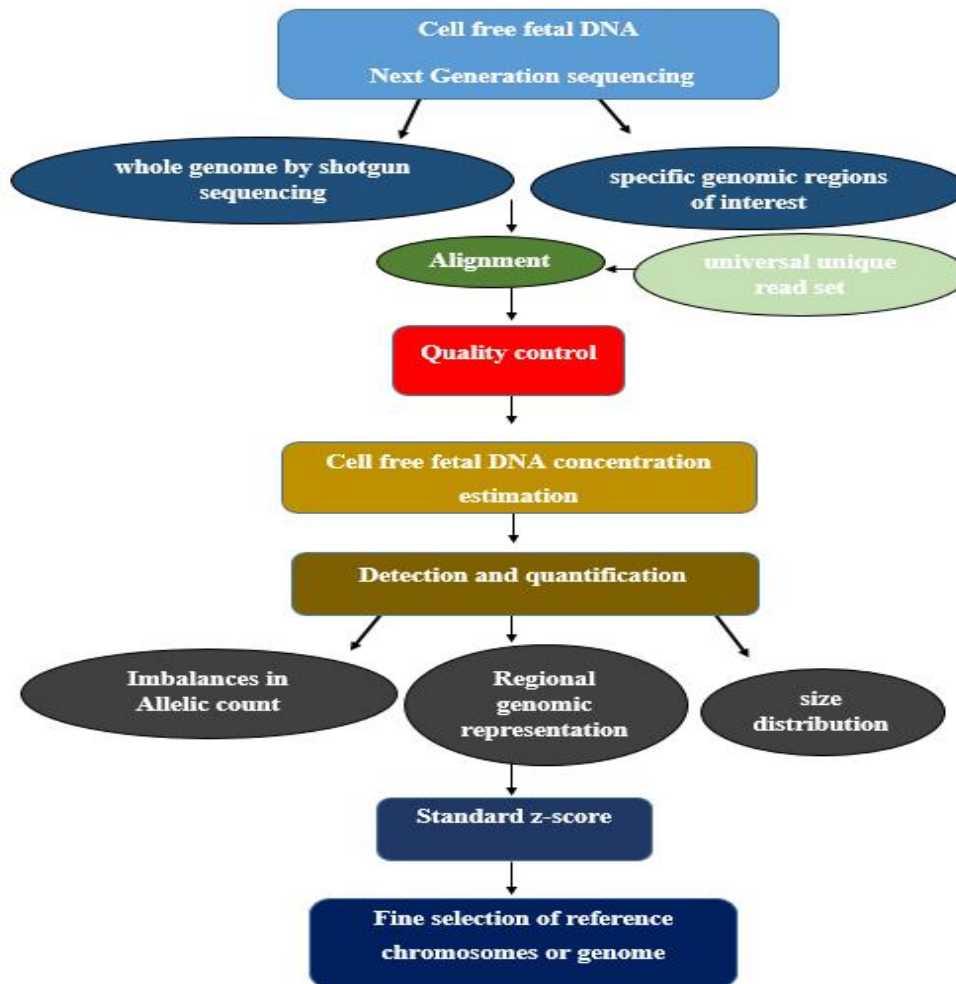


Fig. 2. The flowchart of the whole bioinformatics of cffDNA analysis, including effective alignment, quality control for data correction, cffDNA concentration estimation, quantification and then aneuploidy or size distribution detection. Further standard Z-score is calculated for reference genome selection

polymorphism (SNP) based sequencing of the fetal fraction and its comparison with known genotypes of the parents may revolutionize genomic biobanking in the coming era of bioinformatics [36].

8. ETHICAL AND LEGAL CONCERNS

Currently there are no rules regulating what should be conveyed to the medical community or potential patients concerning the performance characteristics of this screening method prior to being publicly marketed. Continued competition between various commercial groups led to the release of new tests before clinical use could be demonstrated. Since the sequencing techniques

used for the analysis of cfDNA have been developed long after the training of most practitioner providers, there is also a knowledge gap. Many suppliers receive information from commercial laboratories about the test. Although cfDNA testing has resulted in a decrease in invasive procedures, it has led to there being lesser opportunities for individuals undergoing training to learn how to perform the procedures. Another result is a reduction in consultations to genetic counseling practices for couples impacted by single - gene disorders due to some practitioners erroneously believing that all genetic conditions are screened with cfDNA. Determination of fetal sex is also ethical issue in clinical application of this modern technique [37].

9. SIGNIFICANCE OF DEVELOPING QUALITY CONTROL MATERIALS FOR cfDNA ANALYSIS

CfDNA based NIPT phenomenon is not new to the world nowadays. It has become the standardized part of prenatal screening for trisomies 21, 13, and 18. However, its rapid development and clinical introduction circumvent crucial area i.e. ensuring the quality of NIPT. The reasons for this hasty introduction of a clinical screening test are the 2 main advantages compared with the current existing screening tests, first the invasive testing with threats to the pregnancy and second is the first trimester combined screening test (FCT) with low sensitivity and specificity [38,39]. Besides that, FCT can be only carried out during a limited time frame, whereas NIPT can be performed after 10 weeks of gestation.

The introduction of NIPT in clinical settings is not the same as implementation lacking not only in pretest and posttest counseling but also in follow-ups by clinics and laboratories themselves. Another aspect that requires attention is proper validation and calibration of the testing method prior to start offering the test [40]. Lack of proper controls due to specific intrinsic properties of both maternal and fetal cell-free DNA (cfDNA) and enough quantity of blood samples for processing are the other challenges that are faced due to non-standardization of the procedure. Laboratories participation in external quality assessment (EQA) is also lagging behind as through this any lab can assess entire process from sample handling to reporting. International EQAs for NIPT for aneuploidies recommendations have been provided recently by Genomics External Quality Assessment (GenQA, www.genqa.org) and the European Molecular Genetics Quality Network (EMQN, www.emqn.org) [41,42].

10. IMPORTANCE OF THE PREANALYTICAL PHASE OF cfDNA TEST

There are three phases of clinical laboratory analysis; pre-analytical, analytical and post-analytical, where multiple factors have an effect on each of them. The lack of standardisation, particularly in the pre-analytic phase, is one of the hurdles faced in obtaining accurate results. Pre-analytical errors (biological, environmental, or technical) tend to be frequent in clinical laboratory results i.e. 46%- 68.2% (20–22).

Nevertheless, owing to discordant findings that are often difficult to replicate, few liquid biopsy tests have advanced towards clinical use till date. Reproducibility can therefore be improved by establishing and enforcing standard operating procedures and reference materials to properly identify pre-analytical variables [43]. cfDNA is a peculiar sample supposed to be handled by highly skilled personnel preferably in well-established genetic testing laboratories. However, they underline the importance of comprehensive pre-analytical standardization. Impact of pre-analysis influences on the sample collection and its integrity should be thoroughly investigated to increase the success rate and its efficiency of the tests [44]. Pre-analytical phase constitutes of: the patient (demographical and biological status), collection process, sample transport, sample processing, and sample storage/stability. All these factors lead to a possibility of inaccurate results from the laboratory in general. However, specifically in case of preanalytical phase of cfDNA analysis, there are concerns such as

- Heterogeneity of blood contents hampers isolation of cfDNA due to different cells as well as free macromolecules as well as contamination of cfDNA from blood cells. Therefore, recommendation is the use of plasma is, as this helps to minimize contamination with DNA from leukocytes, thus optimizing sensitivity and data homogeneity.
- Complexity of blood as a biofluid and its sensitivity to the duration of the assay, especially involving enzymatic degradation or blood clotting and fragility of naked DNA in a biological environment [45].

Hence, careful management of bio specimens is important in the clinical field for the effective implementation of cfDNA analysis.

11. FUTURE PROSPECTS OF cfRNA

Moreover, new researches on non-coding RNAs, cell free RNA (cfRNA) are captivating more attention like microRNAs (miRNAs), Long non-coding RNAs (lncRNAs), circular RNAs (circRNAs) are in the focus of the clinical research [46]. Measuring circulating RNA may provide insights into the placental transcriptome without the need for invasive testing. RNAs from the placenta are released into the maternal blood from early in pregnancy and may reflect changes

in gene expression. The cfRNA is in the experimental phase to verify the clinical applicability of non-invasively used genetic disease prenatal detection using NGS. The cfRNAs also exhibit possible prognostic, diagnostic, and therapeutic applicability according to reports. All of the above RNAs are in research to detect the most common pregnancy-associated diseases, such as preeclampsia [47], congenital cardiac anomalies, restriction of fetal growth and gestational diabetes [48]. The research is at advanced stage on the use of microRNAs, while lncRNAs and circRNAs are still promising targets.

12. SIGNIFICANCE Of cfDNA SCREENING IN PAKISTAN

According to a hospital-based study conducted in Pakistan, out of 3,210 total admissions, 226 (7%) neonates were congenitally malformed [37]. Worldwide it is 3-7% but varies from country to country [49]. Among different body systems affected, anomalies related to the central nervous system were common 46 (20.35%) in comparison to other system related malformations [37]. It is in comparison with a study from Saudi Arabia that also reported Central nervous system (CNS) as the most commonly affected system followed by musculoskeletal and renal [50]. An Indian study revealed first ranking for CNS malformations followed by musculoskeletal and then cardiovascular system (CVS) anomalies [51]. Antenatal care helps in early detection of congenital malformations (CMs). In Pakistan it is reported that 95% of pregnant women receive antenatal care from lady health visitors (LHVs) or nurse and only 5% from physicians. A Pakistani study reported that only 32.3% mothers with CM babies, irrespective of the stage of pregnancy, received antenatal care [6]. Obvious cause for this difference is socioeconomic and education status of the studied population. Availability of health facilities definitely has an impact on this count. During antenatal care, cfDNA screening could be done to find out congenital anomalies before the birth of the baby and to take required actions to lower the incidence of such congenital malformations. A study conducted in Pakistan regarding the perception and attitude of obstetric professionals towards implementing this test in Pakistan showed that 95% agreed with the necessity to diagnose genetic conditions as early as possible and 97% of the health professionals were in favour of providing prenatal screening tests [52].

In this review, comprehensive information is given about the recent developments on this field. Noninvasive prenatal cfDNA screening for multiple purposes is an active area with increasing test volumes and precision. Maternal plasma cfDNA sequencing can clinically have high potential for screening of different diseases at fetal period. However, concerns remain with medical abortions due to genetic disorder is a religious issue in Pakistan thus requiring country specific health policies and approaches to implementing NIPT [52]. It is believed that novel findings and scientific progress will transform screening by NIPT to a final diagnostic test.

13. CONCLUSION

The current drift in prenatal testing by a substantial move from invasive to non-invasive sampling or from the blood as a less-invasive source. cfDNA as NIPT had a global effect and is commercially available in different developed countries for past few years. Clinicians are now introducing maternal plasma cfDNA screening into private prenatal healthcare in Pakistan too, due to commonality of consanguineous marriages and chromosomal aneuploidy but concerns remain regarding the social and ethical implications. Maternal plasma cfDNA screening has turned from a research endeavor to clinical care with more accurate and specific results. Maternal plasma cfDNA sequencing is a new horizon and a major advancement in genomic medicine that has resulted in screening that is more precise and reduced invasive procedures.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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